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Exploring the evolutionary significance of mitochondrial genetic variation in *Drosophila melanogaster*

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Chapter 4: ***Mitochondrial haplotypes exert sex-specific effects on the locomotory activity of Drosophila melanogaster, independent of the thermal environment***

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Thesis abstract

In recent years, the functional significance of genetic variation in the mitochondrial genome has risen to prominence in the fields of evolutionary biology and biomedicine. In particular, recent attention has focused on an evolutionary hypothesis known as *Mother's Curse*, which predicts that maternal inheritance of mtDNA will permit the accumulation of male-harming mutations provided these same mutations are benign or beneficial in their effects on females. Studies have provided support for this prediction, by showing that the genetic variation found across distinct mitochondrial haplotypes confers greater effects on the expression of key life-history traits in males than in females. Yet, our understanding of the proximate mechanisms that link mitochondrial genetic variation to sex differences in the life-history phenotype remains rudimentary. Furthermore, we know little as to whether mitochondrial genotypic effects on trait function may be affected by mitochondrial genotype-by-environment interactions; a salient omission, given that populations invariably live in heterogeneous environments.

I investigated these questions over three research chapters, leveraging genetic strains of *Drosophila melanogaster* that differ only in their mitochondrial haplotype sequence. My first aim was to explore the proximate basis of Mother's Curse effects on life-history. To this end, I studied the effects of mitochondrial haplotype variation on sex differences in a core physiological trait – the metabolic rate. I uncovered a negative genetic correlation between metabolic rate and longevity, across haplotypes, which was specific to males. Furthermore, I found a strong signature of sexual antagonism across haplotypes – haplotypes that conferred high metabolic rate in females, conferred low rate in males. These findings thus indicate that the key predictions of the Mother's Curse hypothesis extend to the metabolic rate, suggesting that mitochondrial genetic effects on core

metabolic functioning might be the proximate driver of the downstream effects on life-history previously observed.

My second aim was to investigate whether life-history trait expression is sensitive to mitochondrial genotype-by-environment interactions and if so, to determine whether the magnitude of these G × E effects was sex-specific. I first studied longevity outcomes across a panel of mitochondrial haplotypes, on diets that differed in ratios of protein-to-carbohydrate. I found that the longevity of flies was affected by the mtDNA haplotype, with these effects contingent on mitochondrial G × E interactions that were larger in males, than females. Thus, the link between mitochondrial genotype and phenotype is sensitive to the dietary environment. I then studied mitochondrial genotype-by-thermal environment effects on the locomotory activity of adult flies, to further explore the capacity for mitochondrial genotypic effects on core physiological function to be moderated by the abiotic environment. While I found effects of mtDNA haplotype on locomotion, which were contingent on the sex of the flies, the mtDNA-mediated effects were not moderated by the thermal environment.

In summary, this thesis advances our knowledge in understanding the physiological mechanisms that might underpin the effects of the mitochondrial genotype on life-history phenotype, confirming the omnipresence of sex-specificity in these effects even at the level of the organismal physiology, and also confirming a role for genotype-by-environment interactions.

General declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

In the case of *data chapters 2 and 3*, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** (published, in the press, accepted or returned for revision, submitted)	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
2	***Mitochondrial haplotypes confer sexually antagonistic effects on metabolic rate in Drosophila melanogaster***	In review – Philosophical Transactions of Royal Society of London: Biological Sciences	55%. Experimental design, collecting data and writing the manuscript.	Ian Aitkenhead, input in running the experiment, 10%. Prof Steven L Chown, input into final manuscript 5% Prof David J Clancy, input into final manuscript 5% Dr Damian K Dowling, Concept, experimental design, data analysis and drafting the chapter, 25%	No No No No
3	***Interactions between mitochondrial haplotype and dietary***	In press – Journal of Gerontology Series A:	65%. Experimental design, collecting data	James Rapkin, input to experiment, 5%	No

	macronutrient ratios confer sex-specific effects on longevity in Drosophila melanogaster	Biological Sciences	and writing the manuscript	Prof John Hunt, Concept and input to final manuscript, 5% Dr Damian K Dowling, Concept, experimental design, data analysis, input into manuscript 25%	No No

I have renumbered sections of the submitted paper in order to generate a consistent presentation within the thesis.

Name: Venkatesh Nagarajan-Radha
Date: 24-July-2019

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors

Name: Assoc. Prof. Damian Kimon Dowling
Date: 25-July-2019

Publications during enrolment

1. **Nagarajan-Radha V**, James Rapkin, John Hunt and Damian K Dowling (2019). Interactions between mitochondrial haplotype and dietary macronutrient ratios confer sex-specific effects on longevity in Drosophila melanogaster. The Journals of Gerontology: Series A. DOI: https://doi.org/10.1093/gerona/glz104 *In press*. [Special Issue on Drosophila and Ageing]

2. McLay K Lucy, **Nagarajan-Radha V**, Green P Mark and Jones M Therésa (2018). Dim artificial light at night affects mating, reproductive output and reactive oxygen species in *Drosophila melanogaster*. Journal of Experimental Zoology Part A. 2018;1–10. DOI: https://doi.org/10.1002/jez.2164 [Special Issue on Light Pollution]

3. Doss DPS and **Nagarajan-Radha V** (2017). Male resource defence behaviour strengthens harem size in promiscuously mating fruit bats. Acta Chiropterologica 19(2): 329-336. DOI:10.3161/15081109ACC2017.19.2.009

4. Doss DPS, **Nagarajan-Radha V** and Kandula S (2016). Daily and seasonal variation in non-acoustic communicative behaviours of male greater short-nosed fruit bats (*Cynopterus sphinx*). Acta Chiropterologica 18(1): 199-207. DOI:10.3161/15081109ACC2016.18.1.010

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I dedicate this thesis and all the other academic achievements made thus far,
and that are to be made, to my Mother, who lived and died with
the belief that I would become a "Scientist", one day.
I think I am getting close to becoming one!

Chapter 1
General Introduction

1.1 Evidence for non-neutral genetic variation in the mitochondrial genome

The coding region of the mitochondrial genome in bilaterian metazoans consists of 37 genes, of which 13 encode for proteins, two for ribosomal RNAs and 22 for transfer RNAs (Boore, 1999). Of these gene products, the 13 proteins encoded by the mitochondrial DNA (mtDNA) form a core part of the mitochondrial electron transport system (mETS) that produces chemical energy in the form of adenosine triphosphate for eukaryotic cells (Boore, 1999, McKenzie et al., 2007).

Since the 1980s, evolutionary biologists and phylogeneticists have used the sequence variation found within the mitochondrial genome to resolve phylogenetic relationships and study the population genetics of closely related species. They have done so by assuming that the mitochondrial genome possesses certain unique properties: principally, that it is a haploid and maternally-inherited genome (Birky, 2001, Castellana et al., 2011), that the occurrence of recombination events in the mitochondrial genome of most eukaryotes is nearly absent (Dowling et al., 2008, Neiman & Taylor, 2009, Rokas et al., 2003); and that the rate at which mutational events occur in the mtDNA is much higher than mutation rates within the nuclear DNA (nDNA) (Brown et al., 1979, Lynch, 1997, Saccone et al., 2000). However, studies in the past decade have shown that these characteristics of the mtDNA cannot be generally assumed across all taxa of eukaryotes and that the mitochondrial genomic sequence variation should be used with caution in studies of phylogenetics. For instance, there are notable exceptions to strict maternal inheritance of mtDNA, such as "doubly uniparental inheritance" in the *Bivalvia* (Breton et al., 2007, Passamonti et al., 2011) and rare occurrences of heteroplasmy due to the leakage of paternal mtDNA in the developing embryo of humans (Luo et al., 2018, Nunes et al., 2013). It has also been shown that the recombination events in bilaterian metazoans occur more frequently than previously thought (Ladoukakis & Zouros, 2017). And lastly, across many cases of bilaterian metazoans, the rate at which mitochondrial mutation events occur compared to the nuclear DNA varies (Allio et al., 2017, Montooth & Rand, 2008).

Furthermore, for many years, the genetic variation accumulating within the coding region of mtDNA was assumed to be neutral to selection (Ballard & Kreitman, 1995, Kimura, 1983, Rand, 2001). The logic underlying this assumption seemed robust – any phenotype-modifying (that is, non-synonymous) genetic variation that would accrue in the mitochondrial genome would threaten the functionality of core mETS enzyme complexes; and such genetic effects on the functionality of mETS machinery would have immediate consequences on organismal function. (Ballard & Melvin, 2010, Ballard et al., 2007, Ballinger, 2005, Blier et al., 2001, Bratic & Trifunovic, 2010). Because of the unique properties of the mtDNA including the assumption of selective-neutrality of the mitochondrial coding genome, biologists have long used mitochondrial gene sequences as prime investigative tools when seeking to resolve phylogenetic relationships between species and to study the genetic structure of animal populations (Avise, 1986, Ballard & Rand, 2005, Moritz et al., 1987).

Strikingly, however, this traditional assumption of selective neutrality of the mtDNA has been challenged by a growing body of evidence that has shown the mitochondrial genome to often accrue and express mildly or severely deleterious genetic variation that encodes for differences in the expression of core phenotypes in bilateral metazoans (Castellana et al., 2011, Dowling et al., 2008, Lynch & Blanchard, 1998, Nachman, 1998, Nachman et al., 1996, Wallace, 1994). While the efficiency of purifying selection on mtDNA is dependent on the effective population size of mtDNA, *in silico* sequence analysis and *in vivo* mutation screening experiments have, however, demonstrated that the efficiency of purifying selection on mtDNA is generally poor, compared to selection on nDNA [(Castellana et al., 2011, Elson et al., 2004, Fan et al., 2008, Rand & Kann, 1996, Stewart et al., 2008) although see (Cooper et al., 2015)]. In particular, it has been proposed that maternal inheritance and haploidy of the mitochondrial genome will lower the effective population size of the genome, and lead to this lowered efficacy of purifying selection. These effects would be exacerbated

by the high mutation rate of the mtDNA and lack of recombination within the mitochondrial genome, which would render the genome prone to accumulation of deleterious mutations in populations via a process akin to the Muller's ratchet model of mutation accumulation (Castellana et al., 2011, Elliott et al., 2008, Muller, 1964, Neiman & Taylor, 2009).

The earliest evidence for phenotype-modifying genetic variation in the mtDNA was provided by studies in humans, which found that certain harmful mutations in the mtDNA are associated with the etiology of complex genetic disorders, such as Leber's hereditary optic neuropathy (Wallace et al., 1988, Wallace, 1992). In addition to human genetic disorders, studies on animal models, such as the vinegar flies *Drosophila melanogaster*, seed beetles *Callosobruchus maculatus*, marine copepods *Tigriopus californicus*, freshwater snails, and mice have found that the naturally occurring mitochondrial genetic variation affects the expression of a suite of life-history and physiological traits (Ballard & Melvin, 2010, Dobler et al., 2014, Dowling, 2014, Dowling et al., 2008, Ellison & Burton, 2006, Moreno-Loshuertos et al., 2006, Pichaud et al., 2012, Sharbrough et al., 2017). Notwithstanding, intriguing evidence has emerged to demonstrate that not all genetic variation in the mtDNA of metazoans are necessarily deleterious, but some are adaptive and are under positive selection (Dowling et al., 2008, Foote et al., 2011, Meiklejohn et al., 2007, Wolff et al., 2016c).

It is noteworthy that among the earlier studies that have found association between mitochondrial genotype and phenotypic effects in animal models, such as the vinegar flies and mice, some have specifically used genetic strains of these animals that differ only in their mitochondrial genome but harness a common isogenic nuclear genome (Clancy, 2008, Moreno-Loshuertos et al., 2006, Pichaud et al., 2012). These genetic strains are generally created by chromosome replacement techniques that enable the researchers to experimentally prize apart coevolved combinations of mitochondrial and nuclear genotype, placing the mtDNA haplotypes of these populations alongside controlled nuclear

backgrounds. Experimenters have thus been able to use such strains to directly partition the magnitude and patterns of mitochondrial genetic effects on the phenotype, removing any potential confounding effects contributed by nuclear genomic variation. In sum, taking all the evidence from studies on humans and animal models together, it is now becoming clearer that the once-thought 'evolutionary silent' mtDNA is significantly contributing to phenotypic expression in eukaryotes.

1.2 Maternal inheritance of mtDNA invokes sexually asymmetrical effects on the expression of core organismal traits

One of the most interesting aspects of mitochondrial biology is the uniparental inheritance of mitochondria, which occurs strictly through the maternal lineage in most bilaterian metazoans (Birky, 2001, Castellana et al., 2011, Frank & Hurst, 1996, Gemmell et al., 2004). The theory posits that maternal inheritance will render selection incapable of purging mtDNA mutations whose negative effects are limited to males (Frank & Hurst, 1996). Thus, mutations that are benign or beneficial in their effects on females may accumulate in the mtDNA sequence even when these same mutations confer severe costs on male function (Beekman et al., 2014, Connallon et al., 2018, Cosmides & Tooby, 1981, Frank & Hurst, 1996, Gemmell et al., 2004, Vaught & Dowling, 2018). This has now become to be known as the "*Mother's Curse*" hypothesis (Gemmell et al., 2004). One of the key predictions of this hypothesis is that this process will lead to the accumulation of male-biased mutation loads within the mitochondrial genomes of distinct populations; that is, a genetic load of mutations whose effects on phenotypic expression are larger in males than in females. As such, if a set of mitochondrial haplotypes is screened for their associated phenotypic effects in each of the sexes, under the Mother's Curse hypothesis, it is predicted that the effects associated with these haplotypes will be larger in males than in females.

Several studies have now provided experimental support for this prediction. Indeed, studies in plants, vinegar flies, roosters, hares and humans have shown that naturally occurring genetic variation within the mitochondrial genome exerts disproportional effects on reproductive function in males, and several studies have also identified candidate mtDNA mutations associated with incidences of male-limited sterilization (Beekman et al., 2014, Chase, 2007, Clancy et al., 2011, Froman & Kirby, 2005, Holyoake et al., 1999, Kao et al., 1995, Nakada et al., 2006, Patel et al., 2016, Ruiz-Pesini et al., 2000, Smith et al., 2010, Vaught & Dowling, 2018, Xu et al., 2008). Furthermore, mtDNA mutations have been discovered that are associated with male-biases in the onset of blindness and complex disorders in humans (Hudson et al., 2014, Milot et al., 2017, Wallace et al., 1988).

Remarkably, some of the earlier studies that have demonstrated male-specific fertility effects associated with the mitochondrial genome, have found that the impaired male fertility is underpinned by a single non-synonymous genetic substitution in the mitochondrial coding genome. For instance, the Ala278→Thr amino acid substitution in Cytochrome *b* gene of the Brownsville haplotype of *D. melanogaster* affects sperm morphology conferring complete male-sterility (Clancy et al., 2011), where the allele has been shown to be under positive selection across heterogeneous nuclear genetic backgrounds and thermal environments (Wolff et al., 2017); a A→G transition at 11177 bp position in the gene encoding $tRNA^{Arg}$ is associated with low sperm motility in the roosters *Gallus domesticus* (Froman & Kirby, 2005); a mutation in the subunit II of Cytochrome oxidase gene that results in an amino acid substitution from glycine to serine (Gly177→Ser) and encodes for a temperature-specific reduction in male fertility (Patel et al., 2016); and a T→C transition at 8821 bp position in the ATPase6 gene is associated with impaired sperm maturation in humans (Holyoake et al., 1999).

In addition to the findings of Mother's Curse effects on male reproductive functions highlighted above, studies have shown that the genetic variation delineating mitochondrial haplotypes of a

species is strongly associated with male biases in the level of variation in phenotypes, such as longevity, fertility, mitochondrial quantity and functioning of mETS enzymes (Beekman et al., 2014, Camus et al., 2012, Correa et al., 2012, Pichaud et al., 2012, Wolff et al., 2016b, Yee et al., 2013). Particularly, much of this knowledge about the association between mitochondrial genetic variation and phenotypic variation stems from studies conducted on a large panel of thirteen mitochondrial haplotypes of *D. melanogaster* (Clancy, 2008, Wolff et al., 2016a). This panel is unique - where the strains differ only in their mitochondrial genetic variation expressed against a common isogenic nuclear background derived from a w^{1118} laboratory strain that is kept isogenic; each haplotype exists across independent biological duplicates, and these strains are free of *Wolbachia* infection (Camus et al., 2015, Clancy, 2008, Wolff et al., 2016a). In one study, Innocenti *et al.* (2011) leveraged five strains from this panel, reporting that patterns of gene expression throughout the nuclear transcriptome in males are much more sensitive to interference from mtDNA allelic variation than are patterns of expression in females. Strikingly, the genes that were subjected to strong mtDNA-mediated interference were those that encode for proteins that are localized within the male reproductive tissues and are thus actively involved in male reproductive functions (Innocenti et al., 2011).

Second, using the whole panel of thirteen haplotypes, Camus *et al.* (2012) showed that the genetic variation delineating these mitochondrial haplotypes affects longevity and rate of ageing in males, but not in females. Third, using disrupted and coevolved mitochondrial-nuclear (mitonuclear) combinations of six of the thirteen haplotypes, Yee *et al.* (2013) demonstrated that the mtDNA genetic variation existing across these haplotypes affects outcomes of male fertility and that the disruption of coevolved mitonuclear genotypes confers negative effects on male reproductive fitness (Yee et al., 2013). These findings of Yee *et al.* corroborated the findings of mitochondrial genetic effects on transcriptomic changes in male reproductive tissues reported by Innocenti *et al.* (2011).

Fourth, Wolff *et al*. (2016b) showed that mitochondrial haplotype variation confers larger effects on the number of mitochondria per milligram of tissue in young male flies than in young female flies. And finally, a recent study by Camus and Dowling (2018) demonstrated signatures of sexual antagonism across the panel of mitochondrial haplotypes, whereby haplotypes associated with high reproductive performance in females are associated with low reproductive performance in males.

Notwithstanding, while strong support for the Mother's Curse hypothesis comes from the studies highlighted above, not all studies have found support for the predictions of the hypothesis. A number of studies in *Drosophila* have created genetic strains comprised of inter-species combinations of mito-nuclear genotype, in which mtDNA haplotypes sourced from *D. melanogaster* or *D. simulans* were expressed alongside nuclear backgrounds from *D. melanogaster*. These studies reported that mitochondrial genotypic effects on phenotypic expression were larger in females than males, when assayed across different environments, contrary to predictions of the Mother's Curse hypothesis (Mossman et al., 2016a, Mossman et al., 2017, Mossman et al., 2016b, Zhu et al., 2014)

1.3 The mechanistic basis for Mother's Curse effects on organismal traits

While it is now clear that variation across mitochondrial genotypes is often associated with sex-differences on the expression of fertility and other core life-history traits in bilaterian metazoans, the mechanisms by which genetic variation in the diminutive and highly streamlined mitochondrial genome could exert such sex-specific effects at the level of life-history remain unclear.

Some emerging evidence has indicated that mitochondrial haplotypes could regulate patterns of life-history trait expression through moderating the expression of genes within the mitochondrial transcriptome (Camus et al., 2015, Camus et al., 2017). Yet, mitochondrial haplotype-mediated effects on regulation of the mitochondrial transcriptome do not appear to differ between the sexes;

haplotypes associated with low expression of particular mtDNA proteins coding genes tend to confer low expression in males and in females (Camus et al., 2015, Camus et al., 2017). On the other hand, mitochondrial genotypic effects on the regulation of gene expression in the nuclear transcriptome have been shown to exhibit strong sex differences, with sequence variation in mtDNA affecting expression patterns of numerous nuclear genes that are localised in their expression to the male reproductive tissues (Innocenti et al., 2011), and likely to be affecting *in vivo* male reproductive outcomes (Innocenti et al., 2011, Yee et al., 2013).

Thus, the link between mitochondrial genotype and sex-specific life-history phenotype is likely to involve mtDNA-mediated interference of the nuclear transcriptome in males, leading to expression dysregulation with ultimate effects on male life-histories. But, currently, the intermediary links between these effects on gene regulation and the consequent sex-specific effects on life-history trait expression currently remain unclear. Traditionally, evolutionary ecologists have worked under the assumption that investment trade-offs between life-history traits revolve around a currency of energy limitation (Sheldon & Verhulst, 1996); with traits such as the whole-organism level metabolic rate underpinning the expression of these trade-offs (Hulbert et al., 2007, Zera & Harshman, 2001). Currently, however, it is unclear whether physiological traits such as the metabolic rate are generally affected by mitochondrial genetic variation in the first instance (Arnqvist et al., 2010), and if so, whether the effects of this variation on the metabolic rate are larger in males than females, consistent with predictions of the Mother's Curse. Ultimately, if signatures of Mother's Curse were to extend to core organismal physiology, this could have potentially profound consequences for our understanding of the mitochondrion's involvement in the evolution of sex differences in life-history. This is because it would suggest that the entire life-history of males is likely to be shaped by the accumulation of male-harming mtDNA mutations that have accumulated under maternal inheritance of mitochondria.

Studies in fruit flies, marine copepods, freshwater snails and mice have tested for mitochondrial haplotype effects on the fine-scaled functioning of enzyme complexes involved in the mETS machinery (Ballard et al., 2007, Correa et al., 2012, Ellison & Burton, 2006, Moreno-Loshuertos et al., 2006, Pichaud et al., 2012, Sharbrough et al., 2017, Wolff et al., 2016b). While these studies have shown that the enzymatic capacities of each of the five core mETS complexes are affected by naturally occurring mtDNA alleles, only one of these screened for sex biases in effects. Specifically, Wolff *et al*. (2016b) reported mitochondrial haplotype effects on mitochondrial quantity in young, but not old, flies, but did not detect clear signatures of male-bias in mitochondrial genetic effects on the respiratory rate of the individual mETS complexes across both young and old cohorts (Wolff et al., 2016b).

To date, few studies have sought to determine whether genetic variation across mtDNA haplotypes can confer effects on the whole organism metabolic rate, as gauged by levels of CO_2 respired per individual. One study reported that interactions between the mtDNA haplotype, nuclear background and temperature affected metabolic rate in the seed beetle, *Callosobruchus maculatus* (Arnqvist et al., 2010). However, these effects were measured during juvenile development, and thus the authors were unable to determine whether the effects differed in their magnitude across the sexes. Another study of *D. subobscura* found that variation in the expression of whole-organism metabolic rate in adult flies could be mapped to genetic variation across the mtDNA haplotypes. However, the authors did not find clear male-bias in mitochondrial genetic effects on the metabolic rate; albeit their inferences are likely sensitive to sampling error because the authors sampled only three mitochondrial haplotypes of the flies (Novicic et al., 2015). Thus, currently it remains an open question whether the Mother's Curse effects might extend to the core of organismal physiology; and

whether such effects on key physiological traits are involved in sex-specific trade-offs with life-history phenotypes.

1.4 Mitochondrial genetic effects on phenotypes are sensitive to heterogeneity in the environment

While it is clear that sequence variation within the mtDNA affects phenotypic expression, few studies have sought to test whether these mtDNA-mediated effects on trait expression will vary across environmental contexts, via Gene-by-Environment (G × E) interactions. Studies to date that have screened for mitochondrial genotype-by-environment effects have focused on the role of only two environmental factors, temperature and diet, in moderating the link between mitochondrial genotype and phenotype.

It has long been known that mitochondrial functioning and homeostasis are heavily dependent on temperature (Portner et al., 2007). This could mean that the thermal environment has the capacity to alter life-history functions via selection at the level of mitochondrial functioning. Evidence suggests that the heterogeneity in thermal environment is associated with differences in the functioning of core mETS enzymes across mitochondrial haplotypes of a range of species (Abele et al., 2002, Guderley & St-Pierre, 2002, Pichaud et al., 2011, Pichaud et al., 2010, Rand, 1994, Rawson & Burton, 2002). For instance, in the marine copepod, *Tigriopus californicus*, differences in the activity of mETS enzymes across 18° and 25°C were found to be associated with the within-population genetic variation in the mitochondrial genome (Rawson & Burton, 2002). And in the fruit fly, *D. simulans*, effects of temperature on the activity of core mETS enzymes were dependent on the mitochondrial haplotype of the flies (Pichaud et al., 2010). Expanding on these studies that found G × E effects on the mitochondrial functioning, recent studies found similar levels of G × E and more complex G × G × E interactions (among mitochondrial, nuclear haplotypes and temperature) on the

expression of traits such as egg-to-adult development time and whole-organism level metabolic rate across mitonuclear strains of seed beetles (Arnqvist et al., 2010, Dowling et al., 2007); and larval metabolic rate and male-specific fertility effects across mitonuclear strains of fruit flies (Hoekstra et al., 2013, Wolff et al., 2016c).

Furthermore, emerging evidence shows that the mitochondrial bioenergetic functioning is also regulated by the availability of macronutrients, such as protein and carbohydrate, in the diet (Pichaud et al., 2013, Solon-Biet et al., 2014). Similar to the genotype-by-temperature effects on organismal traits, studies have found mitochondrial genotype-by-diet effects on longevity, egg-to-adult development time and *in vitro* functioning of mitochondrial enzymes across genetic strains of fruit flies expressing distinct mitochondrial haplotypes (Aw et al., 2017, Holmbeck & Rand, 2015, Mossman et al., 2016a, Pichaud et al., 2013, Zhu et al., 2014). Collectively, these findings of mitochondrial genotype by environment interactions, suggest that particular mtDNA haplotypes might find themselves under strong selection in particular environments if they encode for optimal trait values in the prevailing dietary or thermal environments, but that these same haplotypes might find themselves to be disfavoured in other environments. (Arnqvist et al., 2010, Ballard & Youngson, 2015, Dowling et al., 2007).

Regardless, the sex-specificity of G × E interactions for organismal phenotypes remains poorly understood, simply because previous studies probing for mitochondrial genotype by environment interactions have focused on one sex only. Exceptions come from recent studies, one of which reported a male bias in the magnitude of G × E interactions for longevity across strains of *D. melanogaster* in which each of two mitochondrial haplotypes were expressed against an isogenic nuclear background (Aw et al., 2017); and from another study that utilised strains of *Drosophila* harbouring inter-species combinations of mitonuclear genotype, which reported female biases in the

magnitude of the G × E interaction (Mossman et al., 2016a). Currently, the role of the environment in moderating the link between mitochondrial genotype and sex-specific life-history phenotype remains largely unexplored, and thus it is unclear whether previous evidence for the Mother's Curse hypothesis, outlined above, would be upheld across a diverse range of heterogeneous environments.

1.5 Positive selection on the mitochondrial genetic variation

While to some degree, the evidence for mitochondrial haplotypic involvement in the G × E interactions helps us to understand the maintenance of non-neutral mitochondrial alleles in natural populations, it remains poorly understood whether the accumulation of phenotype-modifying alleles in the mtDNA happens through non-adaptive or adaptive processes. Until recently, it was generally assumed that the mitochondrial genome could accumulate deleterious mutations via non-adaptive processes because of its high mutation rate, compared to the nDNA, combined with its presumed lower effective population size and lack of recombination (Lynch & Blanchard, 1998). Because of these reasons, it was presumed that the process of mutation-accumulation could largely explain the accumulation and maintenance of phenotype-affecting mutational loads in the mtDNA (Ballard & Whitlock, 2004, Dowling et al., 2008, Lynch, 1997, Lynch & Blanchard, 1998, Neiman & Taylor, 2009).

An alternative hypothesis to explain the non-neutral variation in the mitochondrial genome has, however, emerged over the past decade; based on the premise that sequence variation within the genome can accrue adaptively under positive selection. Indeed, this notion is supported by recent findings of mitochondrial G × E interactions, which would suggest that some mitochondrial haplotypes within a population could increase in frequency and outcompete other haplotypes under particular environmental conditions (James et al., 2016, Kivisild et al., 2006, Nachman et al., 1994, Nachman et al., 1996). Indeed, several studies have reported strong associations between spatial

patterns of genetic variation in the mtDNA and the climatic regions of various metazoan species (Camus et al., 2017, Consuegra et al., 2015, Fontanillas et al., 2005, Foote et al., 2011, Mishmar et al., 2003, Morales et al., 2015), suggesting that climatic selection might be a key selective force in shaping the standing genetic variation in the mitochondrial genomes of natural populations (Mishmar et al., 2003, Ruiz-Pesini et al., 2004).

The earliest evidence for the contention that climatic selection might shape and maintain the spatial distribution of standing genetic variation in the mtDNA comes from studies that conducted *in silico* analysis of sequence variation across the mtDNA sampled from different lineages of humans (Mishmar et al., 2003, Ruiz-Pesini et al., 2004). These studies found that human mtDNA sequences sampled from populations across tropical, temperate and sub-arctic lineages exhibit region-specific genetic substitutions in particular mitochondrial genes that encode core mETS enzymes, prompting the authors to suggest that these alleles could be contributing to better functioning mETS complexes under prevailing climatic environments in those regions (Mishmar et al., 2003, Ruiz-Pesini et al., 2004). A study that followed up on these correlations, reported evidence that levels of mitochondrial sequence divergence across human populations covaried with the differences in temperature that those populations had evolved in (Balloux et al., 2009). Together, these studies provided the first support for an intriguing evolutionary hypothesis; that spatial patterns of mtDNA sequence variation has been shaped by climatic selection -- which is now called the *mitochondrial climatic adaptation* hypothesis (Camus et al., 2017). Further empirical support for the mitochondrial climatic adaptation hypothesis has recently come from a range of studies reporting similar patterns of mtDNA haplotypes or mutational patterns in the mtDNA associated with climatic differences along latitudinal clines (Camus et al., 2017, Cheviron & Brumfield, 2009, Consuegra et al., 2015, Morales et al., 2015); and studies that showed evidence for positive selection on mitochondrial genetic sequence across a range of species (Cheviron & Brumfield, 2009, Fontanillas et al., 2005, Foote et al., 2011, Lamb et al.,

2018). Very few of these studies have, however, moved beyond correlations, to attempt to establish causative links between patterns of mitochondrial haplotype variation and climate.

