



MONASH University

Effects of Mitochondrial Genomic Variation on Life History Trait Expression in *Drosophila melanogaster*

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B.Sc (Hons)

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ABSTRACT

The mitochondria are essential for life in eukaryotes, taking centre-stage in the process of cellular respiration. This process is regulated via a series of finely coordinated interactions encoded by two obligate genomes – nuclear and mitochondrial. Both genomes are required for the production of cellular energy, and thus their harmonious interaction is vital for the maintenance of mitochondrial integrity and the viability of eukaryote life. Recently many studies have shown an abundance of phenotype-changing genetic variation segregating within the mtDNA genome – and these results run counter to the traditional paradigm in which mitochondrial genetic variation was expected to be evolving neutrally. It remains unclear how this variation is accumulating – either adaptively under selection, or non-adaptively under mutation-selection balance. Furthermore, maternal inheritance of the mitochondrial genome predisposes this genome to accumulation of mutations that have male biased effect, and the existence of these male-harming mutations has recently been empirically substantiated.

The aim of my thesis is to explore and elucidate the nature of the evolutionary processes that shape the molecular composition of the mitochondrial genome. My goal was to understand how much of the genetic variance accumulating within the genome is sex-specific, and in particular male-biased. This would support the idea that mitochondrial variation consisted largely of deleterious mutation loads that accumulate under maternal transmission. Secondly I was interested in understanding how much genetic variation is adaptive – occurring in both sexes, and exhibiting phenotypic responses to thermal stresses that concord with expected predictions based on where the mtDNA haplotypes have evolved. Finally, I aimed to elucidate the molecular mechanisms that bridge the link between mitochondrial genotype and phenotype.

GENERAL DECLARATION

I hereby declare that 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.

This thesis includes 1 original paper published in a peer reviewed journal and 2 publications to be submitted. The core theme of the thesis is Mitochondrial Evolutionary Genomics. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Biological Sciences under the supervision of Dr. Damian Dowling

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

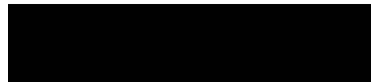
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Other work produced during this my thesis period:

- J.N. Wolff*, **M.F Camus***, D.J. Clancy, D.K. Dowling (2015) “Complete mitochondrial genome sequences of thirteen globally sourced strains of fruit fly (*Drosophila melanogaster*) that form a powerful model in mitochondrial research” *Mitochondrial DNA – In Press*
- J.N. Wolff, N. Pichaud, **M.F. Camus**, G. Côté, P.U. Blier, D.K. Dowling “Evolutionary Implications of Mitochondrial Genetic Variation: Mitochondrial Genetic Effects on OXPHOS Respiration and Mitochondrial Quantity Change with Age and Sex in Fruit Flies” *Journal Of Evolutionary Biology - In Press*
- **M.F. Camus**, D.J. Clancy, D.K. Dowling (2012) “Mitochondria, maternal inheritance and male ageing” *Current Biology* 22 (18), 1717-1721

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

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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

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CHAPTER 1

Introduction

1.1 General Introduction

Mitochondria are the eukaryotic organelles responsible for energy production. These organelles are thought to have evolved as the result of a symbiotic event between an α -proteobacteria and a secondary free-living protoeukaryotic bacteria (Gray et al. 1999). The change from symbiont to organelle represents a pivotal moment in the evolution of life, forming the basis for the Domain of life *Eukaryota* (Margulis and Bermudes 1985; Lane 2007).

The mitochondria are the primary energy producers for the eukaryotic cell, but in addition to this primary role, mitochondria are involved in a range of other vital processes that control key aspects of cellular growth and regulation; such as signalling (Chandel 2015), cellular differentiation (Vega-Naredo et al. 2014) and cell death (apoptosis) (Wang and Youle 2009). Mitochondria produce energy in an extremely efficient manner through the mitochondrial energy machinery known as the Electron Transport Chain (ETC). The ETC encompasses a series of protein complexes within the inner mitochondrial membrane, which produces Adenosine-Triphosphate (ATP) via the process of Oxidative Phosphorylation (OXPHOS) (Mookerjee et al. 2010). This process is regulated via a series of finely coordinated interactions encoded by the two obligate cellular genomes - nuclear and mitochondrial (Rand et al. 2004).

During the course of evolution, the circular mitochondrial genome has experienced the loss of mitochondrial-encoded genes via translocation to the nuclear genome of the host (Bratic and Trifunovic 2010). This relocation process resulted in the transfer of most of the ancient mitochondrial genome into the nucleus (Timmis et al. 2004), with mitochondria now generally harbouring a small genome of around 20,000 base pairs (depending on the

particular species), with a very compact gene organization (Anderson et al. 1981; Clayton 2000). High levels of packaging include overlapping open-reading frames of genes, lack of introns, and a lack of stop codons that are instead added post-transcriptionally (Goddard and Wolstenholme 1980; Wolstenholme 1992; Taanman 1999; Clayton 2000). Although small in size, the mitochondrial genome encodes 13 critical OXPHOS subunits out of the approximately 85 subunits that encompass the whole of the OXPHOS system. The process of OXPHOS uses the oxidation of metabolites and co-enzymes to produce ATP (Hatefi 1985; Chance and Williams 2006) by a pathway composed of five enzyme complexes, four of which are encoded by both mitochondrial and nuclear genomes (Figure 1) (Liu et al. 2002; Zuryn et al. 2010).

1.2 Mitochondrial Genetic Variance

The mitochondrial genome is considered a fundamental tool for modern evolutionary genetics, due to its high mutation rate, maternal inheritance and negligible recombination in most species (Lynch 1997; Pesole et al. 2000; Saccone et al. 2000; Ballard and Whitlock 2004). For decades, mitochondrial DNA (mtDNA) was used widely to reconstruct genealogies and describe population genetic structure (Moritz et al. 1987) under the assumption that mutations accumulate in a random manner (a neutral marker) (Brown et al. 1979; Kimura 1983; Cann et al. 1984; DeSalle et al. 1986; Kocher et al. 1989).

However, this assumption of selective neutrality has been repeatedly challenged over the past two decades (Ballard and Kreitman 1995; Dowling et al. 2008). First, fitness consequences have been associated with naturally occurring levels of mitochondrial genetic variation that are found both within and between natural populations (Maklakov et al. 2006; Melvin and Ballard 2006). More specifically, mitochondrial genetic effects on life-history traits have

been demonstrated in studies using *Drosophila melanogaster* (Camus et al. 2012; Yee et al. 2013). These studies used *Drosophila* strains harbouring an isogenic nuclear background alongside varying mitochondrial haplotypes, thus variation in phenotypic expression could be directly attributed to genetic variation within the mitochondrial genome.

Second, several studies have found signatures of positive selection on the mitochondrial genome by examining the mutational profile of mtDNA sequences and comparing the ratio of non-synonymous (dN – changes amino acid) to synonymous (dS – does not change amino acid) mutations (Mishmar et al. 2003; Ruiz-Pesini et al. 2004; Meiklejohn et al. 2007). The ratio between these two types of mutations (dN/dS) indicates the potential for functional mutations to be accumulating within the gene (or genome) of interest. If the dN/dS ratio of a gene is above the value of 1, then generally it provides evidence that the gene has been shaped by positive selection. Early studies by Lynch & Blanchard (1998) found that mitochondrial genes had higher ratios of non-synonymous to synonymous mutations in relation to the nuclear genome of plants, invertebrate and fungal taxa (Lynch and Blanchard 1998). There have been numerous studies since that have found signatures of positive selection acting within the mitochondrial genome. Most recently, Morales et al (2015) found evidence for positive selection on several amino acids in the mtDNA of the Australian eastern yellow robin (*Eopsaltria australis*) populations. The authors additionally found nuclear genome homogeneity within the robin populations sampled indicating that there was high levels of gene flow, thus the signatures of positive selection were unique to the mtDNA (Morales et al. 2015). The combined outcomes of these studies suggest that certain mtDNA-coding genes of natural populations might have been shaped by positive selection. However, the basic population genetic characteristics of the mitochondrial genome, also suggest that mutation accumulation is likely to be a key contributor to the accumulation of mitochondrial genetic variation seen within and across populations. The reasons are outlined below.

1.3 Mitochondrial Mutation Accumulation and Effective Population Size

From a population genetics perspective, there are two key differences between the mitochondrial and nuclear DNA; maternal inheritance and haploidy of the mtDNA relative to the biparental inheritance and diploidy of most nuclear genome regions (sex chromosomes excluded) (Ballard and Kreitman 1994). This means that for every mitochondrial genome copy being transmitted from one generation to the next, there are four copies of autosomal nuclear DNA transmitted. This process results in the effective number of mtDNA alleles being one-quarter of that of the nuclear genome. Thus the mtDNA has a smaller effective population size (N_e) than the nuclear genome, and is thus predicted to accumulate mutations more quickly than the nuclear genome (Rand, 2001). This is because natural selection is less efficient in shaping genetic variation at smaller N_e , with the effects of genetic drift amplified. Random genetic drift refers to the fluctuation of allele frequencies as a result of stochastic events (Kimura 1983), and is thought to be an important force involved in the process of accumulating mutations of minor effect.

Non-neutral genetic variation is also expected to accumulate in the mitochondrial genome as a result of the negligible levels of recombination within this genome (Ladoukakis and Eyre-Walker 2004). The lack of recombination in the mitochondrial genome means that mutations that appear within the mtDNA sequence cannot be purged from the genome without sending the entire mtDNA lineage to extinction – a process akin to Muller's Ratchet (Muller 1964).

Another important feature that increases the capacity for mutation accumulation in the mitochondrial genome is the increased mutation rate in comparison to the nuclear genome. In addition to producing energy in the form of ATP, the process of OXPHOS also produces reactive oxygen species (ROS) as by-products (Barja 2004). ROS are chemically reactive molecules containing oxygen (oxygen ions and peroxides) (Muller et al. 2007), and are

highly mutagenic. The close proximity of the mtDNA to ROS production has traditionally been thought of a possible cause of high mutation rate (Barja 2004). However, there is a lack of experimental evidence to support reactive oxygen species increasing mitochondrial mutation rate (Siede and Doetsch 2005). Interestingly, ROS causing mtDNA mutations is one of the backbone assumptions of the ‘Mitochondrial Theory of Ageing’. This theory predicts mutations accumulate within the mtDNA following ROS-induced damage, and will in-turn result in disruption to OXPHOS function. Such OXPHOS disruption augments the production of ROS – leading to a vicious mitochondrial cycle resulting in ageing and eventually cell death (Harman 1972; Balaban et al. 2005; Chen et al. 2007).

Furthermore, the mtDNA is not associated with histone proteins, which in the nuclear genome protect the nuclear DNA (Ljungman and Hanawalt 1992). Thus, it has been thought historically that the mtDNA is less protected against mutational degradation than is the nuclear DNA. Mitochondrial DNA replication is thought to cause the vast majority of the mutations within the mtDNA. A nuclear gene (*POLG*) encodes the DNA polymerase responsible for the process of replication within the mitochondrial genome. A recent study suggests that mitochondria may have a nucleotide imbalance within the mitochondrial matrix, meaning that the ratio of the DNA building blocks; adenine, cytosine, guanine and thymine are not balanced within the mitochondria. This nucleotide imbalance is thought to decrease *POLG* fidelity when replicating DNA, by using more readily available nucleotides even if these are incorrect (Song et al. 2005). Additionally, the mitochondrial genome has independent and increased DNA replication rate due to the replication cycle of the mitochondrial organelle. As a consequence of the low replication fidelity and high replication rate, mutations get incorporated into the mtDNA during replication, increasing the mutation frequency within the mtDNA. Mutations within the mtDNA are already vulnerable to

accumulation due to the combined effects of low recombination and low effective population size. By increasing the mutation rate within the mitochondrial genome, the effects of these evolutionary forces are amplified, and mutation accumulation is therefore increased.

1.4 Accumulation of Male Detrimental Mitochondrial Mutations Variation Resulting from Uniparental Inheritance

Mitochondria are asymmetrically inherited (maternal inheritance only) in most species, meaning that they get passed solely from mother to offspring (Neiman and Taylor 2009). Due to this uniparental mode of inheritance, natural selection acting on the mitochondrial genome is effective only in females (Rand 2001), and thus males are considered an evolutionary ‘dead-end’ when it comes to mitochondrial genome evolution. Mutations in the mtDNA that are beneficial, neutral or even slightly deleterious to females can be selected for in the population, whereas mutations that are detrimental to females should be removed from the population via strong purifying selection (Figure 2) (Frank and Hurst 1996; Gemmell et al. 2004). The uniparental inheritance means that males are prone to inherit mutations that are selected through the female lineage, even if these mutations are detrimental to males (Innocenti et al. 2011). This process will in theory result in the fixation of many mutations that are benign or only slightly deleterious to females, but deleterious to males. Thus, through evolutionary time, we expect males to accumulate mitochondrial mutation loads consisting of male-biased deleterious mutations. The process leading to sex-biased mutation accumulation (mutation load) has been termed the “sex-specific selective sieve”(Innocenti et al. 2011), or “Mother’s Curse” (Gemmell et al. 2004).

The Mothers Curse hypothesis was first described in the 1990s (Frank and Hurst 1996) and was further elaborated through a seminal review the following decade (Gemmell and

Allendorf 2001; Gemmell et al. 2004). Efforts have been made to formulate testable predictions to experimentally validate this hypothesis (Friberg and Dowling 2008; Innocenti et al. 2011). Firstly, if populations harbour mitochondrial genomes comprised of male-biased mitochondrial mutation loads, then we should observe greater levels of mitochondrial genetic variation underpinning the expression of male, than female, phenotypes. This prediction should hold if sampling mitochondrial haplotypes from within the same population, and also if sampling haplotypes across distinct populations. When it comes to the inter-population prediction, mitochondrial haplotypes will evolve along their own population-specific trajectories, and accumulate their own distinct pools of male-biased mtDNA mutations of deleterious effect. Purifying selection, however, should remove any such mutations from mtDNA haplotypes that exert deleterious effects on females. Thus, when sampling mtDNA haplotypes from distinct populations, we expect greater levels of mitochondrial haplotypic variance underlying the expression of male than female phenotypes (Figure 2).

The second prediction suggests that not all traits will be equally as susceptible to the accumulation of male-biased mitochondrial mutation loads (Friberg and Dowling 2008; Innocenti et al. 2011). In particular, metabolically-reliant traits that exhibit sexual dimorphism in expression are most likely to be the targets of Mother's Curse. This is because the mitochondrial genome underpins most metabolic traits, given the important role in energy production that the mtDNA plays. Under increasing levels of sexual dimorphism, levels of sex-specific gene expression increase and the intersexual genetic correlation erodes (Innocenti et al 2011). When it comes to optimizing mtDNA-encoded mitochondrial function for the male homologues of sexually dimorphic traits, males will presumably be no longer able to rely on the female-mediated adaptation of the mtDNA sequence. In other words, optimization of mtDNA-mediated mitochondrial function for homologues of sexually

dimorphic traits in females, might not deliver optimized mitochondrial function for the male homologue of that trait.

The first conclusive empirical validation of Mothers Curse was recently obtained in a study that examined the effects of mitochondrial variation on genome-wide patterns of nuclear gene expression. By placing five different mitochondrial haplotypes alongside an isogenic nuclear background, sexual asymmetry was found in sensitivity of the nuclear transcriptome to mtDNA genetic variation, with approximately 10% of the nuclear transcripts being differentially expressed in males relative to females (Innocenti et al. 2011). Interestingly, these differentially expressed transcripts were mostly localized in expression to the male reproductive system (testes, accessory glands, ejaculatory duct), while having no major effect on male non-reproductive and female tissues. These findings support both predictions of the Mother's Curse hypothesis, formulated above; greater mtDNA variation underlying trait expression in males, and in particular high sensitivity of sexually dimorphic male traits involved in reproductive function.

Recently the scope of Mothers Curse has broadened to not only reproductive traits (Smith et al. 2010), but also other life history traits. Ageing is a sexually dimorphic trait (with female *D.melanogaster* outliving males by up to 10% of the female longevity (Lints and Soliman 2013)) and is heavily linked to metabolic activity (Khazaeli et al. 2005), making it an optimal trait to test the Mothers Curse hypothesis. Mitochondrial genetic variation has been found to affect patterns and rates of ageing in male, but not female, *Drosophila melanogaster*, and evidence was presented that these sex-specific effects were underpinned by many mutations of small effect as opposed to few mutations of large effect (Camus et al. 2012).

1.5 Implications of a Sex-Specific Selective Sieve on Antagonistic Co-Evolution between the Sexes

Males and females have very different routes to maximizing their reproductive fitness, and thus selection for optimal expression of a phenotype involved in sexual reproduction, is often sex-specific or sometimes overtly sexually antagonistic (Cox and Calsbeek 2009). The expression of phenotypic traits can differ between the two sexes even when these traits are encoded by the same genes (Bonduriansky and Chenoweth 2009), due to many genes exhibiting sex-biases in expression and in splicing products (Telonis-Scott et al. 2009; Innocenti and Morrow 2010). In response to divergent selection, the sexes of most species have diverged in many physical, biochemical and behavioral attributes. However, the evolution of dimorphism is complicated by the fact that both sexes share most of the genome (Chapman et al. 2003; Bonduriansky and Chenoweth 2009). Despite the large phenotypic differences between males and females, genetic evidence is accumulating for an ongoing adaptive tug-of-war between the sexes that slows or even prevents optimal adaptation (Bonduriansky and Chenoweth 2009). Thus, alleles that are favored in one sex (moving the phenotype closer to the optimum) are regularly transmitted to the other sex, where they can have deleterious effects (moving the phenotype away from the optimum). For example, in the fruit fly *Drosophila melanogaster*, laboratory populations were shown to harbour genetic variation with sexually antagonistic effects on adult fitness, where genotypes that performed well in one sex tended to have below-average fitness in the other sex (Chippindale et al. 2001; Innocenti and Morrow 2010; Griffin et al. 2013).

Theoretically, three pathways may facilitate the build-up of male-harming mitochondrial mutations, but evidence as to the realized importance of each is missing. First, male-harming

mutations in the mtDNA might accumulate under mutation-selection balance, when these mutations exert greater harm on males than on females (Frank and Hurst 1996). Second, genetic drift might drive these mutations to fixation given that the effective population size of, hence efficacy of selection on, mitochondrial genomes is reduced relative to nuclear genomes (Lynch 1997; Gemmell et al. 2004). Third, male-harming mutations might accumulate if they benefit females (Rand et al. 2001; Unckless and Herren 2009; Innocenti et al. 2011). Given the mitochondrial genome is haploid, such mutations will be fully exposed to selection, and it is plausible that female-benefiting sexually antagonistic mutations could readily accumulate within the mitochondrial genome even in cases where there are substantial costs to males. Indeed, given this process involves positive selection, it will conceivably proceed much more rapidly than the aforementioned alternatives. Although several theories have been developed that aim to explain how male-detrimental mutations arise and accumulate in the mtDNA, we do not know to what extent these mutations are sexually antagonistic in their associated fitness effects.

1.6 Adaptive Mitochondrial Variation

It was previously thought that mitochondrial genetic variation accumulated under genetic drift and founder effects, creating diversity between newly established populations (Richards et al. 1998; Gamache et al. 2003). An increasing number of studies have, however, found evidence for positive selective sweeps in the mitochondrial genome (Meiklejohn et al. 2007; Foote et al. 2011; Morales et al. 2015), with many linking temperature adaptations to mitochondrial sequence and function (Cheviron and Brumfield 2009; Teacher et al. 2012; Silva et al. 2014; Morales et al. 2015). The assumptions regarding the role of genetic drift and founder effects versus selection were tested by Mishmar and colleagues (2003), who examined 104 complete mtDNA human sequences from diverse locations around the globe

and varied ethnic backgrounds. They examined the rate of evolution for all mtDNA protein coding genes and found ATP6 to have the highest levels of dN/dS of all mitochondrial genes despite this gene being the most evolutionary conserved mtDNA protein. Furthermore, they associated climatic zones with certain mitochondrial amino acid substitutions, thus suggesting that mitochondrial genome evolution was in part driven to climatic selection, rather than drift alone (Mishmar et al. 2003). Following this study, Ruiz-Pesini and colleagues (2004) further supported Mishmar et al.'s findings, by using a database of 1125 global human mitochondrial genomes. This study identified highly conserved amino acid substitutions at the root of several human mitochondrial lineages derived from colder climates. These results suggest that certain mitochondrial polymorphisms arose early in human evolution that were selected for because they conferred increase tolerance to colder environments. Hence, the authors concluded that specific mtDNA lineages permitted human ancestors to adapt to cold climatic regions (Ruiz-Pesini et al. 2004).