1.6 Does mtDNA evolve adaptively under selection to the environment?

In particular, it is unclear how different mitochondrial haplotypes would be able to make an adaptive response to changes in the prevailing climate. For such a response to take place, the mtDNA haplotype would need to be involved in the regulation of traits that are sensitive to changes in the climatic environment (i.e., traits that are highly sensitive to mitochondrial genotype by environment interactions). To this date, two studies have examined differences in the expression of phenotypic traits associated with thermal tolerance, across mitochondrial haplotypes of the greater white-toothed shrew (Fontanillas et al., 2005) and vinegar flies (Camus et al., 2017). Firstly, Fontanillas *et al.* (2005) found differences in the capacity for non-shivering thermogenesis, a trait associated with thermoregulation in hibernating mammals, across two naturally occurring mitochondrial haplotypes of the greater white-toothed shrew, *Crocidura russula*. Intriguingly, their data showed that the difference in trait values across the two haplotypes was depended on the sex, with males showing a greater difference in the trait value compared to females, which the authors reported that it could be a consequence of sexually-antagonistic selection on the mtDNA (Fontanillas et al., 2005).

Secondly, Camus *et al.* (2017) reported latitudinal variation in frequencies of two major mitochondrial haplotypes along the Australian east coast in *D. melanogaster*. One of these haplotypes denoted 'A1', was present at higher frequencies in populations of the northern sub-tropics, while a second haplotype, denoted 'B1' (which encompasses four subhaplotypes they denoted as B1-A to B1-D) were present at higher frequencies in southern temperate regions of east coast Australia. The authors then created genetic strains that differed only in the mtDNA haplotype (A1 and B1 haplotypes) and subsequently investigated the capacity of flies of each strain to tolerate extreme heat

and cold stresses. They confirmed that strains harbouring the A1 haplotype conferred greater resilience to heat stress but less resilience to cold stress, compared to the strains harbouring the B1 haplotype. In this same study, the authors reported that the thermotolerance capacity of one particular B1 sub-haplotype (B1-D) was strongly sex-specific -- this sub-haplotype conferred high relative tolerance to heat stress in females, but low tolerance in males; a finding that reinforced conclusions of Fontanillas *et al.* (2005) and implies that adaptive trajectories of mitochondrial genome evolution under climatic selection might be sexually-antagonistic selection.

Finally, a recent study by Labjner *et al.* (2018) extended the findings of Camus *et al.* (2017), by submitting replicated populations of *D. melanogaster*, carrying each of the A1 and B1 haplotypes, to experimental evolution under different thermal regimes. The authors replicated their selection experiment across mass-bred populations that were either treated or untreated with antibiotics to remove *Wolbachia* infection. In support of the conclusions of Camus *et al.* (2017), the authors reported that the B1 haplotype increased in frequency under colder temperatures, and decreased in frequency under warm conditions, reinforcing the spatial distribution of these haplotypes in nature, where the B1 haplotype is at higher frequencies in cooler temperate latitudes. Intriguingly, however, these evolutionary responses of the mtDNA haplotype to thermal selection were only observed in populations that were uninfected with *Wolbachia* (Lajbner et al., 2018).

1.7 Structure of the thesis

Our understanding of the evolutionary significance of the genetic variation found within the mitochondrion is incomplete. In particular, I have highlighted knowledge gaps that are in need of empirical attention. Firstly, while it is clear that the mitochondrial genetic variation routinely exerts phenotype-modifying effects on a range of life-history traits, and that the magnitude of these effects is commonly higher in males than females, the mechanistic routes through which these genotypic

effects are manifested are less clearly understood. Secondly, it is not clear whether previously-reported patterns of mitochondrial haplotype effects on sex-specific life-history phenotypes are stable across environments, or subject to genotype-by-environment interactions. Furthermore, although evidence exists that non-neutral genetic variation in the mtDNA could have accumulated via adaptive processes and could be maintained in populations via interactions with the environment, the extent to which positive selection could shape the spatial distribution of mtDNA haplotypes, across heterogeneous environments observed in nature, is poorly understood.

These open questions in the field of mitochondrial evolutionary biology have formed the core of my thesis. Through three research chapters, I have sought to probe the mechanistic basis for previously-reported associations between mtDNA haplotype and sex-specific life-history phenotype, to determine whether these links are affected by genotype-by-environmental interactions, and to assess whether the outcomes of these interactions could facilitate the accumulation of adaptive variation in the mitochondrial genome. Below, I provide a brief outline of each of my research chapters.

1.8 Summary of research chapters

Chapter 2: *Sexually antagonistic effects of mitochondrial haplotype on metabolic rate*

While previous studies reporting male biases in the magnitude of mitochondrial genetic effects on life-history trait expression are intriguing and have greatly advanced our understanding of the sex-specificity of mitochondrial genetic effects, the mechanistic route through which the mitochondrial genotype can exert these sex-specific effects remains poorly understood. While it has been proposed that the mitochondrial genetic effects on the expression of nuclear transcriptome, especially on the genes involved in gonadal function in males (Innocenti et al., 2011), and the mitochondrial transcriptome (Camus et al., 2015, Camus et al., 2017) could provide an avenue through which the mitochondrial genotype could regulate the life-history phenotype, our understanding of the

mechanistic basis of the Mother's Curse process is currently limited by a lack of studies to have examined the effect of mitochondrial genetic variation on variation in core physiological traits such as the metabolic rate. Although variation in the metabolic rate is thought to underpin energy allocation across different axes of life-history (Hulbert et al., 2007, Stearns, 1989, Wikelski & Ricklefs, 2001, Wolff et al., 2016b, Zera & Harshman, 2001), it remains unclear whether the pattern or magnitude of mitochondrial genotypic effects on metabolic rate can diverge across the sexes.

Accordingly, in this chapter, I sought to explore the magnitude of mitochondrial genetic effects on the metabolic rate of male and female vinegar flies, *D. melanogaster*, to answer three key questions: 1) do different mtDNA haplotypes affect the metabolic rate, 2) are any such effects male-biased in their magnitude, and 3) does the genetic variation that accumulates in the mitochondrial genome expert pleiotropic effects that link the effects on physiology to effects on life-history, and that can thus provide insights into the proximate basis of Mother's Curse effects?

To achieve my aims, I harnessed a panel of thirteen strains, each of which harboured a distinct and naturally occurring mtDNA haplotype, placed alongside an isogenic nuclear genetic background. I then measured the *in vivo* metabolic rate, as gauged by the amount of CO_2 released by individual males and females of each strain, using a flow-through respirometry setup (LICOR 7000, Sable Systems, Las Vegas, NV, USA). Using this approach, I demonstrate that the genetic variation delineating the mitochondrial lineages of the thirteen strains of vinegar flies affects the expression of metabolic rate in adult flies, with the effects more pronounced in males than females. This finding shows that the Mother's Curse effects have permeated to the level of organismal physiology. Furthermore, while comparing genetic correlations across haplotypes between metabolic rate and life-history trait expression, across each sex, I uncovered a negative mitochondrial genetic correlation between the metabolic rate and longevity of males, and also a negative mitochondrial genetic

correlation for metabolic rate across each of the sexes. These results suggest the mitochondrial genome is involved in the evolution of sex-specific life-history trade-offs and may well be a hotspot for the enrichment of sexually antagonistic fitness variation, which may accumulate as a consequence of the maternal inheritance of the mitochondrial genome.

Chapter 3: ***Interactions between mitochondrial haplotype, dietary macronutrient ratios and sex affect longevity in Drosophila melanogaster***

It has been shown that the longevity outcomes in each of the sexes are affected to some degree by the ratio of macronutrients in the diet (Jensen et al., 2015), and by the genetic variation delineating the mitochondrial lineages of a species (Camus et al., 2012, Dato et al., 2004, Niemi et al., 2003). Yet, it is still less clear whether the expression of genetic variation in mtDNA is sensitive to variation in the dietary macronutrient ratios, and thus whether epistatic interactions between mitochondrial genome and dietary macronutrients manifest as genotype-by-environment (G × E) interactions for longevity; and furthermore, whether any such G × E interactions on longevity are sex-specific.

To date, only two studies have examined the effects of G × E interactions involving the mitochondrial haplotypes and dietary macronutrient ratios (ratios of protein [P] to carbohydrate [C]), on the longevity of both or one of the sexes (Aw et al., 2017, Zhu et al., 2014). Zhu *et al.* (2014) found pervasive effects of dietary macronutrient content and the nuclear background in moderating the link between mitochondrial genotype and phenotype in strains of *Drosophila* possessing different combinations of mito-nuclear genotype. This indicates that the effects associated with genetic variants in the mitochondrial genome hinge on complex intergenomic and G × E interactions. Whether or not, the outcomes of these interactions may be specific to one or other of the sexes remains elusive, however, given that Zhu *et al.* (2014) studied these effects in females only. Recently, using two genetic strains of *D. melanogaster* that differ only in their mtDNA haplotype, Aw *et al.*

(2017) found that the longevity of males, but not females, was sensitive to a mitochondrial G × E interaction involving dietary P:C ratio. Although these findings are based on just two genotypes, they are interesting in light of the core prediction of the Mother's Curse hypothesis, which predicts that the genetic variation that accrues within the mitochondrial genome will be male-biased in its phenotypic effects (Frank & Hurst, 1996).

Here, inspired by the findings of Aw *et al.* (2017) and Zhu *et al.* (2014), I sought to further explore the capacity for interactions between mitochondrial genotype and dietary macronutrient ratios to confer sex differences on longevity. I tested: 1) whether G × E interactions attributable to interactions between mitochondrial haplotypes and dietary P:C ratio affect longevity outcomes in each of the sexes; 2) whether such G × E interactions on longevity are male-biased, as previously reported by Aw *et al.* (2017); and 3) whether levels of mitochondrial genetic variation for longevity in each sex are moderated by the dietary P:C ratio.

Longevity was assayed across a panel of thirteen genetic strains of *D. melanogaster*, each of which differed only in its mtDNA haplotype, in an otherwise standard nuclear genetic background. Adult male and female flies sourced from this mitochondrial panel were placed on a diet containing either a high protein – low carbohydrate content (protein: carbohydrate ratio = 2:1) or a low protein – high carbohydrate content (P:C = 1:8). I found that longevity in each sex was affected by the interactions between mitochondrial genetic variation and dietary P:C ratio. Consistent with the results of Aw *et al.* (2017), male longevity outcomes were more sensitive to G × E effects compared to female outcomes. Furthermore, contrary to prediction, levels of mitochondrial genetic variation for longevity were larger in females than males. We discuss these findings in light of the predictions of Mother's Curse hypothesis.

Chapter 4: ***Mitochondrial haplotypes exert sex-specific effects on the locomotory activity of Drosophila melanogaster, independent of the thermal environment***

Empirical data linking patterns of mitochondrial sequence variation and climatic zones have recently emerged, consistent with an evolutionary hypothesis that has been named the "Mitochondrial Climatic Adaptation" hypothesis (Balloux et al., 2009, Camus et al., 2017, Mishmar et al., 2003, Ruiz-Pesini et al., 2004). The majority of evidence for this hypothesis is based on correlations between mutational patterns in the mtDNA sequence across climatic zones in various species of animals. However, it is unclear whether such patterns have been driven by climatic selection on segregating variation in the mitochondrial genome, or via non-adaptive processes, such as demographic effects or founder effects (Foote et al., 2011, Mishmar et al., 2003, Morales et al., 2015, Ruiz-Pesini et al., 2004). Recently, two studies have taken an experimental approach to test the Mitochondrial Climatic Adaptation hypothesis (Camus et al., 2017, Lajbner et al., 2018), leveraging the mitochondrial haplotype variation that exists in populations of vinegar flies, *D. melanogaster*, along with the east coast of Australia.

Together, these studies have demonstrated that the Australian population of *D. melanogaster* consists of two main haplotypes, denoted A1 and B1 and that these exhibit latitudinal clines in their population frequencies. A1 is more predominant in subtropical low latitude populations and B1 in temperate high latitude populations. Camus *et al.* (2017) created genetic strains of flies, in which they placed each of these haplotypes alongside an isogenic nuclear background and used these strains to measure the thermotolerance capacity associated with each haplotype. Consistent with their spatial distributions along the Australian east coast, A1 haplotype was indeed associated with the heightened capacity to tolerate extreme heat stress, and diminished capacity to tolerate cold stress, relative to the B1 haplotype. Lajbner *et al.* (2018) then showed that, at least in experimental populations that are

free of *Wolbachia* infection, the B1 haplotype is able to outcompete the A1 haplotype when evolving under cool thermal conditions but is outcompeted under warm conditions. These studies provide direct experimental evidence for the Mitochondrial Climatic Adaptation hypothesis.

Yet, while evidence is emerging in support of this hypothesis, the mechanisms by which climatic selection may target polymorphisms within the mtDNA sequence remain poorly understood, especially in connection to the type of life-history and physiological traits that might be direct targets of variation in the thermal climate. Inspired by recent support for the Mitochondrial Climatic Adaptation hypothesis, here I examine: whether locomotory activity – a trait at the interface between organismal physiology and life-history – is shaped by interactions between the mitochondrial haplotype and thermal environment; whether any such mitochondrial genotypic effects or G × E interactions differ in magnitude across the sexes; and whether the A1 haplotype, which previous studies suggest is a warm-climate adapted haplotype (Camus et al. 2017, Lajbner et al. 2018) is able to maintain higher activity than its B1 counterparts in the face of heightened thermal conditions. We used a panel of five mitochondrial haplotypes that differed only in their mitochondrial genetic variation but harness the same isogenic nuclear background, similar to the panel used by Camus *et al*. (2017), to assay the locomotory activity of adult flies of each sex, across a thermal gradient. We found that the mtDNA haplotype affects the locomotory activity of adult vinegar flies and that the magnitude of mitochondrial effects was stronger in females than males. Contrary to predictions, we did not find a significant interaction between mtDNA haplotype and temperature on the locomotory activity of flies, suggesting that mitochondrial genotypic effects on locomotion in this species are not shaped by thermal selection.

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Chapter 2

Sexually antagonistic effects of mitochondrial haplotype on metabolic rate

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2.1 Abstract

The *Mother's Curse* hypothesis predicts that maternal inheritance of mitochondria will facilitate the accumulation of mitochondrial DNA (mtDNA) mutations that are male-biased in their effects. This prediction was supported by reports of male-biases in levels of mitochondrial genetic variation underpinning life-history traits, such as longevity. However, it is unclear whether Mother's Curse effects could permeate to the level of organismal physiology, resulting in sex differences in the metabolic rate. Such an effect would be striking, given the metabolic rate is often assumed to lie at the heart of investment trade-offs into upstream life-history functions. Here, we report male-biases in mitochondrial genetic variation for metabolic rate across a replicated panel of genetic strains of *Drosophila melanogaster*, which harbour unique mtDNA haplotypes in an otherwise standardised nuclear genetic background. The effect of mtDNA haplotypes on metabolic rate is sexually antagonistic; haplotypes that confer high trait values in females, confer low values in males. Finally, we explore mitochondrial genetic correlations involving metabolic rate, body size and other physiological and life-history traits in each sex, revealing a negative correlation across haplotypes between metabolic rate and longevity, found only in males. These results indicate that maternal mitochondrial inheritance enables the accumulation of sex-specific, even sexually antagonistic, variation in the mitochondrial genome, with pervasive consequences for the evolution of sex differences and life-history trade-offs.

Keywords: antagonistic pleiotropy, circadian rhythm, CO_2 production, flow-through respirometry, life-history theory, mutation-selection balance, sex-specific selective sieve

2.2 Introduction

Mitochondria are involved in the production of the majority of Adenosine Triphosphate in eukaryotes, through oxidative phosphorylation (OXPHOS). OXPHOS takes place in the electron transport system, which is comprised of five multi-subunit enzyme complexes that depend on the concerted action of polypeptides encoded by the genes spanning both mitochondrial and nuclear genomes (McKenzie et al., 2007, Wolff et al., 2014a). Given the mitochondrial genome encodes core OXPHOS genes, and given the products of these genes are essential for cellular metabolic function, it was traditionally assumed that the mitochondrial DNA (mtDNA) sequence would evolve under strong purifying selection, thus preventing the accumulation of non-neutral mutations (i.e., phenotype-changing genetic polymorphisms) within mitochondrial genomes (Dowling et al., 2008). However, empirical evidence has challenged this assumption of neutrality of mitochondrial sequence variation (Ballard & Kreitman, 1995, Ballard & Whitlock, 2004, Rand, 2001). For example, studies that used experimental designs that can partition mitochondrial from nuclear genetic contributions to phenotypic expression, have shown that mtDNA haplotypes routinely harbour polymorphisms that affect the expression of organismal physiological and life-history traits (Dowling et al., 2009, Dowling et al., 2010, Immonen et al., 2016, Lovlie et al., 2014).

Furthermore, several studies have also observed that patterns of mitochondrial genetic variance underpinning phenotypic trait expression are often sex-specific, with the general pattern being one of male bias (Camus et al., 2012, Innocenti et al., 2011, Vaught & Dowling, 2018). Observations of male-biases in the expression of mitochondrial genetic variance are intriguing because such results are consistent with an evolutionary hypothesis known as *Mother's Curse* (Gemmell et al., 2004b). The Mother's Curse hypothesis predicts that maternal inheritance of the mitochondria will render natural selection effective in shaping the mtDNA sequence only when carried by females (Cosmides & Tooby, 1981, Frank & Hurst, 1996b). Consequently, mtDNA mutations that are neutral, or slightly

deleterious to females may accumulate within the mitochondrial genomes of natural populations, even if the same mutations are explicitly harmful to males (Frank & Hurst, 1996b). Furthermore, if mtDNA mutations arise that are overtly sexually antagonistic, benefitting females but harming males, then such mutations should be favoured under positive selection (Beekman et al., 2014, Camus & Dowling, 2018, Innocenti et al., 2011, Unckless & Herren, 2009). While Mother's Curse effects are common in plants, via cases of mtDNA mutations conferring Cytoplasmic Male Sterility (Chase, 2007), their possible relevance to bilaterian metazoans, which harbour diminutive and vastly streamlined mitochondrial genomes have been identified in vinegar flies, mice, hares and humans, where mtDNA mutations confer male-specific fitness effects on fertility (Clancy et al., 2011, Holyoake et al., 1999, Milot et al., 2017, Nakada et al., 2006, Patel et al., 2016, Smith et al., 2010, Xu, 2008).

Much of the previous work examining patterns of mitochondrial genetic variation underpinning phenotypic expression has focused on traits pivotal to organismal life-histories; reproductive outcomes and longevity (Dowling et al., 2010, James & Ballard, 2003, Maklakov et al., 2006, Rand et al., 2006, Santoro et al., 2006, Vaught & Dowling, 2018, Zhu et al., 2015). Furthermore, much of the evidence to date for Mother's Curse effects on life-history comes from the study of the one panel of *D. melanogaster* strains, involving mitochondrial haplotypes sampled from diverse geographic locations spanning the global distribution of *D. melanogaster*. Using this panel, Camus *et al.* (2012) identified strong male-biases in mitochondrial genotypic effects on longevity, and Camus *et al.* (2018) uncovered signatures of sexual antagonism on reproductive outcomes; the haplotypes that conferred highest reproductive outcomes in females typically conferred the lowest outcomes in males. Such signatures of inter-sexual pleiotropy, tractable to genetic variants in the mitochondrial genome, have been substantiated by other examples, and are noteworthy because they suggest mitochondrial genetic involvement in the dynamics of evolutionary conflict between the sexes and

the expression of genetic trade-offs between life-history traits. For example, one particular non-synonymous mutation that lies in the Cytochrome B gene of the mitochondrial DNA of *D. melanogaster*, has been associated with infertility but high longevity in males; high fertility, but low longevity in females, and high juvenile viability (Camus & Dowling, 2018, Camus et al., 2015, Clancy et al., 2011, Dowling et al., 2015, Wolff et al., 2016c). Furthermore, haplotypes bearing this mutation appear to have a competitive advantage, and increase in frequency in populations under positive selection, despite the associated negative effects on male fertility (Wolff et al., 2017).

The aforementioned examples raise the question of how genetic variation in the streamlined mitochondrial genome, which is responsible for encoding some of life's most important products, can exert sex-specificity, and mediate patterns of inter- and intra-sexual pleiotropy, at the level of life-history. In this regard, our understanding of the proximate basis of Mother's Curse effects remains rudimentary. While emerging studies suggest the link between the mitochondrial genotype and sex-specificity of the life-history phenotype is regulated, at least in part, through modifications to patterns of gene expression both within the mitochondrial (Camus et al., 2015, Camus et al., 2017) and nuclear transcriptomes (Innocenti et al., 2011), what remains less understood is whether these sex-specific mitochondrial genetic effects permeate to the level of core organismal physiology. Traits such as the metabolic rate are often assumed to underpin the pace-of-life, determining allocation patterns into different axes of life-history function, and thus are often presumed to sit at the heart of life-history trade-offs (Hulbert et al., 2007, Zera & Harshman, 2001). Thus, if sex-specific genetic variation routinely accrued in the mitochondrial genome affect core metabolic phenotypes, these effects could plausibly resonate across the entire organismal life-history, with mitochondrial genetic variation potentially modulating trade-offs among a manifold array of life-history traits in a sex-specific manner.

Evidence for this contention, however, remains limited. Indeed, most previous studies to examine the capacity for mitochondrial genetic regulation of physiology have examined only one or other of the sexes, or pooled both sexes together in their analysis (Arnqvist et al., 2010, Correa et al., 2012, Hoekstra et al., 2013, Immonen et al., 2016, Pichaud et al., 2012), precluding inferences of sex-specificity. In one recent study, Wolff *et al.* (2016b) observed male-biases in levels of mitochondrial genetic variation for mitochondrial quantity, across the same panel of *D. melanogaster* used by Camus et al. (2012) and Camus and Dowling (2018). These effects were, however, only observed in young, not old flies, and the authors did not detect clear signatures of male-bias in mitochondrial genetic effects on the respiratory rate of the individual OXPHOS complexes. In another study of a different species of Drosophila, *D. subobscura*, Novičić *et al.* (2015) reported that as much as 20% of the variation in whole-organism metabolic rate (gauged as CO_2 production), across adult *Drosophila subobscura* could be mapped to variation across mtDNA haplotypes. Yet, although these mitochondrial genetic effects exhibited some degree of sex-specificity, the general pattern was not one of clear male-bias. Furthermore, only three haplotypes were examined, and thus the inferences are sensitive to sampling error (Novicic et al., 2015).

Thus, currently, it remains unclear whether mitochondrial genetic variation affects the expression of physiological traits in a male-specific pattern similar to the previously reported effects for longevity and reproductive success; and if so, whether such mitochondrial effects on key physiological traits are involved in sex-specific trade-offs with life-history phenotypes. To address this question, we screened for effects of mitochondrial genetic variation on the metabolic rate (volume of CO_2 produced per fly) of each sex, across a panel of genetic strains in *D. melanogaster*, which differ only in their mtDNA haplotype and which has been previously used to study sex-specific patterns of mitochondrial variation mediating the expression of life-history phenotypes (Camus et al., 2012, Camus & Dowling, 2018). We specifically tested whether the level of mitochondrial genetic variation

in the metabolic rate is larger in males, compared to females. Such data would provide evidence for male-biased mitochondrial genetic effects on the metabolic rate of fruit flies. We then leveraged data from other studies to have utilised this same panel of strains, to test whether mitochondrial variation for metabolic rate is involved in a trade-off between physiology and longevity, and more broadly whether mitochondrial genetic effects on other traits such as body size and reproductive success exhibit signatures of pleiotropy (i.e. with effects on one trait linked to effects on another) – specifically testing the direction and strength of the mitochondrial genetic correlations and determining whether any such correlations are sex-specific. .

2.3 Methods

Mitochondrial panel

We utilised a panel of thirteen strains of *D. melanogaster*, each of which is characterised by a distinct and naturally occurring mtDNA haplotype, placed alongside an isogenic nuclear background w^{1118} (Bloomington stock number: 5905) (Clancy, 2008, Camus et al., 2012). The strains are labelled according to the location from which the mtDNA haplotypes were initially collected (**ALS** - Alstonville, Australia; **BAR** - Barcelona, Spain; **BRO** - Brownsville, USA; **DAH** - Dahomey, Benin, **MAD** - Madang, Papua New Guinea; **MYS** - Mysore, India; **HAW** - Hawai'i, USA, **ISR** - Israel; **JAP** - Japan; **ORE** - Oregon, USA; **PUE** - Puerto Montt, Chile; **SWE** - Sweden and **ZIM** - Zimbabwe) (Camus et al., 2012). The strains were obtained from David Clancy in 2007, at which point we created a duplicate copy of each strain, such that each haplotype has been maintained in independent replicate for over a decade. These replicates are denoted as "mitochondrial strain duplicates". During this time, the strain duplicates were maintained by back-crossing five virgin females from each duplicate to five males of the w^{1118} strain. The w^{1118} strain was itself propagated each generation via a solitary full-sibling mating pair. Thus, any new mutations that appeared in the w^{1118} strain were quickly purged, or if fixed would be immediately donated to each of the

mitochondrial strain duplicates, thereby ensuring the nuclear background of these strains was maintained as strictly isogenic.

Each of the mitochondrial strains and their respective duplicates had undergone at least 80 generations of backcrossing at the time of the respirometry experiments described below. Back-crosses were always conducted at low adult densities (5 pairs), and only eggs produced by parents that were four days old at the time of egg-laying, were used to propagate the next generation. All strains were treated with tetracycline hydrochloride (0.3 mg/mL) to eliminate *Wolbachia* infections before their receipt from David Clancy in 2007. Also, the absence of *Wolbachia* across the strain duplicates has been confirmed on numerous occasions using diagnostic PCR, most recently in September 2017 with PCR primers specific for the *Wolbachia CoxA* gene (Simoes et al., 2011, Baldo et al., 2006). We further confirmed the absence of *Wolbachia* by searching the Illumina short-reads data derived from all the strains for the presence of *Wolbachia* sequence reads from Wolff *et al.* (Wolff et al., 2016a) in Geneious v9.0.4 (Kearse et al., 2012).

Experimental design

The experiment was designed to assay the *in vivo* metabolic rate (VCO_2 production) of individual adult flies of each sex across the panel of thirteen mtDNA haplotypes. The experiment was conducted over three temporally-separated "sampling blocks" (i.e. the experiment was run three times over, across three subsequent generations of flies).

Generating focal flies

To control for non-genetic sources of variation, we ensured that all focal flies (i.e., flies that were assayed for metabolic rate) were created under highly standardised conditions. Specifically, all focal flies were produced by parents that were four days of adult age at the time at which they laid the

eggs. Similarly, the focal flies were descended from grandparents that were also four days of age at the time of ovipositioning. In the two generations leading up to the assay, all flies were reared under carefully controlled densities (each vial propagated by ten pairs of adult flies over 24 h, and egg numbers per vial reduced to 80), at constant laboratory conditions (25°C). We ensured we had a steady daily supply of standard-aged focal flies for the VCO_2 measurements, by allowing the great-grandparents of the focal flies to lay eggs that produced the grandparental flies over several successive days (five days in sampling blocks one and two, and eight days in block three). Thus, although all focal flies had parents and grandparents of standard age, they had been produced by great-grandparents that differed in age by up to seven days.

Metabolic rate assay

The focal flies were collected under mild CO_2 anesthesia within six hours of their eclosion into adulthood, thus ensuring their virginity, and were then housed in single-sex groups of ten flies per vial. These flies remained in these vials for four days before measurement of their metabolic rate. For any given sampling day, we maintained one vial of ten focal flies per strain duplicate per sex. The use of virgin flies removed any physiological effects on metabolic rate caused by mating *per se* and post-mating inter-sexual harassment. Additionally, the four-day recovery period following CO_2 anesthesia ensured that the impact of CO_2 anesthesia on the metabolic rate had dissipated by the time of the assay (Colinet & Renault, 2012).

A standard Sable Systems International (SSI, www.sablesys.com, Las Vegas, USA) flow-through CO_2 respirometry system connected to four LI-COR 7000 infrared CO_2/H_2O gas analysers (LICOR, Lincoln, USA), was used to measure carbon dioxide production as a proxy of metabolic rate (VCO_2) of adult flies. Two identical setups were created, each underpinned by two LI-COR 7000s (SSI, www.sablesys.com, Las Vegas, NV, USA). For each configuration, compressed air was directed

through Bev-A-Line tubing to three scrubber columns (silica gel, soda lime, 1/3 Drierite 2/3 soda lime respectively), where the air was scrubbed of atmospheric CO_2 and water vapour (H_2O) to facilitate a dry, CO_2 free-flow. The airstream was then split using a PVC T-piece to direct the flow to one of two LI-CORs in the set-up, with a flow rate of 25ml/min using a mass flow controller (Sierra 840 series). Each LI-COR was connected to a MUX2 intelligent multiplexer (Sable Systems), which housed eight 5×65 mm^2 polycarbonate chambers (Trikinetics, Waltham, USA). We placed one focal fly within each chamber, the ends of which were sealed with 5 mm of foam, such that each fly was left with a 5×55 mm^2 maneuverable space. Seven of the chambers contained flies while the eighth chamber remained empty and served as a baseline to account for machine drift throughout the experiment.

The MUX2 was interfaced with a computer using a UI-2 universal interface (Sable Systems, NV, USA) and was programmed to sequentially measure each chamber using the software Expedata (Sable Systems). Each chamber was measured once for 10 minutes, with a two-minute pause period between every measurement to allow time for the CO_2 readings to stabilise. The assaying chambers were flushed with a humidified air flow (80% RH) in the pause-period of 2-min between VCO_2 measurements, to reduce potential detrimental effects of desiccation. This was achieved using a LICOR-610 portable dew point generator. The assay was conducted within a temperature- and light-controlled constant temperature cabinet (Panasonic MLR-352H-PE environmental growth cabinet, Panasonic Healthcare Co., Ltd, Sakata, Japan). The temperature of the cabinet was set to 25°C and was continuously recorded in the baseline chamber using a type-T thermocouple (Omega Engineering Inc., Stamford, USA) attached to a TC-2000 thermocouple meter (Sable Systems).

The respirometry assays were run over five consecutive days in block one and two, and over eight consecutive days in block three. We ran four "experimental trials" per day at approximately 0900 h;

1130 h; 1400 h and 1630 h. These four time-trials were included as a fixed effect in the statistical model to account for circadian effects on the metabolic rate. We were able to assay 26 flies per experimental trial, and we ensured a balanced design, with every possible combination of mitochondrial strain duplicate × sex represented once per trial. In total, we measured the metabolic rate of 72 focal flies for each combination of mitochondrial strain × sex (36 per strain duplicate), over the three blocks.

The mean metabolic rate data from the 10-min assay for each fly was extracted using the Expedata software (Sable Systems). Firstly, all data were "nearest-neighbour smoothed" to remove noise from the VCO_2 trace, and baseline corrected to account for machine drift over time (Rezende et al., 2005). Following this, the mean VCO_2 values were extracted for each fly over the 10-minute assay period using a macro in the Expedata software. Secondly, we used a different macro in Expedata to extract activity intensity of each fly from the VCO_2 trace file. Here, the activity intensity (that is, intensity of activity of the focal fly while the CO_2 is flushed in the assay chamber) was measured as the cumulative sum of absolute differences in deflection (ADS) of VCO_2 signal (Jensen et al., 2014, Lighton & Turner, 2004, Stevens et al., 2010). In essence, the ADS was calculated by adding the absolute differences between adjacent data points in the VCO_2 trace file (DeVries et al., 2016, Klok et al., 2010). Although ADS is not an absolute quantification of locomotor activity (Jensen et al., 2014), the measure has been used across eco-physiological studies to correct for overall variability in the metabolic rate due to the deflection in the activity intensity of the assayed organism (Jensen et al., 2014, Klok et al., 2010, Lighton & Turner, 2004, Lighton et al., 1993, Stevens et al., 2010, Vorhees & Bradley, 2012). Thus, from the VCO_2 traces, high ADS values were indicative of flies being more active during the assay; and *vice versa*, small values of ADS indicative of the flies being less active. The ADS was extracted for each focal fly, and this served as a measure of activity intensity in the subsequent statistical analysis.

Finally, to account for metabolic rate variation attributable to body mass of the fly, we measured the body mass of each focal fly immediately after the metabolic rate assay. We transferred each focal fly into a labelled microcentrifuge tube and stored the tubes in a freezer at -20°C for 45 min. We then placed the focal fly on a microbalance to measure its mass to the resolution of nearest 0.0001 mg (Cubis series MSA2.7s-000-DM microbalance, Sartorius AG, Goettingen, Germany).

Statistical analyses

Linear mixed effect modelling of the full dataset

All analyses were performed in the R statistical environment (v3.4.4) (R Development Core Team, 2013). The metabolic rate data from individual focal flies were analysed within a linear mixed-effect regression (*lmer*) framework in the *lme4* package (Bates et al., 2015). We used the mean metabolic rate of each focal fly as the response variable, with sex (2 levels), time of day of the assay (4 levels), and the interaction between these factors as fixed effects in the statistical model. To estimate variation in the metabolic rate accountable to the genetic variation existing across the thirteen haplotypes, we modelled the mtDNA haplotype (13 levels) as a random factor. Other variables that accounted for the hierarchical structure of the dataset were also included as random factors. These included the mitochondrial strain duplicates (13 strains × 2 replicates = 26 levels), experimental blocks (3 blocks = 3 levels), assay-day (8 levels; note that this variable was also an indicator of the great grandparental age), assay-day nested within experimental block (18 levels) and experimental trial nested within assay-day and block (70 levels). All possible higher-order interactions among random factors, and between random and fixed factors were modelled as random effects in the model.

We used centre-scaled body mass and ADS of the individual fly as fixed covariates in the same model given that they were not collinear with each other. This non-collinearity was confirmed by

determining the variance inflation factor (vif function in *car* package) of statistical models in which both ADS and body mass were included in the same model and comparing this to models containing just one of the covariates (vif value was 1.02, which is less than the threshold for collinearity of 10). A full model was built with all the fixed effects, higher-order interactions between fixed effects, random effects, higher-order interactions between random effects, higher-order interactions between fixed and random factors as random effects, body mass and ADS as covariates.

We then derived a final reduced model by performing a step-wise model reduction process. First, we progressively eliminated higher-order interactions involving random effects that accounted for negligible variance in the metabolic rate. The variance accountable to each random effect in the full model was estimated from the *summary* function in the *lme4* package. We assessed each progressively simplified model to the previous model, using *log-likelihood ratio* (LLR) tests, and Maximum Likelihood estimation. If the LLR test returned a non-significant p-value ($p>0.05$), we removed the higher-order random effect from the model and proceeded to remove the next higher-order random effect which contributed the least variance to the data. In this way, we derived a final model with the reduced set of random factors, and interactions involving random factors, that explained non-zero variance in the mean metabolic rate of the flies.

Furthermore, we followed the same LLR-test approach to remove or retain non-significant interactions involving the two fixed factors in the full model. We estimated the variance attributable to each random effect in the final model, using restricted maximum likelihood estimation in the *lme4* package. Parameter values of fixed effects and their significance were estimated in the final model, using Type III Wald's Chi-square tests, using the *car* package (Fox, 2011).

Linear mixed effect model for testing mtDNA haplotype effects in sex-specific datasets

The magnitude of the relationship between body mass and metabolic rate was sex-specific (Pearson's correlation coefficient: $\rho_{male} = 0.18$, $p=0.057$; $\rho_{female} = 0.41$, $p<0.0001$). Given that females were on average 30% heavier than males, body mass and sex were clearly not independent of each other, and thus it was difficult to partition the effects of body mass from those of sex in the previously explained full model. Thus, in a second step, we analysed the metabolic rate data of each sex separately, to ensure that any effects identified in the full dataset were upheld in sex-specific analyses. Furthermore, by analysing the data separately for each sex, we could further tease apart and scrutinise for levels of sex-specific mitochondrial genetic variance for metabolic rate.

To do this, we built separate *lmer* models for each sex with precisely the same designation of fixed and random effects as described above, except 'sex' not being included in the models. We took the same previously explained step-wise elimination approach to retain or reduce non-significant random effects that explained the least variance in metabolic rate, from the model and derived a reduced model using the LLR test. Also, we confirmed the significance of mtDNA genetic effects on metabolic rate through the LLR test in each of the sex-specific models. To do this, we compared a model in which mtDNA haplotype was removed from the random effects, with the model that retained mtDNA haplotype using the LLR test. Furthermore, the variance attributable to each random effect and their higher order interactions were estimated using the restricted maximum likelihood estimation from each of the sex-specific final models. The significance of the fixed effect, such as time of the assay and covariates, such as ADS and body mass, were estimated from the Type III Wald's *Chi*-squared test. Additionally, the 95% confidence intervals for variance attributed to the mtDNA haplotype was calculated from the final model. The R codes for estimating CIs of variance is provided in the **supplementary information**.

Least-squared mean metabolic rate adjusted for body mass and ADS in each sex

We calculated the least-squared mean (LSmeans) for metabolic rate, adjusted for body mass and ADS of the flies, for each of the thirteen mtDNA haplotypes, separately for each sex, using the *lsmeans* package (Lenth, 2016). Here, we built a linear model for each sex separately with factors, such as mtDNA haplotype, time of day of the assay, and covariates, such as body mass and ADS. We followed the methods outlined in the *lsmeans* package and accordingly stored the output of the linear models into reference grids using the *ref.grid* function. These reference grids contain the information required for calculating least-square means for all specified independent factors. The LSmeans metabolic rate for each mtDNA haplotype was estimated from these reference grids, separately for each sex.

Likewise, we calculated ADS adjusted mean metabolic rate from sex-specific linear models in the *lsmeans* package, by retaining ADS as the only covariate in the model. This specific ADS-adjusted LSmeans of metabolic rate was used in our correlation analyses, explained below, to deduce the correlation between body mass and ADS-adjusted metabolic rate.

Estimating sex-specific variance in mean metabolic rate attributable to mtDNA haplotypes

From the sex-specific *lmer* analysis mentioned above, we estimated that variance in metabolic rate attributable to the random effect term 'mtDNA haplotype' was greater than zero in males but equal to zero in females. We further scrutinised the levels of mitochondrial genetic variation for the mean metabolic rate in each sex, over two further analyses that incorporate a bootstrapping approach.

To determine whether mtDNA haplotype contributed significant variance to the metabolic rate in each sex, we analysed a sex-specific *lmer* model with mtDNA haplotype as the only random effect term. We built *lmer* models with one random effect per model, separately for each sex and analysed

the models over 10000 simulations using the exact Restricted Likelihood Ratio Test (*exactRLRT*) function in *RLRsim* package (Scheipl et al., 2008). The *exactRLRT* function tests whether the variance attributable to a random effect term (that is, mtDNA haplotype in this case) is either zero or greater than zero. Refer to the **supplementary information** for R codes to run the *exactRLRT* test in R.

Furthermore, we estimated the median and 95% confidence intervals for point variance in the mean metabolic rate of each sex through a parametric bootstrapping approach in R. Using the sex-specific datasets, we built separate linear mixed effect (*lmer*) statistical models for each sex by including mtDNA haplotype as the only random factor in the model. We then bootstrapped this single-factor model (1 ~ (1|mtDNA_haplotype) over 10000 iterations and estimated median point variance along with the 95% confidence interval for the median variance. The R codes to run this parametric bootstrapping is explained in the **supplementary information.**

Genetic correlations between traits

The full panel of thirteen mtDNA haplotypes used in this study has also been used in three earlier studies that have explored mitochondrial genetic effects on longevity in both sexes (Camus et al., 2012), components of reproductive fitness in both sexes (Camus & Dowling, 2018), and mitochondrial respiration and mitochondrial quantity in both sexes from young and old aged flies (Wolff et al., 2016b). We obtained haplotype-specific trait means for each trait from all three earlier studies, and the trait means for metabolic rate and body mass from our study. We then tested for correlations between all possible pair-wise combinations of traits within and across the two sexes.

We estimated the Pearson's correlation coefficient and 95% confidence interval for the correlation coefficient independently for each pairwise comparison of trait means, through a non-parametric

bootstrapping approach in *boot* package (Davison, 1997) in R. Here, given that not all the sampled trait values adhered to a Gaussian distribution, and given that these trait means were extracted from a sample of mtDNA haplotypes that provided a broad representation of global levels of mitochondrial haplotype variation in the species (Wolff et al., 2016a), such bootstrapping provided an appropriate means to analyse correlations between the trait means. All correlation analyses included the trait means of all thirteen haplotypes, except for mitochondrial metabolic traits, which lacked data on the Zimbabwe mtDNA haplotype (Wolff et al., 2016b). In case of the male reproductive functions, we included the reproductive output of males from Brownsville haplotype as zero, as the males were completely sterile (Camus & Dowling, 2018, Clancy et al., 2011). In each correlation test, trait means were resampled with replacement across 10000 replicates and the confidence intervals of the correlation coefficient was estimated from the bias corrected and accelerated (BC_a) method in the *boot* package. The R codes for running the bootstrapped correlation test are provided in the **supplementary information**.

2.4 Results

Mitochondrial genetic variation for the metabolic rate is male-biased

There was an interaction between mtDNA haplotype and sex on the metabolic rate (*Log-likelihood ratio* test, $\chi^2 = 4.6616$, $p = 0.0308$, Table 1). This means that levels of genetic variance for the metabolic rate, across mitochondrial haplotypes, differed across the sexes (Figure 1). We confirmed this pattern of variation in metabolic rate to be male-biased, by partitioning the dataset separately for each sex, and applying a pipeline of further analyses outlined in the methods. First, the variance in metabolic rate attributable to the mtDNA haplotype was greater than zero in males, but not in females (Table 2; variance in metabolic rate attributable to the mtDNA haplotypes in males = 0.00253, bootstrapped 95% confidence intervals = (0.0002, 0.0101), and females = 0, bootstrapped 95% confidence interval = (-0.001, 0). Second, the RLRT tests confirmed the mitochondrial genetic

variance for metabolic rate was statistically significant in males (RLRT score = 4.0464, p = 0.0172), but not in females (RLRT = 1.4858, p = 0.0903). Third, the parametric bootstrapping analysis estimated the median point variance of the metabolic rate of males as approximately two-fold larger than females; albeit the confidence intervals associated with the male median overlapped with those of the female median [bootstrapped variance in males = 0.0297 (0, 0.0547) and females = 0.0145 (0, 0.0292)].

The metabolic rate of males is sensitive to diurnal effects

There was a significant interaction between sex and time of day of the assay on the metabolic rate of the fruit flies (*lmer* analysis: $\chi^2 = 38.0679$, $p < 0.0001$, Table 1). Specifically, the metabolic rate of males declined throughout the day but remained stable in females (effect of time of day on metabolic rate: Table 2a males, $\chi^2 = 43.472$, $p<0.0001$; and Table 2b females, $\chi^2 = 3.058$, $p = 0.3827$). This diurnal variation in male metabolic rate resulted in a sign shift in the direction of sexual dimorphism for this trait between morning (being male-biased) and afternoon (becoming female-biased) measurements (Figure 2).

Mitochondrial genetic correlations involving metabolic and life-history traits

We found signatures of intra- and inter-sexual pleiotropy, across mitochondrial haplotypes, underpinning the expression of metabolic rate and some life-history traits (Figure 3). The inter-sexual mitochondrial genetic correlation for metabolic rate (adjusted for body mass and ADS) was strongly negative (Pearson's correlation coefficient (r_p) = -0.65, bootstrapped 95% confidence intervals (95% CI) = -0.859, -0.348; Figure 4A), while the inter-sexual correlation for body mass was strongly positive (Figure 4B). Also, we found evidence for negative pleiotropy between male metabolic rate (adjusted for body mass and ADS) and a trait that is typically associated with a measure of juvenile fitness in insects, egg-to-adult viability (Figure 4C).

Furthermore, we observed several intra-sexual correlations involving metabolic and life-history traits. These correlations were striking in their sex-specificity (Figures 5 and 6). In males, the mitochondrial genetic correlation for metabolic rate (adjusted for body mass and ADS) and longevity was negative (Figure 7A), but positive for body mass and longevity (Figure 7B). In contrast, there was no mitochondrial genetic correlation between metabolic rate and longevity in females (Figure S1), and the correlation between body mass and longevity was negative (Figure 8A), which is the opposite sign to that observed in males. The genetic correlation between body mass and the absolute metabolic rate (uncorrected for ADS or body mass) was positive only in females (Figure 8B). The mitochondrial genetic correlation between metabolic rate (adjusted for body mass and ADS) and mitochondrial quantity of young flies was negative in females (Figure 8C). Also, the female reproductive traits showed signatures of positive genetic correlation with body mass (Figure 6).

2.5 Discussion

The goals of this study were to investigate whether genetic variation harboured within the mitochondrial genome contributes to the expression of the metabolic rate, determine whether any such effects are sex-biased, and to explore whether the genetic variation found across natural mtDNA haplotypes contributes to the dynamics of life-history trade-offs. We uncovered strong male-biases in levels of genetic variation across mitochondrial haplotypes for the metabolic rate, a result consistent with the key prediction of the Mother's Curse hypothesis (Frank & Hurst, 1996b). Furthermore, we uncovered a clear signature of sexual antagonism in effects of mitochondrial haplotypes on the metabolic rate; mitochondrial haplotypes that conferred the greatest metabolic rate in females are the haplotypes that conferred the lowest metabolic rate in males. Our results suggest that maternal inheritance of the mitochondrial genome has consequences for the evolution of sex differences in life-history; facilitating the accumulation of sexually antagonistic genetic variation for

the metabolic rate, with effects that resonate to the level of organismal life-history and that contribute to sex differences in the expression of trade-offs between core physiological and life-history traits.

Previous studies that utilised the same panel of strains of *D. melanogaster* used here, identified male-biases in the effects of mitochondrial genetic variation on longevity (Camus et al., 2012), and on the mitochondrial quantity of flies of young age (Wolff et al., 2016b). A third study used a subset of five of these strains and demonstrated that variation across these haplotypes had manifold effects on patterns of gene expression across the nuclear transcriptome of males, but virtually no effects on gene expression within the female transcriptome (Innocenti et al., 2011). Furthermore, in that study, approximately one-third of the differentially expressed nuclear genes in males were localised in expression to the male reproductive tissues and involved in encoding male reproductive phenotypes (Innocenti et al., 2011). Finally, a fourth study that utilised the full panel of thirteen strains to examine mitochondrial genetic effects on a range of reproductive traits in each of the sexes, uncovered a signature of intersexual negative pleiotropy in the effects of the mitochondrial haplotypes on the reproductive traits (Camus & Dowling, 2018). In combination, the aforementioned studies on this panel have provided some of the strongest support for the Mother's Curse hypothesis; a hypothesis that predicts that male expression-specific polymorphisms will accumulate within the mitochondrial genome, given the sex-specific selective sieve invoked by maternal inheritance of the genome (Frank & Hurst, 1996b, Gemmell et al., 2004b).

However, the mechanistic underpinnings of the mitochondrial genetic effects on life-history phenotypes have to date remained incompletely investigated, and poorly understood. Previously, studies investigating effects of mitochondrial haplotype variation on physiological traits have generally not studied both of the sexes (Hoekstra et al., 2013, Immonen et al., 2016), with only one exception (Novicic et al., 2015). Thus, our findings that male-biases in mitochondrial genetic

variation extend to the metabolic rate and are involved in the expression of sex-specific life-history trade-offs, shed new light on the candidate mechanisms that could link the mitochondrial genotype to the life-history phenotype. Key hypotheses that extend from life-history theory often centre on the prediction that metabolic rate is the major currency on which the life-history trait expression depends (Dowling & Simmons, 2009, Sheldon & Verhulst, 1996, Stearns, 1989, Zera & Harshman, 2001). Given the products of the mitochondrial genome are all involved in encoding core components of OXPHOS respiration, metabolic rate, therefore, stands at the nexus between mitochondrial function and life-history function (Rand, 1994, Wolff et al., 2016b). In this context, our results of male-biases and signatures of sexual antagonism in linking the mitochondrial genotype to phenotype are striking, because they indicate that the Mother's Curse effect permeates to the level of core organismal physiology, and suggest that these mitochondrial effects on the metabolic rate are likely to drive sex differences in a broad range of upstream components of life history, such as the previously-reported male-biased mitochondrial effects on longevity (Camus et al., 2012), and sexual antagonism across mtDNA haplotypes for reproductive outcomes (Camus & Dowling, 2018).

Currently, our knowledge of the genetic architecture of life-history trade-offs remains limited to the influence of genetic variation in the nuclear genome, with little attention paid to the potential for mitochondrial genetic variation to influence the evolution of these trade-offs. This is especially true in the case of the "rate of living" hypothesis, which predicts a negative correlation between metabolic rate and longevity of eukaryotes (Arking et al., 1988, Johnston et al., 2013, Khazaeli et al., 2005a, Pearl, 1928, Rubner, 1908). We found a negative genetic correlation across mtDNA haplotypes between metabolic rate and longevity, whereby mtDNA haplotypes that conferred higher metabolic rates were associated with reduced longevity. This mitochondrial correlation was only found in males. This result is striking because it suggests that genetic variation might accumulate within the mitochondrial genome in a manner consistent with the rate of living hypothesis, albeit with a twist.

Maternal inheritance of the mitochondrial genome is plausibly rendering selection efficient at removing the pool of mutations that reduce female metabolic fitness and longevity, but less efficient in removing the pool of mutations that exert male-specific effects on each of these traits. The male-specificity of the mitochondrial correlation between metabolic rate and longevity would, therefore, suggest that the mtDNA mutations that underpin this correlation are non-adaptive, accumulated under a selection shadow (that is, a scenario in which mutations are blind to selection), and not associated with fitness benefits to males. This contention is supported by the lack of a positive genetic correlation, across haplotypes, between metabolic rate and reproductive fitness in males.

Additionally, in males, we found a positive genetic correlation, across haplotypes, between body mass and longevity. This pattern of positive correlation is generally conserved across most bilaterian metazoans independent of the sexes (Austad & Fischer, 1991, Fox et al., 2003, Lindstedt & Calder, 1976, Wagener et al., 2013), and previously thought to be modulated in full by genes found within the nuclear genotype (Khazaeli et al., 2005b). Our result clearly highlights mitochondrial genetic involvement in this correlation and highlights the direction of correlation is indeed sex-specific. Remarkably, however, the direction of the correlation between these traits was negative in females, providing further evidence for mitochondrial genetic involvement in the evolution of sex-specific, and sexually antagonistic, trade-offs between different axes of life-history. The magnitude of correlation between body mass and longevity in females decreased when we omitted one particular outlying haplotype from the analysis – the Brownsville haplotype (correlation between body mass and longevity with the Brownsville haplotype included in the analysis: r_p = -0.699, CIs = -0.979, 0.083; and correlation analysis without the Brownsville haplotype; r_p = -0.275, bootstrapped 95% CIs = -0.902, 0.424). This haplotype has received substantial research attention over the past decade, because it is the only haplotype known to harbour a nonsynonymous mutation in the Cytochrome B gene, which has previously been shown to exert sexually antagonistic effects on fertility (males are

either fully infertile, or sub-fertile depending on the nuclear background in which this mtDNA mutation is expressed, but females are fully fertile), and longevity (females that carry this mutation are associated with shortened longevity, while males enjoy an increase in longevity relative to flies that carry other haplotypes) (Camus et al., 2015, Clancy et al., 2011, Wolff et al., 2017, Wolff et al., 2016c, Yee et al., 2013). Here, we extend the catalogue of sexually antagonistic effects associated with this mutation, and with the Brownsville haplotype generally, by highlighting its involvement in mitochondrial genetic correlations between body size and metabolic rate that differ in sign in males and females.

The panel of haplotypes used here provides an excellent toolkit in which to examine the role of mitochondrial genetic variation in driving sex-differences in trait expression and life-history trade-offs. The panel consists of thirteen mtDNA haplotypes that represent the entire global distribution of *D. melanogaster*, and therefore capture much of the mitochondrial genetic variation present in the species (Wolff et al., 2016a). Furthermore, because the panel consists of a large number of haplotypes, it provides an excellent opportunity to home in on levels of mitochondrial genetic variation underpinning trait expression, which are likely to reflect true effects, rather than be prone to sampling error, which is a limitation of experimental design that utilize smaller panels of haplotypes. Furthermore, because the nuclear background in which the haplotypes are expressed is completely isogenic, and each of the haplotypes replicated across independent duplicates, the panel offers a powerful platform on which to unambiguously partition true mitochondrial genetic effects, from effects of cryptic and residual nuclear variation, or other sources of environmental variance. However, we acknowledge that the approach of replicating our strains within a solitary nuclear background (w^{1118}) carries a caveat too. From a theoretic standpoint, mitochondrial genes must work in intimate coordination with nuclear genes to encode key processes such as OXPHOS, and thus it is likely that mitochondrial genetic effects on the phenotype will be at least in part shaped by epistatic

mitochondrial-nuclear interactions (Rand et al., 2004, Wolff et al., 2014a). A recent meta-analysis across animal and plant kingdoms suggests that while effect sizes associated with cyto (mitochondrial and/or chloroplast)-nuclear epistasis are generally larger than those associated with additive cytoplasmic effects, the additive effect size is nonetheless moderate to large (Dobler et al., 2014); suggesting that the sex-differences in mitochondrial effects we have uncovered here, are likely to extend across more than just the one nuclear background used here. Nonetheless, our study and previous studies conducted to date on this panel of flies should, at this stage be seen as providing proof-of-concept for the Mother's Curse hypothesis. It is important that future studies screen patterns of sex-specific mitochondrial genetic variance for metabolic rate, and other life-history traits, across a range of nuclear genetic backgrounds, to determine whether patterns of male-bias are upheld across a broad array of nuclear contexts.

Finally, our study uncovered new insights into the magnitude and context-dependency of sexual dimorphism in the metabolic rate of *D. melanogaster*. The metabolic rate across the haplotypes was generally higher in females, than in males (LSmeans metabolic rate of males: mean = 1.685, SD = 0.07; and females: mean = 2.031, SD = 0.035). The existing literature indicates that sexual dimorphism in the expression of metabolic rate in fruit flies is context-dependent (Burggren et al., 2017, Van Voorhies et al., 2004). In this regard, the genotype of flies, number of flies assayed in the respirometer (single fly vs group of flies), type of respirometry setup (open vs flow-through), mating status of the focal flies (virgins vs mated), age of the focal fly (young vs old), and the type of assaying area (confined vs unconfined) have been shown to influence patterns of sexual dimorphism in metabolic rate of fruit flies (Burggren et al., 2017). Furthermore, our results showed a sign-shift in the direction of sexual dimorphism across the time course of a day. Trait values were male-biased during the morning assays and became female-biased in the afternoon. This sign-shift in the magnitude and patterns of metabolic rate between the sexes was mainly attributable to high plasticity

in males over the course of the day. In contrast, female trait values were robust to the time of day. Such circadian effects are known to affect mating behaviours of male and female *Drosophila* and moths (Groot, 2014, Partridge et al., 1987, Sakai & Ishida, 2001), but have not been previously found for physiological traits such as metabolic rate. These findings, highlighting diurnal variation in the sexual dimorphism in metabolic rate, have implications for the design of future eco-physiological studies that aim to scrutinise levels of sex-differences in physiological traits of fruit flies.

In summary, our study uncovers sex-specific genetic variation in the mitochondrial genome for a core physiological trait, showing a male-bias that is consistent with that predicted by the Mother's Curse hypothesis. Furthermore, we have demonstrated that genetic variation, harboured within the panel of mtDNA haplotypes used here, affect the magnitude and direction of mitochondrial genetic correlations between physiological and life-history traits and that many of these correlations are sex-specific, even sexually antagonistic. This extends to a negative intersexual correlation, across haplotypes, for metabolic rate. The direction of this correlation suggests that maternal inheritance of the mitochondria has enabled mutations that augment female fitness but harm male fitness, to accrue within the mtDNA sequence, presumably under the direct action of positive selection. Furthermore, the negative mitochondrial correlation observed between metabolic rate and longevity in males suggests mitochondrial involvement in the Rate of Living hypothesis; a hypothesis that has proved to be controversial since its inception. Our study adds to previous evidence that mitochondrial genetic variation affects OXPHOS functionality (Correa et al., 2012, Katewa & Ballard, 2007, Pichaud et al., 2012, Wolff et al., 2016b), with effects that resonate across the entire biology and evolutionary trajectories of the organism, ultimately affecting life-history outcomes. Future research should now explore whether the signatures of male-bias and sexual antagonism, detected across haplotypes in our study, are upheld, across a broader range of nuclear genetic and environmental contexts.

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2.7 Tables and Figures

Table 1: Results from the model of the full dataset, which includes both sexes. The final model was derived by sequentially eliminating non-significant higher-order interaction terms across both fixed and random effects using the *log-likelihood ratio* test in R. Thus, the final model displayed here, represents all fixed and random effects, and any higher-order interactions that were statistically significant at $p < 0.05$. Both body mass and ADS were retained as covariates in the final model. Statistical significance of each fixed effect in the final model was estimated using the Type III Wald's *Chi*-squared test. The variance attributable to each random effect in the final model was estimated using the restricted maximum likelihood method. In this table, the random effect – Day[Block] (experimental day nested within block) is a term that explains the hierarchical structuring of the data.