The previous two studies have generated interest within the scientific community, given they were the first to provide empirical evidence that global patterns of mtDNA hapotypic variation had been shaped under thermal selection. Several studies that followed, however, were not able to replicate the patterns found by the Mishmar et al and Ruiz-Pesini et al studies (Elson et al. 2004; Kivisild et al. 2006; Sun et al. 2007). For example, Kivisild et al. (2006) found that a surplus of non-synonymous mutations was a general feature of young phylogenetic branches, which also applied to African populations (Kivisild et al. 2006). Elson et al (2006), Ingman & Gyllensten (2007) and Sun et al (2007) all independently concluded that the most parsimonious explanation for accumulated mitochondrial polymorphisms is relaxed purifying selection and the action of genetic drift (Elson et al. 2004; Ingman and Gyllensten 2007; Sun et al. 2007).

Balloux *et al.* (2009), however, took a different approach to the previously mentioned studies, by testing the degree to which climate (minimum temperature) and past demography (distance from sub-Saharan desert) have shaped the current distribution of human mtDNA sequences, rather than relying on dN/dS ratios as a proxy for positive selection. Firstly, their results suggest a decrease in haplotypic diversity for populations living in colder environments. Furthermore, they found that genetic divergence between pairs of populations correlated with differences in temperature, and these results were found in the mtDNA, but not the nuclear DNA (Balloux *et al.* 2009). Thus, their results further suggest that the mtDNA genome is under climatic selection. Although the phenotypic patterns observed in this study are intriguing, an experimental approach is now required to validate the capacity for mtDNA haplotypes to evolve under thermal selection

1.7 This Study: Aims and Structure

In this thesis I first examine the extent of antagonistic selection present in the mitochondrial genome, and then identify sexually antagonistic SNPs present within the mitochondrial genome. Finally I examine the adaptive role the mitochondrial genome plays in a changing climate. The overall aim of all chapters is to explore the adaptive and non-adaptive forces that contribute to the accumulation of non-neutral genetic variation within the mitochondrial genome. Each research chapter in this thesis (Chapters 2 to 4) is written in the style of a journal article, and contributes to addressing the overall aim of my thesis. Chapters are presented in the correct format for the target journal, with tables and figures at the end of each chapter. I finalise this thesis with a general conclusions, and discuss future directions of this thesis.

Chapter 2: Mitochondrial Genetic Variance for Male and Female Fitness Components in *Drosophila melanogaster*: signatures of within-sex positive pleiotropy and between-sex antagonism

The small set of genes located within the mitochondria has been the subject of much attention by evolutionary biologists, as previous studies have documented that allelic variance within the mitochondrial DNA confers phenotypic effects. Furthermore there is evidence for both within- and between-population mitochondrial effects on fitness traits. The maternal inheritance of mitochondrial genomes invokes a sex-specific selective sieve, whereby mutations in the mtDNA can only respond to selection acting directly on females. In theory, this enables male-harming mutations to accumulate in mitochondrial genomes when these same mutations are neutral, beneficial, or only slightly deleterious in their effects on females. Ultimately, this evolutionary process could result in the evolution of male-specific mitochondrial mutation loads, an idea previously termed Mother's Curse.

Many studies have found that genetic variation found within the mitochondrial genome affects the expression of both male or female reproductive traits (James and Ballard 2003; Ballard et al. 2007; Dowling et al. 2007; Jelic et al. 2015). However, to my knowledge, no study has looked at mitochondrial genetic effects on expression of reproductive success concurrently across both sexes, to determine patterns of within- and between-sex pleiotropy across mtDNA haplotypes.

This chapter seeks to explore i) the effects of mitochondrial genetic variation on various measurements of reproductive success, ii) the extent to which such mitochondrial genetic effects on reproductive phenotypes are sex-specific in nature, consistent with the Mother's Curse hypothesis, and iii) signatures of pleiotropy across mtDNA haplotypes, in their effects on different components of reproductive success, both within and between the sexes. In this

chapter, I document mitochondrial effects on the expression of male and female reproductive success. Furthermore I find signatures of mtDNA-mediated positive pleiotropy between different components of reproductive success measured within each of the sexes, and signatures of mtDNA-mediated negative pleiotropy in effects on reproductive success between the sexes.

Chapter 3: Single nucleotides in the mtDNA sequence modify mitochondrial molecular function and are associated with sex-specific effects on fertility and ageing

The small mitochondrial genome has recently been the subject of increasing attention, with evidence that mitochondrial genetic variance may shape evolutionary trajectories and exert large effects on life-history traits considered key human health indicators (Reinhardt et al. 2013; Wolff et al. 2014). In several cases, effects have been shown to be heavily male-biased, which is presumably linked to the maternal transmission of the mitochondria and the resulting implication that selection on the genome can only act through females (Innocenti et al. 2011; Camus et al. 2012). However, it is not currently understood how genetic variance across a diminutive genome, which in most species harbours only about a dozen protein coding genes, can exert such large-scale modifications to the organismal phenotype. This chapter aims to test two hypotheses for the mechanistic regulation of mtDNA-mediated effects. First, that mitochondrial allelic variation directly alters cellular mitochondrial DNA abundance, and second that mitochondrial allelic variation alters expression patterns across single protein-coding mtDNA genes.

Using *Drosophila melanogaster* strains in which diverse mitochondrial haplotypes were placed alongside an isogenic nuclear background (Clancy 2008), I show that naturally-occurring sequence variation within the mitochondrial DNA (mtDNA) affects the copy number of mitochondrial genomes as well as the expression of mitochondrial-encoded gene

products. I observed mtDNA haplotype-mediated variation in the expression of key mitochondrial protein-coding genes, including differences in expression patterns of genes belonging to the same transcriptional units. This indicates post-transcriptional processing and variation in mitochondrial abundance are important regulators linking the mitochondrial genotype to the striking variation observed in life-history trait expression. These haplotype-mediated effects could be traced both backwards to the level of individual SNPs, and forwards to sex-specific effects on fertility and longevity. These findings elucidate how small-scale sequence changes in the mitochondrial genome can achieve broad-scale regulation of health-related phenotypes, and contribute to the sex gap in longevity.

Chapter 4: Experimental Evidence for Mitochondrial Genomic Adaptation to Climate

Latitudinal gradients are highly informative when examining the expression of traits that progressively change along an environmental gradient and this continuous trait change with latitude is called a “cline”. Clinal studies are a powerful means of identifying traits and genes that are under climatic selection given that a cline encompasses a wide range of climatic conditions. The Australian eastern cline has been very well studied over the last decade, with significant phenotypic and molecular clines reported in ectothermic species (Hoffmann et al. 2002; Hoffmann and Weeks 2007; Turner et al. 2008; Sgrò et al. 2010; Kolaczowski et al. 2011). Ectothermic organisms are particularly susceptible to thermal stress as they cannot internally regulate their body temperature. Almost all physiological and biochemical functions (as well as crucial behavioural traits) of ectotherms are impacted by temperature. These functions include cell membrane structure (Overgaard et al. 2008; Su et al. 2009), locomotion (Lachenicht et al. 2010), metabolism (Holzman and McManus 1973; Berrigan

and Partridge 1997), immune function (Le Morvan et al. 1998; Karl et al. 2011), and mating ability (Saeki et al. 2005; Amin et al. 2010).

Previous studies have shown that non-neutral genetic variation exists in the mitochondrial genome, and that some mito-nuclear genotypes are thermally sensitive (Mishmar et al. 2003; Ruiz-Pesini et al. 2004; Silva et al. 2014; Morales et al. 2015). The effect of thermal selection on the mitochondrial genomic adaptation has not yet been thoroughly examined, and mitochondrial genetic involvement in the dynamics of thermal adaptation remains an open question. In this chapter I aim to answer whether thermal selection might have shaped patterns of mitochondrial genetic variation observed along the Australian eastern coast, using *D. melanogaster* as an ectothermic model organism.

I first find two main mtDNA haplogroups distributed along the Australian eastern coast, with one haplotype predominant in the northern latitudinal populations, and the other predominating in southern populations. I then introgressed northern and southern haplotypes into a standard nuclear background, and then examined the effects of each of these haplotypes on the expression of traits linked to thermal tolerance. I found that flies harbouring the northern haplotype exhibit greater tolerance to a heat stress than counterparts with southern haplotypes, while southern haplotypes confer enhanced tolerance to an exposure to cold stress. Furthermore, I was able to locate SNPs that differed between these two main haplotypes, and associate them to the phenotypic differences observed. These findings thus implicate mitochondrial genomic variation in the dynamics of thermal adaptation.

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1.9 Figures

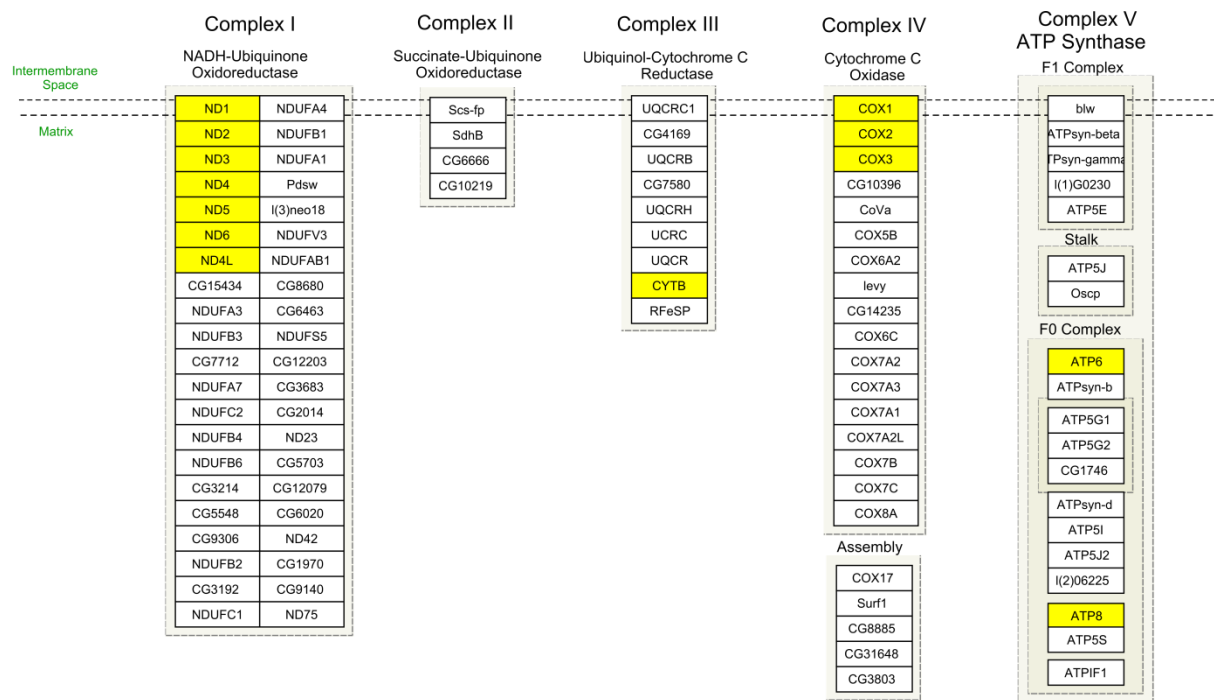


Figure 1: *Drosophila melanogaster* OXPHOS system. This figure shows the five complexes associated with energy production, and within each complex all sub-units involved in the formation of each complex. Highlighted in yellow are the products encoded by the mitochondrial genome.



Figure 2: The ‘sex-specific selective sieve’.

Mitochondrial DNA is maternally inherited in most species. Given males do not usually transmit mitochondrial DNA, mutations that are male detrimental, but more-or-less benign in females, will be overlooked by selection, which occurs directly through females because of mitochondrial maternal inheritance. In this figure, mutations that are detrimental to females only or to both sexes (orange) are trapped in the sieve and are removed from the population via strong purifying selection. Mutations that are beneficial to females only or both sexes (green) go through the sieve and remain in the population. Mutations that are only deleterious to males, not females, will be expected to pass through the sieve (blue).

Declaration for Thesis Chapter 2

Monash University

“Mitochondrial genetic variance for male and female reproductive success in *Drosophila melanogaster*: signatures of positive intra-sexual, but antagonistic inter-sexual, pleiotropy”

This thesis chapter is in submission format for the peer reviewed journal *Evolution*

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design, execution of experimental work, analysis, manuscript writing	75%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Damian K. Dowling	Experimental design, manuscript writing	25%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's
Signature



Date 23/03/2016

Main
Supervisor's
Signature

Date

CHAPTER 2

Mitochondrial genetic variance for male and female reproductive success in *Drosophila melanogaster*: signatures of positive intra-sexual, but antagonistic inter-sexual, pleiotropy

2.1 Abstract

Mitochondria contain their own DNA, and numerous studies have reported that genetic variation in this (mt)DNA sequence modifies the expression of life history phenotypes. Maternal inheritance of mitochondria adds a layer of complexity to trajectories of mtDNA evolution, because in theory it will facilitate the accumulation of mtDNA mutations that are male-biased in effect. While it is clear that mitochondrial genomes routinely harbor genetic variation that affects reproductive performance, the extent to which this variation is sex-biased or even sex-specific in effect remains elusive. Previous studies have not examined mitochondrial genetic effects on both males and female reproductive performance within the one and same study. Here, we show that variation across naturally-occurring mitochondrial haplotypes affects components of reproductive success in both sexes, in *Drosophila melanogaster*. However, while we uncovered evidence for positive pleiotropy across haplotypes, in effects on separate components of reproductive success when measured within the same sex, such patterns were not evident across sexes. Indeed, we found a signature of sexual antagonism across haplotypes in one of two measured components of reproductive success. This finding suggests the pool of polymorphisms that delineate global mtDNA haplotypes is likely to be shaped by maternal transmission of mtDNA, resulting in the accumulation of sex-specific mitochondrial polymorphisms.

2.2 Introduction

Eukaryotic cells are thought to have arisen from the ancient symbiotic union between two prokaryote cells; one an α -proteobacterium and the other an archaean-like bacterium. The α -proteobacterium would evolve into the mitochondrion, and the archaea-like bacterium into the eukaryote (Gray 1992). Moreover, each of these entities harbored their own genomes, and their symbiosis kick-started more than a billion years of inter-genomic coevolution that delineates contemporary eukaryotes from the organisms of other domains (Embley and Martin 2006). Almost without exception, eukaryotes from fungi to animals have retained these two genomes – one mitochondrial (comprised of mtDNA), the other nuclear, and interactions between genes spanning each of these genomes coordinate closely to regulate critical biological processes tied to cellular metabolism via oxidative phosphorylation (OXPHOS) (Rand et al. 2004; Lane 2007; Wolff et al. 2014).

Over the course of evolutionary history, most of the genes of the mitochondrial genome were translocated to the host nuclear genome, leaving only a small number of genes in the mtDNA, including thirteen protein-coding genes that are salient to OXPHOS function (Lane 2007; Havird et al. 2015). Evolutionary biologists long assumed that purifying selection would eliminate any non-neutral (i.e., phenotype-modifying) genetic variation from accumulating within these mtDNA-encoded genes, given these genes encode essential subunits of the electron transport chain, and given that the mitochondrial genome is haploid and therefore all alleles within it are invariably exposed to natural selection (Dowling et al. 2008). As such, the mitochondrial genome was harnessed as the quintessential molecular marker upon which to base evolutionary and population genetic inferences, facilitated by its high mutation rate, maternal inheritance and general lack recombination (Lynch 1997; Pesole et al. 2000; Saccone et al. 2000; Ballard and Whitlock 2004).

However, over the past two decades, the results of an increasing number of studies have challenged this assumption of neutrality (Ballard and Kreitman 1994; Rand 2001; Dowling et al. 2008). In particular, numerous studies have used multigenerational breeding schemes with the power to partition mitochondrial genetic from nuclear genetic effects, and revealed that the genetic polymorphisms that delineate distinct mitochondrial haplotypes, sourced from separate populations, contribute to the expression of life-history traits tied to reproductive success, development, and longevity (James and Ballard 2003; Maklakov et al. 2006; Melvin and Ballard 2006; Dowling et al. 2007a; Dobler et al. 2014; Wolff et al. 2014; Zhu et al. 2014; Camus et al. 2015; Jelic et al. 2015).

Currently, however, it is unclear how these phenotype-modifying genetic polymorphisms accumulate within mitochondrial genomes. One alternative is that they constitute adaptations, fixed under natural selection. This is consistent with the results of some studies that examined mutational profiles of mtDNA sequences, and found signatures of positive selection in the form of elevated ratios of non-synonymous (dN – changes amino acid) to synonymous (dS – does not change amino acid) mutations (Mishmar et al. 2003; Ruiz-Pesini et al. 2004; Meiklejohn et al. 2007). Alternatively, such polymorphisms might rise to appreciable frequencies within populations under mutation-selection balance, and potentially then be fixed by drift. This alternative is plausible; firstly, given the mitochondrial genome has a high mutation rate relative to its nuclear counterpart (Lynch et al. 2006). Secondly, it is reasonable to predict there will be a diminished efficiency of selection in shaping the mtDNA sequence relative to nuclear DNA sequences, because of a theorised fourfold reduction in the effective population size of the mitochondrial genome that stems from it being haploid and maternally inherited (Ballard and Whitlock 2004; Dowling et al. 2008).

Maternal inheritance of mitochondrial genomes adds a further layer of complexity to the dynamics of mtDNA sequence evolution, because it means that selection can only act on non-neutral mtDNA polymorphisms directly through the female lineage (Frank and Hurst 1996; Gemmell et al. 2004; Beekman et al. 2014). This hypothesis, which has been called “Mothers Curse” (Gemmell et al. 2004), predicts that mutations that are neutral, beneficial or even slightly deleterious to females may accumulate in the mtDNA sequence even if these very same mutations are harmful in their effects on males (Frank and Hurst 1996). Recent studies in *Drosophila* uncovered evidence for the existence of a pool of male-harming, but female-neutral polymorphisms that have accrued within mtDNA haplotypes, and which affects genome-wide patterns of gene expression in males, particularly of genes involved in encoding male-specific reproductive tissues (Innocenti et al. 2011), and shapes patterns of male, but not female, longevity (Camus et al. 2012; Camus et al. 2015).

However, the extent to which mitochondrial haplotypes exhibit sex-biases in their effects on the expression of life history phenotypes remains unclear, because very few studies have measured phenotypic effects associated across sets of mtDNA genotypes in both males and females, respectively. The sparsity of studies reporting sex-specificity in effects is particularly true for phenotypes tied to reproductive performance. Indeed, we are unaware of a single study to yet measure mtDNA-mediated effects on components of reproductive success in both males and females. This represents a striking gap in understanding, given that traits and tissue types exhibiting strong sexual dimorphism (such as the testes, sperm and reproductive glands involved in male reproductive outcomes) are hypothesized to be the key candidates for susceptibility to Mother’s Curse effects (Innocenti et al. 2011; Beekman et al. 2014; Dowling 2014).

Furthermore, very few studies have measured multiple traits across the same set of mtDNA genotypes, to examine levels of mtDNA-linked pleiotropy in effects across traits, within and across the sexes. Studies that have achieved this, have however reported interesting patterns, which provide insights into the evolutionary processes by which genetic variation can accumulate within mitochondrial genomes. For example, Dowling et al (2009) found a strong positive association in effects of two mtDNA haplotypes segregating within a population of *D. melanogaster*, on two life history traits in females - reproductive performance and longevity (Dowling et al. 2009). The haplotype conferring higher female reproductive success conferred higher female lifespan. Camus et al (2015) reported that a SNP found within the mtDNA-encoded CYTB gene of *D. melanogaster*, which is known to cause low fertility in males (Clancy et al. 2011; Yee et al. 2013; Dowling et al. 2015), but not in females, confers higher male lifespan but shorter female lifespan relative to haplotypes harbouring other variants of this gene (Camus et al. 2015). This SNP is therefore associated with antagonistic pleiotropic effects both within and across the sexes, consistent with the idea that some mtDNA SNPs might accumulate under positive selection in females, even if they are associated with suboptimal male phenotypes (Beekman et al. 2014). If so, then maternal inheritance of mitochondria could potentially lead to overtly sexually antagonistic trajectories of mtDNA evolution (Dean et al. 2014; Rogell et al. 2014).

To address patterns of sex-specificity and pleiotropy, here we screen thirteen naturally occurring and geographically distinct mitochondrial haplotypes of *D. melanogaster* for two measurements of reproductive output in each sex. We used strains in which each of these haplotypes had been placed alongside an isogenic nuclear background prior to the phenotypic assays (Clancy 2008; Camus et al. 2012), such that all phenotypic effects observed could be traced directly to genetic polymorphisms separating each haplotype. Firstly, we measured

reproductive success of males and females who had abstained from sexual interactions until the peak of their fertility, and were then provided with a 24 h opportunity to mate and reproduce (“short-burst” reproduction). Secondly, we measured reproductive success of each sex over a prolonged period of time, from eclosion into adulthood to 8 (male) and 12 (female) days of age (“sustained” reproductive success). Thus, we had two measures of reproductive success for each sex – one representing success based on a limited opportunity at the peak of an individual’s reproductive lifespan; the other based on reproductive stamina when faced with multiple opportunities and partners across early phase of adult life (“sustained” reproductive success).