Fixed effects	*d.f.*	*Chi.sq*	*p - value*
(Intercept)	1	3165.0979	<0.0001
Sex	1	5.9349	0.0148
Time of assay	3	3.1943	0.3626
Body mass	1	117.4666	<0.0001
ADS	1	114.221	<0.0001
Sex × time of assay	3	38.0679	<0.0001

Random effects	*Variance*
mtDNA haplotype	0
Strain duplicate	0
Day[Block]	0.002051
mtDNA haplotype × sex	0.001572
Strain duplicate × sex	0
Day[Block] × sex	0.003415
Residual	0.134445

Table 2: Results from the sex-specific *lmer* models are summarised here. The final models of each sex are presented - a) males, b) females. The significance of fixed effects and covariates was derived through the Type III Wald's *Chi*-squared test and random effects through the restricted maximum likelihood method. In this table, the random effect – Day[Block] (experimental day nested within block) explains the hierarchical structuring of the data.

a) Male metabolic rate

Fixed effects	*d.f.*	*Chi.sq*	*p - value*
(Intercept)	1	2547.645	<0.0001
Time of assay	3	43.472	<0.0001
Body mass	1	21.507	<0.0001
ADS	1	127.131	<0.0001

Random effects	*Variance*
mtDNA haplotype	0.00253
Strain duplicate	0
Day[Block]	0.007273
Residual	0.167978

b) Female metabolic rate

Fixed effects	*d.f.*	*Chi.sq*	*p - value*
(Intercept)	1	6368.625	<0.0001
Time of assay	3	3.058	0.3827
Body mass	1	148.856	<0.0001
ADS	1	3.346	0.067

Random effects	*Variance*
mtDNA haplotype	0
Strain duplicate	0
Day[Block]	0.004766
Residual	0.094063

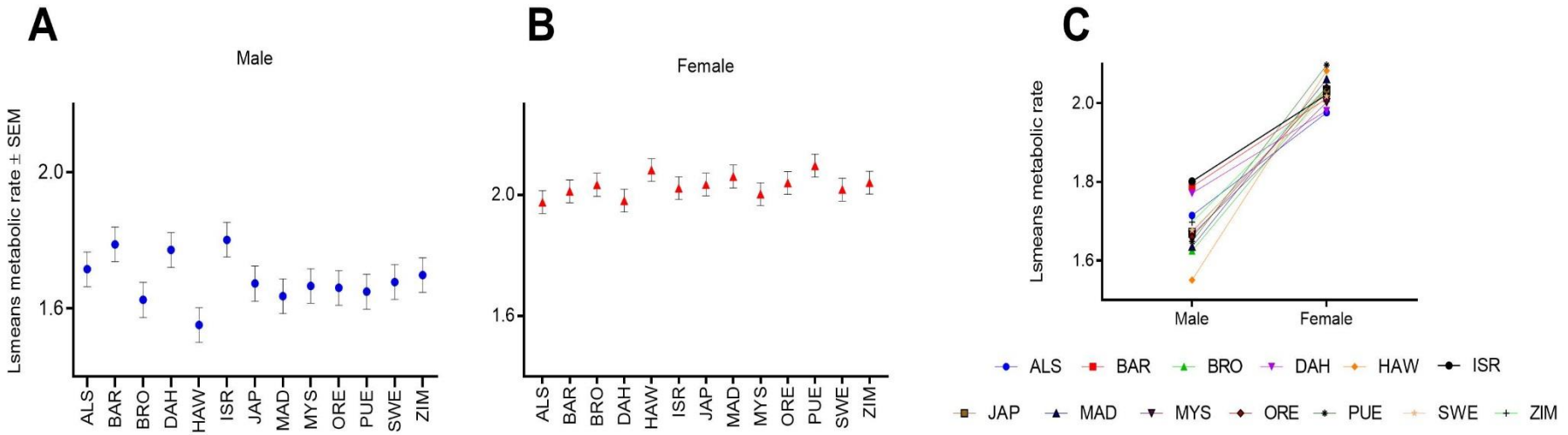

Figure 1. Effects of mtDNA haplotype on the metabolic rate of A. male; and B. female flies. In panels A and B, the least-squared means (LSmeans) ± 1 Standard Error of metabolic rate for each mtDNA haplotype in each sex were derived from the linear models, using the *lsmeans* function in R. The LSmeans take account of variation in body mass and ADS. C. Interaction plot showing variation in LSmeans metabolic rate (adjusted for body mass and ADS) between the sexes, across the thirteen mtDNA haplotypes.

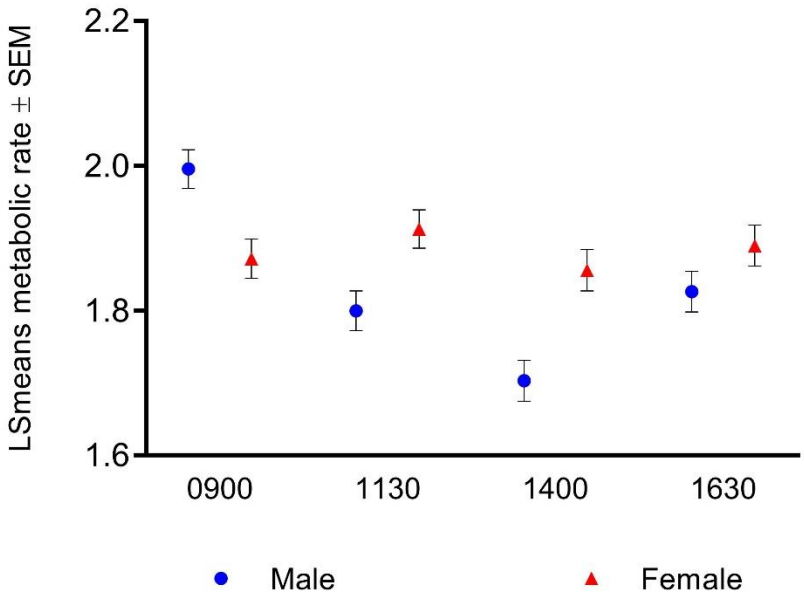

Figure 2. Diurnal variation in metabolic rate (LS means ± 1 S.E.) in each of the sexes. For each assaying-time, LSmeans were estimated using the *lsmeans* function in R. Four assaying-times are shown in the horizontal axis – 0900, 1130, 1400 and 1630 h. These four-separate assaying-times represent the approximate (± 5 min) time of the day at which the metabolic rate assays were initiated in this study.

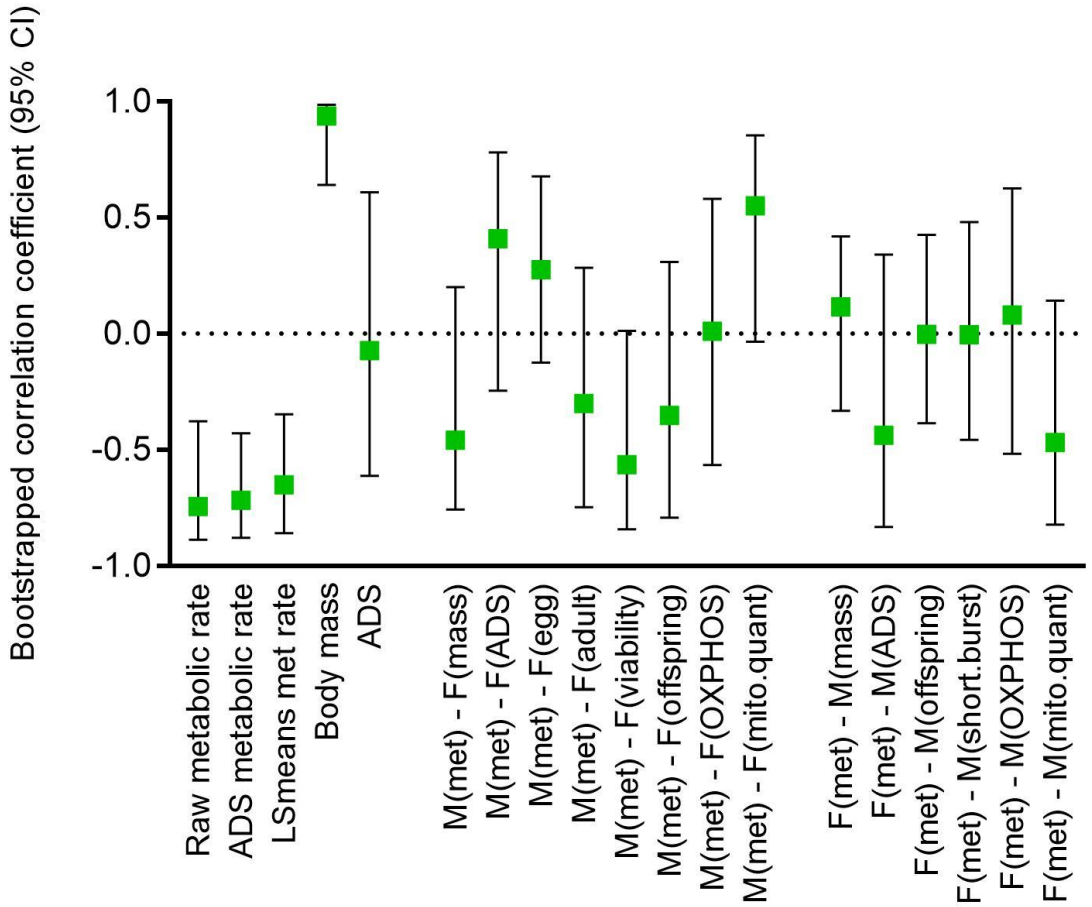

Figure 3. Inter-sexual mitochondrial genetic correlations across traits estimated using the non-parametric bootstrapping approach in *boot* function in R. Each Pearson's correlation test was resampled over 10000 iterations, and the 95% confidence interval (CI) was calculated using the bias corrected and adjusted method. The horizontal axis shows all pair-wise comparisons of life-history and physiological traits. In the horizontal axis, M refers to males and F is females. All measurements of metabolic rate, body mass and ADS were made in this study. Met in the horizontal axis refers to LSmeans metabolic rate. In the horizontal axis, the labels of reproductive traits have been changed from Camus *et al.* (2018). Here, M(offspring) is male sustained offspring production and M(short.burst) is male short-burst offspring production; F(egg) is female short-burst fecundity, F(adult) is female short-burst offspring production, F(viability) is female short-burst viability and F(offspring) is sustained offspring production. The same applies to metabolic traits reported in Wolff *et al.* (2016b), where OXPHOS is the PC1 estimate, and mito.quant is the PC2 estimate of young male and female flies.

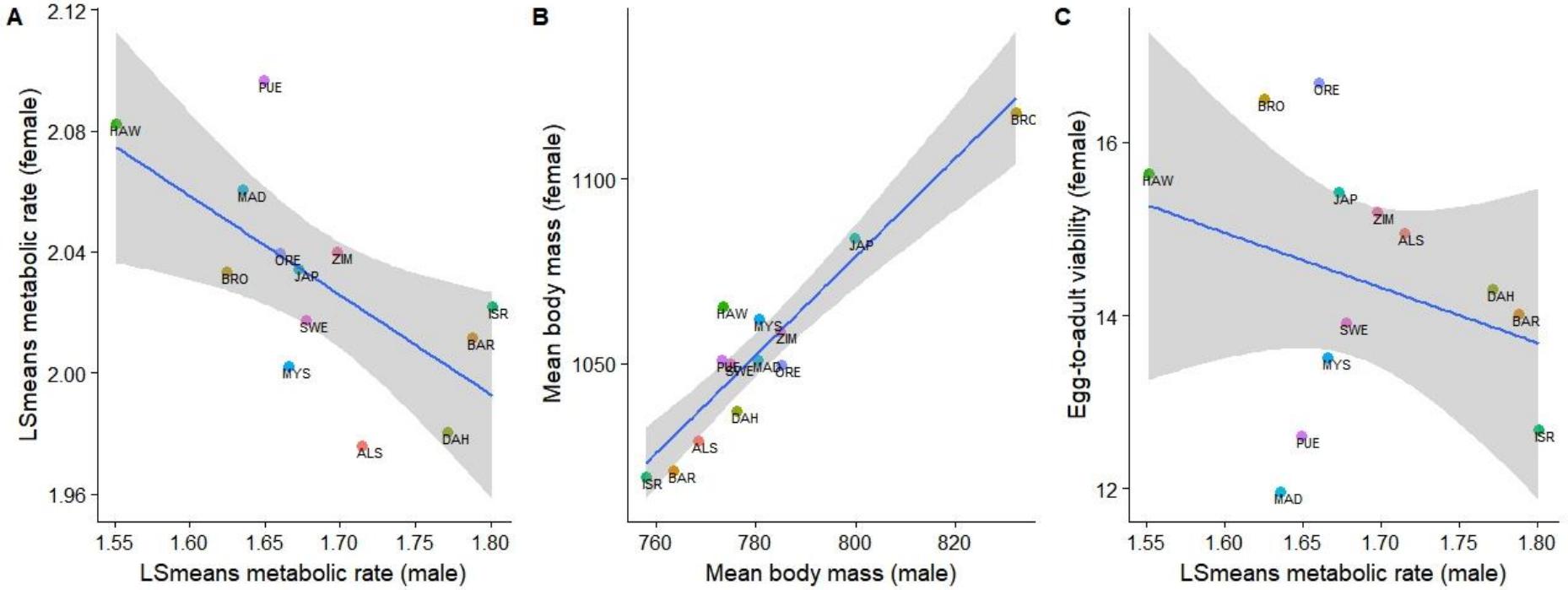

Figure 4. Inter-sexual mitochondrial genetic correlation across life-history and physiological traits. A. The inter-sexual correlation for LSmeans metabolic rate (adjusted for body mass and ADS) was negative (Pearson's correlation coefficient (r_p) = -0.65, bootstrapped 95% confidence intervals (95% CI) = -0.859, -0.348), B. The direction of correlation between body mass of both sexes was positive (r_p = 0.937, 95% CI = 0.641, 0.986), and C. LSmeans metabolic rate of males was negatively correlated with female reproductive trait, egg-to-adult viability (r_p = -0.564, 95% CI = 0.012, -0.843). The scales and both axes are adjusted to show the direction of the relationship between each pair-wise comparison of traits.

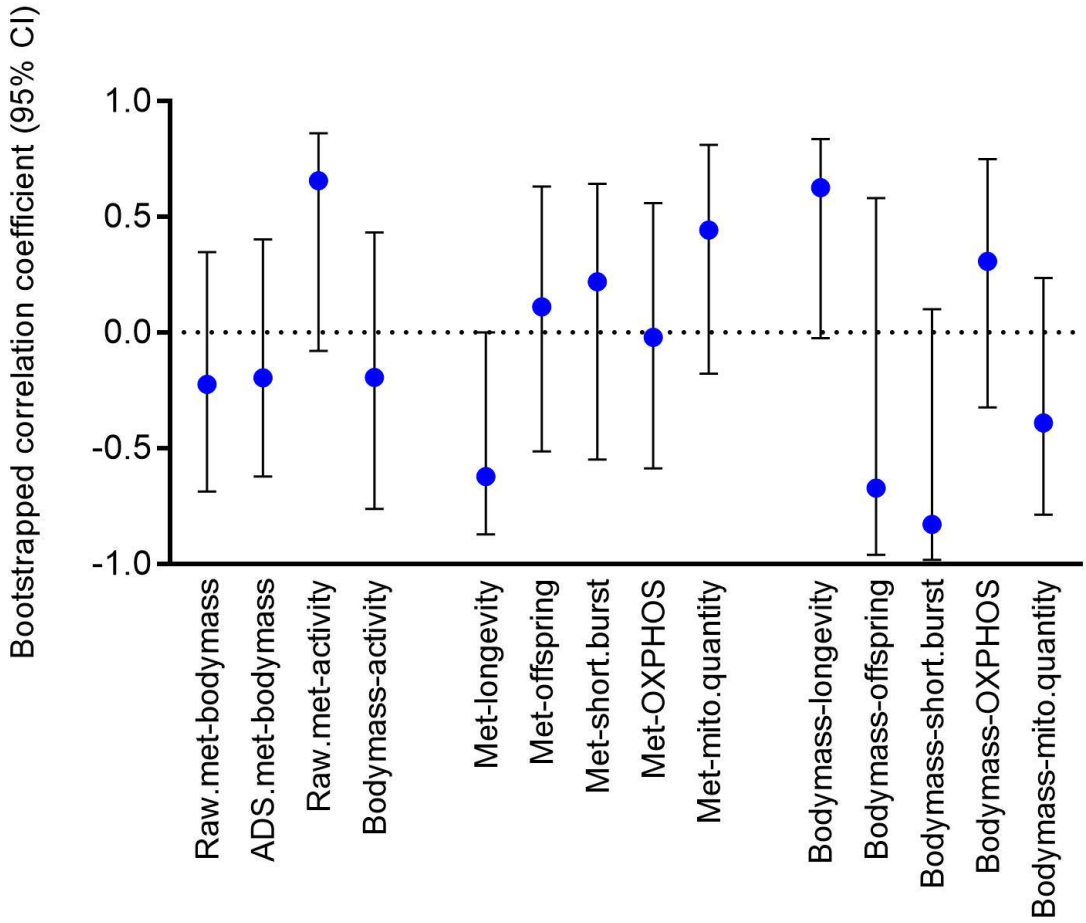

Figure 5. Mitochondrial genetic correlations across traits in males, estimated using a non-parametric bootstrapping approach in R. Each Pearson's correlation test was resampled over 10000 iterations, and the 95% confidence interval (CI) was calculated using the bias corrected and adjusted method in *boot* function. The horizontal axis shows all pair-wise comparisons of life-history and metabolic traits. The labels in the horizontal axis are as follows, Raw.met – raw metabolic rate, ADS.met – ADS-adjusted metabolic rate, Met – LSmeans metabolic rate (adjusted for body mass and ADS), and activity is ADS. In the horizontal axis, the labels of reproductive traits have been changed from Camus *et al.* (2018). Here, offspring is male sustained offspring production and short.burst is male short-burst offspring production. The same applies to metabolic traits reported in Wolff *et al.* (2016b), where OXPHOS is the PC1 estimate, and mito.quantity is the PC2 estimate of young male flies.

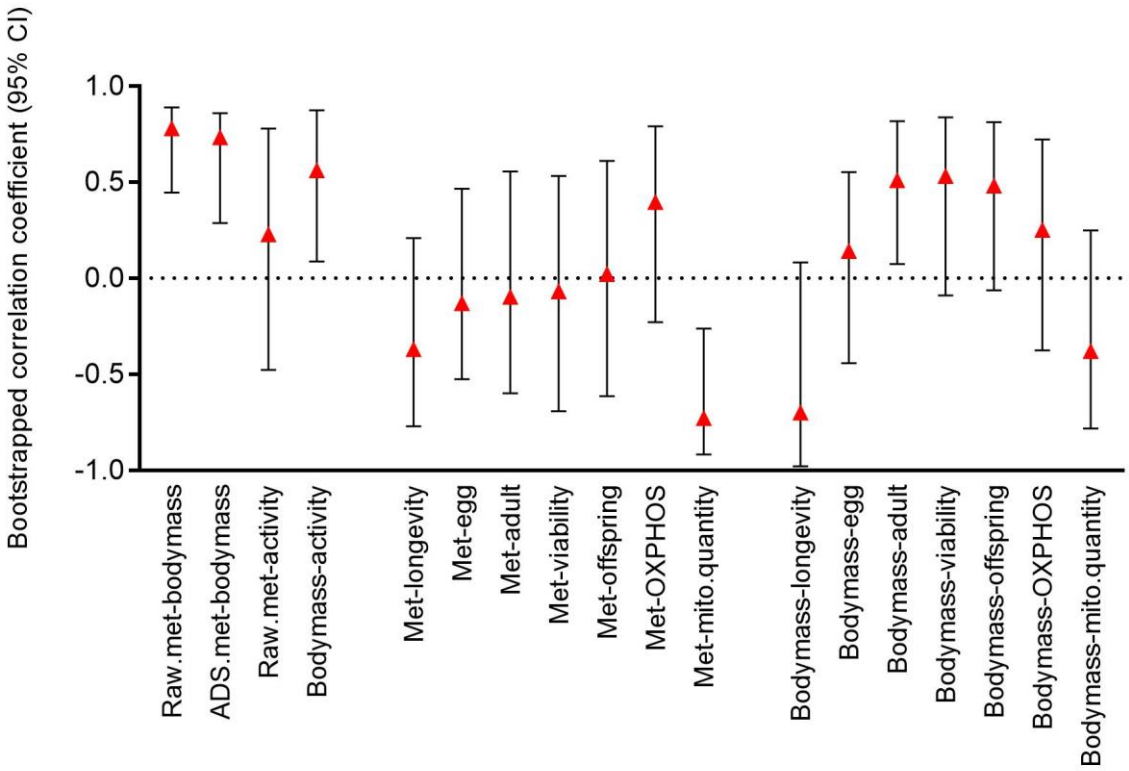

Figure 6: Genetic and phenotypic correlation between traits in females estimated using the non-parametric bootstrapping approach in *boot* function in R. Each Pearson's correlation test was resampled over 10000 iterations, and the 95% confidence interval (CI) was calculated using the bias corrected and adjusted method. The horizontal axis shows all pair-wise comparisons of life-history and metabolic traits. The label in the horizontal axis are as follows, Raw.met – raw metabolic rate, ADS.met – ADS-adjusted metabolic rate, Met – LSmeans metabolic rate adjusted for body mass and ADS, and activity is ADS, all of which were measured in this study. Here, in the horizontal axis, the name of female reproductive traits has been changed from Camus *et al.* (2018): egg is short-burst fecundity, adult is short-burst offspring production, viability is short-burst viability and offspring is sustained offspring production. The same applies to metabolic traits harnessed in Wolff *et al.* (2016b), where OXPHOS is the PC1 estimate, and mito.quantity is the PC2 estimate of young female flies.

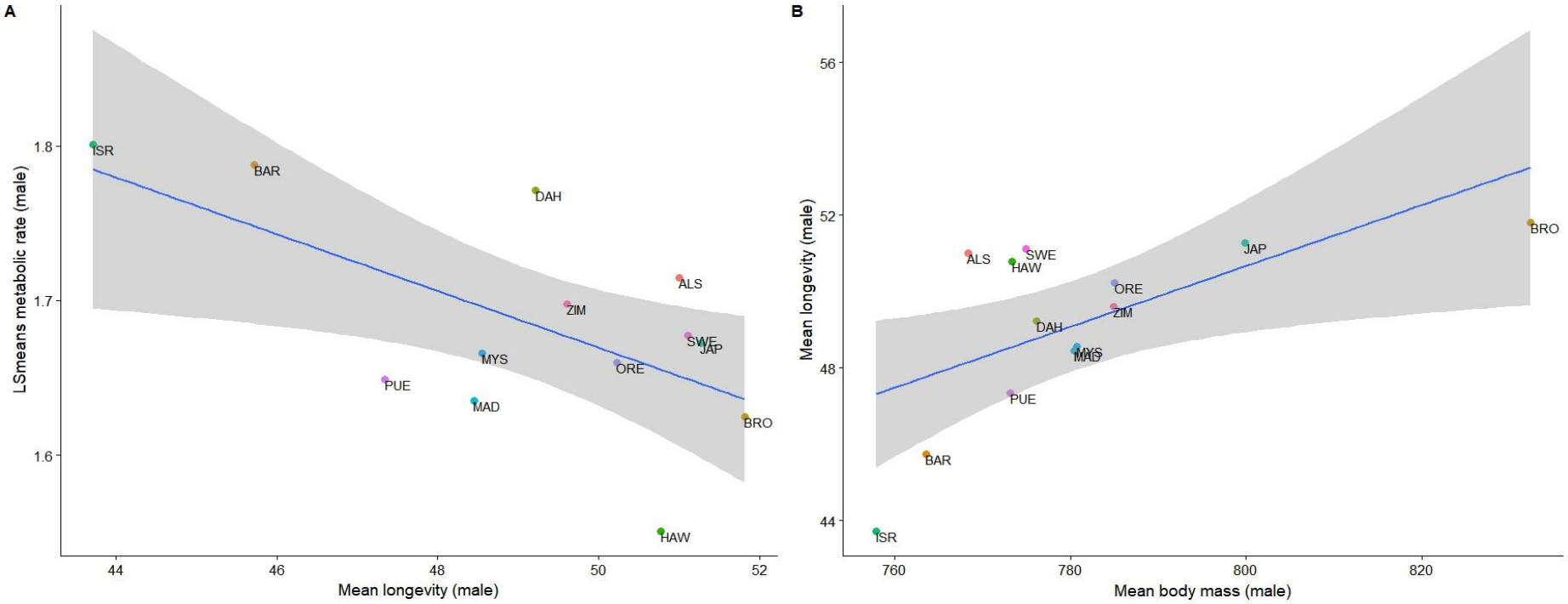

Figure 7. Intra-sexual mitochondrial genetic correlation across life-history and physiological traits in males. A. Longevity was correlated negatively with LSmeans metabolic rate (Pearson's correlation coefficient (r_p) = -0.623, bootstrapped 95% confidence intervals (CI) = 0, -0.872), B. but positively correlated with body mass (r_p = 0.626, 95% CI = -0.025, 0.836) in males. The scales in both vertical and horizontal axes are adjusted to show the direction of correlation in each pair-wise comparison. For annotations of the mtDNA haplotypes, refer to the Methods section.

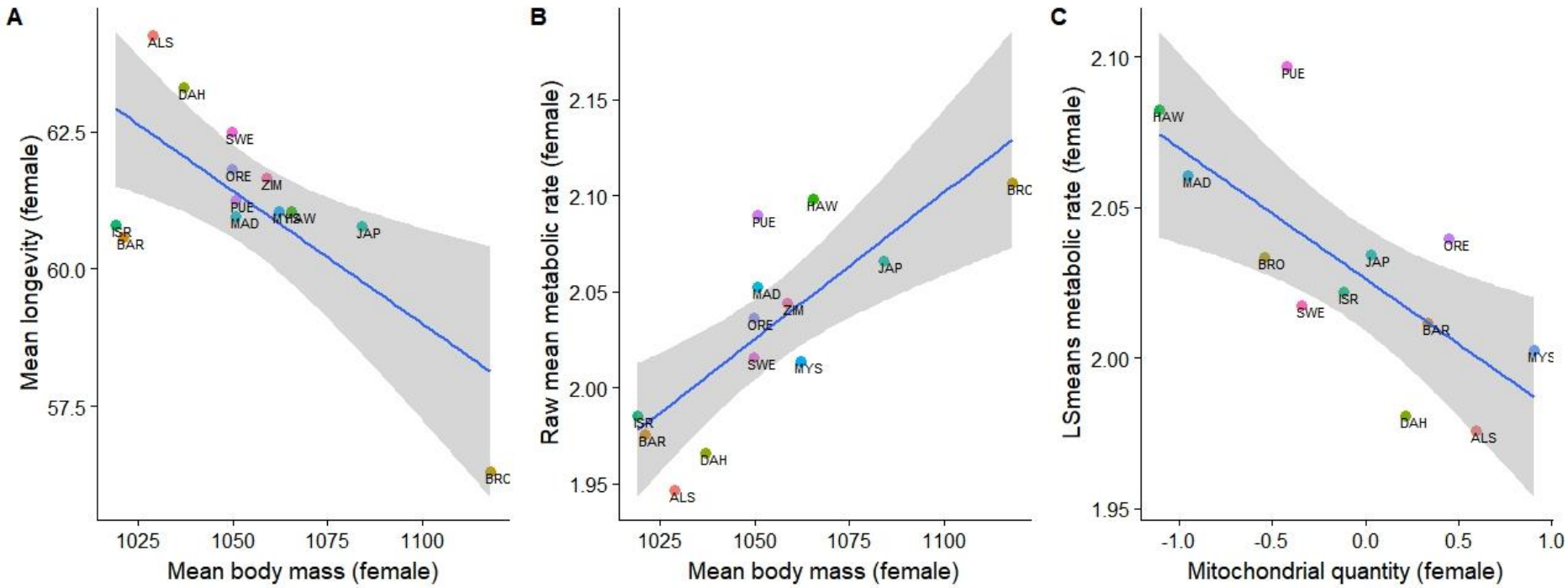

Figure 8. Intra-sexual mitochondrial genetic correlation in female fruit flies. A. Longevity was correlated negatively with body mass (Pearson's correlation coefficient (r_p) = -0.699, bootstrapped 95% confidence intervals (95% CI) = -0.979, 0.083), B. Raw metabolic rate was correlated positively with body mass (r_p = 0.781, 95% CI = 0.445, 0.889) and B. LSmeans metabolic rate (adjusted for body mass and ADS) was correlated negatively with mitochondrial quantity (r_p = -0.727, 95% CI = -0.918, -0.262) in females. The scales in both horizontal and vertical axes are adjusted to show the direction of correlation between each pair-wise comparison of traits. Refer to the Methods section for annotations of the mtDNA haplotypes.

2.8 Supplementary information

Methods

ExactRLRT() test of RLRsim package in R

To test if mtDNA haplotype contributes to zero or non-zero variance in the metabolic rate of each sex, we ran exactRLRT tests on sex-specific metabolic rate datasets. Below, the response variable is the mean metabolic rate (met.rate).

```
library(RLRsim)
mod1<- lmer(met.rate ~ 1 + (1|mtDNAhaplotype), data=data, REML=T)
exactRLRT(m=mod1, mA=NULL, m0=NULL, nsim=10000)
```

Parametric bootstrapping of point median variance and confidence intervals in R

To extract point variance from the sex-specific dataset, we used the R codes provided by Dr Bjorn Rogell, Stockholm University. This method is limited to extracting variance attributable to one random effect term, and so we used only the mtDNA haplotype term in our model to estimate the median and 95% confidence intervals of the point variance attributable to mtDNA haplotypes. This parametric bootstrapping method was run separately for each sex dataset.

```
data$sim <- NA
dataout<- NULL
mod<- lmer(y ~ 1 + (1|mtDNAhaplotype), data=data)
for(i in 1:10000){ data$sim <- simulate (mod, nsims=1) $sim_1
mod.temp<- lmer(sim ~ 1+ (1|mtDNAhaplotype), data=data)
dataout[i]<- sqrt(as.numeric(VarCorr(mod.temp)[1]))/as.numeric(fixef(mod.temp))}
hist(dataout)
quantile(dataout, prob=c(0.025, 0.975))
median(dataout)
```

Estimating 95% confidence intervals for variance attributed to random effects in the *lmer* model

```
model1<- lmer(y ~ fixef1 + covariate1 + (1|mtDNAhaplotype) + (1|ranef2), data=data, REML=T)
summary(model1)
m1<- confint(varianceProf(profile(model1, which=c("theta_", ".sigma"), ranef=TRUE)))
cbind(as.data.frame(VarCorr(model1), order="lower.tri"),m1)
```

If the above confint() code returns an error because of negative variance, use confint.merMod function to extract the bootstrapped 95% CI for standard deviation attributed to each random effect term in the final model. Then, calculate 95% CI for the variance by squaring the values of 2.5% and 97.5% CIs of the standard deviation. Use the code below,

```
confint.merMod(model1, "theta_", level=0.95, method ="boot", nsim=1000, boot.type = "basic",
FUN=NULL, oldNames=FALSE)
```

Estimating Pearson's correlation coefficient and its bootstrapped 95% confidence intervals in *boot* package using bias corrected and accelerated method

```
library(boot)
varA<- data$varA
varA<- as.numeric(varA)
varB<- data$varB
varB<- as.numeric(varB)
xy<- data.frame(cbind(varA, varB))
pearson<- function(data, i=c(1:n)){
  d1<- data[i,]
  return(cor(d1$varA, d1$varB))
}
bootcorr1 <- boot(data=xy, statistic=pearson, R=10000)
bootcorr1
boot.ci(bootcorr1, type = "bca", conf=.95)
```

Supplementary figure

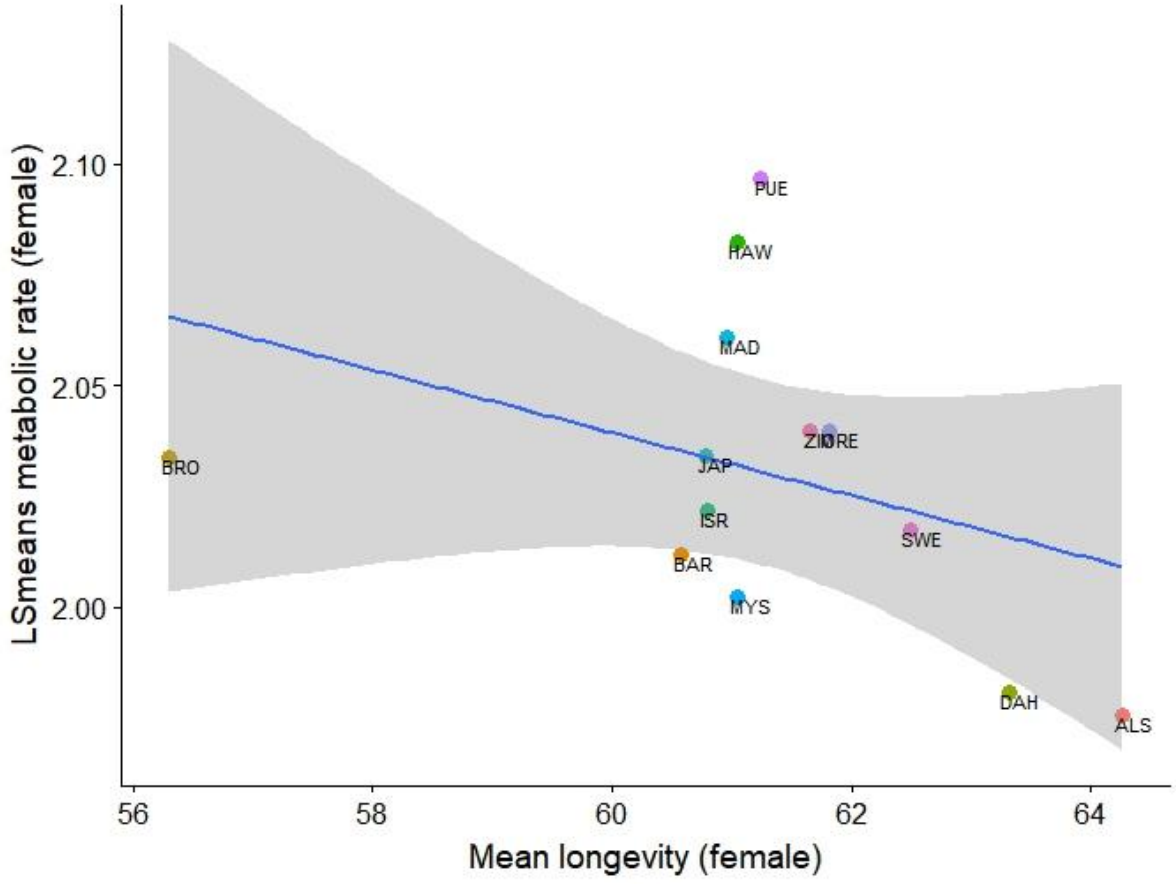

Figure S1: Intra-sexual mitochondrial genetic correlation between metabolic rate (body mass and ADS adjusted) and longevity in females (Pearson's correlation coefficient (r_p) = -0.369, bootstrapped 95% confidence intervals (CI) = -0.771, 0.209). For annotations of the mtDNA haplotypes in the figure, refer to the Methods section.

Chapter 3

Interactions between mitochondrial haplotype and dietary macronutrient ratios confer sex-specific effects on longevity in *Drosophila melanogaster*

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3.1 Abstract

Recent studies have demonstrated that modifications to the ratio of dietary macronutrients affect longevity in a diverse range of species. However, the degree to which levels of natural genotypic variation shape these dietary effects on longevity remains unclear. The mitochondria have long been linked to the ageing process. The mitochondria possess their own genome, and previous studies have shown that mitochondrial genetic variation affects longevity in insects. Furthermore, the mitochondria are the sites in which dietary nutrients are oxidized to produce adenosine triphosphate, suggesting a capacity for dietary quality to mediate the link between mitochondrial genotype and longevity. Here, we measured longevity of male and female fruit flies, across a panel of genetic strains of *Drosophila melanogaster*, which vary only in their mitochondrial haplotype, when fed one of two isocaloric diets that differed in their protein-to-carbohydrate ratio. The mitochondrial haplotype affected the longevity of flies, but the pattern of these effects differed across the two diets in males, but not in females. We discuss the implications of these results in relation to an evolutionary theory linking maternal inheritance of mitochondria to the accumulation of male-harming mitochondrial mutations, and to the theory exploring the evolution of phenotypic plasticity to novel environments.

Keywords: gene-by-environment, Mother's Curse, nutrition, protein:carbohydrate ratio, sexual conflict

3.2 Introduction

Individual investment into the various components of life-history is expensive and relies on the acquisition of resources, including the regular uptake of dietary macronutrients (Simpson & Raubenheimer, 2009). Indeed, one of the core facets of life-history is longevity; a trait whose expression is explicitly tied to variation in dietary quality and quantity (Fontana & Partridge, 2015). For many years, it was assumed that extensions in longevity could be achieved simply through reductions in total caloric intake (Masoro, 2005). However, research advances over the past two decades have determined that variation in the balance of specific dietary macronutrients ingested, rather than total calories *per se*, may be the main contributor to longevity (Simpson et al., 2015, Simpson & Raubenheimer, 2009). These insights came from studies that harnessed experimental designs able to decouple the influence of macronutrient ratios from the total caloric intake, which demonstrated that ratios of dietary protein-to-carbohydrate (P:C) are the primary contributors to longevity outcomes across crickets, *Drosophila* flies, and mice (Simpson & Raubenheimer, 2009, Simpson et al., 2015). As such, the association between macronutrient ratios and longevity appears to be conserved across a range of bilaterian metazoans.

Recently, further progress has been made in understanding the link between macronutrient balance and longevity in metazoans. Firstly, several studies have demonstrated that the effects associated with modifying macronutrient ratios on longevity are largely consistent across the sexes, in insects and mice, albeit with minor differences in the optimal ratios of P:C in each sex (Jensen et al., 2015, Solon-Biet et al., 2015). Secondly, studies that have focused on the molecular pathways that mediate longevity responses to dietary intake have identified a key role for the Insulin-like Growth Factor 1 and mechanistic Target of Rapamycin (IGF-1/mTOR) network in the regulation of these effects (Fontana & Partridge, 2015). However, one area of research that remains less explored when it comes to the links between diet and longevity, is the role that natural genetic variation plays in mediating

the magnitude of longevity response to dietary manipulation. While it is clear that the phenotypic expression of longevity is underpinned by genetic variation within the nuclear (Pawlikowska et al., 2009, Willcox et al., 2008) and mitochondrial genomes (Camus et al., 2012, Dato et al., 2004), little is known as to whether the expression of this genetic variation is sensitive to variation in the dietary environment, manifesting as macronutrient-mediated genotype-by-environment (G × E) interactions for longevity. Temperature-mediated G × E interactions for longevity have been previously demonstrated in *D. melanogaster* (Vieira et al., 2000), and given the widespread prevalence of G × E interactions underpinning the expression of quantitative traits (Flint & Mackay, 2009), this would suggest considerable scope for genotype-specific responses in levels of macronutrient mediated plasticity for longevity. Furthermore, such G × E interactions could conceivably differ in their sensitivity across the sexes (De Block & Stoks, 2003).

The mitochondria have long been at the forefront of hypotheses that seek to explain why ageing occurs (Harman, 1972). As the organelles that generate energy reserves used for organismal and somatic maintenance, their functionality is a key prerequisite for sustaining healthy life (Wallace, 2005). Somewhat paradoxically, they generate highly Reactive Oxygen Species, as by-products, implicated in oxidative stress, and progressive physiological deterioration with advancing age (Dowling & Simmons, 2009, Monaghan et al., 2009). Furthermore, mitochondrial dysfunction is a known pathology of normal ageing, as well as late-onset diseases such as Alzheimer's and Parkinson's (Fivenson et al., 2017). From an evolutionary standpoint, the mitochondria are of particular interest to the ageing process given that they have retained their own genome, comprised of mitochondrial DNA (mtDNA), in which a set of 13 core protein-coding genes encode polypeptide subunits that interact intimately with nuclear-encoded subunits to assemble the five enzyme complexes that underpin oxidative phosphorylation (OXPHOS). As such, interactions between genes

encoded by the mtDNA and nuclear genes that contribute to the mitochondrial proteome underpin biological processes that are essential for eukaryotic life (Rand et al., 2004, Wolff et al., 2014b).

Recent studies investigating the role of genetic variation in dietary-mediated effects on longevity have focused explicitly on the role of genetic variation across mitochondrial haplotypes, and epistatic combinations of mitochondrial-nuclear (mito-nuclear) genotype. For example, Zhu *et al.* (2014) examined effects of caloric and macronutrient modification on the longevity of female flies harbouring different combinations of mito-nuclear genotype (two mtDNA haplotypes from *D. melanogaster* and two from *D. simulans*, placed against two different isogenic nuclear backgrounds from *D. melanogaster* = eight mito-nuclear combinations). They reported evidence that longevity was affected by interactions between mtDNA haplotype, the nuclear background and the dietary regime, thus highlighting a prominent role for mitochondrial genetics in regulating the longevity response to the dietary modification. Mito-nuclear effects on ageing were moderated both by changes in the caloric content of the diet, and the macronutrient content (Zhu et al., 2014). While Aw *et al.* (2017) examined sex-specific effects on a range of mitochondrial biochemical, physiological and life-history traits including survival, associated with interactions between two mtDNA haplotypes and variation in macronutrient ratios, in *D. melanogaster*. They reported the effects of the mitochondrial haplotype, and interactions between the mitochondrial haplotype and diet, on survival in males, but not females (Aw et al., 2017).

The findings of male-specific mitochondrial genotype by dietary environment interactions for survival, reported by Aw *et al.* (2017), are interesting in light of an evolutionary hypothesis known as Mother's Curse (Frank & Hurst, 1996a), which predicts that mitochondrial genetic effects on the phenotype will be male-biased in magnitude. This prediction is based on the premise that maternal inheritance of mitochondria will facilitate the accumulation of mutations within the mitochondrial

genome that exert male-biases in effects on the expression of life-history and physiological traits (Frank & Hurst, 1996a). Maternal inheritance means that the evolutionary fate of any mutation in the mtDNA sequence will be determined by its fitness effects in females. If a mutation arises in the mitochondrial genome that is benign or positive in its effects on female fitness, this mutation can accumulate and even fix within the mtDNA sequence, even if the same mutation incurs significant costs to male fitness (Beekman et al., 2014). In theory, this will lead to the accumulation of mutation loads in the mitochondrial genome that exert male-biases in their associated phenotypic effects. A key prediction of the hypothesis, therefore, is that the genetic variation that delineates different mtDNA haplotypes will exert larger effects on the expression of phenotypes in males than females. That is, levels of mitochondrial genetic variation underpinning phenotypic trait expression should be male-biased. This prediction has received some empirical support from studies of genetic strains of *D. melanogaster*, which differ only in their mtDNA sequence. These studies have revealed male-biases in levels of mitochondrial genetic variation underpinning several traits, such as longevity and ageing rates (Camus et al., 2012), as well as nuclear genome-wide patterns of gene expression (Innocenti et al., 2011). These studies have been supported by other studies reporting specific mtDNA mutations, or haplotypes, associated with negative phenotypic outcomes in males, but not females (Clancy et al., 2011, Milot et al., 2017, Nakada et al., 2006)

In this study, we aimed to extend on previous studies that have linked mitochondrial genetic variation to longevity (Maklakov et al., 2006, Rand et al., 2006, Trifunovic et al., 2004), and that have examined the role of mitochondrial genotype-by-diet interactions for longevity (Aw et al., 2017, Zhu et al., 2014). We explored three questions. Firstly, whether the genetic variation that occurs across a panel of 13 mtDNA haplotypes, sourced from distinct global localities, affects longevity, and whether any such mitochondrial haplotypes effects are male-biased in magnitude (that is, the level of mitochondrial genetic variation for longevity in each of the two diets would be larger in males,

compared to females), as was previously observed by Camus *et al.* (2012). Secondly, whether this variation is sensitive to macronutrient-mediated mitochondrial G × E interactions, and finally, whether these G × E interactions are male-biased (that is, whether the magnitude of G × E effects is larger in males than in females) in their manifestation as previously indicated by Aw *et al.* (2017) in their study of two haplotypes. To address these questions, we utilized the same panel of haplotypes as previously used by Camus *et al.* (2012), who uncovered clear male-biases for longevity and ageing rates across the 13 haplotypes. This panel also includes the two haplotypes used by Aw *et al.* (2017). Specifically, we measured longevity of replicated cohorts of male and female flies that were maintained on one of two isocaloric diets that differed only in their levels of protein and carbohydrate content but not total nutritional content (South et al., 2011). We also investigated whether mitochondrial haplotype effects on longevity, measured on artificial diets used in our study, matched those reported by Camus *et al.* (2012), who measured longevity of flies kept on standard yeast-based diets.

3.3 Methods

Mitochondrial panel

We measured the longevity of male and female flies, across a panel of 13 genetic strains of *D. melanogaster*, on each of two different diets. Each strain is characterised by a distinct and naturally occurring mtDNA haplotype in an otherwise isogenic nuclear background, derived from the w^{1118} line (Clancy, 2008). These strains are annotated based on the geographical location from which they were originally sourced, thus - **Als**tonville, Australia; **Bar**celona, Spain; **Bro**wnsville, USA; **Dah**omey, Benin, **Mad**ang, Papua New Guinea; **Mys**ore, India; **Haw**ai'i, USA, **Isr**ael; **Jap**an; **Ore**gon, USA; **Pue**rto Montt, Chile; **Swe**den and **Zim**babwe (Camus et al., 2012). The breeding scheme used to create the mitochondrial strains is outlined in Clancy (2008). The mitochondrial strains were obtained in 2007 from Dr David J Clancy and immediately divided into two biological

duplicates, which have since been independently maintained through more than 100 generations of backcrossing of virgin females of each strain to males of the isogenic w^{1118} line. Additionally, the w^{1118} line has been propagated each generation via a single full-sibling pair to maintain isogenicity throughout the nuclear genome. By backcrossing the strain duplicates into the w^{1118} nuclear background over ~100 successive generations, we ensured that the nuclear backgrounds of each of the mitochondrial strains were truly isogenic and devoid of any cryptic nuclear genetic variation that may have accumulated over multiple generations of laboratory maintenance.

The strains were cleared of infection from the bacterial symbiont *Wolbachia* via antibiotic (tetracycline hydrochloride) treatment (Clancy, 2008). Before the start of our experiment, the absence of *Wolbachia* among the mitochondrial strains was confirmed via a diagnostic PCR, using whole genomic DNA isolated from two female flies per strain duplicate. This PCR amplified the *Wolbachia* Cytochrome Oxidase subunit I (*CoxA*) gene (Simoes et al., 2011). We used a separate true-positive control (DNA sample extracted from *Wolbachia*-positive wild-type strain) and a true-negative control (DNA sample obtained from a tetracycline treated laboratory strain) in our diagnostic PCRs to confirm the *Wolbachia* status of the mitochondrial strain duplicates. Using a secondary analysis, we further confirmed the absence of *Wolbachia* in the mitochondrial strains by screening for the presence of *Wolbachia* gene sequences in the pool of Illumina paired-end reads obtained from the mitochondrial panel NGS data (Wolff et al., 2016a).

In summary, this panel of mitochondrial strains serves as a valuable genetic resource to explore mitochondrial genetic effects on the phenotype, because the 13 haplotypes of the panel provide a broad representation of the total levels of mitochondrial genetic variation found across the globe for this species (Wolff et al., 2016a). Furthermore, because each of the haplotypes persists across two independent biological duplicates, mitochondrial genetic effects on longevity can be statistically

decoupled from confounding sources of environmental variance, as well as from possible effects mediated by residual nuclear variation that might have accumulated across the strains despite our best efforts to maintain these as isogenic.

Generating experimental flies

The flies of each strain duplicate were subjected to a stringent breeding scheme for three generations leading up to the generation of the focal flies, to control for potential sources of environmental variance (such as parental and grandparental age effects, and density-dependent effects). In the first generation, for each strain duplicate, 15 pairs of flies were housed in vials with access to standard laboratory food (see **supplementary material**) until two days of adult age. The protein:carbohydrate ratio of the lab food is estimated to be 1:2.5 (that is, P = 22.5% and C = 57.4%). The mating pairs were then transferred to a second vial for 24 h, during which time females deposited eggs onto the surface of the food. These egg densities were reduced to 80 eggs per vial, by removing excess eggs with a clean spatula. This process was continued in the subsequent generation, with flies collected within 24 h of eclosion used to propagate the next generation. In the second generation, 15 pairs of flies per vial were stored until four days of adult age, then transferred to fresh vials with access to fresh lab food supplemented with *ad libitum* dry yeast. The females were allowed to oviposit for 24 h, and egg density again trimmed to 80 eggs. The same procedure was followed for a third generation, which produced the parents to our "focal" flies used in our experiments.

In the fourth generation, the focal flies were collected as virgins, within 6 h of eclosion into adulthood, and then housed separately by sex, across two vials (10 flies per vial) per strain duplicate, for 24 h. This 24-h period enabled us to confirm the virginity of the female flies (as gauged by the absence of viable eggs over this time-period). When the focal flies were two days of adult-age, we added a group of 10 two-day-old "tester" flies of the opposite sex, collected from the w^{1118} line, to

each vial. The flies were allowed to cohabit for 24 h, during which time the flies will have mated (Camus et al., 2012), and then the tester flies removed from these vials under light CO_2 anaesthesia. Focal flies were transferred to fresh vials with standard laboratory food in their respective same-sex cohorts for 24 h. This 24 h resting period allowed focal flies to recover from post-mating stress and CO_2 anaesthesia.

Isocaloric diets

When five days of age, each cohort of focal flies was placed onto one of two isocaloric, solid diets that varied only in the ratio of protein and carbohydrate, but otherwise had standardised quantities of Wesson's salts, ascorbic acid, cholesterol and vitamin mix (South et al., 2011) and the same total nutritional content (i.e. diets were isocaloric). One of the diets was protein biased (2P:1C), while the other was carbohydrate biased (1P:8C). The protein used in these diets consisted of a 3:1:1 mixture of casein, peptone and albumen, whereas the carbohydrate was a 1:1 mixture of sucrose and dextrin. The constituents of the solid diets were added to warm distilled water that was boiled with 1% agar. This boiled food mixture was allowed to cool down to room temperature, before propionic acid and 10% nipagin were added. Two millilitres of food was dispensed into individual vials using a peristaltic pump (Watson Marlow Limited, UK), allowed to solidify overnight, before use in the longevity assay. Fresh food was prepared *ad hoc*, and the excess food was stored in the refrigerator for no longer than four days, before deployment into the longevity experiment. Refer to **supplementary material** for full methods on the preparation of the isocaloric diets.

Longevity assay

Each cohort of focal flies remained on the diet to which it had been assigned for the duration of the longevity experiment. Flies were transferred to fresh vials, every 48 h. This method of transferring flies to vials with fresh food ensured the availability of fresh food to the adult flies, free of fungal or

bacterial growth and free from advanced stages of larval activity; and controlled for accidental deaths caused by flies getting stuck to the old food, which becomes stickier with age (Camus et al., 2012). We housed each of these technical replicates in separate trays, and the trays were kept in separate parts of the temperature-controlled cabinet (maintained at 25°C and ~30% relative humidity, Panasonic MLR-352H-PE environmental growth cabinet) to ensure that micro-environmental variation across the vials did not confound our capacity to accurately home in on mitochondrial genetic effects on longevity. When transferring the flies between vials, every 48 h, we recorded the number of dead flies in each vial. The longevity assay was run over four independent 'experimental blocks' that were temporally separated from each other by one generation time (14 days). The experimental units (combinations of mitochondrial strain duplicate × sex × diet) were balanced in their representation across all the blocks, with each unit represented twice per block. That is, across all the experimental blocks, we maintained two independent copies of each strain duplicate-by-sex combination in separate vials for the two isocaloric diets. These vial replicates were denoted as the "technical replicates" in the statistical analysis.

Statistical analyses

We constructed a linear mixed effects model using the *lme4* package in R (Bates et al., 2015) in R v3.4 to analyse sources of variation affecting longevity. Here, we treated the mtDNA haplotype as a fixed effect in the models, because we were interested in characterising the nature of the mitochondrial genotype × dietary environment (G × E) interactions for longevity, rather than estimating sex-specific levels of mitochondrial genetic variance across diets. Thus, mtDNA haplotype (13 levels), sex (2 levels), diet (2 levels), and the higher-order interactions among these factors were included as fixed effects. In the same model, strain duplicates (26 levels), technical replicates (104 levels), experimental blocks (4 levels), and higher-order interactions between these

factors were included as random effects. We included all possible interactions (up to second order) between fixed and random factors as random effects in the full model.

We derived a reduced model by progressively eliminating higher-order random effects whose removal did not explain a significant ($p<0.05$) amount of change in the model (estimated using log-likelihood tests, and maximum likelihood estimation), commencing first with the highest-order interactions between random effects. We used this step-wise elimination approach to first simplify the random effects component of the model, before proceeding to the fixed effects component of the model. Once we had derived our final model, we estimated the variance attributable to each random effect using restricted maximum likelihood (REML) estimation. We then used the *Anova* function of the *car* package (Fox, 2011) to estimate the significance of the fixed effects in the final model, using a Type III model and Chi-square distribution.

In the above analysis of the full dataset, we found that the second-order interaction term mtDNA haplotype × sex × diet explained statistically significant variation in longevity (log-likelihood ratio test, $\chi^2 = 61.114$, $p<0.0001$). To further probe this three-way interaction, we divided the dataset into two and analysed the male and female data separately. In this second step of the analysis, we built separate *lmer* models for each sex-specific dataset with mtDNA haplotype (13 levels), dietary P:C ratio (2 levels) and the first-order interaction between these two variables as fixed effects. In each sex-specific model, we included strain duplicates (26 levels), experimental blocks (4 levels), technical replicates (52 levels), higher-order interactions between random effects, and higher-order interactions between fixed and random effects as random effects. We took the above described step-wise elimination approach to retain or reduce random effects from the model and derived a final reduced model using the log-likelihood ratio tests in R. The standard deviation attributable to each random effect and their higher-order interactions were estimated using the restricted maximum

likelihood method from the final reduced model. The significance of the fixed effects was estimated from the Type III Wald's Chi-squared test using the maximum likelihood estimation approach in the *car* package.

In a third analysis, we estimated levels of mitochondrial genetic variance for longevity across haplotypes separately for each sex and diet combination. In this analysis, for each combination of sex and diet, we built a random effect *lmer* model with longevity as the response variable, and mtDNA haplotype, strain duplicate, technical replicate, and first-order interactions between mtDNA haplotype and block, and strain duplicate and block as the random effects in the models. We estimated the variance attributable to each random effect from each of the sex and diet random effects models, using the restricted maximum likelihood (REML) estimation. The proportion of variance attributable to the mtDNA haplotypes was estimated from the variance of each random effect using a formula = (Var(X_1)/(Var(X_1) + Var(X_2) +…+ (Var(X_n)) *100, where X_n is the random effect and 'Var' is the variance of each random effect estimated from the *lmer* model. In addition, we estimated the 95% confidence intervals for the variance of each random effect from the same model using a parametric bootstrapping approach in *confint.merMod* function in R.

The panel of 13 mitochondrial haplotypes used in this study was also used in an earlier study that examined the effects of mitochondrial haplotype on male and female longevity on a standard yeast-based diet (Camus et al., 2012). We obtained the mean longevity of all possible combinations of mtDNA haplotype × sex from Camus *et al.* (2012) and estimated the mitochondrial genetic correlations for mean longevity assayed on the isocaloric diets (used in this study) and yeast-based diet (used by Camus *et al.* 2012). We ran these correlation tests in *boot* package in R (Puth et al., 2015). For all possible pair-wise comparisons between trait means, we estimated the Pearson's correlation coefficients (r_p), and the 95% confidence intervals for the r_p. In each correlation test, trait

means were resampled with replacement across 10,000 replicates, and the confidence intervals of the correlation coefficient were estimated from the bias-corrected and accelerated method in *boot* package (Puth et al., 2015).

3.4 Results

Sources of variance affecting longevity

Both the mtDNA haplotype and dietary treatment affected the longevity of flies (*lmer* analysis, mtDNA haplotype: $\chi^2 = 116.115$, $p < 0.0001$, diet: $\chi^2 = 199.907$, $p < 0.0001$, Table 1). Moreover, longevity was affected by interactions between the mtDNA haplotype, sex and diet ($\chi^2 = 61.524$, $p < 0.0001$, Table 1). To further probe this interaction, we analysed the data separately for each sex and observed that the interaction between mtDNA haplotype and dietary P:C ratio was statistically significant only in males (*lmer* analysis, $\chi^2 = 41.038$, $p < 0.0001$, Table 2). Thus, in males, but not in females (*lmer* analysis, mtDNA haplotype × diet: $\chi^2 = 12.089$, $p = 0.4386$, Table 2), the magnitude of the dietary-mediated longevity response was affected by the mtDNA haplotype (Figure 1A and 1B).

Our analyses of levels of mitochondrial haplotype variation for longevity, per sex-by-diet combination, showed substantial mitochondrial variation underpinning longevity in females across both diets, but lower levels of mitochondrial variation in males (Table 3). These sex differences in mitochondrial variation for longevity appear to be primarily attributable to low female trait values of two haplotypes (Brownsville and Oregon) relative to the other haplotypes on both of the diets, and a low female trait value of the Hawai'i haplotype on the high P:C diet (Figure 1C and 1D).

Generally, changes in dietary macronutrient balance affected the longevity of flies. In general, the diet with high P:C ratio caused early death in flies, with the longevity of both sexes reduced by

approximately 35% across the mtDNA haplotypes, compared to the low P:C diet (Figure 1C and 1D). Moreover, levels of sexual dimorphism in longevity were larger on the low P:C diet, with the level of dimorphism, eroded on the high P:C diet (Figure 1C and 1D).

Mitochondrial genetic correlations for longevity, across diets

Generally, intra- and inter-sexual mitochondrial genetic correlations for longevity were positive across the two isocaloric diets used in our study (Figure 2). This means that haplotypes that conferred high longevity on the high P:C diet generally conferred high longevity on the low P:C diet relative to other haplotypes, with the rank order of haplotypes generally consistent across the sexes. In contrast, mitochondrial genetic correlations were weak to absent, when comparing longevity on the isocaloric diets, in which the protein content was determined primarily by the casein content, to longevity on the yeast-based diets of Camus *et al.* (2012). However, there was a signature of a negative mitochondrial genetic correlation involving male longevity when assayed on the high P:C casein diet and male longevity when assayed on the yeast-based diet (Figure 2). Furthermore, there were signatures of negative correlations, across haplotypes, for female longevity when sampled on the isocaloric diets and male longevity when sampled on the standard yeast-based diet.

3.5 Discussion

Here, we examined the contribution of mitochondrial genetic variation to longevity in male and female fruit flies subjected to diets differing only in macronutrient balance. We had two aims. First, to determine whether previously observed male-biases in the magnitude of mitochondrial genetic variance for longevity, measured using the same panel of haplotypes, would be replicable across novel dietary contexts that differed in their ratios of proteins and carbohydrates, but not calorie content. Second, to test whether longevity was affected by G × E interactions involving the mitochondrial haplotype and dietary P:C ratio, and to test whether any such interactions were male-

biased in their manifestation, as reported by Aw *et al.* (2017) who studied mtDNA haplotype-by-diet interactions across two of the haplotypes used in our current study. While we found evidence that mitochondrial genetic variation for longevity was sex-specific in its pattern, unexpectedly this pattern was seemingly attributable to lower levels of mitochondrial genetic variance for longevity in males maintained on the high P:C diet relative to males or females maintained on the other diets. This decrease in mitochondrial genetic variance in males on high P:C diets, resulted in a G × E effect that was apparent only in males, consistent with the results of Aw *et al.* (2017) over an extended mitochondrial panel that included eleven additional haplotypes than used by Aw and colleagues. We discuss the implications of our findings, in light of previous research into the Mother's Curse hypothesis, and recent developments in the study of phenotypic plasticity, and outline suggested avenues of future research enquiry.