2.3 Materials and Methods

Mitochondrial lines

Thirteen *Drosophila melanogaster* strains were used, and these strains have been previously described (Clancy 2008; Camus et al. 2012). In brief, the isogenic nuclear background from the w^{1118} strain (Bloomington stock number: 5905) was coupled to mitochondrial haplotypes from thirteen distinct geographic locations using a crossing scheme that is outlined in Clancy (2008). These strains have been maintained in duplicate since 2007, with the duplicates propagated independently, and at standardized and low adult and egg densities, to enable us to partition mitochondrial genetic effects from cryptic nuclear variance that might have accumulated among the strains and other sources of environmental variation. Each generation, virgin females are collected from each duplicate of each mitochondrial strain (hereafter *mitochondrial strain duplicate*) and backcrossed to males of the w^{1118} strain, to maintain isogenicity of the nuclear background. Furthermore, w^{1118} is itself propagated by one pair of full-siblings per generation. Thus, if mutations arise in the w^{1118} strain, they will

be swiftly fixed and passed to all mitochondrial strain duplicates, thus maintaining the critical requirement of isogenicity of the nuclear genome.

One of the mitochondrial strains (Brownsville) incurs complete male sterility in the w^{1118} nuclear background, whereas females who harbour this haplotype remain fertile (Clancy et al. 2011). This strain was therefore excluded from assays of male reproductive success (n=12 haplotypes in these assays), but included in assays of female reproductive success (n=13 haplotypes).

All mitochondrial strains and w^{1118} flies were reared at 25°C, under a 12h: 12h light: dark photoperiod regime, on potato-dextrose-agar food medium and with *ad libitum* access to live yeast. All strains had been cleared of any potential endosymbionts, such as *Wolbachia*, through tetracycline treatment at the time that the strains were created (Clancy and Hoffmann 1998). Diagnostic PCR with *Wolbachia*-specific primers confirmed all lines are free of *Wolbachia* (ONeill et al. 1992).

Male Reproductive Success

Two separate components of male reproductive success were measured, via two separate experiments. The first experiment measured male reproductive success following an exposure to a single female at the peak age of male reproductive fertility. This assay measures the ability of a male to convince a virgin female to mate, and then measures the number of offspring produced from sexual interaction with that female, which is likely to be a function of the males ejaculate quality (number and quality of sperm, and content and quality of reproductive proteins, transferred). The second experiment gauged male reproductive success across the first eight days of adult life, during which time males had constant access to new

and virgin females. This assay thus represents a measure of male reproductive stamina (a function of male mating rate across time, and ability to replenish sperm and ejaculate stores). Each assay is described below.

Male reproductive success following exposure to a single female (short-burst reproductive success)

This experiment measured offspring produced by a single male after a one-off mating opportunity with a virgin female when 4 days of adult age. Prior to the experiment, each mitochondrial strain duplicate was propagated across 3 vials by 10 pairs of parents of standardised age (4 day old) and at controlled larval densities (approximately 80 eggs per vial) for 3 generations. Twenty virgin males from each of these duplicates were then collected and stored individually in a separate 40 ml vials containing 5mL of food medium. At the same time, virgin females were collected from the isogenic w^{1118} strain (sourced from 10 vials, and stored in vials of 10 females per vial), which had been propagated and stored under the same experimental conditions as described for the mitochondrial strain focal males. When four days old, each focal male was then combined with an equivalently-aged tester female, and these flies then cohabited the same vial for a 24 h period. Following this, focal males were removed from the mating vial and discarded. Females were then transferred into fresh vials with food substrate every 24 h over a 4 d period. The total number of offspring eclosing across these four vials was recorded for each focal male.

Male reproductive across 8 days (sustained reproductive success)

Male reproductive success was assayed following methodology described in (Yee et al. 2015). In brief, individual males collected from each mitochondrial strain duplicate were provided with the opportunity to mate with eight different virgin females over eight

consecutive 24 h long exposures. To initiate the assay, twenty virgin males were collected from each duplicate, and each placed in a separate vial. Twenty four hours later, one 4-day-old, virgin w^{1118} female was added to each vial, and male and females then cohabited for 24 h. Following this 24 h exposure, males were removed and placed with another 4-day-old virgin w^{1118} female for another 24 h period. This process was repeated until day eight of the experiment (8 separate exposures). After each exposure, the w^{1118} females were retained and themselves transferred into fresh vials every 24 h for a period of four consecutive days, thus providing each female with 96 h to oviposit. Thirteen days following the 96h oviposition period, the number of eclosed adult offspring emerging from each vial was counted.

Female reproductive success

Two separate components of female reproductive success were measured. In the first experiment, female fecundity and fertility was measured as the number of eggs, and proportion of eggs that ultimately eclosed into adulthood, following a 24 laying opportunity at the peak age of female fecundity (4 days of age). In the second experiment, female reproductive success was measured over a 13 day period of reproductive success, and thus represents a measure of female reproductive stamina.

Female fecundity and fertility at peak fecundity (short-burst reproductive success)

The assay was run in five blocks, each separated in time by one generation. Female focal flies from each mitochondrial strain duplicate were collected as virgins, and stored individually. These were collected over numerous 40mL vials, each of which had been propagated by 10 pairs of age-controlled parents (4 day old), and at controlled larval densities (approximately 80 eggs per vial). When 4 days of age, each female was exposed to one 4 d old tester virgin male, collected from w^{1118} strain, for a period of 12 hours and then the females transferred to

a fresh vial for 24 h to oviposit. Following this 24 hour oviposit period, females were discarded. We counted the eggs oviposited per female over this 24 hour period (short-burst fecundity), plus the offspring that emerged from these eggs, and female short-burst fertility was estimated as the proportion of adults emerging from these eggs (i.e., egg-adult viability).

Female reproductive success across 13 days (sustained reproductive success)

Forty females from each mitochondrial strain duplicate were collected as virgins, and placed in individual vials. One day later, two 4 d old virgin w^{1118} males were placed into each female vial. Females, and the two males with which she cohabited, were then transferred into fresh vials every 24 hours, for 13 days. Every 4 days the accompanying males were discarded, and two 4 d old virgin males of the w^{1118} strain were added. This ensured that females were not sperm-limited throughout the duration of the experiment. Female reproductive success was determined by counting the total number of adult offspring produced by each female, per vial, over the 13-day assay.

Statistical Analysis

A general linear mixed model using a Gaussian distribution was fitted to the male short-burst reproduction data. Female short-burst fecundity data was modelled by fitting a general linear mixed model using a Poisson distribution, given it is count data. Female fertility data was modelled using a binomial distribution, with female fertility a two-vector response variable composed of the number of adults and number of eggs minus number of adults per vial. Mitochondrial strain was modelled as a fixed effect, and the duplicate nested within mitochondrial strain and the sampling block (Block) included as random effects, using either the *glmer* (for female short-burst fertility) or *lmer* (for male short burst fertility and female short-burst fecundity) function in the *lme4* package (Bates 2012) in R (Fox 2002). The fitted

models were evaluated by simplifying a full model, by sequentially removing terms that did not change the deviance of the model (at $\alpha = 0.05$); starting with the highest order interactions, and using log-likelihood ratio tests to assess the change in deviance in the reduced model relative to the previous model (Fox 2002).

For the experiments gauging sustained reproductive success, the overall total number of offspring (for both males and females) was zero-inflated, and the resulting models over-dispersed. We therefore analysed both datasets using a negative binomial distribution (Mullahy 1986), in which the zero values are a blend of sampling and structural effects (negative binomial parameter; variance = $\phi\mu$). These models were performed using the R (v. 3.0.2) package glmmADMB (<http://glmmadmb.r-forge.r-project.org/glmmADMB.html>). The response variable was reproductive success for each sex (total number of offspring), with mitochondrial strain and day sampled, plus their interaction as fixed factors. The random effect in the model was mitochondrial duplicate nested within mitochondrial strain.

A matrix of mitochondrial genetic correlations (Pearson's correlation coefficients) was created by obtaining mtDNA haplotype-specific means for each reproductive trait across all mitochondrial strains. Thus, we had 13 means (one per haplotype) for female measures of short burst and sustained reproductive success, and 12 means for male measures (since BRO was excluded from the male assays). Inter-sexual correlations across haplotypes were thus based on 12 means, and were obtained using the Hmisc package (<https://cran.r-project.org/web/packages/Hmisc/index.html>) in R (3.0.2).

2.4 Results

Male Mitochondrial Reproductive Success Assays

The identity of the mitochondrial strain affected male short-burst reproductive success ($\chi^2 = 30.992$, $p = 0.001$, Table 1A, Figure 1A). Male sustained reproductive success was affected by an interaction between mitochondrial strain and day of mating (haplotype \times day, $\chi^2 = 183.039$, $p < 0.001$, Table 1B, Figure 2A, Figure 2A). Male reproductive success tended to increase up to day 4 of adult age, and then incrementally decrease to day 8. However, the magnitude of increase was contingent on the mtDNA haplotype, with only two haplotypes exhibiting a clear peak in reproductive success at day 4 (MYS and ORE). The reaction norms per haplotype crossed-over across the eight days of the experiment, with several haplotypes that exhibited the highest relative reproductive success at the peak of the assay (day 4) generally associated with low reproductive success relative to the other haplotypes at Day 1 and 8 of the experiment (Figure 3A).

Female Mitochondrial Reproductive Success Assays

Polymorphisms within the mitochondrial genome affected female short-burst fertility ($\chi^2 = 22.746$, $p = 0.0296$, Table 1C, Figure 1B), but not short-burst fecundity ($\chi^2 = 7.4573$, $p = 0.826$, Table 1D). An interaction between mitochondrial strain and day of the mating assay affected sustained female reproductive success (haplotype \times day, $\chi^2 = 256.3$, $p < 0.001$, Table 1E, Figure 2B). All haplotypes exhibited a similar trend, with reproductive success incrementally increasing up until day 4 of the assay, following which point, reproductive success began to decline. Again, however, these patterns were contingent on the mtDNA haplotype, with norms of reaction crossing per haplotype across Days 1, 4 and 8 of the assay (Figure 3B).

Mitochondrial Genetic Correlations

We screened for genetic correlations between traits across the mtDNA haplotypes, both within and between the sexes. Intra-sexual correlations between reproductive traits tended to be strongly positive in direction ($r_{\text{female short-burst vs sustained}} = 0.76$, $p < 0.004$; $r_{\text{male short-burst vs sustained}} = 0.61$, $p < 0.02$, Figure 4A&B). However, inter-sexual correlations tended to be negative in direction, and in particular the correlation between female and male short-burst reproductive success was strongly negative ($r_{\text{female vs male short-burst}} = -0.67$, $p = 0.034$, Figure 4C).

2.5 Discussion

We explored mitochondrial genetic effects, across distinct and naturally-occurring mitochondrial haplotypes, on components of reproductive success in male and female *D. melanogaster*, using an approach that enabled us to unambiguously trace the genetic effects to the level of the mtDNA sequence. Notably, genetic polymorphisms located across these haplotypes affected almost all components of reproductive success measured – in females and in males. Furthermore, we uncovered strong pleiotropy in the reported effects. These patterns of pleiotropy were positive for intra-sexual correlations across haplotypes (e.g. for associations between short-burst and sustained reproductive success in each of the sexes), but strongly negative for the inter-sexual correlation between male and female short-burst reproductive success.

This negative inter-sexual correlation is striking because it indicates that, at the level of whole haplotypes, those haplotypes that confer relatively high reproductive success in one sex, confer low success in the other. Furthermore, we note that our estimate of this negative

correlation is conservative, because it excluded the Brownsville mtDNA haplotype, which is completely male-sterile in the nuclear background assayed here (w^{1118}), and which we have previously implicated as a repository for sexually antagonistic polymorphisms (Camus et al. 2015). The negative correlation between male and female short-burst reproductive success is consistent with evolutionary theory first developed by Frank and Hurst (1996), and which is routinely called “Mother’s Curse” (Gemmell et al. 2004), which proposes that maternal inheritance of the mitochondria will lead to the accumulation of male-biased mutation loads within the mtDNA sequence (Innocenti et al. 2011). Specifically, however, while Frank and Hurst (1996) envisaged that such mutations would accumulate under mutation-selection balance (i.e. the mutations would be largely benign in their effects on females), our results suggest a role for sexually antagonistic selection (Unckless and Herren 2009; Beekman et al. 2014), with mutations accumulating in the mtDNA sequence that augment female reproductive success, but that come at cost to male reproductive performance.

Under strict maternal inheritance, female harming but male benefiting mtDNA mutations should be efficiently purged by purifying selection. In contrast, if mtDNA mutations appear that are female benefiting, but male-harming, they will presumably increase in frequency under positive selection. Furthermore, the pool of sexually antagonistic mutations accumulating within the mitochondrial genomes will differ across populations – in terms of the identity of the mutation sites at which they occur, the associated nucleotides, and total number of mutations accrued. Consequently, at the level of whole haplotypes sourced from different global populations, we should then expect to observe a negative genetic correlation, with haplotypes that harbour numerous female-benefiting but male harming mutations (or alternatively harbouring a few mtDNA mutations of major sexually antagonistic effect) conferring higher relative female, but lower male, reproductive success. Conversely, those

haplotypes harbouring few such mutations (or alternatively mutations of only minor effect) will confer lower female reproductive success relative to other haplotypes, but relatively higher success in males.

A key prediction of Mother's Curse theory is that levels of mitochondrial genetic variation will be larger in males than in females (Innocenti et al. 2011; Beekman et al. 2014). Previous studies supporting this theory have found statistically significant effects of the mtDNA haplotype on male, but not female, phenotypes (Innocenti et al. 2011; Camus et al. 2012). In our study, however, we found sizable and mtDNA-mediated effects in both sexes, which at face value might be perceived as at odds with previous results. This prediction, however, is explicitly based on the assumption that the mtDNA mutations accumulate under a Frank and Hurst (1996) model, under mutation-selection balance. That is, the prediction assumes most of the male-harming mutations that accumulate under maternal inheritance are effectively benign in females. However, our results suggest that when it comes to mtDNA-mediated effects on reproductive success, the effects of the underpinning mutations might be of similar magnitude in each of the sexes, but antagonistic (male-harming, but female benefiting). Furthermore, we note that the prediction of male-biased mtDNA variance, empirically substantiated in recent studies (Innocenti et al. 2011), assumes that the sampled traits in males and females are "homologous" – encoded by the same tissues and underpinning genes. This assumption is, however, clearly not fulfilled when it comes to the genes and traits that determine reproductive outcomes across the sexes. For example, components of the accessory glands, testes and spermatozoa contribute heavily to shaping male reproductive outcomes, but all of these traits are sex-limited in expression (Chandley and Cooke 1994; Ding et al. 2010). Our findings provide further empirical evidence for the emerging realization that polymorphisms that accumulate within the mitochondrial genome will typically have

pleiotropic effects on key life-history traits. Here, in this study, we limited our investigation to correlations across different components of reproductive success in each of the sexes. But, previous studies have reported mitochondrial genetic associations between longevity and reproductive success, or traits associated with juvenile components of fitness. Consistent with the signature of intra-sexual positive pleiotropy identified in our experiment here, Dowling et al. (2009) reported a positive genetic association between female longevity and female reproductive success, between two mtDNA haplotypes that were segregating within a population of *D. melanogaster*. The haplotypes used in that study were delineated by a single nonsynonymous SNP within the mtDNA *CYTB* gene (Dowling et al. 2007b).

Congruent with the intersexual negative correlation between short-burst reproductive success found in our current study, Rand et al. (2001) reported an negative correlation between the sexes for a measure of juvenile viability in *D. melanogaster* (based on a chromosome segregation assay), across two of three mtDNA haplotypes measured. Recently, Camus et al (2015) identified sex-specific effects tied to polymorphisms at key protein-coding mtDNA genes, *ND5* and *CYTB*. In particular, a single nonsynonymous mutation (Ala-278-Thr) in the CytB gene was associated with patterns of antagonistic pleiotropy both within and between the sexes. Females with the Brownsville haplotype, which carries this SNP were fully fertile, but suffered short longevity relative to females with other haplotypes. Males with this SNP, however, suffer reduced fertility (Clancy et al. 2011; Yee et al. 2013; Dowling et al. 2015) and are in fact sterile when this SNP is placed in the w^{1118} nuclear background used here), but experience higher longevity than males with other haplotypes (Camus et al. 2015). This is the first identified SNP within the mitochondrial genome associated with overtly sexually antagonistic effects (Camus et al. 2015), however our study here suggests there are numerous other such SNPs segregating within the mitochondrial genome.

The aforementioned Ala-278-Thr SNP in *CYTB* is an example of a SNP in the mtDNA that is putatively entwined in regulation of the classic life-history trade-off between investment into reproduction versus survival. Our current study reinforces a growing body of evidence that supports this contention, by indicating that numerous relationships between multifaceted components of life-history will be affected by genetic polymorphisms located within the mtDNA sequence. Additionally, our findings substantiate the emerging hypothesis that maternal inheritance of the mitochondrial genome will have consequences on the evolution of life-histories, by leading to the accumulation of mutations that contribute to the evolution of sex differences in key components of life-history.

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2.7 References

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2.8 Tables and Figures

Table 1: Mitochondrial effects on male (A) short-burst and (B) sustained reproductive success, and female (C) short-burst fecundity, (D) short-burst fertility, and (E) sustained reproductive success. Haplotype denotes the effect of mitochondrial strain (hence mtDNA haplotype), and Duplicate[Haplotype] denotes the mitochondrial strain duplicate. In the short-burst assays, each experiment was conducted over consecutive sampling blocks (Block). In the sustained reproductive success assays, each experiment was conducted over a number of consecutive days (Day; 8 in males, 13 in females). For all models, chi-square test statistics (χ^2), degrees of freedom, and p values are reported for fixed effects, and standard deviation (SD) for random effects.

A) Male short-burst reproductive success

	χ^2	d.f.	P
(Intercept)	611.758	1	< 0.001
Haplotype	30.992	11	0.001104
SD			
Duplicate [Haplotype]	2.969		
Block	0.000		
Residual	21.546		

B) Male sustained reproductive success

	χ^2	d.f.	P
Haplotype	25.299	12	0.04738
Day	14.221	12	0.00824
Haplotype \times Day	183.039	144	<0.001
SD			
Duplicate [Haplotype]	0.0081		

C) Female short-burst fecundity

	χ^2	d.f.	P
(Intercept)	66.641	1	<0.001
Haplotype	7.457	12	0.826

	SD
Duplicate [Haplotype]	1.838
Block	6.039
Residual	14.506

D) Female short-burst fertility

	χ^2	d.f.	P
(Intercept)	90.837	1	<0.001
Haplotype	24.746	12	0.0221

	SD
Duplicate [Haplotype]	7.04e-05
Block	4.42e-01
Residual	0.1762

E) Female sustained reproductive success

	χ^2	d.f.	P
Haplotype	9.6678	12	0.6451
Day	189.923	12	<0.001
Haplotype \times Day	256.327	144	<0.001

	SD
Duplicate [Haplotype]	0.1009

Table 2: Estimates of intra- and inter-sexual genetic correlations (using Pearson's correlation coefficients) for male and female reproductive traits across 12 mitochondrial haplotypes. Emboldened values are significant at an alpha criterion of 0.05.

	Female fecundity short-burst	Female fertility short-burst	Female sustained	Male short- burst	Male sustained
Female fecundity short-burst	-				
Female fertility short-burst	0.0044628	-			
Female sustained	0.2588207	0.7558424	-		
Male short-burst	-0.4302265	-0.6695821	-0.5377190	-	
Male sustained	-0.43997458	-0.0255083	-0.1988325	0.6143892	-

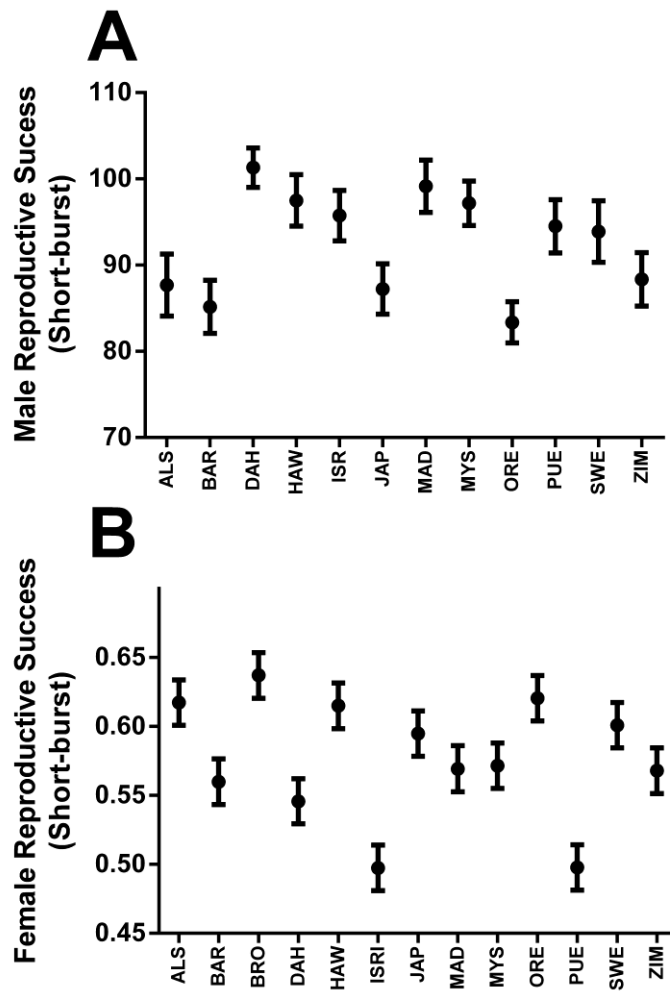


Figure 1: Short-burst reproductive success (Means \pm 1 S.E.) across all mitochondrial strains for (A) males and (B) females.

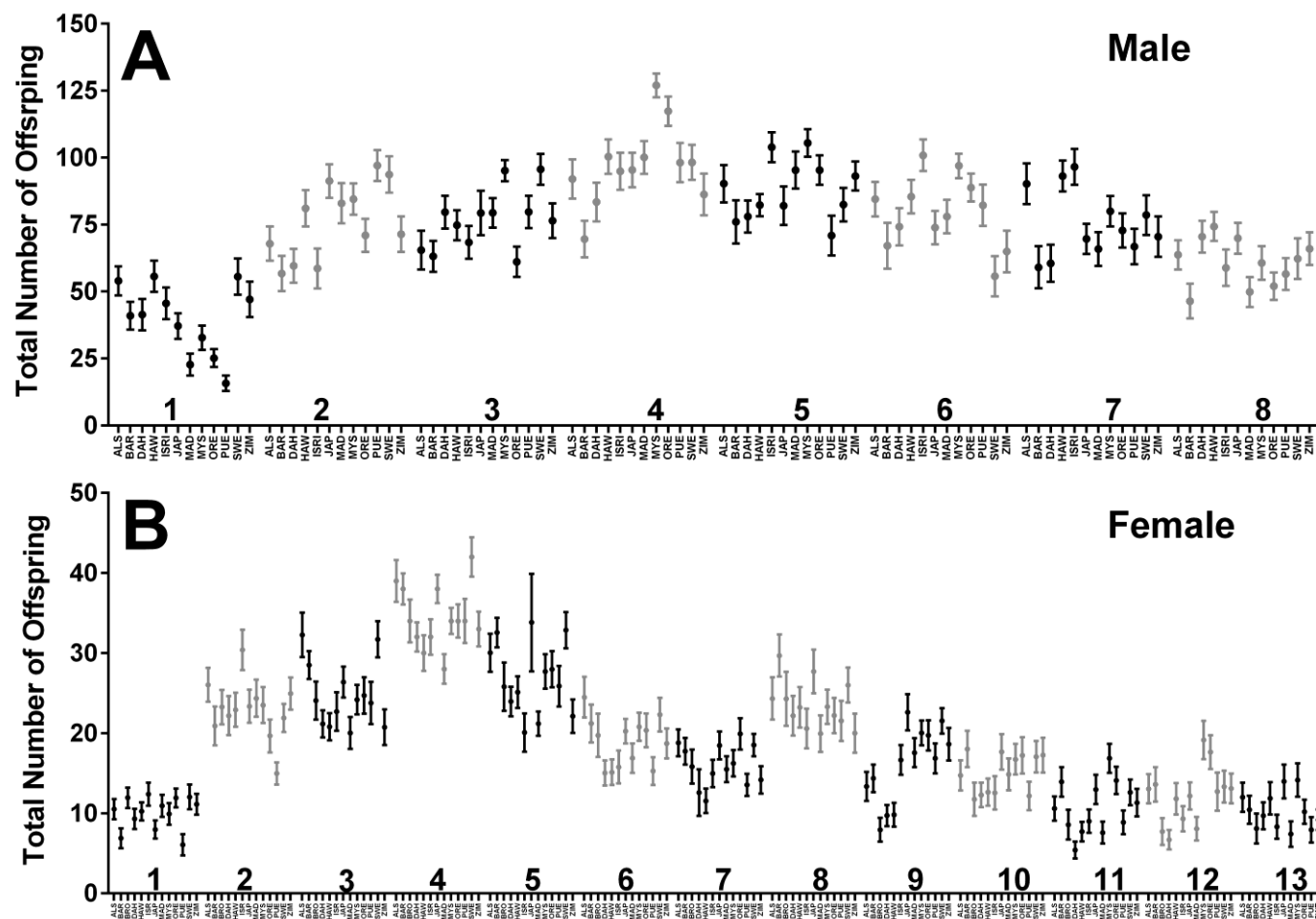


Figure 2: Total number of offspring produced per day (mean \pm 1 S.E.) for (A) males and (B) females across the mitochondrial strains per day of the “sustained reproductive success” experiment. Male experiment ran for 8 days, whilst female productivity assay ran for 13 consecutive days.

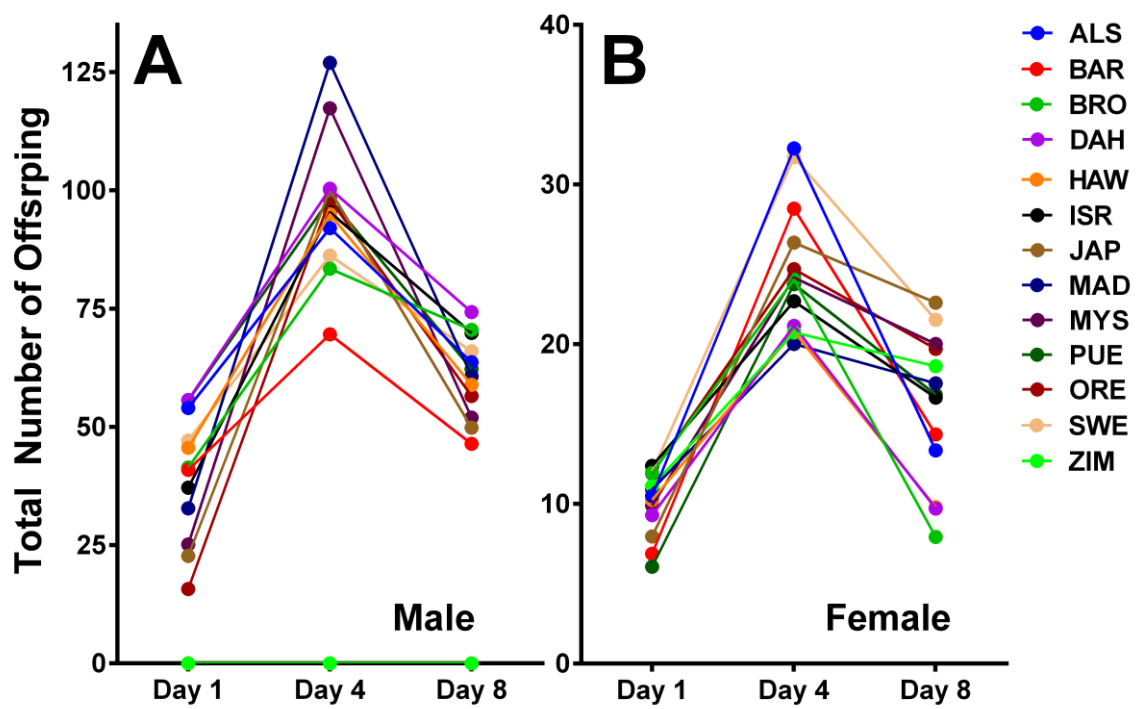


Figure 3: Total number of offspring produced (reproductive success) for (A) males and (B) females across the mitochondrial strains, and at 3 different age points of the sustained reproductive success experiment.

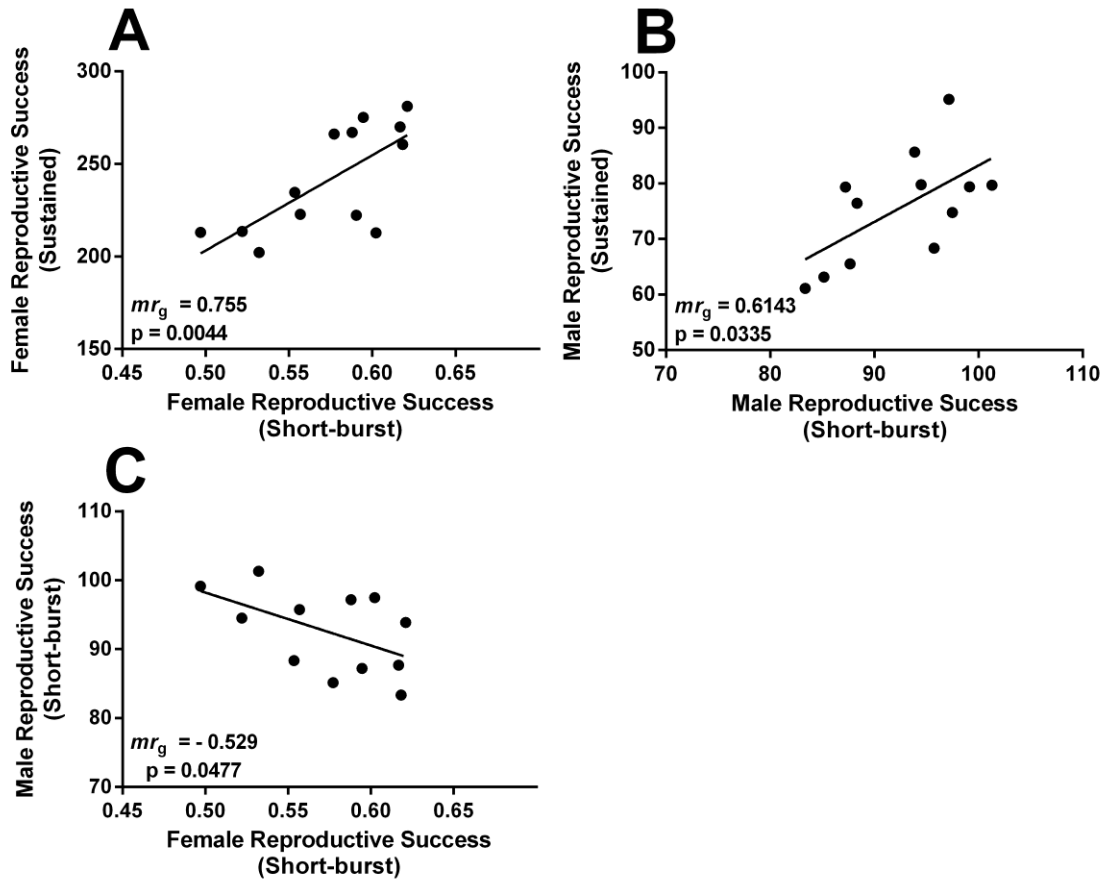


Figure 4: **A)** Intra-sexual association between mean female short-burst fertility and sustained reproductive success across mitochondrial strains; **B)** intra-sexual association between male short-burst and sustained reproductive success across mitochondrial strains; **C)** inter-sexual association between male short-burst reproductive success and female short-burst fertility across mitochondrial strains.

Declaration for Thesis Chapter 3

Monash University

“Single nucleotides in the mtDNA sequence modify mitochondrial molecular function and are associated with sex-specific effects on fertility and aging”

This thesis chapter is in the same format as the manuscript currently Published in the peer reviewed journal *Current Biology*

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design, execution of experimental work, analysis, manuscript writing	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Edward. H. Morrow	Manuscript editing	5%
Jochen B.W. Wolf	Experimental design and manuscript editing	5%
Damian K. Dowling	Experimental design, manuscript writing	20%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's
Signature



Date 23/03/2016

Main
Supervisor's
Signature

Date

CHAPTER 3

**Single nucleotides in the mtDNA sequence
modify mitochondrial molecular function, and
are associated with sex-specific effects on
fertility and aging**

3.1 Summary

Mitochondria underpin energy conversion in eukaryotes. Their small genomes have been the subject of increasing attention, with evidence that mitochondrial genetic variation can affect evolutionary trajectories, and shape the expression of life-history traits considered key human health indicators [1, 2]. However, it is not understood how genetic variation across a diminutive genome, which in most species harbours only about a dozen protein-coding genes, can exert broad-scale effects on the organismal phenotype [2, 3]. Such effects are particularly puzzling given the mitochondrial genes involved are under strong evolutionary constraint, and mitochondrial gene expression is highly conserved across diverse taxa [4]. We use replicated genetic lines in the fruit fly, *Drosophila melanogaster*, each characterised by a distinct and naturally-occurring mitochondrial haplotype placed alongside an isogenic nuclear background. We demonstrate that sequence variation within the mitochondrial DNA (mtDNA) affects both the copy number of mitochondrial genomes, as well as patterns of gene expression across key mitochondrial protein-coding genes. In several cases, haplotype-mediated patterns of gene expression were gene-specific, even for genes from within the same transcriptional units. This invokes post-transcriptional processing of RNA in the regulation of mitochondrial genetic effects on organismal phenotypes. Notably, the haplotype-mediated effects on gene expression could be traced backwards to the level of individual nucleotides, and forwards to sex-specific effects on fertility and longevity. Our study thus elucidates how small-scale sequence changes in the mitochondrial genome can achieve broad-scale regulation of health-related phenotypes, even contributing to sex differences in longevity.

3.2 Results and Discussion

We quantified cellular mtDNA copy number, and levels of mitochondrial gene expression (for 9 of 13 protein-coding mtDNA genes) across thirteen lines of *D. melanogaster*, each of which is characterised by a mitochondrial haplotype sourced from a distinct global locality, expressed alongside a completely isogenic nuclear background, *w*¹¹¹⁸ [5, 6] (Table S1). The assays were replicated separately for each sex, and at both younger (6 days) and older (35 days) ages (Experimental Procedures, Fig. S1).

We uncovered an effect of mitochondrial haplotype on mtDNA copy number variation, which was contingent on interactions involving the sex and age of the flies (Table S2A). Furthermore, mtDNA copy number was found to be sexually dimorphic (generally exhibiting higher values in females) for flies of most, but not all haplotypes; with the greater levels of dimorphism in older flies (Fig. 1, Table S2A: age \times sex \times mtDNA haplotype: $p < 0.001$). This effect was haplotype dependent, and in two cases (Dahomey and Hawaii mtDNA at older age) the pattern of dimorphism was one of male-bias (Fig. 1, Fig. S2A).

Similarly, mitochondrial gene expression exhibited strong signatures of sexual dimorphism, in this case generally male-biased (Fig. 2, Fig. S2A). Expression patterns were contingent on interactions involving the mtDNA haplotype, sex and age of the flies (Table S2B, age \times sex \times mtDNA haplotype: $p < 0.001$), and mtDNA-mediated effects on expression also varied across the sampled mitochondrial genes (Table S2B: gene \times mtDNA haplotype, $p < 0.001$, Fig. 1). Notably, some genes exhibited strong differential expression across mtDNA haplotypes, while others did not (Fig. 2).

These results are noteworthy at several levels; firstly, they demonstrate that molecular phenotypes such as gene expression and copy number are not only affected by naturally occurring variation in mitochondrial haplotypes, but that these mtDNA-mediated effects are contingent on the context of sex and life stage. Secondly, it was previously believed that mtDNA copy number regulation was purely under the control of the nucleus, given that the DNA polymerase γ responsible for mitochondrial replication is nuclear-encoded [7]. Our finding adds to a recent study, which showed that an mt-tRNA point mutation in *Drosophila* alters mtDNA copy and organelle number, also in a context-dependent manner, being contingent on interactions between the mitochondrial and nuclear genome [8, 9]. The context-dependence of effects, arising even in conditions of isogenicity of the nuclear background, demonstrates that the links between mitochondrial genotype and phenotype will be difficult to predict in many cases. This, in turn, might help in understanding the complex epidemiology of mitochondrial diseases in humans, where simple links between candidate mtDNA mutations and disease penetrance often remain elusive [2, 10].

Notably, the effects of mitochondrial haplotypic variation on patterns of mitochondrial gene expression extended to cases in which the genes were nested within the same transcriptional units [11], and were therefore expected to exhibit the same pattern of expression. The most striking example pertains to the mt-*ND4* and mt-*ND5* genes, each of which is transcribed as part of a larger polycistronic precursor in *D. melanogaster* [11]. Expression of *ND4* was stable, but expression of *ND5* varied substantially, across haplotypes (Fig. 3A). Expression of *ND5* clustered into two groups of haplotypes, each delineated by an eightfold difference in expression. Males of the *ND5* “high expression” group of haplotypes were associated with shorter longevity than males of the “low expression” group, while female longevity did not differ across groups (sex \times expression group: $p = 0.039$, Table S2C, Fig. 3B). Furthermore,

these groups correlate closely to the molecular phylogeny of the haplotypes (Fig. 3A), and thus effectively represent mitochondrial clades differing in expression. The Mysore haplotype was the only exception. It shows high gene expression, yet closely clusters with the “low expression” Swedish haplotype. Together, these two haplotypes are more closely related to the clade of low expression haplotypes (18 synonymous SNPs between these and Japan) than the clade of high expression haplotypes (38 synonymous SNPs between these and Oregon). The Mysore haplotype differs from the Swedish haplotype by one non-synonymous SNP (Ala-318-Asp) within the protein-coding region, and this SNP is located in the *ND5* gene itself. This Ala-318-Asp SNP is thus putatively responsible for high ND5 expression at the Mysore gene (mt-GWAS p -value < 0.001 , Table S2D), despite the observation that this haplotype appears to be phylogenetically aligned within the low expression clade. Given that sequence polymorphism in the ND5 gene seems to directly affect its mRNA abundance, this indicates parallel evolution at the gene level, brought about by different underlying polymorphisms [12].

At the mt-*CYTB* gene, a haplotype sourced from Brownsville, USA, exhibited a fourfold decrease in expression relative to the other haplotypes, an effect observed in both sexes (Fig. 3C). This effect was traced to a unique non-synonymous SNP in the *CYTB* gene itself (Ala-278-Thr), which delineates Brownsville from the other haplotypes (mt-GWAS, $p < 0.001$). The downstream effects of this SNP are far-reaching, with the Brownsville haplotype associated with sex-specificity in the expression of the core life-history phenotypes – fertility and longevity. The Brownsville haplotype, which harbours the SNP, confers complete male sterility when expressed alongside w^{1118} [13], representing to our knowledge the only known case of mitochondrially-induced cytoplasmic male sterility in metazoans. It is, however, associated with greater male longevity relative to the other haplotypes (Fig. 3D), suggesting

the Ala-278-Thr SNP has antagonistic pleiotropic effects, with sterile males effectively released from the costs of producing highly viable sperm and enjoying longer lives. Females who harbour this haplotype remain fertile (at least under the standardized and non-competitive conditions in which we culture the females), however exhibit shorter longevity relative to females harbouring other haplotypes (sex \times SNP: $p = 0.002$, Table S2E, S2F, Fig. 3D). This SNP therefore represents the first documented example of a candidate sexually antagonistic polymorphism segregating within the mitochondrial genome of a metazoan. Currently, it remains unclear whether or not the mitochondrial genome might generally be enriched for sexually antagonistic fitness variation; a question that deserves further experimental attention. Evolutionary theory predicts that maternal inheritance of mitochondria will render the mitochondrial genome prone to accumulation of mutations of sex-biased effect [14-17]. In particular, any *de novo* mutations that appear in the mtDNA sequence that are overtly sexually antagonistic - being explicitly female-benefitting, but male-harming - would seem particularly likely to accrue quickly, given these mutations can only directly respond to selection through females [16, 17].

Intriguingly, the CYTB Ala-278-Thr SNP is associated with negatively pleiotropic effects on fertility and ageing, both within and between the sexes. This observation has potential to complicate tests of evolutionary theory based on the mitochondrial maternal inheritance, which predicts that mitochondrial genomes will accrue mutations that are explicitly male harming. While in our case it is clear that the negative male sterilizing effects would clearly outweigh any benefits recouped by enhanced male longevity, our findings nonetheless highlight the need for future studies to take several phenotypes into account when assessing evolutionary predictions tied to mitochondrial maternal inheritance [18].