A key prediction to arise from the Mother's Curse hypothesis is that the genetic variation that delineates the naturally occurring mtDNA haplotypes within any given species will confer male-biases in the magnitude of its effects on phenotypic expression (Innocenti et al., 2011). Of those studies that have sought to test this prediction, the evidence for male-biases in levels of mitochondrial genetic effects has been mixed (Camus et al., 2012, Camus & Dowling, 2018, Innocenti et al., 2011, Mossman et al., 2016b, Wolff et al., 2016b). Notwithstanding, in many studies, the number of haplotypes surveyed has been too few to accurately home in on true levels of intra-specific mitochondrial variance underpinning the focal traits, whilst overcoming effects of sampling error (Aw et al., 2011, Aw et al., 2017, Pichaud et al., 2013). While in other cases, inferences have been deduced following inter-specific crosses which placed mtDNA haplotypes of one species alongside the nuclear background of a congeneric species with which the haplotypes have no recent evolutionary exposure (Mossman et al., 2016b, Zhu et al., 2014), potentially unmasking cryptic mitochondrial genetic variation (Chevin & Hoffmann, 2017), and complicating inferences.

In particular, few studies have screened for sex differences in levels of mitochondrial genetic variation for longevity, or the capacity for sex differences in patterns of G × E interactions, between mtDNA haplotypes and different dietary contexts, to affect longevity. In 2012, Camus *et al.* screened the same panel of 13 haplotypes in *D. melanogaster* used in our study and reported that the effects of mitochondrial haplotype variation on longevity were specific to males. While, Aw *et al.* (2017) utilised two of the haplotypes used in our study, to reveal mitochondrial genotype-by-diet interactions for longevity in males but not females, across four diets differing in the ratios of protein to carbohydrates. Inspired by these two studies, we set out to screen for sex-biases in effects of mitochondrial haplotype variation for longevity across two isocaloric diets that differed only in their P:C ratios. Contrary to previously reported findings by Camus *et al.* (2012), we did not detect a general male-bias in the magnitude of mitochondrial genetic variation for longevity in our study. Instead, levels of mitochondrial genetic variation were specific to particular combinations of diet in each sex, and in particular, were lower for males maintained on the diet with high P:C ratio. We contend that the discrepancy in our results relative to those of Camus *et al.* (2012) may stem directly from the novel protein sources used in our study (casein, peptone, albumen). Fruit flies of the species *D. melanogaster* have evolved over long timescales to derive their protein from the yeast of fermenting fruits, which is the protein source used in our standard laboratory food and that used by Camus *et al.* (2012). In contrast, the casein-based protein provided to flies in our current study is derived from bovine milk, and likely to represent a novel protein source, whose constitution and relative contributions of essential amino acids, as well as vitamins and minerals, is potentially mismatched to that found in yeast on which flies have evolved. This point is particularly pertinent in light of recent research that has demonstrated that optimal life-history trait expression in fruit flies and mice can be achieved on diets in which the amino-acid constitution is matched to the relative

representation of the amino acids found within a species' exome; a paradigm known as "exome-matching" (Piper et al., 2017).

Theory proposes that exposure to stressful, previously-unencountered environments can disrupt adaptive responses in plasticity, unmask cryptic genetic variation, and take individuals away from their fitness optima (Chevin & Hoffmann, 2017, Ghalambor et al., 2007). This may then account for the failure of our current study to replicate previously observed male biases in the effects of mitochondrial haplotype variation on longevity. Mother's Curse theory proposes that maternal inheritance will remove mtDNA mutations that harm female fitness but fail to screen the set of male expression-specific mutations and that this will lead to male biases in levels of mitochondrial genetic variation underpinning the expression of life history traits (Frank & Hurst, 1996a). Notwithstanding, the environmental arena in which selection acts on the mtDNA sequence should be key to this process. Novel environmental conditions, such as those provided by the isocaloric diets used here, will plausibly unmask cryptic genetic variation in the mtDNA sequence, which while benign or adaptive to females on the yeast-based diets in which the flies have evolved, may incur fitness costs on a novel diet. This contention is supported by our finding of positive correlations, across mtDNA haplotypes, for longevity when measured across the two isocaloric diets used here (both for intra- and inter-sexual mitochondrial genetic correlations), but an absence of mitochondrial genetic correlations when comparing longevity on the isocaloric diets to longevity on the yeast-based diet of Camus *et al.* (2012). That is, the mitochondrial polymorphisms that affect the longevity of females reared on isocaloric diets do not affect the longevity of females when reared on yeast-based diets. Accordingly, we propose that the mitochondrial genetic variation affecting female and male longevity on the isocaloric diets is likely to be non-adaptive, given the lack of prior exposure of fruit flies to these isocaloric diets means that these environments will plausibly represent extreme environments (Chevin & Hoffmann, 2017). A priority for future research will be to test this idea by

measuring levels of sex-specific mitochondrial haplotype variation across diets that differ in their macronutrient ratios, using yeast-based diets that are more closely aligned to the protein and carbohydrate sources that the flies have evolved to feed on in the wild.

As has been shown previously in a number of invertebrate and vertebrate species (Hunt et al., 2004, Jensen et al., 2015, Le Couteur et al., 2016, Lee et al., 2008, Solon-Biet et al., 2015), diets of high P:C ratio conferred striking reductions in longevity; an effect that was upheld across each of the sexes in our study. However, the genetic architecture of such longevity responses to dietary modifications was generally thought to be strictly associated with genetic variation in the nuclear genome of fruit flies, nematodes and mice (Hansen et al., 2005, Liao et al., 2010, Tatar et al., 2014). Here, we have contributed to emerging studies that suggest that mitochondrial genetic variation plays an important role in mediating the link between dietary quality and longevity (Aw et al., 2017, Zhu et al., 2014). Finally, we point out while the strength of our experimental design is that we have been able to assess sex-specific patterns of phenotypic plasticity in longevity responses to dietary quality across a large panel of mitochondrial haplotypes, a limitation of the design is that these effects have all been assessed within the one nuclear genetic background. Mitochondrial functionality hinges on interactions between polypeptides encoded by nuclear and mitochondrial genomes, and this point alone suggests that mito-nuclear interactions will be important in regulating the longevity phenotype (Rand et al., 2004, Wolff et al., 2014b). Indeed, several studies have provided evidence to support this contention (Rand et al., 2006, Zhu et al., 2014). The next frontier will, therefore, be to explore whether previously reported evidence for Mother's Curse effects (Camus et al., 2012), and male-biases in levels of mitochondrial gene-by-environment interactions (Aw et al., 2017), are upheld or change when surveyed across multiple nuclear genetic backgrounds. Such research could reveal hidden complexity in the evolutionary trajectories of life-history traits if it confirms that the

expression of traits such as longevity is routinely underpinned by epistatic interactions spanning two genomes, whose outcomes are moderated across environmental contexts.

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3.7 Tables and Figures

Table 1. Results from the final *lmer* model of the full dataset. The final model included only the significant fixed and random effects including their significant higher-order interactions. We used the Type III Wald's Chi-squared test to estimate the significance of each fixed effect and higher-order interactions in the final model. Furthermore, we used the restricted maximum likelihood method to estimate the variance attributable to each random effect in the final model.

Fixed effects	*d.f.*	*Chi.sq*	*P - value*
Intercept	1	1200.023	<0.0001
mtDNA haplotype	12	116.115	<0.0001
Sex	1	63.698	<0.0001
Diet	1	199.907	<0.0001
mtDNA haplotype × sex	12	74.732	<0.0001
Sex × diet	1	9.127	<0.005
mtDNA haplotype × diet	12	15.664	0.207
mtDNA × sex × diet	12	61.524	<0.0001

Random effects	*Variance*
Block	2.5347
Strain duplicate	0
Strain duplicate × diet	1.0653
Strain duplicate × block	0.7578
Technical replicate	2.1564
mtDNA haplotype × block	1.2199
Residual	78.5497

Table 2. Results from the final sex-specific *lmer* models. The final reduced model for each sex was derived separately using log-likelihood ratio test in R. The models included only the significant fixed and random effects, along with their second-order interactions within and between fixed and random effects. We used the Type III Wald's Chi-squared test in *car* package to estimate the significance of fixed effects and their higher-order interactions from the final reduced model. The restricted maximum likelihood method was used to estimate the variance attributable to each random effect in the final reduced model.

a) Male longevity

Fixed effects	*d.f.*	*Chi.sq*	*P - value*
(Intercept)	1	631.772	<0.0001
mtDNA haplotype	12	45.289	<0.0001
Diet	1	107.83	<0.0001
mtDNA haplotype × diet	12	41.038	<0.0001

Random effects	*Variance*
Block	4.069
Strain duplicate	0
Technical replicate	1.763
MtDNA haplotype × block	0.69
Strain duplicate × diet	1.661
Strain duplicate × block	2.220
Residual	81.729

b) Female longevity

Fixed effects	*d.f.*	*Chi.sq*	*P - value*
(Intercept)	1	889.794	<0.0001
mtDNA haplotype	12	82.801	<0.0001
Diet	1	163.063	<0.0001
mtDNA haplotype × diet	12	12.089	0.4386

Random effects	*Variance*
Block	2.574
Strain duplicate	0
Technical replicate	2.288
mtDNA haplotype × block	2.753
Strain duplicate × diet	2.208
Strain duplicate × block	1.670
Residual	70.954

Table 3. Results from the random effects model analysed for each combination of sex and diet datasets. The proportion of variance was estimated only for the mtDNA haplotype in each model.

a) Male longevity on low P:C diet

Random effect	Variance	95% Confidence interval	Proportion of variance of mtDNA haplotype
mtDNA haplotype	3.001e-13	(-5.53, 0)	0%
Strain duplicate	0	(-5.22, 0)	
Technical replicate	5.23	(2.39, 11.79)	
mtDNA haplotype × block	14.26	(7.17, 31.62)	
Strain duplicate × block	11.55	(5.96, 21.11)	
Residual	88.44	(82.5, 93.59)	

b) Male longevity on high P:C diet

Random effect	Variance	95% Confidence interval	Proportion of variance of mtDNA haplotype
mtDNA haplotype	0.09	(-0.47, 0.34)	0.14%
Strain duplicate	0	(-1.49, 0)	
Technical replicate	1.06	(0.36, 4.23)	
mtDNA haplotype × block	2.19	(0.38, 7.84)	
Strain duplicate × block	2.39	(0.85, 6.22)	
Residual	57.80	(54.94, 62.04)	

c) Female longevity on low P:C diet

Random effect	Variance	95% Confidence interval	Proportion of variance of mtDNA haplotype
mtDNA haplotype	10.51	(1.62, 42.05)	9.2%
Strain duplicate	8.27e-12	(-8.56, 0.3e-10)	
Technical replicate	6.46	(3.51, 14.1)	
mtDNA haplotype × block	9.76	(5.07, 21.16)	
Strain duplicate × block	5.29	(1.78, 11.9)	
Residual	82.2	(77.58, 87.28)	

d) Female longevity on high P:C diet

Random effect	Variance	95% Confidence interval	Proportion of variance of mtDNA haplotype
mtDNA haplotype	12.78	(2.75, 29.11)	17.18%
Strain duplicate	0	(-4.98, 0)	
Technical replicate	3.28	(1.72, 6.44)	
mtDNA haplotype × block	2.85	(0.49, 10.23)	
Strain duplicate × block	5.43	(2.89, 11.78)	
Residual	50.07	(47.18, 53.46)	

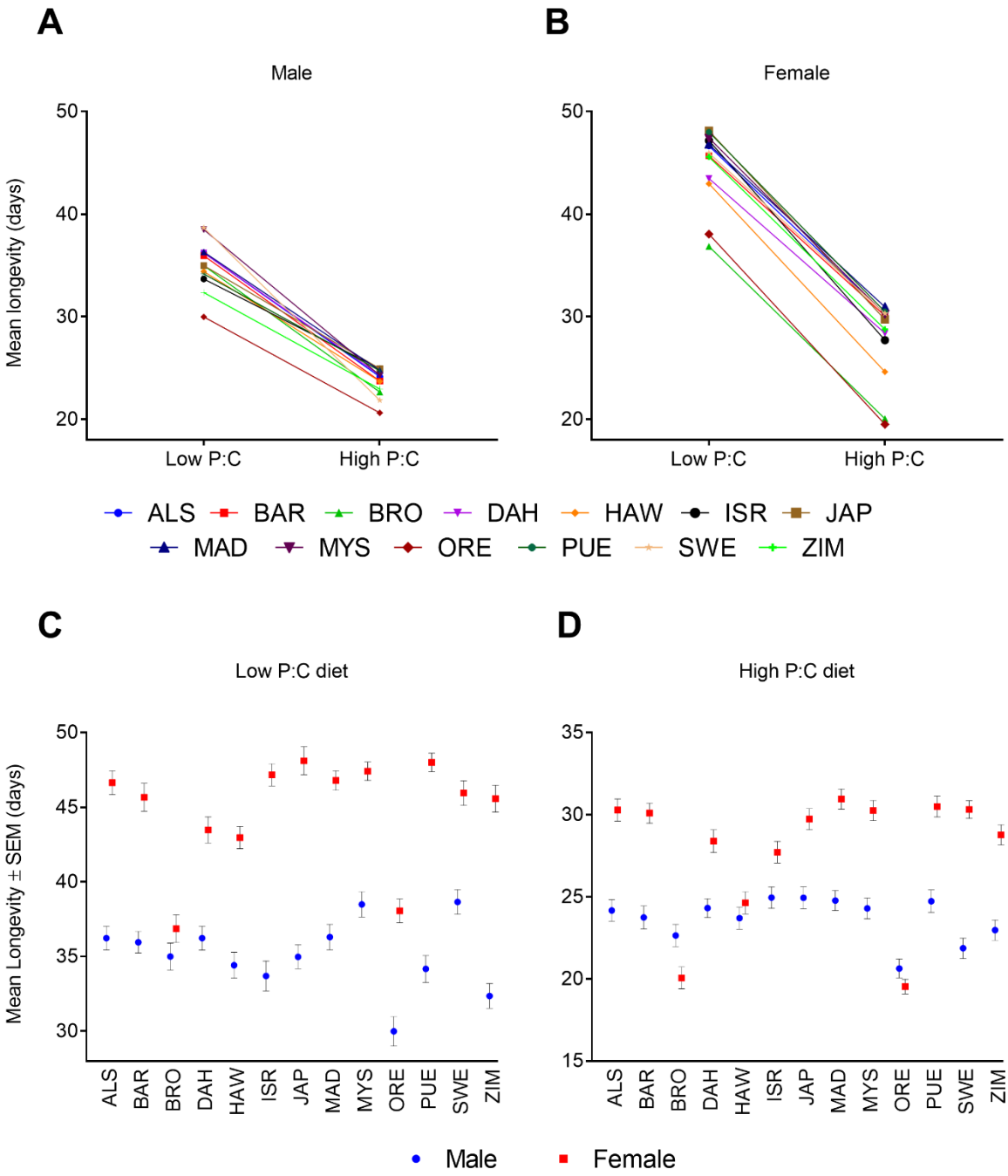

Figure 1. The effects of interactions between mtDNA haplotype and dietary P:C ratios for longevity are shown as reaction norms for A) male and B) female longevity on low P:C (1:8) and high P:C (2:1) diets. Mean longevity ± Standard Error for each combination of mtDNA haplotype and sex on C) the low P:C diet and D) the high P:C diet. The scales of Y-axis are adjusted for each diet in panels C and D to highlight the variation in longevity within each diet. In legend: low P:C indicates an isocaloric diet with P:C ratio = 1:8, high P:C is an isocaloric diet with P:C ratio = 2:1.

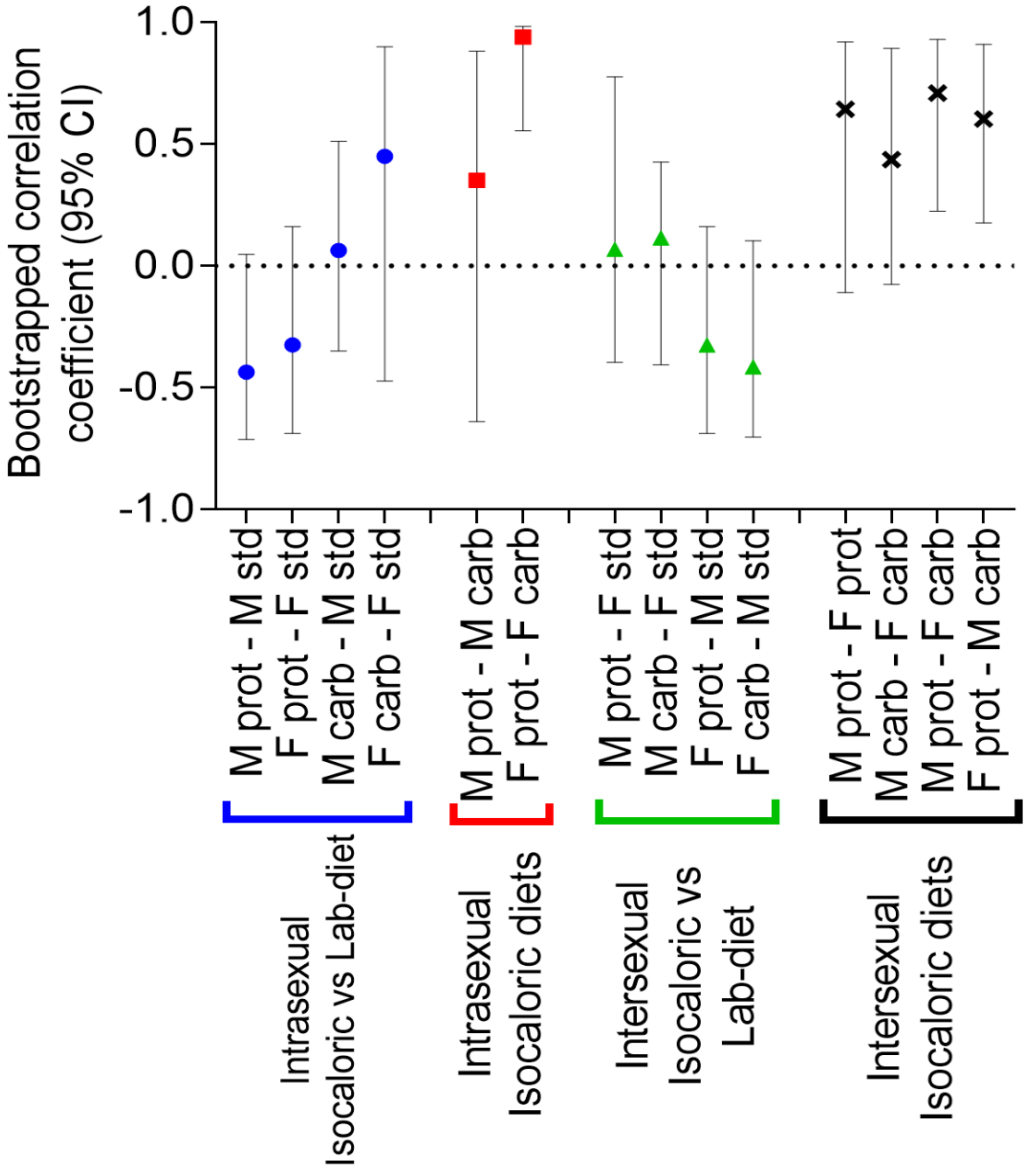

Figure 2. Intra- and inter-sexual mitochondrial genetic correlations for longevity across the different diets used in our study, and those of Camus *et al.* (2012) are shown here. Sex-specific longevity means for each mtDNA haplotype on isocaloric diets and yeast-based lab diet was estimated separately. Pearson's correlation tests were performed on all combinations of pairwise comparison between longevity means. In the horizontal axis, M refers to male, F is female, std – refers to standard laboratory food (yeast-based) used in Camus et al. (2012), carb refers to the low protein high carbohydrate diet (P:C = 1:8) and prot is the high protein low carbohydrate diet (P:C = 2:1) used in this study.

3.8 Supplementary information

Protocol for preparing isocaloric diets

We used the following proportion of materials in each of our cooks.

Ingredients	gram per vial
Water (mL)	7
Agar (g)	0.07
Nipagin (g)	0.007
Dry diet (g)	0.7

We required 102 Drosophila vials supplemented with fresh isocaloric diets for a given day of longevity assay, within an experimental block. That is, 102 experimental units = 26 strain replicates × two sexes × two technical replicates. These 102 vials of fresh food were prepared 48 h before being used in the longevity assay.

During each of our cooks, both the high and low P:C dry diets (designed based on South et al. (2011)) were prepared separately without contaminating one another. The necessary amount of distilled water required to dissolve the solid food ingredients was allowed to boil in a sterile pot. The boiled water was added with agar and was stirred until the agar was dissolved. This liquid was allowed to cool down to approximately 40ºC, was added with nipagin, and stirred until the nipagin was dissolved entirely. Once the nipagin was completely dissolved, we added the dry diets into this mixture and allowed it to cool down to 40ºC, after which the liquid food was dispensed into the Drosophila vials with precisely two millilitres of food in each vial. These vials were allowed to sit overnight at 24ºC to solidify and were inspected for fungal or other contamination before using the vials for longevity assay. Only sterile vials were used for the longevity experiment. Excess vials that were not immediately used for the longevity assay were stored at 4ºC until further use. The vials that were

used for the longevity assay were no older than four days throughout which the excess vials were stored at constant 4ºC.

Protocol for preparing the standard lab diet

The mitochondrial panel has been evolving on a standard lab diet that contains 37.32% yeast, 31.91% dextrose, 23.40% potato medium and 7.45% agar combined with 98.48% H2O, 0.97% ethanol, 0.45% propionic acid and 0.11% nipagen. In case of collecting eggs from adult flies, the vials with standard lab diet were substrate-laced with live yeast to stimulate oviposition in females.

Chapter 4

Mitochondrial haplotypes exert sex-specific effects on the locomotory activity of *Drosophila melanogaster*, independent of the thermal environment

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4.1 Abstract

The "*mitochondrial climatic adaptation*" hypothesis predicts that the regional distribution of mitochondrial haplotypes is shaped by climatic selection on the mitochondrial DNA (mtDNA) sequence. Recent studies have tested this hypothesis in *Drosophila melanogaster*. In this species, the latitudinal distribution of mtDNA haplotypes along the east coast of Australia follows a clinal pattern, with one haplotype (denoted A1) occurring at a higher frequency in the northern sub-tropics, and another haplotype (denoted B1) predominating in the temperate south. Recently, it was reported that flies carrying the A1 haplotype confer greater resilience to heat stress but decreased resilience to cold stress than flies carrying the B1 haplotype; a result that suggests that the latitudinal distribution of these haplotypes has been shaped by thermal selection. A second study reported that the B1 haplotype outcompeted the A1 haplotype, when evolving under cool but not warm temperatures, across replicated populations of the fruit fly, but only when populations were free from *Wolbachia* infection. Here, we further probe the contribution of thermal selection to shaping the Australian distribution of these haplotypes, by examining whether the haplotypes encode differences in locomotory activity in each of the sexes and whether any such mitochondrial genetic effects on locomotion are contingent on the thermal environment. We found that the mitochondrial haplotype affected the locomotory activity of females, but not males. However, we did not find any evidence that interactions between the mitochondrial haplotypes and temperature affected locomotory activity in either sex, suggesting that mitochondrial genetic effects on locomotion in this species are not shaped by thermal selection. We discuss avenues for future research into the role of climatic selection in shaping the dynamics of mitochondrial genome evolution.

Keywords: mitochondrial climatic adaptation; sexual dimorphism; starvation; thermal selection; *Zebrabox*

4.2 Introduction

Our understanding of the mitochondrial genome has changed considerably over the past two decades. The traditional assumption of "selective neutrality" of the sequence variation within the mitochondrial DNA (mtDNA) has been challenged by studies that provided evidence of "phenotype-modifying" effects associated with this variation on a wide range of life-history and physiological traits in metazoans, including genetic diseases in human populations (Dowling, 2014a, Dowling et al., 2008, Hill et al., 2019, Meiklejohn et al., 2007, Wallace, 1992). Yet, while it is clear that the mitochondrial genome often harbours non-neutral sequence variation, the evolutionary processes responsible for shaping the patterns of variation observed across haplotypes remain unclear. One process that could explain the accumulation and maintenance of this sequence variation is mutation accumulation, which would imply that much of the variation comprised a load of deleterious variants that interfere with organismal function (Lynch, 1997). It was traditionally assumed that the mitochondrial genome will exhibit heightened sensitivity to mutation accumulation owing to its high mutation rate, lack of detectable recombination, and combination of maternal inheritance and haploidy, which combined were suggested to decrease the effective population size of, hence efficacy of selection on, the genome (Ballard & Whitlock, 2004, Dowling et al., 2008, Lynch, 1997).

Alternatively, at least some of the mitochondrial sequence variation typically observed within and across populations could have accumulated under adaptive selection, if certain variants increase performance and fitness under prevailing environmental conditions (James et al., 2016, Kivisild et al., 2006, Nachman et al., 1994, Nachman et al., 1996). A growing body of evidence has reported associations between spatial patterns of mtDNA variation and climatic conditions, suggesting that climatic variation may be a key selective force in shaping the standing genetic variation in the mitochondrial genomes of natural populations (Mishmar et al., 2003, Ruiz-Pesini et al., 2004). Notable early evidence for climatic selection on the mtDNA was provided by studies analysing

patterns of mitochondrial sequence variation within and among the human populations, which uncovered correlations between climatic regions and regional variation in the amino acid substitutions; and associations between mitochondrial sequence divergence and temperature across populations (Balloux et al., 2009, Ingman & Gyllensten, 2007, Mishmar et al., 2003, Ruiz-Pesini et al., 2004). These results support the hypothesis that spatial patterns of mtDNA sequence variation, and mtDNA haplotypes, has been shaped by climatic selection. This hypothesis, which has been called the "*mitochondrial climatic adaptation*" hypothesis (Camus et al., 2017), has received further support via studies of other metazoan taxa reporting associations between mutational patterns in the mtDNA and climatic region (Cheviron & Brumfield, 2009, Consuegra et al., 2015, Fontanillas et al., 2005, Foote et al., 2011, Morales et al., 2015).

These association studies inspired other lines of enquiry that have attempted to confirm a causative relationship between climate and patterns of mtDNA variation. To this end, two recent studies examined patterns of mitochondrial variation in vinegar fly populations along the eastern coast of Australia (Camus et al., 2017, Lajbner et al., 2018). Firstly, Camus *et al*. (2017) reported a latitudinal association in population frequencies of two major mtDNA haplotypes segregating within populations. One of these haplotypes, which they denoted A1 was present at higher frequencies in low latitude in the northern sub-tropics, while the other (B1 haplotype, which encompasses four sub-haplotypes they denoted as B1-A to B1-D) predominated in high latitude populations in the southern temperate regions of east coast Australia (Camus et al., 2017). Subsequently, the authors created genetic strains of the A1 and B1 haplotypes that differed only in their mtDNA haplotype but shared a common isogenic nuclear background, thus, enabling them to map variation in thermal tolerance phenotypes to the level of mtDNA sequence across the strains. They found that flies carrying the A1 haplotype exhibited greater resilience to heat stress but less resilience to cold stress, than flies carrying B1 haplotypes (Camus et al., 2017). Secondly, using an experimental evolution approach,

Lajbner *et al.* (2018) tracked changes in the frequency of the A1 and B1 haplotypes in replicate mass-bred populations evolving under four different thermal selection regimes. They replicated their experiment in populations that were either infected or uninfected with *Wolbachia* and showed a consistent response of mtDNA haplotypes to temperature, but only in populations lacking *Wolbachia* infection (Lajbner et al., 2018). Specifically, and consistent with the findings of Camus *et al.* (2017), the B1 haplotype increased in frequency under cooler temperatures, but decreased under warmer temperatures, in populations that had been cured of *Wolbachia* infection (Lajbner et al., 2018). Combined, these two studies provided direct experimental evidence of a role for climatic selection in shaping regional distribution of mtDNA haplotypes in natural populations of vinegar flies, lending support to the hypothesis that similar patterns of latitudinal variation in mtDNA haplotype frequencies observed in other species might also be shaped under climatic selection.

While intriguing, currently we do not understand the mechanisms through which climatic selection can target the mtDNA sequence, in the context of which phenotypic traits underpin the adaptive response. While Camus *et al.* (2017) demonstrated that flies harbouring distinct mtDNA haplotypes differ in their capacity to recover from extreme thermal stresses (0°C and 39°C), Lajbner *et al.* (2018) showed complementary responses of the same haplotypes upon multigenerational exposure to more moderate differences in temperature (18°C and 39°C). To this end, it is important to understand the degree to which sequence variation in the mitochondrial genome affects the expression of key physiological and life-history traits that are likely to be at the centre of adaptive evolutionary processes and to test whether any such mitochondrial genetic effects on these traits are sensitive to heterogeneity in the climatic environment.