It was previously thought there was little capacity for individual mitochondrial genes to respond to particular SNPs, as observed in the case of *ND5* and *CYTB*, given these genes are transcribed as part of broader transcriptional units [11]. Remarkably, the candidate SNPs we identified were located at non-synonymous sites and nested within the very same genes whose expression they affected. These candidate SNPs are therefore likely to exert their effects post-transcriptionally, potentially by altering the stability of transcripts [11]. Furthermore, although the effects of these SNPs on patterns of gene expression were generally of similar magnitude across the sexes, their effects on longevity and fertility were strikingly sex-specific.

In conclusion, we have been able to trace effects of naturally-segregating single nucleotides to the expression of protein-coding mitochondrial genes, and then link these effects to patterns of longevity and fertility. Our study documents previously unrealized levels of context-dependence in the expression of mitochondrial molecular phenotypes, which are plausible mechanistic mediators of the link between mitochondrial genotype and life-history phenotype. Furthermore, it provides novel insights into the contribution of the mitochondrial genome to life-history trait evolution, and suggests that mitochondrial polymorphisms can contribute to the sex differences observed in traits such as longevity, in which the females of many animal species generally outlive the males [18, 19].

3.3 Experimental Procedures

3.3.1 *Drosophila* lines

We used thirteen ‘mitochondrial lines’ of *Drosophila melanogaster*, reflecting the global diversity of mtDNA genetic variation [5, 6] (Supplemental Experimental Procedures). These lines were created by Clancy [5], via a chromosomal substitution procedure (Table S1) that replaced the nuclear backgrounds associated with each of the 13 mtDNA haplotypes with that of a standardized isogenic, homozygous nuclear background (w^{1118}). Since their generation, the lines have been maintained by back-crossing virgin females from each line to males of the isogenic w^{1118} line for a further 70 generations. The w^{1118} line is itself propagated by only one full-sibling pair mating, per generation, ensuring that any cryptic mutations that accrue within w^{1118} will be swiftly purged, or otherwise transmitted to all of the mitochondrial lines, thus maintaining the critical requirement for nuclear isogenicity across the lines. As a final safeguard of isogenicity, each mitochondrial line has been independently maintained and propagated in duplicate since 2007, which enables effects attributable to allelic variation across mtDNA haplotypes to be statistically partitioned from effects of residual cryptic nuclear genetic variance or other environmental sources of variance (Fig. S1). Thus, placement of the mtDNA haplotypes alongside the isogenic w^{1118} nuclear background provides a powerful model in which to probe for mitochondrial genetic variance for traits such as copy number and gene expression. A caveat is that by controlling for the effects of the nuclear background, we curtail the ability to test for the role of mito-nuclear epistasis in affecting the traits under study [20]. Thus, future studies should be designed to test for mito-nuclear interactions, and also to test for the additive mitochondrial genetic effects under a broader range of nuclear genetic backgrounds.

All lines had been cleared of any potential bacterial endosymbionts, such as *Wolbachia* [21], as confirmed by diagnostic PCR [22].

3.3.2 Experimental design

We tested for mitochondrial genetic variation in mtDNA copy number (an indicator of mitochondrial DNA abundance) and gene expression across nine mitochondrial protein-coding genes, spanning all four mitochondrial polycistronic transcripts [11]. Copy number and gene expression were measured at two age classes (6 and 35 days), separately in males and females. The first age class represents flies in their reproductive prime, while the latter age represents the age at which population-level mortality rates start to increase exponentially when kept under single-sex conditions, thus indicating the onset of physiological senescence [6, 23].

The experimental design was fully factorial ($13 \text{ mito-lines} \times 2 \text{ sexes} \times 2 \text{ age classes} = 52$ experimental units), with each experimental unit represented by three biological replicates of eight flies (Supplemental Experimental Procedures, Fig. S1). All known environmental variables (*e.g.* food source, larval density, temperature, light, age, parental effects and mating status) were carefully standardized when rearing the experimental flies.

3.3.3 Total RNA/DNA extraction and cDNA Synthesis

Total RNA was jointly extracted from all 8 flies of each biological replicate using a combination of TRIzol® Reagent coupled with the Roche *HighPure* RNA extraction kit (Roche Applied Science, Indianapolis, IN), following the manufacturer's instructions. By using TRIzol® Reagent, we were able to separate and independently store DNA and RNA from the one sample. This resulted in 40µL of purified RNA per sample and 30µl of purified

DNA, which was quantified using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA/DNA integrity was assessed with 1% agarose gel electrophoresis. Purity of total RNA was determined as the 260/280 ratio with expected values between 1.8 and 2.0. cDNA was synthesized from 1 μ g of RNA using the Roche Transcriptor First Strand *cDNA Synthesis* Kit. We used a mixture of random hexamers and oligodT primers for cDNA synthesis to capture mitochondrial transcripts both in the transitory polycistronic stage and as individual polyadenylated single transcripts [24].

3.3.4 Gene expression quantification

Nine out of the thirteen mitochondrial protein-coding genes were amplified: COXI, COXII, COXIII, ATPase6, ND1, ND3, ND4, ND5, and CYTB (see Table S2G for complete list of primers used). The other four genes were either too small to amplify using quantitative real time (qRT)-PCR (ND6, ND4L, ATPase8) or were too A-T rich to make suitable primers (ND2). Gene expression of each biological replicate was measured with a Roche Lightcycler 480 (Roche Applied Science, Indianapolis, IN). Reactions occurred in a 384-well plate, which was designed using a sample maximization layout [25]. Each plate measured the expression of a single mitochondrial gene (nine genes corresponding to nine plates in total), with all 52 experimental units and their three biological replicates (156 independent reactions) assayed per plate, with each independent reaction itself subjected to a further level of replication – i.e., a technical replicate (Supplemental Experimental Procedures).

Reactions were performed using the Roche *SYBRGreen I* Mastermix (Roche Applied Science, Indianapolis, IN). Each well contained 5 μ l of SYBR buffer, 4 μ l of 2.5 μ M primer mix and 1 μ l of diluted cDNA. The following amplification regime used was: 90°C (10s),

60°C (10s), 72°C (10s) for 45 cycles, followed by a melt curve analysis to verify the specificity of the primer pair (See Supplemental Experimental Procedures).

For standardization, three nuclear housekeeping genes (HKGs) were chosen from an initial candidate list of 50 commonly used HKGs in *Drosophila* [26] (See Supplemental Experimental Procedures). For each experimental sample, the expression values of the mitochondrial target genes were standardized as follows:

$$2^{-\Delta Ct}$$

in which the cycle threshold ' $\Delta Ct = Ct_{GOI} - Ct_{GEOM}$ ' is a relative measure of gene expression, GOI the gene of interest and GEOM the geometric mean of the three housekeeping genes. The values provided from this equation indicate relative gene expression for each experimental sample in relation to the housekeeping genes.

Gene expression levels of all nine mitochondrial genes were obtained by determining the ΔCt per sample, measured at the maximum acceleration of fluorescence, using the Second Derivative Maximum Method [27] in the Roche Lightcycler Software V1.5.0 (Roche Applied Science, Indianapolis, IN).

3.3.5 mtDNA copy number quantification

mtDNA copy number is a factor known to differ across sexes [28, 29] and may be expected to covary with transcript abundance [30]. We saved the DNA fraction from all RNA extractions and purified the DNA using the *Gentra Puregene Tissue* kit (Qiagen, Valencia, CA). mtDNA copy number was then measured per biological replicate, relative to a single copy gene in the nuclear genome [31]. The parameter thus reflects the average number of mitochondrial DNA copies per cell (or nucleus). Mitochondrial quantification was done by

amplifying a 113 bp region of the large ribosomal subunit (CR34094, FBgn0013686) using quantitative real-time PCR. No nuclear copies of this gene are found in the *Drosophila melanogaster* genome. Nuclear DNA was quantified by amplifying a 135 bp region of the single-copy [32] subunit of the RNA polymerase II gene (CG1554, FBgn0003277).

3.3.6 Longevity analyses

Mean longevity data was obtained for the mitochondrial lines from Camus *et al.* [6].

Longevity data points were extracted as mean longevity estimates for a ‘cohort’ of on average 90 flies per mitochondrial line (as per the original analysis in Camus *et al* [6]); hence we had several longevity data points per mitochondrial haplotype.

Statistical analyses are outlined in the Supplemental Experimental Procedures.

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 32. Aoyagi, N., and Wassarman, D.A. (2000). Genes encoding *Drosophila melanogaster* RNA polymerase II general transcription factors: diversity in TFIIA and TFIID components contributes to gene-specific transcriptional regulation. *The Journal of Cell Biology* 150, F45-50.

3.6 Figures

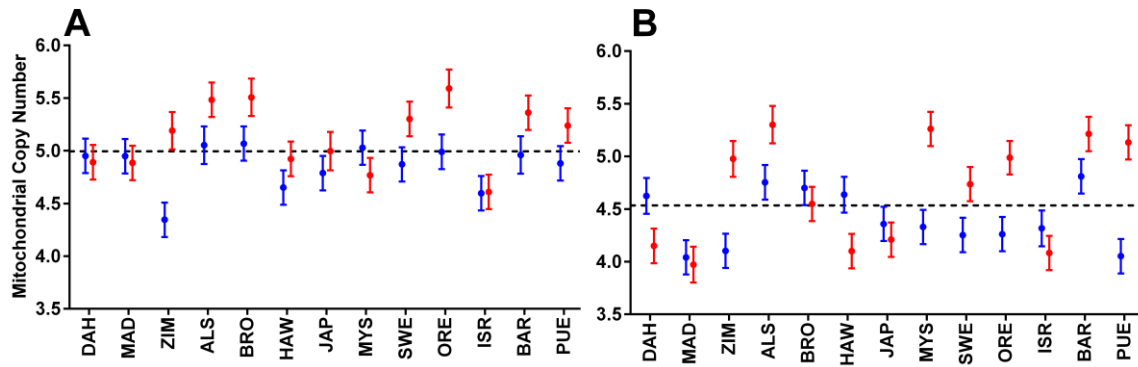


Figure 1. Mitochondrial genetic effects on mtDNA copy number variation

Least-square means (± 1 S.E.) of \log_{10} transformed mtDNA copy number across mtDNA haplotypes, for males (blue points) and females (red points), at **A)** younger (day 6) and **B)** older age (day 35). Dashed line represents the mean for all lines and both sexes at that specific age category.

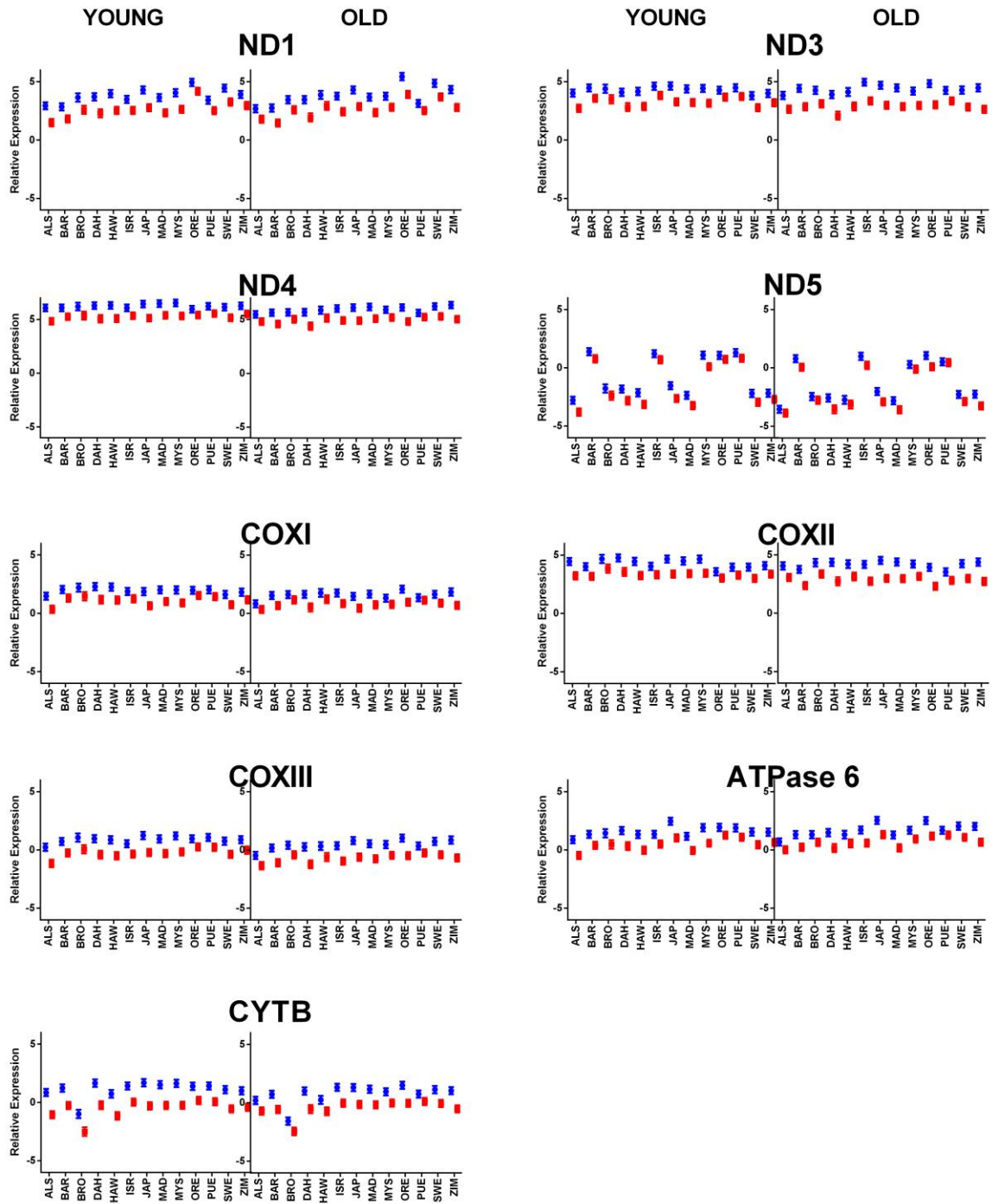


Figure 2. Mitochondrial genetic effects on mitochondrial gene expression. Least-square means (± 1 S.E.) of \log_{10} transformed gene expression values of nine mtDNA protein-coding genes, across 13 mtDNA haplotypes, with males (blue points) and females (red points), in Young (6d) and Old (35d) flies.

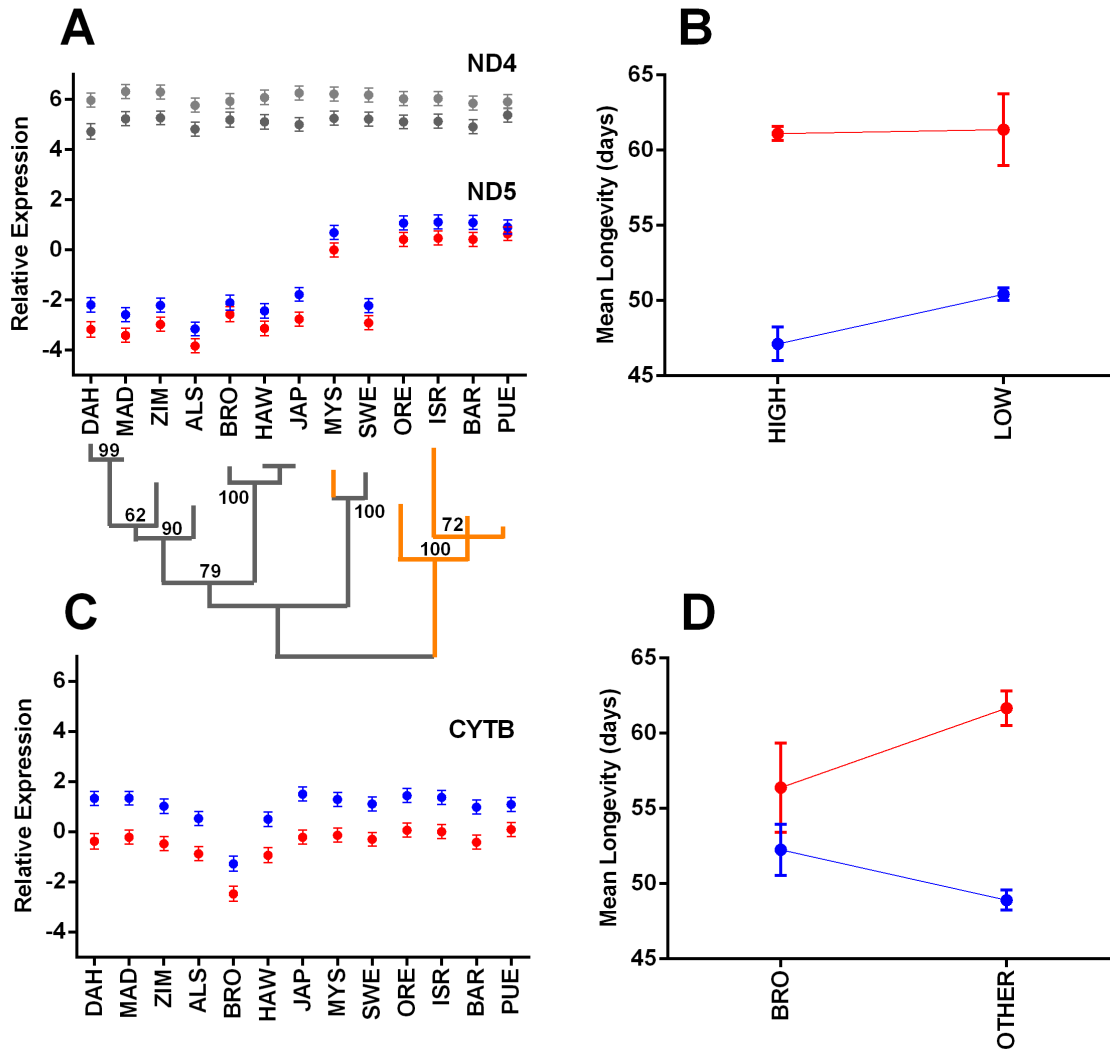


Figure 3. Mitochondrial genetic effects on gene expression map to patterns of sex-specific longevity

A) Least-squares means (± 1 S.E.) of \log_{10} transformed gene expression across mtDNA haplotypes for the *ND4* and *ND5* gene. For the *ND4* gene, males are denoted by light grey data points, and females by dark grey. For the *ND5* gene, males are denoted by blue points and females by red. Drawn below the axes is the mitochondrial phylogeny (neighbour-joining) using the complete genome (excluding D-loop) of all thirteen mtDNA haplotypes. Least-square means for all plots are derived from the multilevel models, which take into account mtDNA copy number as a covariate (Table S2B) .

B) Mean longevity comparisons between the low and high *ND5* expression clades of haplotypes, with females denoted in red and males in blue.

C) Least-squares mean (± 1 S.E.) of \log_{10} transformed gene expression across mtDNA haplotypes, with males (blue points) and females (red points) for the *CYTB* gene.

D) Mean longevity (± 1 S.E.) comparisons between the Brownsville mtDNA haplotype (BRO) and the other (OTHER) haplotypes in females (red) and males (blue).