Recent evidence indicates that the magnitude and rank order of effects of different mtDNA haplotypes on the expression of metabolic rate, juvenile development rates, and fertility can change

across thermal contexts, at least in insects (Arnqvist et al., 2010, Doi et al., 1999, Dowling et al., 2007a). Here, we extend on this body of research by investigating whether locomotory activity – a composite trait that sits at the nexus between organismal physiology and life-history, is affected by the genetic variation that delineates the A1 and B1 haplotypes in *D. melanogaster* that have previously been implicated in the dynamics of climatic adaptation by Camus *et al.* (2017) and Lajbner *et al.* (2018). Furthermore, we test whether any such mitochondrial haplotype effects on this trait are contingent on the sex of the flies and whether the magnitude or rank order of these effects changes along a thermal gradient of six different temperatures between 18° and 33°C. Notably, these assaying temperatures are broadly representative of the maximum daily temperature experienced along the east coast distribution of the species. The mean maximum daily temperatures along the east coast in the Autumn months of 2014, which is when the flies were collected from the field, range between 15° and 33°C (source: Bureau of Meteorology, Australia). Locomotory activity is a promising trait to study in the context of mitochondrial fitness effects and thermal plasticity. In *D. melanogaster*, the trait is closely aligned with lifetime fitness, but exhibits signatures of sexually antagonistic selection and intralocus conflict. High locomotory activity increases male reproductive fitness but decreases female fitness (Long & Rice, 2007). Locomotory activity should also closely align with variation in mitochondrial respiratory capacity and metabolic rate; traits whose expression has previously been shown to be affected by mitochondrial sequence variation (Anunciado-Koza et al., 2011, Arnqvist et al., 2010, Gianni et al., 2004, Horan et al., 2012, Pichaud et al., 2012, Wolff et al., 2016b), and which are also highly sensitive to the ambient temperature in poikilothermic species (Arnqvist et al., 2010, Blier & Guderley, 1993, Blier & Lemieux, 2001, Kjaersgaard et al., 2010, Pichaud et al., 2010).

4.3 Methods

Australian mitochondrial panel

Previously, Camus *et al*. (2017) created a panel of genetic strains of *D. melanogaster* that differed only in their mtDNA sequence. They harnessed eight isofemale lines (each isofemale line represents a population of flies originating from a single matriline), four of which carried the A1 haplotype, and the other four the B1 haplotype. Two of the lines harbouring the A1 haplotype, and two harbouring the B1 haplotype, were derived from a field-collection from Melbourne (latitude 37.99°, longitude 145.27°), and the other two A1 and two B1 isofemale lines were derived from a geographically disjunct population in Brisbane (27.61°, 153.30°) in Australia. Virgin females from each of these eight isofemale lines (two haplotypes × two populations × two replicates) were then sequentially backcrossed to males from a near-isogenic laboratory stock population originally derived from Puerto Montt (hereafter, PUE) in Chile (41.46°S, 72.93°W), for 27 consecutive generations, to replace the wild-type nuclear genome of the mitochondrial haplotypes with the isogenic nuclear genome derived from the PUE strain. The nuclear genome of the PUE strain had been maintained as isogenic by propagating the strain through single-pair full-sibling mating for 20 continuous generations. Thus, the genetic panel of haplotypes created by Camus *et al.* (2017) consisted of four strain replicates of A1 and four strain replicates of the B1 haplotype, all of which were placed alongside a common isogenic PUE nuclear background.

The A1 haplotype differs from the B1 haplotype at 15 distinct single nucleotide polymorphisms (SNPs) in the protein-coding region of mtDNA. Resequencing of the eight strains revealed an extra level of mtDNA sequence variation. While all of the four A1 strains shared the same sequence, each of the four B1 strains could be further distinguished from each other by the presence of one to four extra SNPs, prompting the authors to denote each B1 strain replicate as a distinct subhaplotype, B1A-B1-D (Camus et al., 2017). Accordingly, each of the B1 sub-haplotypes lacked a truly independent

replicate strain. The existence of independent replication is, however, necessary for studies seeking to partition phenotypic variance into genotypic, from other environmental sources of variation. Therefore, we created a further tier of replication, by splitting a clutch produced by a single female per strain into two duplicates, which were thereafter maintained as independent replicates (thus, creating 16 strain replicates), by backcrossing five virgin females of each strain replicate to five PUE males over an additional 10 generations (Figure 1). Throughout this process, we ensured the PUE strain, used as the nuclear background, remained isogenic by propagating it through a full-sibling pair in each generation. Throughout the backcrossing procedure, the egg density of each vial was maintained at 100 eggs by removing the excess eggs with a sterile spatula; and the age of the females and males used, as well as the time given for mating and egg laying, was also controlled.

Prior to the start of the experiment, each mitochondrial strain replicate had undergone 37 generations of backcrossing to males of the isogenic PUE strain (10 generations of the 16 strain replicates in our study, preceded by 27 generations of the eight strains by Camus *et al*. (2017)). At this point, we then genotyped the mtDNA haplotypes of each of the strain replicates, to ensure that there had been no contamination event throughout the process of their creation. Genotyping was conducted using a Sequenom MassARRAY iPLEX platform (Agena Bioscience, San Diego, CA, USA) by Geneworks (Thebarton, SA, Australia). We designed a custom-made array that enabled us to simultaneously assign genotypes across 16 distinct mtDNA SNP sites (12 of which were diagnostic of the 15 SNPs found across all the A1 and B1 haplotypes, and four that were diagnostic of the B1A-D subhaplotypes). Furthermore, we screened for the presence of the bacterial endosymbiont, *Wolbachia*, across all the 16 mitochondrial strain replicates, using a diagnostic PCR that was designed to detect the presence of the *Wolbachia* Cytochrome *c* oxidase subunit I (*coxA*) gene in the genomic DNA isolated from two adult females per strain replicate (Simoes et al., 2011). Through this method, we confirmed that all mitochondrial strain replicates were free of *Wolbachia* infection.

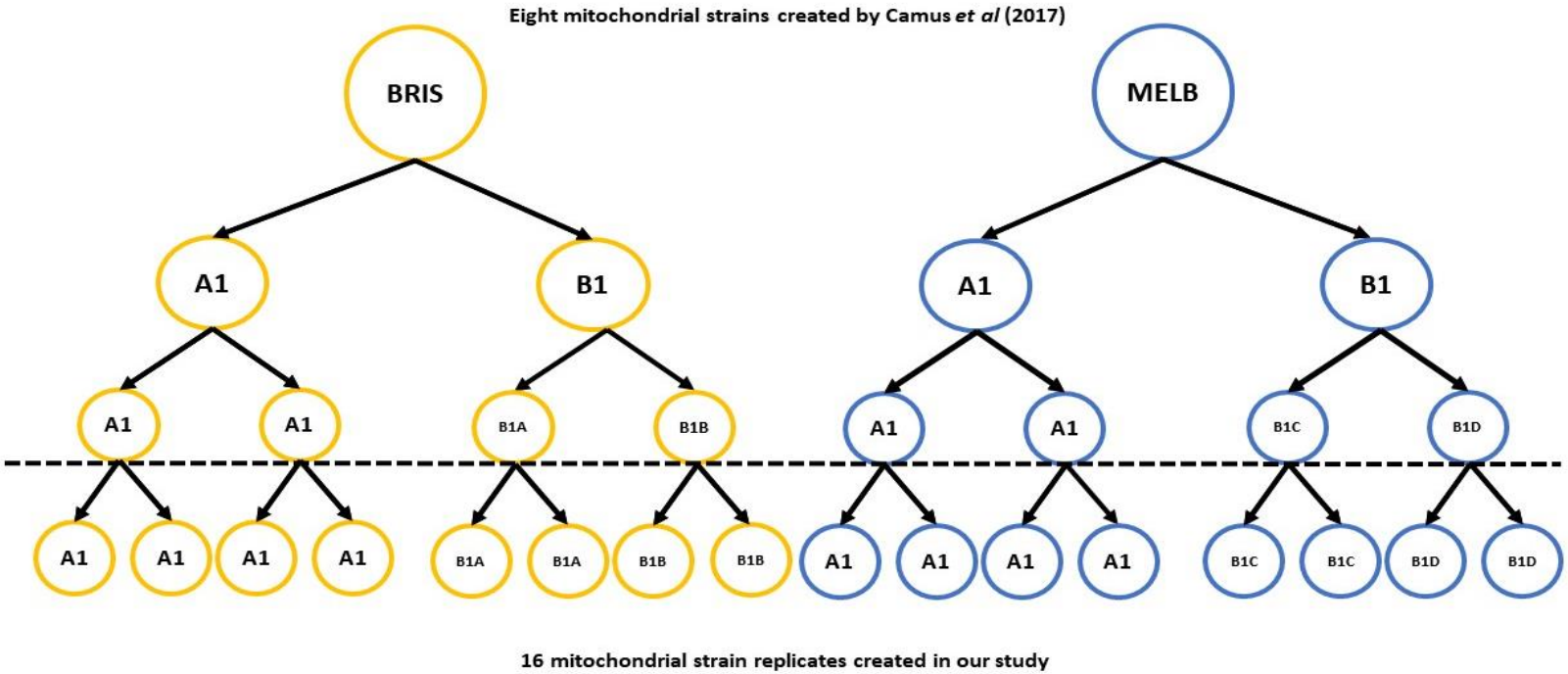

Figure 1. The creation of 16 strain replicates of the Australian mitochondrial panel used in this study is shown here. The panel consists of two distinct mitochondrial haplotypes A1 and B1 sourced from Melbourne (latitude 37.99°, longitude 145.27°) and Brisbane (27.61°, 153.30°) of Australia. The A1 and B1 haplotypes differ in 15 SNPs in the coding mitochondrial genome. The B1 haplotype encompasses four subhaplotypes denoted as B1-A, B1-B, B1-C and B1-D, each of which harbours one to four extra SNPs along with the 15 SNPs that delineates the A1 and B1 haplotypes (Camus et al., 2017). Each of the mitochondrial strains has been replicated into two biological replicates, and the strain replicates backcrossed into a common isogenic nuclear background derived from the PUE strain.

Propagation of focal flies

The locomotory activity of adult flies was sampled across three "experimental blocks" each separated in time by one full generation (that is, 14 days). Within each of these experimental blocks, we conducted the locomotory activity assays across six consecutive "experimental days". We propagated the focal flies used in our experiment through a stringent breeding scheme that ran across three generations, to ensure that all sampled focal flies were of the same age and produced by parents that were all of standard age. Three generations prior to the experiment, we collected eggs from females of each strain replicate, over six consecutive days, with females being transferred to fresh vials every 24 h. These females constituted the great-grandparents of the focal flies assayed in our study. In the following two generations (the grandparents and parents of the focal flies), all flies were 4 days old (since eclosion into adulthood) at the time of ovipositioning. This breeding scheme, therefore, enabled us to precisely control for parental age effects, which have been shown previously to affect phenotypic variation in this species. The age of the great-grandparents at ovipositioning was known and could, therefore, be used in the statistical analyses. We also controlled the density of adult flies (16 pairs per strain replicate), and egg numbers (100), per vial across each of these three generations, and the vials were maintained under standard laboratory conditions, where adult flies were housed on fly food made with 37.32% yeast, 31.91% dextrose, 23.40% potato medium and 7.45% agar combined with 98.48% H2O, 0.97% ethanol, 0.45% propionic acid and 0.11% nipagin, and reared with the constant temperature maintained at 25°C.

Experimental flies

The focal flies were collected from the parents under mild CO_2 anesthesia within 6 h of their eclosion. We collected ten virgin males and ten virgin females from each mitochondrial strain replicate. For each strain replicate, we distributed the 10 flies of each sex across two vials of five flies, housed on standard food (potato-yeast-dextrose) without dry live yeast. These two groups of five flies per sex

per mitochondrial strain replicate served as independent "technical replicates" for the locomotory activity assays. In total, we maintained 64 vials that represented all possible combinations of 16 mitochondrial strain replicates × two technical replicates × two sexes. These vials were maintained for 48 h from the time of eclosion, to allow the flies to recover from any effects of the CO_2 anesthesia (Colinet & Renault, 2012). The flies were then translocated into vials containing access to a diet lacking in nutrients (1% agar added with 10% Nipagin and propionic acid) for another 48-h preceding the assays. We placed the focal flies in this nutrient-deficient diet since a previous study has shown that flies that have been through such treatment exhibit heightened locomotory activity (Dean et al., 2015). We ensured that all focal flies were exposed to this treatment for the same amount of time.

Locomotory activity assay on an automated phenotyping system

We used an automated phenotyping system, consisting of a series of light- and climate-controlled test chambers, called *Zebraboxes* (ViewPoint Lifesciences, France), to assay the locomotory activity of individual focal flies across a thermal gradient. Each Zebrabox consists of a wide-angle lens and infrared light source that tracks the movement of 16 individual flies, each maintained in their own separate tube, in two-dimensional space. The Zebrabox was remotely controlled through the Zebralab tracking software v3.22 (ViewPoint Lifesciences), enabling us to precisely detect the movement of each fly in the assaying area, under conditions of darkness within the box. Furthermore, we used temperature-controllers (JULABO CORIO CD-200F refrigerator/heating circulator, JULABO GmbH, Seelbach, Germany) that were attached to the Zebrabox to regulate the thermal environments within the assaying area. The phenotyping setup employed six Zebraboxes, with two boxes attached to each temperature-controller.

We were able to assay 32 individual flies in each thermal environment, within an "experimental trial". Accordingly, a group of 32 flies was exposed to one of six different temperatures (18, 21, 24,

27, 30 and 33°C), with three temperatures being deployed on any given day of the experiment. Individual flies of each combination of mitochondrial strain replicate and sex were placed into 325 mm^2 tubes (TriKinetics, Waltham, MA, USA), without the use of CO_2 anaesthesia. These tubes were then sealed at each end with 5 mm foam to prevent flies from escaping (thus, the assaying area was restricted to 275 sq.mm). The position of the flies within the Zebrabox's assaying area was randomized across the trials.

We ran two experimental trials in the first experiment Block, and five trials in the second and third blocks, within each day of assay. The trials were run at approximately (± 5 min) 10:30 and 12:00 in block 1; at 10:30, 12:00, 13:30, 14:00 and 15:30 in block 2; and 10:30, 12:00, 13:30, 15:30 and 17:00 in block 3. Thus, across the experiment, we ran six time-trials (10:30, 12:00, 13:30, 14:00, 15:30 and 17:00) across the three experimental blocks. Each trial lasted for 30 min, within which we tracked the overall distance travelled (mm) by each fly inside its tube.

The focal flies were immediately removed from the assay tubes at the end of each trial and were individually transferred into microcentrifuge tubes (Axygen Scientific). These tubes were immediately stored at -20°C for 45 min and the dead focal flies were then individually weighed on a microbalance (Cubis series MSA2.7s-000-DM microbalance, Sartorius AG, Goettingen, Germany), to the nearest 0.0001 mg.

Statistical analyses

Linear mixed effects modelling of data with the locomotory activity of both sexes

The total locomotory activity of each focal fly was calculated using a macro installed in FastData Monitor software (ViewPoint Lifesciences). The data were then analysed in R v3.4.0 (R Development Core Team, 2010) using a linear mixed-effects (*lmer*) model with the total locomotory

activity of an individual fly as the response variable. In the statistical model, we included the mtDNA haplotype (5 levels), temperature (6 levels), and sex (2 levels) as fixed effects, and explored interactions between these fixed effects. The random effects in the same model included variables that explained hierarchical structuring of the data, such as the experimental blocks (3 levels), assaying-day (6 levels, this variable is also indicative of age of the great-grandparents), day nested within the block (18 levels = 6 days × 3 blocks) and trial nested within day and block (68 levels). The random effects also included mitochondrial strains (8 levels), strain replicates (16 levels = 8 mitochondrial strains × 2 biological replicates) and technical replicates (32 levels = 16 strain replicates × 2 technical replicates) that could contribute to environmental sources of variation in our phenotype data. All possible higher-order interactions within random effects and interactions between random and fixed effects were also modelled as random effects. The centre-scaled fly body mass was included in the model as a fixed covariate. Thus, a full model was built with all fixed effects, all possible higher-order interactions within fixed effects, covariate, random effects, higher-order interactions within random effects, and higher-order interactions between fixed and random effects.

We employed a model simplification process that progressively eliminated non-significant higher-order interaction terms across both random and fixed effects of the full model, starting by reducing the higher-order interactions in random effects that explained zero or near-zero variance in locomotory activity. The simplification process compares a reduced model that has one higher-order random effect removed, with the full model that retains the same random effect, using the *log-likelihood ratio* (LLR) test in the *anova* function. If the LLR test returns a non-significant p-value (>0.05), we removed the higher-order random effect from the model and proceeded to remove the next higher-order random effect that explained the least variance. Through this approach, we derived a reduced model that included only random effects which explained significant variance in the

locomotory activity. We followed the same sequential elimination approach for removing non-significant higher-order interactions in fixed effects, commencing with firstly eliminating the non-significant highest-order interactions and proceeding on to removing other interactions from the full model. Thus, a final model with a reduced set of fixed and random effects was derived from which we estimated the standard deviation attributable to each random effect from the summary of the final model, using restricted maximum likelihood estimation in the *lme4* package (Bates et al., 2015). The significance of each fixed effect in the final model, including the fixed covariate was calculated using the maximum likelihood estimation method in the *lme4* package (Bates et al., 2015). The parameter values of fixed effects and their significance were estimated from the final model, using Type III Wald's Chi-square tests in the *car* package (Fox, 2011).

Linear mixed effect model for testing mtDNA haplotype effects on locomotory activity in sex-specific datasets

We were further interested in analysing the locomotory activity separately for each of the sexes, for two specific reasons. Firstly, because the first-order interaction term mtDNA haplotype × sex showed a significant effect on the locomotory activity of flies in our previous step of analysis with the full dataset (*lmer* analysis: $\chi^2 = 20.671$, $p<0.0005$). And secondly, because the magnitude of correlation between body mass and locomotory activity was different in each sex [male data: Pearson's correlation coefficient (r_p) = 0.327, 95% confidence intervals for r_p = (0.294, 0.359); female data: (r_p) = 0.153, 95% confidence intervals for r_p = (0.117, 0.188)], it was difficult to separate out mitochondrial genetic effects on locomotory activity in each sex, from the effects of their body mass. These sex-specific models were identical to the above model, with the exception that 'sex' was not included as a factor. We followed the same model simplification process to derive a final model for each sex.

Estimating least-squared mean locomotory activity adjusted for body mass

Since the magnitude of the correlation between body mass and locomotory activity differed between the sexes, we estimated the least-squared mean (LSmeans) for locomotory activity adjusted for body mass of the flies, separately for each sex using the *lsmeans* package (Lenth, 2016). We built linear models separately for each sex, with factors including the mtDNA haplotype, assaying-temperature, and assaying-time. In these models, body mass was modelled as a covariate. Furthermore, we followed the methods outlined in the *lsmeans* package and accordingly stored the output of the linear models into reference grids using the *ref.grid* function. These reference grids contain the information required for calculating least-square means for all specified independent factors. The Lsmeans locomotory activity adjusted for body mass for all mtDNA haplotypes and Lsmeans for each of the six assaying-temperatures were estimated from the reference grid constructed from each sex-specific dataset.

4.4 Results

The locomotory activity of female flies is affected by the mtDNA haplotype

The mtDNA haplotype affected the locomotory activity of flies, but the magnitude of this effect was dependent on the sex of the flies (Table 1, Figure 2a). We found the mitochondrial genetic effect on locomotory activity to be larger in female, compared to male flies (*lmer* analysis: Table 2a, mtDNA effects on male locomotory activity $\chi^2 = 9.887$, $p = 0.042$; Table 2b, mtDNA effects on female locomotory activity $\chi^2 = 17.793$, $p = 0.001$, Figure 2b). However, the mitochondrial haplotype effects were not moderated by the temperature of the assay, in either sex (Tables 1, 2a and 2b).

The locomotory activity of male flies is sensitive to the thermal environment

The temperature in which the assays were run affected the locomotory activity of adult flies, with the effects contingent on the sex (*lmer* analysis: Table 2a, temperature effects on male locomotory

activity, $\chi^2 = 115.613$, $p<0.0001$; Table 2b, female locomotory activity, $\chi^2 = 42.259$, $p<0.0001$). Male flies were more sensitive to temperature changes, compared to female flies, with the activity of males increasing incrementally with increases in temperature, while the activity of female flies plateaued as temperatures increased above 24°C (Figure 3). Furthermore, these thermally-mediated effects on locomotory activity dissipated throughout the course of the day, being much larger in the morning when the flies were most active, and generally absent by the afternoon when flies were less active (Table 1, 2, Figure S1).

Sex differences in the level of phenotypic expression

The sex-difference in levels of locomotory activity of fruit flies was evident across all assaying temperatures, with males being generally more active than females, at any given temperature (Figure 3). Moreover, the locomotory activity of both sexes was correlated positively with their body mass, albeit the strength of correlation differed across the sexes [male data: Pearson's correlation coefficient (r_p) = 0.327, 95% confidence intervals for r_p = (0.294, 0.359); female data: (r_p) = 0.153, 95% confidence intervals for r_p = (0.117, 0.188)].

4.5 Discussion

The aims of this study were to test whether distinct mtDNA haplotypes, which diverge by only a very small number of SNPs and of which most do not change the amino acid sequence, affect the locomotory activity of *D. melanogaster*, and to determine whether any such effects differ across males and females, and across different temperatures. We confirmed that the mtDNA haplotype affects this trait, with evidence that such effects are stronger in females. We did not, however, find an interaction between haplotype and temperature gradient, which indicates that the mitochondrial effects on locomotion are not sensitive to thermal selection and that this trait is, therefore, unlikely

to be involved in an adaptive mitochondrial genetic response to climatic selection. We discuss these results below.

Studies over the past fifteen years have provided evidence to suggest that the mitochondrial genetic variation that naturally exists within and between populations of animals and plants exerts phenotype-modifying effects on a range of phenotypes (Dobler et al., 2014), therefore challenging the fundamental assumption of "neutral" evolution of mtDNA (Ballard & Melvin, 2010, Ballard & Whitlock, 2004, Dowling et al., 2008, Meiklejohn et al., 2007). The affected phenotypes include broad-scale life history traits such as longevity, fertility, and development rate, to proximate physiological functioning such as the whole-organism metabolic rate and respiratory functioning of the enzyme complexes that regulate OXPHOS (Arnqvist et al., 2010, Chase, 2007, Clancy, 2008, Dowling et al., 2007a, Dowling et al., 2007b, James & Ballard, 2003, Moreno-Loshuertos et al., 2006, Wallace, 1992). Here, we examined a trait at the nexus of life-history and metabolic physiology – locomotory activity, to determine whether this trait is affected by the genetic variation that exists across naturally occurring mtDNA haplotypes. Previous to our study, very few studies had tested for mtDNA haplotype effects on this trait (Roubertoux et al., 2003, Yu et al., 2009). In the studies conducted on mice, Roubertoux *et al.* (2003) and Yu *et al.* (2009), used conplastic strains of mice that differ only in their genotypic combination of mtDNA haplotype and nuclear background, with each showing a clear haplotype effect on a range of complex behaviours including locomotory activity. Our study extends on these findings, showing that such effects likewise exist in *D. melanogaster*, thus providing new insights into a trait whose genetic architecture was previously analysed only from the context of nuclear genome (Jordan et al., 2007).

Despite previous studies indicating that the mtDNA haplotypes used in this study are sensitive to thermal selection (Camus et al., 2017, Lajbner et al., 2018), we did not detect any interactions

between the mtDNA haplotype and thermal regime on locomotory activity. Although the locomotory activity of flies was highly dependent on the thermal environment, increasing with increases in temperature, the magnitude of this increase was unaffected by the particular mitochondrial haplotype an individual harboured. Yet, while we were unable to uncover any evidence that mtDNA effects on locomotion are sensitive to thermal selection, other studies in this and other species have documented cases of gene-by-environment interactions, involving the mtDNA haplotype and environmental temperature, affecting a range of other traits. This includes examples of haplotype-by-temperature interactions affecting male fertility in *D. melanogaster* (Wolff et al., 2016c), and juvenile development and the metabolic rate in seed beetles, *Callosobruchus maculatus*, with the effects further contingent on the nuclear background (Arnqvist et al., 2010, Dowling et al., 2007a). Similarly, Hoekstra *et al.* (2013) has shown that a composite strain of *Drosophila*, comprised of mtDNA from *D. simulans* and nuclear background of *D. melanogaster*, results in a mitochondrial-nuclear incompatibility that affects development rate and larval survival, and that the severity of the incompatibility increases with temperature (Hoekstra et al., 2013).

Finally, we found that the mitochondrial haplotype effects on locomotory activity differed across each of the sexes and were stronger in females than males. Indeed, there was little evidence for sexual antagonism in the effects of the haplotypes. With the exception of subhaplotype B1-C, which was associated with high relative locomotory activity in females, while lower activity in males relative to the other haplotypes, the performance of the other haplotypes was consistent across each of the sexes, but with effect sizes that were larger in females (Figure 2). In a previous study that utilised the same set of strains, Camus *et al.* (2017) reported that subhaplotype B1-D was associated with particularly low tolerance to extreme heat stress in males, but not in females. Here, however, we found that subhaplotype B1-D was associated with the highest locomotory activity in males, suggesting that the

polymorphisms on this haplotype that regulate the link between genotype and phenotype may be exerting pleiotropic effects of the antagonistic sign on different phenotypes.

Generally, the sex-specificity of mitochondrial haplotype effects on locomotory activity that we have detected here is interesting for two reasons. Firstly, previous studies have shown that locomotory activity is under sexually antagonistic selection in *D. melanogaster*, and thus genotypes conferring high activity will result in high male reproductive fitness, but low female fitness (Long & Rice, 2007). This is an interesting point to consider, because it suggests that certain haplotypes, such as B1-C, which exhibited relatively high female activity but low male activity, confers suboptimal trait values in both of the sexes, while haplotypes such as B1-A, which was associated with low activity in both of the sexes, will confer high fitness in females, but at cost to males. These predictions will, however, require formal testing by future studies that measure the reproductive success of flies carrying each of these haplotypes. Secondly, the sex-specificity of mitochondrial genetic effects is interesting in light of a hypothesis, known as Mother's Curse, which predicts that maternal inheritance of mitochondrial genome will facilitate the accumulation of mutations that are male-biased in their phenotypic effects, harming males with little or no untoward effects on females (Beekman et al., 2014, Frank & Hurst, 1996b, Gemmell et al., 2004a). Evidence for this hypothesis has come from the identification of a number of mtDNA mutations that appear to cause effects only on the male phenotype, including male-sterilization in vinegar flies, mice, and hares (Clancy et al., 2011, Dowling et al., 2015, Nakada et al., 2006, Patel et al., 2016, Smith et al., 2010, Trifunovic et al., 2004, Xu et al., 2008), and male-blindness and male-biases in infant mortality rate in humans (Milot et al., 2017).

Furthermore, as mentioned above, Camus *et al.* (2017) found that sub-haplotype B1-D used in our study is associated with low tolerance to extreme heat stress, in males, but normal tolerance levels in

females. One of the key predictions of the Mother's Curse hypothesis is that the genetic variation that exists across naturally occurring mtDNA haplotypes will confer larger effects on the expression of male, than female, phenotypes. Previous studies have tested this prediction in *D. melanogaster*, by leveraging a panel of strains, where a large pool of thirteen mtDNA haplotypes, sourced from distinct global localities, were placed alongside an isogenic nuclear background. These studies have shown that levels of mitochondrial genetic variation for longevity, and genome-wide patterns of gene expression, are larger in males than females (Camus et al., 2012, Camus et al., 2015, Innocenti et al., 2011), and that haplotypes are sexually antagonistic in their effects on reproductive success, with haplotypes that confer high reproductive success in females conferring low success in males (Camus & Dowling, 2018). In contrast, our study found no support for the Mother's Curse hypothesis, given that mitochondrial haplotype effects on locomotion were larger in females. Notwithstanding, it is important to note that our panel was not established in order to test the Mother's Curse hypothesis. The panel contains just a small amount of mitochondrial genetic variation, with fifteen synonymous SNPs separating the A1 and B1 subhaplotypes. Moreover, the panel consists of just two major haplotypes, with the B1 haplotype further delineated into four subhaplotypes. The key prediction of the Mother's Curse hypothesis centres on a test of mitochondrial genetic variance for trait expression, and given the low number of haplotypes, our panel has very low power to test this prediction and therefore our inferences will suffer from sampling error.

In conclusion, we have shown that locomotory activity in *D. melanogaster* is affected by the genetic variation found across naturally occurring mtDNA haplotypes and that the magnitude of these effects differs between males and females. Our results, however, did not support the key prediction that locomotory activity would be shaped by interactions between the mtDNA haplotype and temperature. Previous studies have documented evidence for mitochondrial sequence adaptation to the thermal climate (Balloux et al., 2009, Mishmar et al., 2003, Ruiz-Pesini et al., 2004), including evidence of

involvement of the haplotypes used in the current study in the dynamics of thermal adaptation (Camus et al., 2017, Lajbner et al., 2018). Our study indicates that such thermal adaptation is unlikely to be mediated, however, by thermal selection acting on locomotory activity. While it is clear that the A1 and B1 subhaplotypes used here differ in their capacity to tolerate extreme thermal challenges (Camus et al., 2017), future studies should seek to home in on the core suite of traits through which the mitochondrial DNA sequence can be targeted by, and respond to, climatic selection. Moreover, because mitochondrial function hinges on coordinated interactions between genes spanning both mitochondrial and nuclear genomes (Hill et al., 2019, Rand et al., 2004, Wolff et al., 2014a), it is likely that the epistatic interaction between mtDNA haplotype and nuclear genetic background could be key to determining adaptive trajectories of mtDNA variants under thermal selection. Indeed, several studies have shown that the outcomes of mitochondrial-nuclear interactions on phenotypic expression are altered across different thermal contexts (Arnqvist et al., 2010, Doi et al., 1999, Dowling et al., 2007a, Hoekstra et al., 2013). As such, future studies should seek to further clarify the relative contribution of additive mitochondrial versus epistatic mitochondrial-nuclear contributions to trajectories of thermal adaptation.