3.5 Supplementary Information

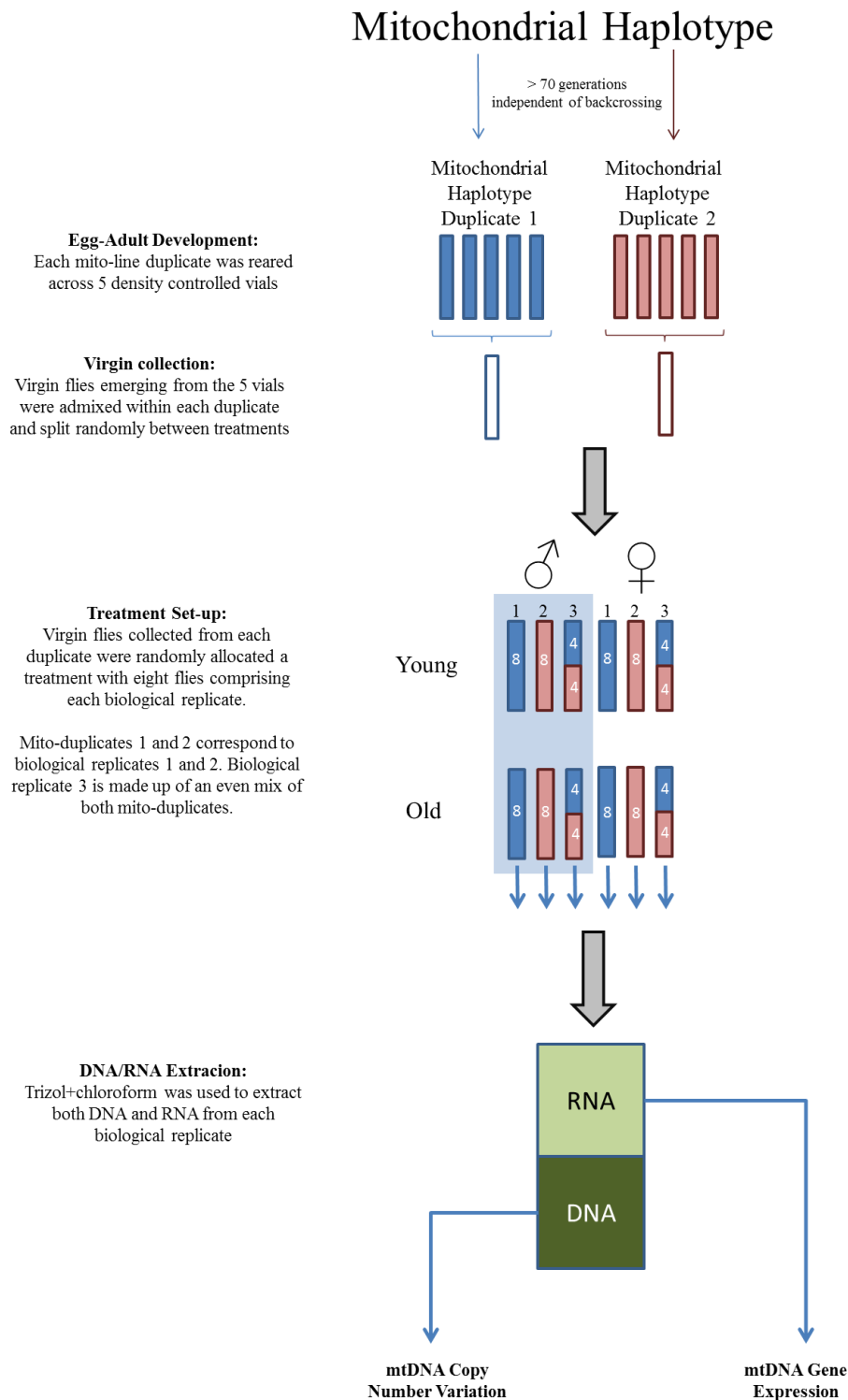


Figure S2: Schematic illustration of experimental set-up of the biological replicates used in the assays. Figure S1 relates to Figures 1-3.

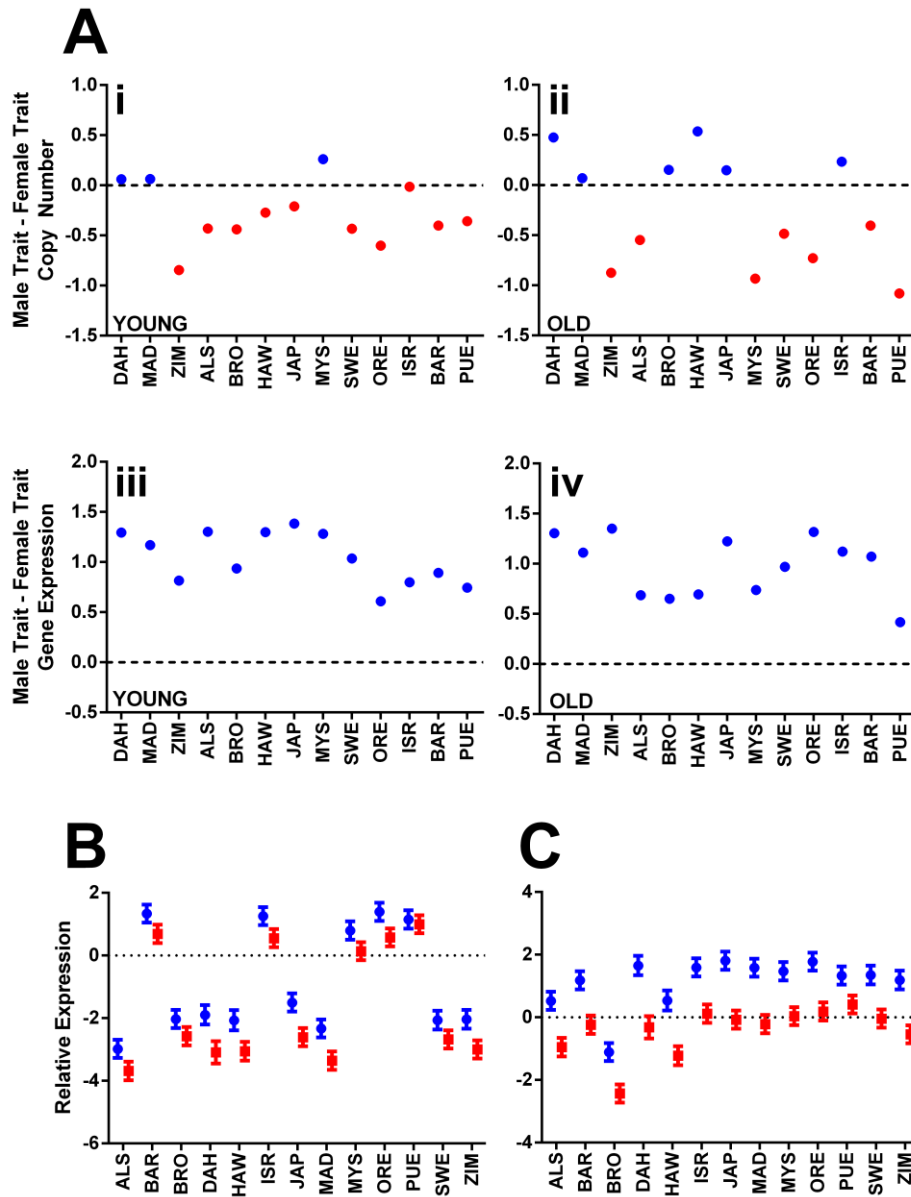


Figure S2. Patterns of trait expression across thirteen sampled mtDNA haplotypes.

(A) Sexual dimorphism of mtDNA copy number variation and mitochondrial gene expression, across age classes; denoted as the difference between mean male and female trait values per mtDNA haplotype, across the thirteen mtDNA haplotypes. Positive values denote male-biased trait expression, and negative values indicate female-bias. Sexual dimorphism per haplotype for copy number expression is shown for (i) young and (ii) old flies; and sexual dimorphism for mitochondrial gene expression (mean across all genes) for (iii) young and (iv) old flies.

(B) Least-squares means (\pm 1 S.E.) of relative gene expression across mtDNA haplotypes and sexes for the ND5 gene, for biological replicates 1 and 2 only.

(C) Least-squares means (\pm 1 S.E.) of relative gene expression across mitochondrial haplotypes and sexes for the CYTB gene, for biological replicates 1 and 2 only.

Table S1. Chromosome replacement crossing scheme utilized by David Clancy [S1] to replace wild type chromosomes from all the founding *Drosophila* mitochondrial lines with isogenic chromosomes. Here we show the consecutive *Drosophila* crosses that were performed in order to place the isogenic nuclear background (w^{1118}) alongside the focal mitochondrial haplotypes.

1	Wild type mitochondrial strain	♀	×	♂	FM7/Y	→ FM7/+(mt) ♀
2a	SM5/+	♀	×	♂	TM6B/+	→ SM5/+;TM6B/+
2b	FM7/+(mt)	♀	×	♂	w^{1118} iso	→ w^{1118} /FM7(mt) ♀
3a	w^{1118} /FM7(mt)	♀	×	♂	w^{1118} iso	→ w^{1118} iso(mt);+/+;+/+ ♀
3b	w^{1118} iso;iso;iso	♀	×	♂	SM5/+;TM6B/+	→ w^{1118} iso/Y;SM5/iso;TM6B/iso ♂
4	w^{1118} iso(mt);+/+;+/+	♀	×	♂	w^{1118} iso/Y;SM5/iso;TM6B/iso	→ w^{1118} iso(mt);SM5/+;TM6B/+ ♀
5	w^{1118} iso(mt);SM5/+;TM6B/+	♀	×	♂	w^{1118} iso/Y;iso;iso	→ w^{1118} iso(mt);SM5/iso;TM6B/iso ♂
6	w^{1118} iso(mt);SM5/iso;TM6B/iso	♀	×	♂	w^{1118} iso/Y;iso;iso	→ w^{1118} iso(mt);iso;iso

Table S2: Statistical analyses and supporting data

S2A. Multilevel general linear model examining the effects of mitochondrial genetic variation, sex and age on mitochondrial DNA copy number. mtDNA haplotype, sex and age were modelled as fixed effects, with mitochondrial biological replicate nested within mitochondrial haplotype as a random effect.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	890.0078	1	< 0.001	***
mtDNA	112.7798	12	< 0.001	***
sex	5.1467	1	0.0082	**
age	0.5752	1	0.3695	
mtDNA × sex	68.5619	12	< 0.001	***
mtDNA × age	37.8474	12	< 0.001	***
sex × age	0.1183	1	0.6977	
mtDNA × sex × age	30.8872	12	< 0.001	***
	SD			
biorep(mtDNA)	0.02148			
Residual	0.40954			

S2B. Multilevel general linear model examining the effects of age, sex, gene and mitochondrial haplotype on gene expression. mtDNA haplotype, sex, gene and age were modelled as fixed effects, with mtDNA copy number as a fixed covariate; whilst mitochondrial biological replicate nested within mitochondrial haplotype was modelled as a random effect.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	210.1585	1	< 0.001	***
age	0.6675	1	0.24663	
sex	0.7773	1	0.3350	
gene	1918.262	9	< 0.001	***
mtDNA	55.6798	12	< 0.001	***
copy number	0.9882	1	0.54837	
sex × gene	91.3104	9	< 0.001	***
age × mtDNA	10.6611	12	0.00308	*
sex × mtDNA	23.2744	12	< 0.001	***
age × sex	10.3113	1	< 0.001	***
gene × mtDNA	1161.466	108	< 0.001	***
age × gene	28.3319	9	< 0.001	***
age × sex × mtDNA	22.403	12	< 0.001	***
age × sex × gene	25.0057	9	0.0203	*
	SD			
biorep(mtDNA)	0.3164			
Residual	0.6140			

S2C. General linear mixed model examining the effects of group expression level of the ND5 gene (HIGH or LOW) on longevity. Expression level and sex were modelled as fixed effects, while mitochondrial haplotype identity, mtDNA line duplicate (2 per haplotype), mtDNA \times sex, and mtDNA duplicate \times sex were modelled as random effects.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	6239.2852	1	< 0.001	***
sex	159.2255	1	< 0.001	***
expression group	0.0489	1	0.85896	
sex \times expression group	4.5489	1	0.03857	*
SD				
mtDNA haplotype	1.5228			
Duplicate[mtDNA haplotype]	0.2922			
mtDNA haplotype \times sex	1.6127			
Duplicate[mtDNA haplotype] \times sex	0			
Residual	0.5479			

S2D. ND5 molecular composition across the thirteen mtDNA haplotypes. Nucleotide changes are displayed, and amino acid changes are shown in bold. The SNP at aa318 is unique to the Mysore haplotype, and is also correlated with ND5 expression.

Position	220	237	241(81)	255	828	945	952(318)	1068	1137	1254	1500(500)
Consensus	A	T	C(H)	C	A	T	A(N)	C	C	C	T(I)
ALS											
MAD											
ZIM											
DAH											
BRO				T							
HAW				T					T		
JAP				T					T		
SWE			T(Y)		G			T		T	
MYS			T (Y)		G		G(D)	T		T	
BAR		C				C			T		A(M)
ISR		C				C					A(M)
ORE	G	C				C					A(M)
PUE		C				C					A(M)

S2E. General linear mixed model examining the effects of the Ala-278-Thr SNP in the CYTB gene and sex on longevity. The only mtDNA haplotype that harbours this SNP is Brownsville. Presence of the SNP, and sex were modelled as fixed effects, while mitochondrial haplotype identity, mtDNA line duplicate (2 per haplotype), mtDNA \times sex, and mtDNA duplicate \times sex were modelled as random effects.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	12794.53	1	< 0.001	***
Sex	322.6848	1	< 0.001	***
SNP	6.7059	1	0.009609	**
sex \times SNP	9.2453	1	0.002361	**
SD				
mtDNA haplotype	0.7785			
Duplicate[mtDNA haplotype]	1.47×10^{-5}			
mtDNA haplotype \times sex	0			
Duplicate[mtDNA haplotype] \times sex	3.655×10^{-6}			
Residual	3.644			

S2F. Model, accounting for heteroscedasticity, examining the effects of the Ala-278-Thr SNP in the CYB gene and sex on longevity (see Longevity analyses in Supplemental Experimental Procedures). Presence of the SNP, and sex, were modelled as fixed effects, and SNP was assigned to the *varIdent* variance function structure.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	18872.91	1	< 0.001	***
Sex	498.89	1	< 0.001	***
SNP	3.65	1	0.056	
sex \times SNP	4.70	1	0.03	*

S2G. Amplification efficiency of all primers pairs was calculated by constructing a standard curve using serial dilution of RNA (gene expression) or DNA (copy number). Melting curve analysis followed every qRT-PCR reaction to confirm specificity of the primers, and detect possible instances of contamination.

Primer Name	5'-Forward Sequence-3'	5'-Reverse Sequence-3'	Efficiency
COXI	TCCTGATATAGCATTCCCACG	CAACTGAAGCTCCACCATGA	96.6%
COXII	AGATGTTGATAACCGAGTAGTTTACC	AAGCAGGTGATAACCGAGTAGTTTACC	85.7%
COXIII	TCACCCGCTATTGAATTAGGA	TCTATGGTGGGCTCAAGTTACA	86.5%
ND1	TCATTAGCTGAACTAATCGTACTCC	AAAGCAAAACCCCTCTTCT	83.3%
ND3	GACCGAGAAAAAAGATCCCC	GGTAATCGAGATGAAGATTTTGG	94.4%
ND4	GCTCATGGTTTATGTTCTTCTGG	TTCGACTTCCAAGACGTCA	93.5%
ND5	TATTGTTTATATGTGCTGGG	GCTAAAAAAGGTATTCCACATAAAGC	85.6%
ATPase6	GGAACACCCGCTATTCTTATACC	GCAATTATATTAGCAGTTAATCGAACA	89.2%
CYTB	ACACCTGCCCATATTCAACC	TGATAAACTAATGCAATAACTCCTCC	91.4%
AppI	AGTTCCCGAGGGCTGTCTGTT	GCTTGGGACAGCAGACGAACT	99.96%
Elav	CGCGACAAGTCGCAGGTCTAC	TCATCGTCTTTGGCAGCCCCG	97.99%
Sdha	CATGCTGCTGTGTTCCGCGA	ACCATCCAGGGGCTTGCTGA	99.36%
rrnL	TCGTCCAACCATTCATTCC	ATAAAGTCTAACCTGCCCCTGA	97.3%
RpolII	AGGCGTTTGAGTGGTTGG	TGGAAGGTGTTCAAGTGTATC	98.6%
ND5 (2)	TGCTATAACTAAAAGAGCTCAGATTCC	AAGAATGAACTAAAGCAGAAACAGG	92.5%
CYTB (2)	AAATTTATTGGGAGACCCTGATAAC	GGAATAGATCGTAAAATAGCATAAGCA	94.3%

S2H. Number of synonymous (lower half) and non-synonymous (upper half in bold) polymorphic sites across the mitochondrial haplotypes.

	ALS	BAR	BRO	DAH	HAW	ISR	JAP	MAD	MYS	ORE	PUE	SWE	ZIM
ALS	-	7	1	3	0	5	1	2	4	6	5	4	4
BAR	37	-	8	10	7	2	8	9	11	3	2	11	11
BRO	13	39	-	4	1	6	2	3	5	7	6	5	5
DAH	5	36	14	-	3	8	4	1	7	9	8	7	5
HAW	15	41	2	16	-	5	1	2	4	6	5	4	4
ISRI	37	4	41	38	43	-	6	7	9	1	0	9	9
JAP	13	39	0	14	2	41	-	3	5	7	6	5	5
MAD	5	36	14	0	16	38	14	-	6	8	7	6	4
MYS	13	38	18	16	20	38	18	16	-	10	9	1	8
ORE	37	6	39	36	41	8	39	36	38	-	1	10	10
PUE	37	2	39	36	41	4	39	36	38	6	-	9	9
SWE	13	38	18	16	20	38	18	16	0	38	38	-	8
ZIM	1	36	12	4	14	36	12	4	12	36	36	12	-

S2I. Multilevel model examining the effects of age, sex and mitochondrial haplotype on gene expression. Data encompasses biological replicates 1 and 2 only.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	187.9691	1	< 0.001	***
Age	1.6783	1	0.195152	
Sex	0.1071	1	0.743469	
Gene	1083.802	9	< 0.001	***
mtDNA	80.8823	12	< 0.001	***
CopyN	0.0395	1	0.842397	
Sex × Gene	46.9192	9	< 0.001	***
Age × mtDNA	17.2815	12	0.13931	
Sex × mtDNA	33.0928	12	< 0.001	***
Age × Sex	5.0184	1	0.025079	*
Gene × mtDNA	776.4382	108	< 0.001	***
Age × Gene	18.647	9	0.028367	*
Age × Sex × mtDNA	20.139	12	0.064502	
Age × Sex × Gene	13.1416	9	0.156303	
SD				
Duplicate[mtDNA haplotype]	0.08064			
Residual	0.61047			

S2J. Multilevel model examining the effects of age, sex and mitochondrial haplotype on gene expression. These analyses are limited to first-order interactions only, and use all biological replicates.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	258.9395	1	< 0.001	***
Age	3.4392	1	0.063667	
Sex	3.6863	1	0.05486	
Gene	2261.019	9	< 0.001	***
mtDNA	74.368	12	< 0.001	***
CopyN	2.2352	1	0.134901	
Sex × Gene	85.0522	9	< 0.001	***
Age × mtDNA	28.5343	12	0.004618	**
Sex × mtDNA	30.7243	12	2.17E-03	**
Age × Sex	0.6517	1	0.419501	
Gene × mtDNA	1348.306	108	< 0.001	***
Age × Gene	31.6823	9	< 0.001	***
SD				
Duplicate[mtDNA haplotype]	0.2455			
Residual	0.5854			

S2K. Multilevel model examining the effects of age, sex and mitochondrial haplotype on gene expression. These analyses are limited to two-way interactions only, and biological replicates 1 & 2.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	207.0336	1	< 0.001	***
Age	0.0927	1	0.760802	
Sex	1.6136	1	0.203985	
Gene	1142.017	9	< 0.001	***
mtDNA	80.9477	12	< 0.001	***
CopyN	0.0485	1	0.82564	
Sex × Gene	58.4355	9	< 0.001	***
Age × mtDNA	11.2825	12	0.504873	
Sex × mtDNA	38.4859	12	< 0.001	***
Age × Sex	4.579	1	0.032365	*
Gene × mtDNA	772.9596	108	< 0.001	***
Age × Gene	27.657	9	0.001088	**
	SD			
Duplicate[mtDNA haplotype]	0.06179			
Residual	0.64272			

Supplemental Experimental Procedures

Drosophila lines

We used thirteen ‘mitochondrial lines’ of *Drosophila melanogaster*, reflecting the global diversity of mtDNA genetic variation [S1, S2]. These were: Alstonville, New South Wales, Australia (**ALS**); Barcelona, Spain (**BAR**); Brownsville, Texas, USA (**BRO**); Dahomey - now Benin - West Africa (**DAH**); Hawaii, USA (**HAW**); Israel (**ISR**); Japan (**JAP**); Madang, Papua New Guinea (**MAD**); Mysore, India (**MYS**); Oregon, USA (**ORE**); Puerto Montt, Chile (**PUE**); Sweden (**SWE**); and Zimbabwe (**ZIM**). These lines were created by Clancy [S1], via a chromosomal substitution procedure (Table S1) that replaced the nuclear backgrounds associated with each of the 13 mtDNA haplotypes with that of a standardized isogenic, homozygous nuclear background (w^{1118}).

Constitution of the biological replicates

The biological replicates of each experimental unit were created in the following way. In the generations leading up to the experiment, each of the mitochondrial line duplicates was maintained by 16 pairs of fly per vial, across five density- and age-controlled vials, to mitigate the potential for non-genetic parental effects, or environmental sources of variance (e.g. vial-sharing effects), to influence the results. Rearing densities were controlled by regulating the total number of eggs per vial to a low density of between 80 and 100 eggs. In the generation prior to the experiment, the parents of the ‘experimental’ flies, when 4 d old, were provided with a 24 h opportunity in which to lay eggs. Egg numbers were then culled to 80 per vial. Ten days later, and within 6 h of their eclosion to ensure their virginity, adult offspring emerging from each of the vials per independent duplicate were admixed into the one vial, prior to being re-sorted into single-sex vials at densities of 8 flies per vial. Each of these vials of 8 flies was assigned as a biological replicate in the experiment. Specifically, the first biological replicate of any experimental unit was comprised of flies collected from

duplicate 1 of a given mitochondrial line, the second biological replicate from duplicate 2 of that same mitochondrial line, and the third comprised an equal mix of flies from each of the two duplicates (4 flies from each) [See *Supplemental Statistical analysis* section below for discussion and analysis on the constitution of the biological replicates].

When the experimental flies of each biological replicate were 3 d old, they were exposed to 3 d old ‘tester’ virgin flies of the opposite sex, which were sourced from the w^{1118} line, for a period of 24 h to enable mating. The w^{1118} tester flies had been reared in synchrony with the flies of each mitochondrial line, under the same standardized conditions, controlling for density- and age-effects. The tester flies were then discarded and the experimental flies (8 flies per biological replicate) transferred into a new vial with fresh food substrate and controlled amounts of live yeast (50μg) every second day. Flies comprising the “young” treatment were aged until 6 d old, while flies in the “old” treatment were aged until 35 d post-eclosion. Flies of each biological replicate were flash frozen in liquid nitrogen upon reaching the designated age class at the same time of day (11:00AM) to control for effects of circadian rhythm.

Gene expression analyses

Selection of mitochondrial protein-coding genes

The *D. melanogaster* mitochondrial genome is highly A-T rich, and it is therefore difficult to design suitable primers for qRT-PCR. Furthermore, some mitochondrial genes are extremely small, and suitable amplification products could not be designed. In total, we were able to reliably and successfully amplify nine of the thirteen mitochondrial protein-coding genes.

Investigating the potential for primer-specific effects

To explore the possibility that specific SNPs contained within the primer sequences interacted with the qRT-PCR reaction, we also used a second set of primers for both the ND5 and CYTB genes across the mitochondrial lines. For each gene, primer efficiency was then compared across the two sets of primers, across the mitochondrial lines. We reasoned that if particular SNPs were interacting with the qRT-PCR reaction, then we would observe significantly different efficiency curves across primer sets and across lines. This was not the case, however, and both sets of primers had similar efficiencies and resulted in the same expression profiles across mtDNA haplotypes (Table S2G).

Plate optimisation, calibrators and variation in expression across technical replicates

To facilitate comparisons between plates, calibrators were placed on each plate to minimize plate-to-plate variation. To enable this design, each plate measured the expression of a single mitochondrial gene (nine genes corresponding to nine plates in total). In addition, each independent reaction was itself replicated on the plate, by measuring its expression across two separate wells (i.e., a technical replicate), to account for technical variation in the assay.

When the ΔC_t values between the two technical duplicates for each sample fell within 0.5 units of each other, then the mean gene expression estimates were pooled to form a single data point [S3]. Technical duplicates with a ΔC_t value differences greater than 0.5 were resampled alongside HKGs and calibrators on a separate plate. The technical duplicates of only seven samples, in total, deviated by more than 0.5 ΔC_t units, thus requiring resampling. This is less than one percent of all reactions included in this study. We included these resampled reactions in our final analyses because plate-plate variation, tested using calibrator values, was small (ΔC_t mean per plate = 25.07, ΔC_t S.D. = 0.48), and inclusion of these seven samples did not change the qualitative inferences of the final model.

Selection of housekeeping genes

Three nuclear housekeeping genes (HKGs) were chosen from an initial candidate list of 50 commonly-used HKGs in *Drosophila* [S4] (See Supplemental Experimental Procedures). This list was then scanned for sex- and mtDNA-specific signatures of differential expression across five (of the 13) mitochondrial lines, using a published and publicly-accessible microarray dataset [S5]. The resulting shortlist of 10 genes was tested for mtDNA-, sex- and age-specific effects using quantitative real-time PCR. After quality assessment with the Bestkeeper[®] software [S6], three suitable HKGs that were insensitive to sex and age effects across the mtDNA haplotypes were identified: succinate dehydrogenase A (CG17246), β amyloid protein precursor-like (CG7727), and protein-coding gene CG4262. All three genes had high correlation coefficients (>0.8) against each other, further indicating their suitability for use as housekeeping genes.

Statistical analysis

Statistical analysis of mitochondrial abundance and gene expression variation

We fitted multilevel models, in which mtDNA copy number and gene expression data were modelled separately as response variables. Mitochondrial haplotype, age, sex, and gene identity were modelled as fixed effects, and the biological replicate nested within the mitochondrial line as a random effect. Both copy number variation and expression data were \log_{10} transformed prior to analysis, to attain normally distributed model residuals. Mitochondrial copy number values were added as a fixed covariate to the analysis of gene expression, to account for gene expression patterns that were directly tied to mtDNA copy number. Parameter estimates were calculated using the *lmer* function in the *lme4* package [S7] [1] in R (R Development Core Team 2009). The fitted models were evaluated by simplifying a full model, by sequentially removing terms that did not change the deviance of

the model (at $\alpha = 0.05$); starting with the highest order interactions, and using log-likelihood ratio tests to assess the change in deviance in the reduced model relative to the previous model [S8] [2]. Only the final models are presented in Table S2. Both measures of copy number (single fly extraction or derived from 8 fly Trizol extraction) were highly correlated ($R^2 = 0.7925$), hence we used results from the 8-fly extraction to model copy number variation instead of single fly values.

Longevity analyses

Mean longevity data was obtained for the mitochondrial lines from Camus *et al.* [S2]. Longevity data points were extracted as mean longevity estimates for a ‘cohort’ of on average 90 flies per mitochondrial line (as per the original analysis in Camus *et al* [S2]); hence we had several longevity data points per mitochondrial haplotype. While haplotype effects on expression were pervasive across genes, and generally contingent on the sex and age of the flies (Table S2B), mt-GWAS (Supplemental Experimental Procedures) identified SNP associations of interest at two genes (ND5 and CYTB), and visual inspection of the expression patterns reconciled against the sequence phylogeny confirmed these two genes were unusual (Fig. 3).

Gene expression of ND5 exhibited two distinct clusters (a high and a low expression group, each delineated by an eightfold difference in expression), which corresponded tightly to the two mitochondrial phylogenetic clades, with one exception (MYS). No other sampled gene exhibited this pattern of clade-specific expression. As such, the following analysis could only be applied to the ND5 gene. Our goal was to determine whether the two expression groups of the ND5 gene were associated with any longevity consequences. We fitted a mixed model in which longevity was modelled as a response variable, sex and expression group (High or

Low expression groups, in which the MYS haplotype was included in the high expression group) were modelled as fixed effects, while the identity of the mtDNA haplotype, the mitochondrial line duplicate (2 per haplotype), the mtDNA \times sex interaction, and the mitochondrial duplicate \times sex interaction were modelled as random effects (Table S2C, Supplemental Experimental Procedures).

The other gene of interest was the CYTB gene, in which one particular haplotype (BRO) exhibited a fourfold reduction in gene expression relative to the other mtDNA haplotypes. Notably, the Brownsville haplotype is completely male-sterile when expressed alongside the *w¹¹¹⁸* nuclear background of the mitochondrial lines. This haplotype harbours a non-synonymous SNP (Ala-278-Thr) in CYTB, which delineates it from the other twelve haplotypes [S1], and which was identified by the mt-GWAS as a candidate SNP. A similar model as used above for the ND5 gene was used to examine the effect of the Ala-278-Thr SNP on longevity. Sex and SNP presence (Brownsville has the SNP, whereas the rest of the mtDNA haplotypes do not) were modelled as fixed effects, and the identity of the mitochondrial haplotype, mitochondrial line duplicate (2 per haplotype), mtDNA \times sex, and the mitochondrial duplicate \times sex interaction modelled as random effects (Table S2E, S2F, Supplemental Experimental Procedures).

The longevity analyses fully account for the hierarchical structure of the data, with the mitochondrial line (i.e. the mtDNA haplotype), and the mitochondrial line duplicate nested within the mtDNA haplotype as random effects in these models. Levene's tests for homogeneity of variance, indicated that variance across expression groups in the ND5 analysis was homogeneous ($F=2.02$, $p = 0.16$), but heterogeneous across the SNP classification for the CYTB analysis ($F = 8.86$, $p = 0.004$). While the analyses we present in

the main body of the manuscript do not account for unequal variances, but instead a model that accounts for the full hierarchical structure of the data, we also present a mixed model on the CYTB analysis that does account for these variances and that confirms our results (Table S2F). In that analysis, we added the *varIdent* variance function structure to the linear mixed model, which allows for different variance structures for each level of a factor, and thus accounts for heteroscedasticity [S9]. This analysis was conducted using a linear model in the *nlme* package [S10] of R, using longevity data extracted at the level of replication of the mtDNA haplotype (to simplify the structure of the data. Thus, each data point represented the mean longevity associated with a cohort of flies that shared the same mtDNA haplotype, sex and were sampled from within the same experimental block (there were seven sampling blocks included in the analysis of Camus et al. [S2]). The *varIdent* function was assigned to the SNP factor, to account for heterogeneity of variance across levels of this factor (Table S2F). Finally, Welch's unequal variance t-tests, conducted separately for each sex, for flies carrying versus not carrying the Ala-278-Thr SNP, confirmed the differences in longevity (males, $t = 3.6501$, $p = 0.004$; females. $t = -3.6504$, $p = 0.004$).

Analyses excluding the third biological replicate

The third experimental biological replicate for each mitochondrial line was derived from a balanced combination of flies from mitochondrial duplicates 1 and 2, but inclusion of this replicate did not alter any of our inferences for either copy number variation or gene expression patterns. Reanalysis of the dataset, when including only biological replicates 1 and 2 (Table S2I, K), or when limiting to first-order interactions (Table S2 J) yielded qualitatively similar parameter estimates (Fig. S2B, C).

Molecular Composition, Phylogeny and mtGWAS

Full protein-coding regions for each haplotype have previously been sequenced [S1, S2] and are publicly available. Separate alignments for each gene, transcriptional unit, and whole coding regions were performed using Geneious (version 5.6.3, <http://www.geneious.com>) and MEGA6 [S11]. Analyses revealed two major clades delineating the haplotypes (Fig. 3).

A total of 90 SNPs exist across the mitochondrial haplotypes, 65 of which are synonymous and 25 non-synonymous (Table S2H). All variable sites were used for a mitochondrial genome-wide association study (mt-GWAS / eQTL analysis) to examine correlations between particular SNPs and mitochondrial gene expression levels at a gene-specific level, using mixed linear models in Tassel version 4.0 [S12]. We also used a similar GWAS analysis to screen for associations between the 90 SNPs and mtDNA copy number variation (Supplemental Experimental Procedures). Significance was adjusted for multiple testing via a Bonferroni correction for the number of SNPs tested.

We analysed results at the three molecular scales to examine and compare possible differences in the phylogenetic topologies. The phylogeny of these mitochondrial haplotypes was assembled using maximum likelihood and Bayesian approaches, and nucleotide substitution models assessed using the software jModeltest [S13]. The best fitting substitution model, used for construction of a maximum likelihood (ML) tree, was defined as: HKG+G (BIC = 38446.7394), and this model was constructed in MEGA6 [S10] (bootstrap replications = 1000).

Analyses conducted at the whole haplotype level, transcriptional unit, and the individual gene level indicates two major clades delineating the haplotypes (Fig. 3); the first clade contains

mtDNA haplotypes sourced from: ORE, ISR, BAR, PUE, while the second clade comprises haplotypes sourced from DAH, ZIM, MAD, ALST BRO, HAW, JAP, SWE and MYS.

In the GWAS analyses, kinship or relatedness (K) among mitochondrial lines was calculated using the SNP matrix, and was used in the model to control for background SNP variation and reduce *type I* error due to relatedness. A mixed linear model (MLM) was implemented with trait values (copy number variation and gene expression values) as response variables in separate models, genetic markers (SNPs) as fixed effects and the K matrix as a covariate. This analysis was run for both copy number variation and gene expression, for combined traits as well as separate age and sex categories. Significance was adjusted for multiple testing via a Bonferroni correction for the number of SNPs tested. Significant associations were found only for the gene expression data, and these associations are presented in the main manuscript.

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Declaration for Thesis Chapter 4

Monash University

“Experimental Evidence for Mitochondrial Genomic Adaptation to Thermal Climate”

This thesis chapter is in submission format for the peer reviewed journal *Nature*

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design, execution of experimental work, analysis, manuscript writing	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Jonci N. Wolff	Experimental work, Manuscript editing	10%
Carla M. Sgrò	Experimental design, Manuscript editing	5%
Damian K. Dowling	Experimental design, Manuscript writing	15%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's
Signature



Date 23/03/2016

Main
Supervisor's
Signature

Date

CHAPTER 4

Experimental evidence for mitochondrial genomic adaptation to the thermal climate

4.1 Abstract

Mitochondrial metabolism is regulated by a series of enzyme complexes within the mitochondrion, and functioning of these enzymes is sensitive to heterogeneity in the prevailing temperature^{1,2}. Such thermal sensitivity, coupled with the observation that mitochondrial haplotype frequencies tend to associate with latitude³, altitude⁴ or climatic region across species distributions^{5,6}, led to the hypothesis that thermal selection has played a role in shaping the molecular architecture of the mitochondrial DNA^{3,5,7}. This hypothesis has, however, yet to receive experimental validation. Here, we present such support. First, we describe two major mitochondrial haplogroups in *Drosophila melanogaster*, each characterised by one major haplotype whose frequencies exhibit an opposing pattern of clinal variation along the Australian eastern seaboard. One haplogroup predominates in the north of Australia, the other in the south. Second, we extracted each of these major haplotypes from each of two latitudinally-divergent populations along the cline, and created novel genetic strains by placing them alongside an isogenic nuclear background. Third, we assayed flies of each strain for their capacity to withstand extreme heat and cold events, and investigated candidate molecular mechanisms that might underpin observed thermal responses across the haplotypes. Flies harboring the northern-predominant haplotype exhibited greater resistance to heat stress than flies harboring southern-predominant haplotypes. Conversely, flies harboring southern haplotypes exhibited increased resistance to cold stress than flies with the northern haplotype. The SNPs delineating the two haplogroups do not change the amino acid sequence, but nonetheless they have large effects on thermotolerance phenotypes, affect levels of codon bias and patterns of gene expression at *mt-ND4* and *ND5*. Our results thus highlight a key role for a set of SNPs previously considered to be

non-functional, found inside a genome that was likewise considered to be devoid of phenotype-modifying allelic variation, in the dynamics of thermal adaptation.

4.2 Main

The mitochondria are essential for eukaryote evolution, taking centre-stage in the process of cellular respiration. This process is regulated via a series of finely coordinated interactions encoded by two obligate genomes – nuclear and mitochondrial⁸. Both genomes are required for the production of cellular energy, and thus their harmonious interaction is essential to maintain mitochondrial integrity and the viability of eukaryote life^{8,9}. Indeed, because of the strong dependence of cellular respiration on mitochondrially-encoded gene products, biologists long assumed that strong purifying selection would efficiently prevent function-encoding genetic variation from accumulating within the mtDNA sequence^{10,11}.

The assumption of neutrality has, however, been challenged during the past decade, via studies demonstrating that naturally-occurring mitochondrial genetic variation affects the expression of core phenotypic traits - both metabolic and life-history¹²⁻¹⁵. Furthermore, a series of observations has led to the hypothesis that the genetic variation found within the mitochondrial genome, which delineates the mtDNA haplotypes of spatially-disjunct populations, has been shaped by natural selection imposed by the prevailing thermal climate^{5,7,16}. For instance, some studies have detected positive selective sweeps in the mitochondrial genome¹⁷, while others have found that the frequencies of particular mtDNA haplotypes change with latitudinal³ or altitudinal variation¹⁸. In addition, particular SNPs in core mtDNA protein-coding genes of humans have been linked to geographic regions characterised by climatic extremes^{5,16}, and pairwise differences in genetic differentiation between human mtDNA haplotypes have been correlated with thermal differences between the geographic

regions from which they derive ⁶. Although correlative in nature, the findings of these studies have been substantiated by empirical studies in invertebrates, which indicate that fitness outcomes associated with particular combinations of mitochondrial and nuclear (mito-nuclear) genotype depend on the thermal conditions in which the study subjects are assayed ^{19,20}.

Further evidence that the mitochondrial genome responds to thermal selection comes from studies in *Drosophila* that documented inter-generational shifts in the frequency of distinct mtDNA haplotypes competing within individuals (i.e. in heteroplasmy). The relative frequencies of each of the mtDNA haplotypes were affected by the temperature the experimental flies were reared at, the nuclear background and their interaction²¹. In those studies, heteroplasmy was artificially induced by introducing the mtDNA of one *Drosophila* species into strains of co-generic species^{21,22}. Finally, studies of genetic variation in *Drosophila* have detected signatures of thermal selection on nuclear alleles associated with core metabolic architecture and mitochondrial function along a latitudinal cline spanning the eastern coast of North America ^{23,24}, thus implicating nuclear genes that are putatively involved in mito-nuclear interactions in the dynamics of thermal adaptation.

Collectively, the aforementioned evidence presents a case consistent with a hypothesis that patterns of mitochondrial genetic variation observed in natural populations have been shaped in part by thermal selection. Direct experimental evidence for this hypothesis is, however, lacking, and the contention remains hotly debated²⁵⁻²⁷. Such evidence can be provided via integration of a robust ecological framework with targeted experimental genomics. Firstly, to confirm that the frequencies of mtDNA haplotypes found within a species range exhibit associations with a thermally-dependent environmental gradient. Secondly, to experimentally demonstrate that the focal mtDNA haplotypes in question are sensitive to thermal selection,

and in the predicted direction (i.e. haplotypes found in the tropics exhibiting greater tolerance to warmer temperatures).

Here, we provide this experimental evidence for direct involvement of the mitochondrial genome in climatic adaptation. We demonstrate that the genetic variation delineating northern and southern-predominant Australian mtDNA haplogroups, in *D. melanogaster*, confers striking differences in the thermal performance of flies that harbour these mtDNA genotypes, when faced with extreme climatic stressors. These effects are unambiguously traceable to the mtDNA sequence. We then identify candidate mtDNA protein-coding genes involved in these thermal responses.

We first collected field-inseminated female flies from 11 populations along an eastern Australian latitudinal cline (Table S1), and used these flies to initiate isofemale lines, and ultimately mass-bred populations per latitudinal location (with each population kept in independent duplicates). Previous research has shown strong linear associations between the expression of thermotolerance phenotypes, and allele frequencies of underlying candidate nuclear genes, along this latitudinal cline²⁸, uncovering strong signatures of thermal adaptation.

To gauge levels of mtDNA sequence variation across these populations, we examined full mtDNA sequences across pooled-samples of flies from each mass-bred population. Using this approach we identified 15 SNPs in the mitochondrial genome that appeared to delineate the northern and southern populations, whilst the rest of the genome was highly conserved (Table S2). To probe levels of haplotype diversity and estimate their frequencies within each of the source populations, we designed a custom genotyping assay based on these 15 SNPs, and

used this assay to genotype all founding females of each mass-bred population (N = 312 females). We identified a total of 10 unique haplotypes. All haplotypes fell into one of two main haplogroups, with a total of 12 SNPs delineating these two groups (Figure 1). Both haplogroups were found to segregate across most of the 11 populations, but one haplogroup predominated in the northern tropical populations (hereafter called Northern haplogroup, N), while the other predominated in southern temperate populations (hereafter Southern haplogroup, S, Figure S1). Furthermore, each haplogroup contained one major haplotype (N1 accounting for 93.3% of N haplotypes; & S1 accounting for 77.2% of S haplotypes, Figure 1). The frequency of the N1 haplotype was negatively associated with the latitude of its source population ($R^2 = 0.4924$, $b = -0.02893$, $p \leq 0.05$, Figure 2), while the frequency of S1 exhibited a positive correlation ($R^2 = 0.5009$, $b = 0.02533$, $p \leq 0.05$, Figure 2B).

We next sought to experimentally assess whether these clinal patterns of mitochondrial genetic variance had been shaped under thermal selection. To address this question, it was necessary to disentangle effects attributable to mtDNA genetic variants from those caused by segregating nuclear allelic variation, or other sources of environmental variance. We thus created genetic strains of flies, in which the only genetic differences between strains lay in the mtDNA sequence. All strains were free of *Wolbachia* infection. We sourced four isofemale lines from each of the two main haplotypes (N1 & S1), and placed these alongside an isogenic nuclear background derived from a distinct southern latitudinal population [Puerto Montt (PM), Chile in South America].