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4.7 Tables and Figures

Table 1. Results from the *lmer* model examining effects of mtDNA haplotype, temperature, sex, assay-time and their higher-order interactions on the locomotory activity of flies. The final model was derived through a model simplification process explained in the methods. In the table, the random effects - Day[Block] indicates experimental day nested within block, and Trial[Day[Block]] is experimental trial nested within day and block.

Fixed effects	*d.f.*	*Chi.sq*	*p-value*
(Intercept)	1	106.701	<0.0001
MtDNA haplotype	4	5.905	0.2064
Sex	1	158.447	<0.0001
Temperature	5	55.168	<0.0001
Assay time	5	5.594	0.3478
Body mass	1	297.972	<0.0001
MtDNA haplotype × sex	4	20.671	<0.0005
MtDNA haplotype × temperature	20	29.451	0.0792
Sex × temperature	5	36.021	<0.0001
Sex × assay time	5	21.249	<0.0005
Assay time × temperature	25	69.026	<0.0001

Random effects	*SD*
Mitochondrial strain	312.54
Strain replicate	136.27
Day[Block]	474.10
Trial[Day[Block]]	382.07

Technical replicate	84.93
Mitochondrial strain × sex	0
Strain replicate × sex	158.78
Residual	2385.85

Table 2. Results from *lmer* models examining the effects of mtDNA haplotype, temperature, assay time and their higher-order interactions on the locomotory activity of a) males, and b) females are presented in these tables. In both tables, the random effects - Day[Block] implies experimental day nested within block, and Trial[Day[Block]] is experimental trial nested within day and block.

a) Male locomotory activity

Fixed effects	*d.f.*	*Chi.sq*	*p - value*
(Intercept)	1	268.869	<0.0001
MtDNA haplotype	4	9.887	0.042
Temperature	5	115.613	<0.0001
Assay-time	5	7.565	0.1819
Body mass	1	510.098	<0.0001
Temperature × assay-time	25	51.04	<0.005

Random effects	*SD*
Mitochondrial strain	210.5
Strain replicate	0
Technical replicate	0
Block	310.2
Day[Block]	172.5

Trial[Day[Block]]	486
Residual	2426.9

b) Female locomotory activity

Fixed effects	*d.f.*	*Chi.sq*	*p - value*
(Intercept)	1	126.821	<0.0001
MtDNA haplotype	4	17.793	0.001
Temperature	5	42.259	<0.0001
Assay-time	5	3.931	0.559
Body mass	1	53.579	<0.0001
Temperature × assay-time	25	56.329	<0.0005

Random effects	*SD*
Mitochondrial strain	317.8
Strain replicate	247.7
Technical replicate	288.3
Block	358.6
Day[Block]	549.2
Trial[Day[Block]]	481.3
Residual	2198.3

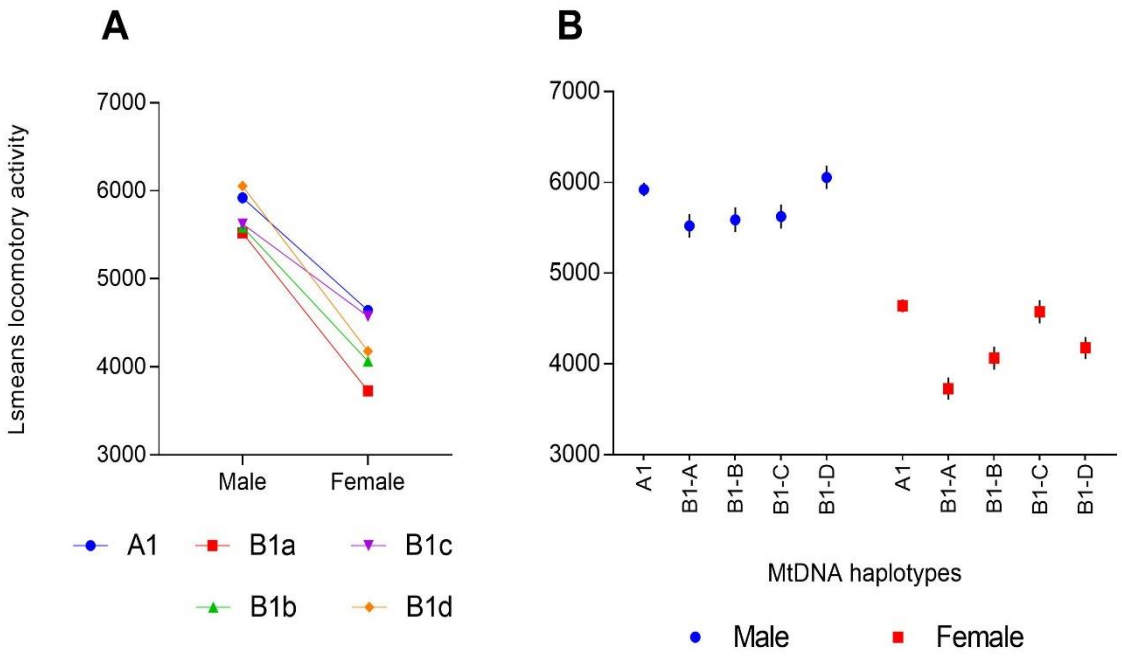

Figure 2. The mitochondrial genetic effects on the locomotory activity of each sex. A) interaction plot showing the variation in Lsmeans locomotory activity between the sexes, across the five mtDNA haplotypes and B) Lsmeans (± 1 SE) of locomotory activity for each mtDNA haplotype in each of the sexes. The locomotory activity was adjusted for body mass and the Lsmeans was calculated separately for each sex in the *lsmeans* package in R.

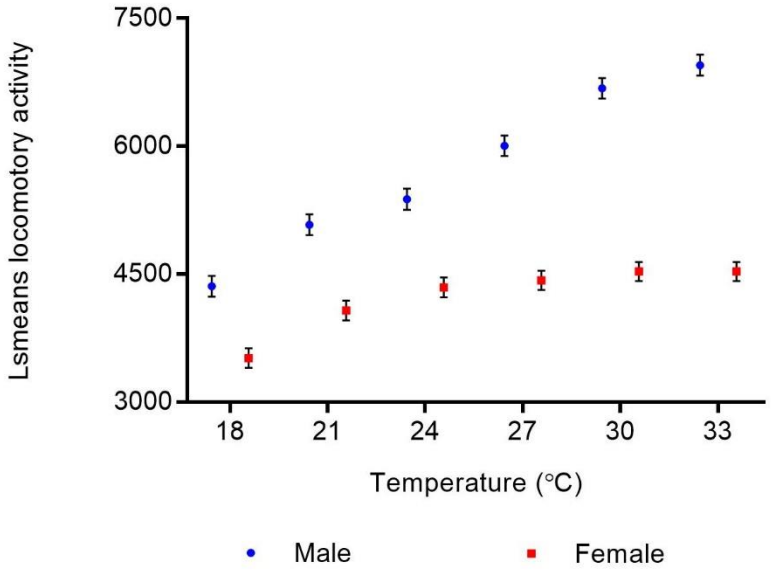

Figure 3. Effect of temperature on the locomotory activity of each sex. The Lsmeans (± 1 SE) of locomotory activity for each sex was estimated using the *lsmeans* package in R, separately from each sex dataset.

4.8 Supplementary information

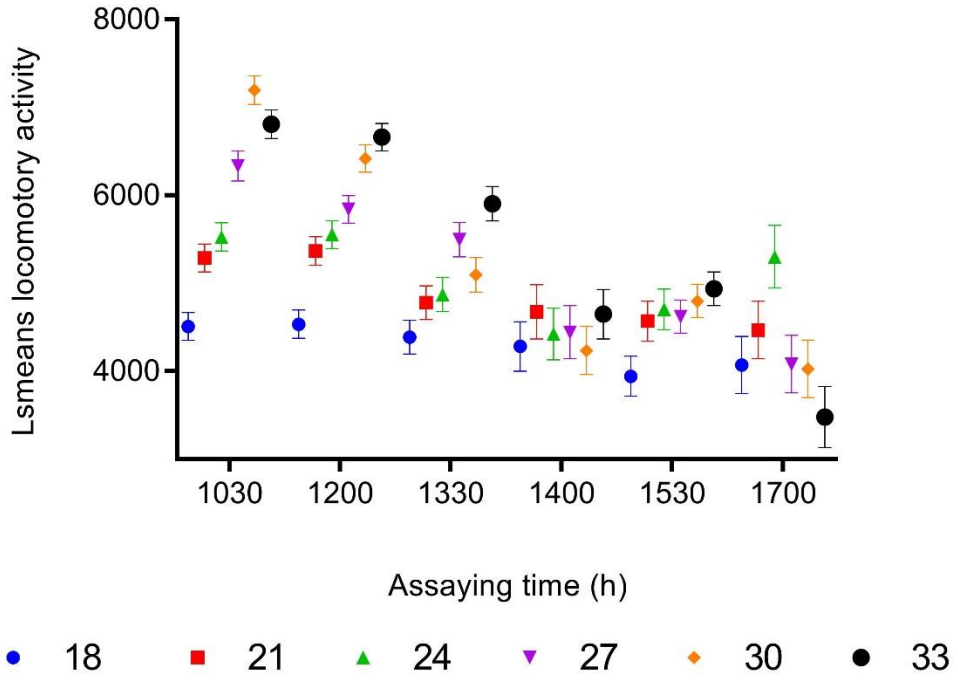

Figure S1. The body mass-adjusted Lsmeans locomotory activity for all combinations of temperature and assaying-time of the day is shown here. The labels in the X-axis indicates the six experimental trials run between 1030 and 1730h on each experimental day.

Chapter 5
General Discussion and Future Directions

5.1 General Discussion

The overall aim of my PhD research was to demonstrate the role of the mitochondrial genome in regulating phenotypic expression in the fruit fly, *Drosophila melanogaster*. I used genetic strains of the fruit flies that differed only in their mitochondrial haplotype but possessed a common and near-isogenic nuclear genomic background, to study the effects of mitochondrial sequence variation on the expression of life-history and physiological traits, across varying environmental conditions. A strength of the strains is that each of these haplotypes exists in a distinct independent replicate, and this enabled me to statistically partition out mitochondrial genetic effects from other sources of confounding variance, be it cryptic residual variation in the nuclear genetic backgrounds of the strains, or environmental sources of variance.

This thesis is structured into three research chapters, each of which reports the results of a stand-alone experiment that probes a specific question pertaining to the evolutionary significance of the mitochondrial genome. Through these experiments, I first aimed to investigate whether previously observed sex-specific effects of mitochondrial genetic variation on the expression of life-history traits, such as lifespan and fertility, extend to the level of organismal physiology, and whether mitochondrial genetic variation is involved in the expression of trade-offs between life-history and physiological traits (Chapter 2). My second aim was to investigate whether mitochondrial genotypic effects on trait expression are subject to genotype-by-environment interactions, thus enabling me to home in on the degree to which the link between mitochondrial genotype and life history phenotype is moderated by heterogeneity in the abiotic environment (Chapters 3 and 4). Currently, it is unclear whether the genes that encode eukaryotic life's most important products – the mitochondrial genes involved in our energy production – exhibit plasticity in their expression in the face of short-term changes in the environment. The existence of mitochondrial genotype × environment interactions would provide new evolutionary insights, for example, into the capacity for environmental

heterogeneity to contribute to the maintenance of mitochondrial sequence variation within populations (Arnqvist et al., 2010, Dowling et al., 2007). Furthermore, my thesis explored novel territory by testing whether the outcomes of mitochondrial genotype × environment interactions might manifest differently across the sexes. I was particularly interested in this idea, given the Mother's Curse hypothesis, which predicts that mitochondrial genomes will be enriched for mutations that are explicitly male-harming in their effects (Frank & Hurst, 1996, Gemmell et al., 2004, Innocenti et al., 2011).

While there has been a significant advance in our level of understanding about the mitochondrial genetic effects on a suite of life-history traits ranging from longevity, fertility, development rate to complex behaviours (Camus et al., 2012, Clancy, 2008, Clancy et al., 2011, Dowling et al., 2007, Patel et al., 2016, Xu et al., 2008, Yee et al., 2013, Yu et al., 2009) and physiological traits including the quantity of mitochondria per cell and *in vitro* functioning of the mitochondrial enzyme complexes that encompass the mitochondrial electron transport system (Moreno-Loshuertos et al., 2006, Pichaud et al., 2012, Sharbrough et al., 2017, Wolff et al., 2016), clear evidence demonstrating the proximate mechanistic pathways through which the mtDNA genotype exerts organismal-level effects is lacking. In Chapter 2, I sought to redress this knowledge gap, by investigating whether mitochondrial genetic effects on *in vivo* metabolic rate could help us to better understand the proximate links between the mtDNA genotype and life-history phenotype. I was specifically interested in testing whether the effects of variation across mtDNA haplotypes on metabolic rate exhibited similar signatures of male bias or sexual antagonism, as previously reported for life-history traits such as longevity and reproductive success (Camus et al., 2012, Camus & Dowling, 2018, Yee et al., 2013). I focused on the metabolic rate because evolutionary hypotheses stemming from life-history theory predict that metabolic rate is the major limiting currency on which the life-history trait expression depends (Dowling & Simmons, 2009, Sheldon & Verhulst, 1996, Stearns, 1989, Zera &

Harshman, 2001). Accordingly, the effects of mitochondrial genetic variation detected at the level of metabolic rate might resonate across the entire life-history of an organism, (Beekman et al., 2014, Camus & Dowling, 2018, Connallon et al., 2018, Frank & Hurst, 1996, Gemmell et al., 2004).

I detected clear male-biases in levels of mitochondrial haplotype variation for metabolic rate, across strains of fruit flies that differed only in their mtDNA haplotype. These findings are consistent with the core prediction of the Mother's Curse hypothesis and aligned closely with previous studies showing similar male-biases in effects of these same strains on longevity and mitochondrial quantity (Camus et al., 2012, Frank & Hurst, 1996, Wolff et al., 2016). Furthermore, the results revealed a strong signature of sexual antagonism in the effects of the mitochondrial genome on metabolic rate, where haplotypes that conferred the highest metabolic rate in females conferred the lowest metabolic rate in males. These results were consistent with the recent findings of sexual antagonism across the same panel of strains for components of reproduction (Camus & Dowling, 2018). To this end, the findings suggest that the maternal inheritance of mitochondrial genome facilitates the accumulation of sexually antagonistic genetic variation; specifically, variants that augment female performance and are therefore fixed under positive selection, despite negative effects on males.

Finally, the results revealed a role for genetic variation in the mitochondrial genome in the regulation of pleiotropic trade-offs between core physiological and life-history traits. Given that the life-history trade-offs have been hitherto thought to be strictly associated with the nuclear genetic variation and given that the evidence for sex differences in trade-offs between various phenotypic traits is scant, the results were striking (Arking et al., 1988, Arnqvist et al., 2017, Johnston et al., 2013). In particular, I found a negative genetic correlation, across haplotypes, between metabolic rate and longevity, which was specific to males. This result is intriguing because it is consistent with a key prediction of the "rate of living" hypothesis, which predicts a negative correlation between metabolic

rate and longevity of eukaryotes (Pearl, 1928, Rubner, 1908). In sum, results from the second chapter have shed new light on mechanisms through which the Mother's Curse process can affect the evolution of sex differences in life-history; and the expression of life-history trade-offs; and have implicated a role for the mitochondrial genome in the dynamics of evolutionary conflict between the sexes.

I then set out to probe for mitochondrial genetic effects on phenotypic traits across different environmental contexts, over the next two chapters. In Chapter 3, the first aim was to determine whether previously observed male-biases in the magnitude of mitochondrial genetic variance for longevity, measured using a panel of thirteen haplotypes on a standard lab diet (Camus et al., 2012), were evident across two dietary environments that differed only in their protein-to-carbohydrate (P:C) ratios but not in their total caloric content. The results showed that levels of mitochondrial genetic variation for longevity differed across the sexes, with these effects contingent on mitochondrial genotype × diet interactions. In general, however, the pattern of variation across haplotypes was not consistent with predictions of the Mother's Curse hypothesis, exhibiting female-biases, at least under some dietary conditions. These results indicate that further work is required to understand the context-dependency of the association between mitochondrial genotype and sex-specific phenotype, in particular, to determine the reasons why previously-observed male-biases in the magnitude of mitochondrial genotypic effects on longevity in the study of Camus *et al.* (2012), were not observed on these two diets. I suggest that the contradicting results observed between the two studies could be associated with the amino acid differences in the source of novel proteins (casein, peptone, albumen) used in this study, compared to the protein source (yeast) that *D. melanogaster* has evolved to consume, and which was used in the Camus *et al.* (2012) study. These novel protein sources used in this study may represent an evolutionary novel, and potentially extreme dietary environments, that this species has not previously been exposed to. These diets may then have

unleashed previously-cryptic and non-adaptive mitochondrial genetic variation for longevity across the sexes (Chevin & Hoffmann, 2017, Piper et al., 2017).

Generally, the results of this Chapter revealed new insights into the association between mitochondrial genotype and diet in regulating longevity outcomes, demonstrating that these G × E interactions may manifest differently in each sex. Notably, I found that the magnitude of G × E effects, attributable to the interactions between mitochondrial haplotypic variation and dietary P:C ratios on longevity, was stronger in males compared to females. Thus, mitochondrial polymorphisms exerted higher levels of plasticity to the dietary environment in males than in females. This result is consistent with the findings of Aw *et al.* (2017), who previously had reported similar male-bias in G × E effect on the longevity of male flies, across two haplotypes of fruit flies housed on four different diets differing in the ratio of protein and carbohydrate. Finally, I confirmed that the diet containing the high concentration of proteins relative to carbohydrates exerted drastic longevity-reducing effects in each sex, a finding that has been reported across several earlier studies on different model animals (Jensen et al., 2015, Le Couteur et al., 2016, Lee et al., 2008, Solon-Biet et al., 2015). Notwithstanding, it is particularly important to note that that low mean lifespan of the haplotypes observed in my study [compared to Camus *et al*. (2012)] is not attributable to the nuclear background used, or the particular mtDNA haplotypes, all of which are naturally occurring in nature, but rather to the synthetic diets used in the current study, that were needed to precisely control the macro and micronutrient intake of individuals. The results extend on earlier findings by demonstrating the magnitude of the cost of high protein on longevity is modified by both the mitochondrial haplotype an individual harbour and the sex of the individual. This study thus highlights a role for mitochondrial genetic variation in regulating the links between diet and longevity.

In Chapter 4, I extended on the research exploring the capacity for mitochondrial genetic variation to be involved in G × E interactions, by exploring the interaction of mtDNA haplotypes with another key abiotic parameter – temperature, which has recently been invoked to shape adaptive trajectories of mtDNA sequence evolution (Mishmar et al., 2003, Ruiz-Pesini et al., 2004). Specifically, I leveraged mtDNA haplotypes from the Australian east coast distribution of fruit flies, *D. melanogaster*, which have previously been implicated in the dynamics of thermal adaptation (Camus et al. 2017, Lajbner et al. 2018). The panel of five distinct mitochondrial haplotypes differed from each other by only a handful of single nucleotide polymorphisms (SNPs), most of which were synonymous substitutions that do not produce an amino acid change (Camus et al., 2017). Akin to the large panel of thirteen mitochondrial haplotypes used in Chapters 2 and 3, these haplotypes shared a common isogenic nuclear background and were present across two biological replicates.

A major aim of this chapter was to test for a role of mitochondrial haplotypes in regulating the expression of a key phenotypic trait, locomotory activity, across a thermal gradient. Evidence of mitochondrial haplotype-by-thermal environmental interactions on activity would add support to an evolutionary hypothesis known as the "*mitochondrial climatic adaptation*", which predicts that the spatial variation in the mitochondrial genetic diversity is shaped by climatic selection (Camus et al., 2017, Mishmar et al., 2003, Ruiz-Pesini et al., 2004). To date, the majority of support for this hypothesis comes from studies reporting clinal associations (both latitudinal and altitudinal clines) in population frequencies of particular mtDNA haplotypes across the geographical distributions of several species (Balloux et al., 2009, Cheviron & Brumfield, 2009, Fontanillas et al., 2005, Foote et al., 2011, Mishmar et al., 2003, Morales et al., 2015, Ruiz-Pesini et al., 2004). One key prediction to arise from this hypothesis is that the mitochondrial genotypes under climatic selection would encode for locally adapted mitochondrial OXPHOS proteins that have a functional advantage over the other variants, in their native climatic environment. This prediction, however, has proven to be difficult to

demonstrate experimentally. It is further unclear as to which organismal traits respond directly to climatic selection on mitochondrial proteins.

The earlier empirical evidence for the hypothesis was recently supported by two experimental studies that leveraged the same haplotypes that I used in my study to provide the first experimental support for the hypothesis (Camus et al., 2017, Lajbner et al., 2018). Camus *et al.* (2017) showed that the A1 mitochondrial haplotype, which is more predominant at subtropical low latitudes is associated with higher tolerance to extreme heat stress, but lower tolerance to extreme cold stress, than B1 haplotypes that predominate at temperate high latitudes of the Australian east coast. Lajbner *et al.* (2018) used an experimental evolution approach and found that the B1 haplotype increased consistently in frequency within replicate populations exposed to cooler thermal regimes than populations exposed to warmer regimes; but that these effects were only observed in populations that were free of infection with the *Wolbachia* endosymbiont.

In this chapter, I expanded the studies of Camus *et al.* (2017) and Lajbner *et al.* (2018) and tested whether the effects of these haplotypes on a key trait – locomotory activity – that stands at the nexus between organismal physiology and life-history is contingent on interactions with the thermal environment and whether the outcomes of any such G × E interactions may be specific to one or other of the sexes. In particular, I explored the contention that locomotory activity of fruit flies would be affected by mtDNA haplotype variation, and that the magnitude of the effects would vary across a thermal gradient, with the A1 haplotype maintaining higher activity than the B1 haplotype at higher temperatures, in line with results of Camus *et al.* (2017) and Lajbner *et al.* (2018). However, I found no evidence for G × E interactions affecting the locomotory activity of either sex, indicating the locomotory activity of each haplotype is not optimised at different temperatures.

Notwithstanding, the analyses uncovered mitochondrial genotypic effects on locomotory activity that was specific to each of the sexes. This indicates that complex phenotypes such as locomotory activity are shaped in part by a small number of SNPs, most of which do not change the amino acid sequence, that delineate each of the five mitochondrial haplotypes used in this study. These findings add new evidence for mitochondrial genomic control of the locomotory activity in fruit flies, which was otherwise thought to be associated with the nuclear genome (Jordan et al., 2007). Consistent with the results of Chapter 3, and contrary to the key prediction of the Mother's Curse hypothesis, I again observed that the magnitude of mitochondrial genetic effects on the expression of locomotory activity in adult flies was female biased. Further attention is required to determine the effects of these Australian haplotypes on a range of life-history traits, to determine whether general patterns of female-bias in effects are maintained across other traits. While previous work has identified signatures of Mother's Curse (male-biases in levels of mitochondrial genetic variation) for adult life-history traits of reproductive success (Camus and Dowling 2018) and longevity (Camus *et al.* 2012, but see Chapter 3), as well as for the metabolic rate (Chapter 2), this is, however, the first study to screen for sex biases on locomotory activity; and thus, it is unclear whether this trait will be generally susceptible to the Mother's Curse process.

The Mother's Curse hypothesis predicts that traits with high intersexual genetic correlations are unlikely candidates to be affected by male-harming mutational variation since selection for optimised mitochondrial function in the female homologues of these traits should lead to optimised mitochondrial function in the male homologue (Frank & Hurst, 1996, Gemmell et al., 2004). The intersexual correlation for locomotory activity is high (Long & Rice, 2007), indicating that this trait may be less prone to effects of male-harming mutational variation relative to traits that are generally sex-limited in expression, such as male versus female tissues involved in reproduction [for example, the testes versus ovaries (Innocenti et al., 2011)]. Further work is thus required to examine the

generality by which Mother's Curse effects are observed across traits. For instance, in this same panel of haplotypes, Camus *et al*. (2017) observed that one particular sub-haplotype (B1-D) was associated with particularly poor tolerance to heat stress – but that this effect was only manifested in males, a result consistent with Mother's Curse. This same sub-haplotype is, however, associated with high male activity in this study – indicating that mitochondrial haplotypes can exert negative pleiotropic effects on different traits, resulting in poor performance of one trait, while maintaining function in another. Finally, it must be noted that the scale at which I tested for mitochondrial genotypic effects differed from other studies that have found evidence for the Mother's Curse hypothesis (Camus et al., 2012, Camus & Dowling, 2018, Innocenti et al., 2011). The haplotype in this study differed by just a few SNPs, most of which do not encode for a change in the amino acid sequence; whereas the haplotypes used in other studies were sourced from the global distribution of haplotypes in *D. melanogaster* and contained numerous non-synonymous SNPs that delineated each haplotype. It is thus, plausible that the theoretical predictions for what I should expect to observe across these two biological scales (intra-population in my study versus inter-continental in other studies) will differ, and that Mother's curse mutations are all fixed at the within-population scale if they have accrued over long periods of evolutionary time, which would lead to no male-bias across mtDNA variants at this scale.

5.2 Future directions

In conclusion, this thesis expands the list of core phenotypic traits that are affected by the sequence variation that have naturally accumulated within the mitochondrial genome. Most importantly, I have provided evidence for context-specificity of these genetic effects on the phenotypic expression that is specific to sex and are sensitive to the heterogeneity in dietary, but not, thermal environments.

While Chapter 2 of my thesis provided strong evidence that the Mother's Curse process affects core physiological function – in the metabolic rate, leading to negative intersexual correlations across mtDNA haplotypes; the results of Chapters 3 and 4 questions the generality of Mother's Curse effects in nature – since these studies revealed a lack of male-bias, and in general a tendency for female-bias in the magnitude of mtDNA haplotype effects across different environmental contexts. Moreover, the nuclear genetic background against which the mitochondrial genotypes were placed was kept isogenic (by serially propagating the strains through an inbreeding scheme) to partition out the true and absolute measurable phenotypic effects exerted by the mitochondrial haplotypic variation from any nuclear genetic effects. While this may be the best possible way to detect mitochondrial effects on phenotypic expression, I acknowledge that this genomic architecture is not a true representative of that of the natural populations of fruit flies. That is, in nature, it is expected that the mitochondrial genotypes are expressed against an outbred nuclear genome that express a lot of genetic variation. Therefore, further research is urgently needed to better understand the context-dependency by which male-biases in mitochondrial genotypic effects manifest in natural populations. Several outstanding questions remain largely unaddressed. These are: what traits should be most susceptible to accumulation of male-harming mtDNA mutations; at what scale do we expect these effects to manifest; under what environmental conditions; and finally, would previously-reported evidence for the Mother's Curse hypothesis be upheld if the haplotypes had been sampled under a diverse range of nuclear backgrounds that are non-isogenic? Currently, there is little to no theory that addresses these questions, and only a small number of empirical studies have turned their attention to these questions. Indeed, most studies that have sought to examine the link between mitochondrial genotype and phenotype have suffered from limitation that they have only examined effects in one sex, or in one nuclear genetic background (Vaught & Dowling, 2018) – and this has resulted in a large constraint on our capacity to derive general conclusions as to the prevalence of the Mother's Curse

process in natural populations. I hope that my thesis will inspire a larger number of researchers to turn their attention to testing the generality of the Mother's Curse hypothesis.

Another key outcome of my thesis was to show that mitochondrial genotypes can exhibit a high degree of plasticity in expression across environmental contexts and are thus involved in G × E interactions. Notwithstanding, while I found mitochondrial by environment interactions affecting longevity in Chapter 3, I was unable to find such interactions affecting locomotory activity in Chapter 4. Given that each of these Chapters utilised different panels of haplotypes, which were sampled over different spatial scales and therefore differed in their level of mitochondrial genetic divergence (larger genetic variation across haplotypes in Chapter 3 compared to the strains used in Chapter 4) and different environmental treatments (diet in Chapter 3 versus temperature in Chapter 4), more research is required to attempt to distinguish whether the differences between studies are primarily mediated by discrepancies in the level of genetic divergence screened between studies, or in the environmental treatments used.

Finally, I note that our knowledge of mitochondrial genetic effects on organismal function is currently limited to studies on model animals, such as mice, fruit flies, and seed beetles. Future studies should seek to embrace these questions across a range of non-model species including extending to non-bilaterian metazoans. We stand at an exciting time for the study of mitochondrial genetic regulation of the phenotype. The last decade has witnessed a wave of research interest in this field and implicated that the genetic variation in the mitochondrial genome, which was previously considered to be selectively neutral, may well be implicit in mediating a range of key ecological processes, from reproductive isolation and speciation to the evolution of sex, to sexual selection, and climatic adaptation (Hill et al., 2019). My thesis has contributed to this growing field, by showing that the link between the mitochondrial genotype and phenotype is complex and likely to depend on

both the scale and the environment under which the experiments are conducted. I hope that my studies will inspire many evolutionary biologists to turn their attention to the evolutionary genomics of the mitochondrial genome, to resolve many of the outstanding questions that have emerged from this body of research. Such studies would not only help biologists to home in on the genetics underpinning complex ecological and evolutionary processes but would be likely to uncover new insights relevant to the health of our own species.

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