To enable statistical partitioning of effects attributable to the mitochondrial haplogroup from other sources of variation, each haplogroup was replicated across two tiers. We did so via a two-step process. Firstly, we backcrossed females *en masse* from each of the independent

duplicates of each population to males of the isogenic PM strain over 20 generations, such that the pool of segregating mtDNA haplotypes per population was expressed against an isogenic background (Figure S2). Secondly, we then extracted one N1 and one S1 haplotype from each of the two independent population duplicates from two of these 11 populations (geographically-disjunct populations Brisbane in the central north, 27.61°S, and Melbourne in the south 37.99°S), and continued to backcross these to males of the isogenic PM for a further seven generations (Figure S3). Thus, each of the N1 and S1 haplotypes was represented across four separate strains, and at two levels of replication (an inter-latitudinal replicate [Brisbane, Melbourne], and an intra-latitudinal replicate, Figure S3).

Once these strains were created, full protein-coding genome re-sequencing of each duplicated haplotype revealed that the strains harbouring the N1 haplotype were indeed all homogeneous; characterised by a single haplotype. The southern haplotype (S1) strains were, however, heterogeneous (Figure 1). That is, resequencing revealed that the S1 haplotype could be further partitioned into four unique “sub-haplotypes” (S1-A, S1-B, S1-C, & S1-D). Each southern sub-haplotype was delineated by 1 to 4 SNPs, but all sharing the same pool of 12 SNPs that delineate them from the northern haplogroup (Figure S3, Table S3). This enabled us to partition mitochondrial genetic effects over two levels – at the level of the haplotype, and the sub-haplotype. The genetic variation differentiating the N1 and S1 haplotypes was comprised completely of 12 synonymous SNPs in the protein-coding genes. Synonymous SNPs have traditionally been considered to be functionally silent because they do not change the amino acid sequence. However, a growing body of empirical evidence suggests that synonymous polymorphisms might routinely modify the phenotype and thus be of functional and evolutionary significance²⁹. On the other hand, the SNPs delineating the

“sub-haplotypes” hubbed within the S1 haplotype consisted of a mixture of synonymous and non-synonymous SNPs (Table S3).

Flies harbouring the N1 haplotype, which predominates in the tropics, exhibited generally greater tolerance to an extreme heat challenge than flies harbouring S1 mtDNA (haplotype, $\chi^2=6.04$, $p = 0.014$, Table S4), but the magnitude of these effects changed across the sexes (haplotype \times sex, $\chi^2=24.7$, $p < 0.001$, Figure 3A-B, Table S4). We also uncovered sex-specific effects that mapped specifically to the level of the mtDNA sub-haplotype (sex \times sub-haplotype[haplotype], $\chi^2=25.04$, $p = <0.001$, Figure 3C). This interaction was primarily attributable to the S1-D sub-haplotype, which conferred inferior heat resistance in males, but not in females, relative to the other sub-haplotypes. Only one synonymous SNP, located in the *ND4* gene, delineates this sub-haplotype from the other S1 sub-haplotypes (Table S3). Thus, this polymorphism is a candidate SNP to have evolved under the overt sexual antagonism generated by strict maternal transmission of the mitochondrial genome, in which female-benefitting polymorphisms can accumulate under positive selection even when they are associated with significant male harm¹⁵.

Flies harbouring the S1 haplotype were superior at withstanding an extreme cold challenge, relative to their N1 counterparts ($\chi^2=34.31$, $p < 0.001$, Figure 3D-E, Table S5). This effect was affected by the SNPs that delineate each of the S1 sub-haplotypes (Table S5). Saliently, the effects of mitochondrial haplotype on all thermal tolerance phenotypes was robust to the source of origin of the haplotypes (i.e. whether they were sourced from Brisbane or Melbourne), providing unambiguous evidence that the phenotypic effects are directly tied to the mtDNA (Figure 3). Critically, all southern sub-haplotypes exhibited decreased heat tolerance and increased cold tolerance when compared to the N1 haplotype, providing

support for the contention that the differences in thermotolerance observed between northern and southern haplogroups are mapped to the 12 SNPs that delineate the N1 and S1 haplotypes, rather than the 4 SNPs that delineate the different sub-haplotypes hubbed within S1 (Table S4, Table S5, and Figure 3F). Previous studies of thermal performance of *D. melanogaster* from the east Australian coast have shown that genetic differences account for between 13% to 60% of the phenotypic divergence in thermotolerance responses between northern and southern populations³⁰. Our results suggest that a considerable fraction of the phenotypic variation in thermal tolerance present along the Australian latitudinal cline might ultimately be traceable to the fine-scale genetic variance that resides within the mtDNA sequence.

We then examined whether thermotolerance phenotypes might be mediated by patterns of differential gene expression of protein-coding mtDNA genes, or codon usage bias, across the N1 and S1 haplotypes. For the gene expression analyses, we examined five genes involved in complex I and complex IV of the electron transport chain (Complex I: *COXI*, *COXII*, *COXIII*, Complex IV: *ND4*, *ND5*). Emerging evidence suggests that genetic variation within complex I genes (both mitochondrial and nuclear) might contribute disproportionately to trajectories of mito-nuclear, and ultimately, life history evolution¹⁵. Complex IV, on the other hand, harbours genes with the lowest levels of *dN/dS* across all mitochondrial genes, across taxonomically diverse organisms³¹. Accordingly, we found that strains harbouring the S1 haplotype exhibited higher gene expression for the Complex I genes – ND4 and ND5, which belong to the same transcriptional unit, than strains with the N1 haplotype (haplotype \times gene < 0.001 , Figure S4, Table S6). We did not detect mitochondrial copy number to differ between the two haplotypes.

Variation in patterns of codon usage bias across DNA sequences can be shaped under natural selection³², given that unfavourable codons can cause polymerases and ribosomes to pause transcription/translation, with downstream effects on protein folding³³. In green algae *Chlamydomonas*, experimental alteration of mitochondrial codons drastically changes translational efficiency, suggesting that mitochondrial codon usage has been optimised for translation of mitochondrial products³⁴. In our study, the N1 and S1 haplotypes differ by 12 synonymous SNPs that are evenly distributed across the mitochondrial genome, with most protein-coding genes harbouring at least one SNP site. SNPs of the N1 haplotype show a high G-C bias, with 80% of the SNPs represented by a guanine or cytosine, whereas those of S1 reveal a G-C content of only 20% (Table S7, Fishers exact test, $p = 0.001$). Additionally, the SNPs delineating the N1 haplotype change the codon bias and produce rarer codons, whereas SNPs in S1 increase the codon bias (Table S8, Fishers exact test $p = 0.002$). These findings suggest that codon bias might play a role in the observed haplotype effects on gene expression of *ND4* and *ND5*, with ultimate effects on thermotolerance.

Our results advance understanding of the role of the mitochondrial genome in mediating population evolutionary trajectories, by providing definitive evidence that genetic variation found within the mtDNA sequence has been shaped by natural selection imposed by thermal stress. By harnessing an experimental genomic approach applied to the mitochondrial genome, within a powerful ecological framework, we have demonstrated that clinal variation in segregating mtDNA haplotypes is unambiguously associated with the capacity of these haplotypes to tolerate thermal stress, and we have elucidated molecular mechanisms that putatively drive these phenotypic effects. In doing so, our findings indicate a new role for a set of SNPs that were previously thought to evolve under neutrality.

4.3 Methods

Field collection, Isofemale line establishment and *Drosophila melanogaster* maintenance

Populations of *Drosophila melanogaster* were sampled from the east coast of Australia during March-April 2012 from 11 locations (Table S1). Samples were collected as close to sea level as possible to avoid altitudinal differences between the populations³⁵. Individual field-inseminated females were isolated into individual vials in the laboratory to initiate independent isofemale lines. At least twenty isofemale lines were generated for each population. Each line was treated with tetracycline to eliminate cytoplasmic endosymbionts, such as *Wolbachia*.

Three generations after the isofemale lines were established in the lab, one massbred population was created from the isofemale lines of each latitudinal location (11 locations). Specifically, the populations were established by combining 25 virgin males and 25 virgin females from each of 20 randomly-selected isofemale lines per latitudinal location. The following generation, each population was divided into two duplicates (11 populations \times 2 duplicates), which were kept separate from this point onward, thus making these independent duplicates of each location. A small sample of flies (~20-50 individuals) from each isofemale line was also collected at this time, and placed at -20°C for sequencing and genotyping purposes. Massbred populations were kept at 25°C under a 12:12h light/dark cycle. Genetic variation was maintained within each duplicate population by rearing flies across two bottles on potato-dextrose-agar food medium, with densities of approximately 300 flies per bottle. Every generation, newly-emerged flies from each duplicate were collected from both bottles and then randomly redistributed into two new fresh bottles.

Next Generation Sequencing and SNP Genotyping

To identify regions of variation between the clinal 11 populations, we first used pooled samples of 100 individuals (both males and females) from each population and used next generation sequencing technologies to obtain full mitochondrial genomes. DNA samples were enriched for mitochondrial DNA in order to get the best coverage possible. This process was achieved by using *Wizard SV* Miniprep Purification Kit (Promega) for DNA extraction, which captures circular DNA. Enriched DNA samples were made into 200bp paired-end libraries and sequenced using the Illumina GAIIx platform (Micromon, Monash University, Australia). Reads were aligned to the *Drosophila melanogaster* mitochondrial reference genome (NCBI reference sequence: NC_001709.1) using Geneious (version 5.6.3, <http://www.geneious.com>), generating mitochondrial genomes for each of the 11 latitudinal locations.

To obtain allele frequencies from each population, variable sites obtained from the mass sequencing were used as markers. DNA from each isofemale line was extracted using the *GenEra Puregene Cell and Tissue Kit* (Quiagen, Valencia, CA). Even though each massbred population was created using 20 randomly-chosen isofemale lines, we genotyped all isofemale lines collected from each latitudinal location. A custom SNP genotyping assay was developed (Geneworks, Australia) for the 15 SNPs identified via mass sequencing, and genotyping was performed on a SEQUENOM MassARRAY platform (San Diego, CA). This genotyping revealed the presence of northern-predominant (i.e. predominating in northern latitude populations) and southern-predominant (i.e. predominating in southern latitude populations) haplogroups, with each haplogroup characterised by one major haplotype (N1 and S1).

Creation of Mitochondrial Strains from Massbred Populations

We created “introgression strains” from each of the population duplicates (11 latitudes \times 2 population duplicates = 11 introgression strains \times 2 duplicates), by introgressing the pool of mtDNA variants of each population duplicate into a standard and isogenic nuclear background originally sourced from Puerto Montt (PM), Chile (41.46°S, 72.93°W), which had been created via 20 sequential generations of full-sibling matings. To initiate each strain, 100 virgin female flies were sampled from each population duplicate and crossed to 120 males from the PM strain. Then, for 20 sequential generations, 100 daughters were collected per strain and backcrossed to 120 PM males. This crossing scheme aimed to maintain the pool of segregating mitochondrial haplotypes within each population, while translocating them alongside that of an isogenic nuclear background, to enable partitioning of mitochondrial genetic effects from cryptic variance tied to the nuclear genome (Figure S2). In order to prevent mitochondrial contamination from the Puerto Montt (PUER) line, all lines were tested every 5 generations during the introgression regime, to ensure there were no instances of contamination of the lines (by rogue females of the PM strain) by using qRT-PCR melt curve analysis that would detect PM-specific mtDNA SNPs.

We then created a new set of isofemale lines from each of the introgression strain duplicates, and re-genotyped females of each line using the custom SNP genotyping assay described above (Geneworks, Australia). From the genotyping results, we were able to identify female lineages that carried individual haplotypes (N1 [northern] or S1 [southern]), and using this information we then selected one isofemale line carrying the N1 haplotype and one isofemale line carrying the S1 haplotype, from each of the two independent population duplicates from two (Brisbane, Melbourne) of the 11 latitudinal locations (Figure S2, S3). We continued to backcross virgin females of each isofemale line to males of the isogenic PM for a further

seven generations. We chose to use isofemale lines from Brisbane (latitude: 27.61°S) and Melbourne (latitude: 37.99°S), because they are geographically-disjunct (Table S1), and because re-genotyping confirmed that both N1 and S1 haplotypes were segregating in each of the introgression strain duplicates following the 20 generations of introgression. Following this process, each of the N1 and S1 haplotypes was represented across four independent “mitochondrial” strains, at two levels of replication; a within-population replicate (Brisbane-Melbourne) and a between-population replicate (Figure S3).

We then obtained full complete mitochondrial genomes for all eight mitochondrial strains, again using the next generation sequencing approach described above. Resequencing results revealed that haplotype N1 was isogenic across all 4 lines (2 populations \times 2 duplicates), whilst we found four unique sub-haplotypes nested within the S1 haplotype. These four southern sub-haplotypes all shared the known SNPs that delineate the north and south haplogroups (and the N1 and S1 haplotypes), however they each carried between one and three additional SNPs (Table S3).

Heat Tolerance Assay

Heat tolerance was measured for 120 flies of each sex from each mitochondrial strain³⁰. Flies were placed in individual 5mL water-tight glass vials and subsequently exposed to a 39°C heat challenge, by immersion of the glass vials in a preheated circulating water bath. Heat knock-down time was recorded as the time taken for each individual fly to become immobilized at 39°C. This experiment was conducted tested over two trials within the same generation. Each trial of the experiment consisted of a fully-balanced replicate of the experimental units (i.e. equal numbers of flies of each sex \times mitochondrial strain), separated in time by 2 hours within the same day. The position of flies of each experimental unit was

randomized within each trial of the experiment. The assay was conducted blind to the genotype or sex of the fly.

Chill Coma Recovery

This assay measures the amount of time it takes a fly to regain consciousness and stand on all legs after succumbing to a cold-induced coma³⁰. In each trial of the assay, 40 flies from each experimental unit (N = 640) were placed individually in 1.7mL microtubes. These tubes were then submerged in a water bath set to 0°C (comprised of water and engine coolant) for 4 h, to place flies into coma. At 4 h, all microtubes were removed from the bath, and laid out on a bench at 25°C, and the time taken (seconds) for each fly to regain consciousness was recorded. The assay was conducted blind to the genotype or sex of the fly.

Statistical Analyses of Thermal Tolerance data

We used separate multilevel linear mixed models to test the effects of mtDNA haplotype and sub-haplotype on each of the heat and cold tolerance responses. The response variable for the heat tolerance assay was the time taken to fall into coma, while the response variable for the cold tolerance assay was the time taken to wake from coma. Fixed effects were the identity of the mtDNA haplotype (N1, S1), the sub-haplotype nested within haplotype (N1, S1-A, S1-B, S1-C, S1-D), sex and their interactions. Random effects described the biological structure of the mitochondrial strains; there were two tiers of replication – with each haplotype replicated across two “duplicates” within each of two latitudinal “populations”. Thus, duplicate nested within population was included as a random effect, as well as other known and random environmental sources of variance (the trial identity, and the identity of the person scoring the response variable [2 people]).

Parameter estimates were calculated using restricted maximum likelihood algorithm in the *lme4* package of R³⁶. The fitted model was evaluated by simplifying a full model, by sequentially removing terms that did not change (at $\alpha = 0.05$) the deviance of the model, starting with the highest order interactions, using log-likelihood ratio tests to assess the change in deviance in the reduced model relative to the previous model³⁷.

Haplotype Network, Divergence and Codon Bias

Relationships among haplotypes were visualized on a median-joining network³⁸ and constructed in the software NETWORK version 4.6.1.2 (www.fluxus-engineering.com).

We obtained divergence estimates between N1 and S1 haplotypes using Geneious (version 5.6.3) and MEGA6³⁹. With Geneious software, divergence was calculated by examining the *%identity function* and subtracted that value from 100 to derive the percentage divergence. In MEGA6, we performed a pairwise distance comparison using a maximum composite likelihood model. Both methods gave concordant estimates of divergence (divergence = 0.001%)

We obtained *Drosophila melanogaster* mitochondrial codon usage bias values from Codon Usage Database (<http://www.kazusa.or.jp/codon/>). For both haplotypes, each SNP site was given the title “preferred” or “unpreferred” based on the codon usage bias score. Results were then analysed as a 2×2 contingency table using Fishers exact tests (Table S7 + S8).

Total RNA/DNA extraction and cDNA Synthesis

We sourced four female flies of each haplotype (three biological replicates per haplotype); by evenly combining a single fly from each sub-haplogroup into a microtube. We then performed a coupled RNA and DNA extraction was performed as per the supplier’s protocols

using TRIzol® Reagent (Life Technologies) to first create a phase separation of RNA and DNA from which the total RNA was then purified using a HighPure *RNA extraction kit* (Roche Applied Science, Indianapolis, IN). In this manner, both the DNA and RNA was independently separated and stored from the one sample. The separated nucleic acids (~100µL of each/sample extracted) *were quantified by UV/Vis spectrophotometry* (NanoDrop Technologies, Wilmington, DE) and the *purity of total RNA was confirmed using the A_{260}/A_{280} ratio with expected values between 1.8 and 2.0. The integrity of both the RNA and DNA was assessed by electrophoresis (1% TBE agarose gel).*

The cDNA was synthesized from 1µg of RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and a mixture of random hexamers and oligodT primers to capture mitochondrial transcripts both in the transitory polycistronic stage and as individual polyadenylated single transcripts ⁴⁰.

Mitochondrial copy number quantification

Mitochondrial copy number was measured for each DNA extraction performed (see *Total RNA/DNA extraction and cDNA Synthesis*) because it is known to differ across sexes ^{41,42} and may be expected to covary with transcript abundance ⁴³. Mitochondrial copy number was calculated relative to a single copy gene in the nuclear genome ⁴⁴. The mitochondrial DNA was determined using quantitative real-time PCR of a 113 bp region of the large ribosomal subunit (CR34094, FBgn0013686). No nuclear copies of this gene are found in the *Drosophila melanogaster* genome. Similarly, nuclear DNA was quantified by amplifying a 135 bp region of the single-copy ⁴⁵ subunit of the RNA polymerase II gene (CG1554, FBgn0003277). The copy number was then determined as the relative abundance of the

mitochondrial DNA to nuclear DNA ratio and thus reflects the average number of mitochondrial DNA copies per cell.

Gene expression quantification

Five of the thirteen total mitochondrial protein-coding genes were amplified to quantify gene expression levels. Quantified genes were: *COXI*, *COXII*, *COXIII*, *ND4*, and *ND5*. Gene expression of each biological replicate (three biological replicates per haplotype) was measured using quantitative real time (qRT)-PCR (Lightcycler 480 – Roche Applied Science, Indianapolis, IN). Reactions were performed in duplicate (technical duplicates) using a SYBRGreen I Mastermix (Roche Applied Science, Indianapolis, IN), whereby each well contained 5µl of SYBR buffer, 4µl of 2.5µM primer mix and 1µl of diluted cDNA. The following amplification regime used was: 90°C (10s), 60°C (10s), 72°C (10s) for 45 cycles, followed by a melt curve analysis to verify the specificity of the primer pair.

The Bestkeeper[®] software ⁴⁶ was used to select nuclear housekeeping genes (HKGs) for quality assessment. Three suitable HKGs were chosen: succinate dehydrogenase A (CG17246), 14-3-3ε (CG31196), and an unknown protein-coding gene (CG7277). All three genes had similar expression levels with high correlation coefficients (>0.8) against each other. For each experimental sample, the expression values of the mitochondrial target genes were standardized as follows:

The cycle threshold was calculated using the gene of interest (GOI) and the geometric mean of the three housekeeping genes (GEOM):

$$\Delta Ct = Ct_{GOI} - Ct_{GEOM}$$

The cycle thresholds were then used to calculate the relative gene expression for each experimental sample in relation to the housekeeping genes.

$$\text{Relative gene expression} = 2^{-\Delta\text{Ct}}$$

Gene expression levels of all five mitochondrial genes were obtained by determining the ΔCt per sample, measured at the maximum acceleration of fluorescence, using the Second Derivative Maximum Method ⁴⁷ in the Lightcycler Software V1.5.0 (Roche Applied Science, Indianapolis, IN). When the ΔCt values between two technical duplicates for each sample fell within 0.5 units of each other, then the mean gene expression estimates were pooled to form a single data point ⁴⁸.

Statistical analysis of gene expression data and copy number variation

We fitted multilevel models, in which mitochondrial copy number and gene expression data were modelled separately as response variables. Mitochondrial haplotype (N1, S1), and gene identity were modelled as fixed effects, and the duplicate nested within population as a random effect. Mitochondrial copy number values were added as a fixed covariate to the analysis of gene expression, and parameter estimates calculated using the *lmer* function in the *lme4* package ³⁶ in R (R Development Core Team 2009). Mitochondrial copy number variation was modelled with haplotype (N1 and S1) and the duplicate nested within population as a random effect. The fitted models were evaluated by simplifying a full model, by sequentially removing terms that did not change (at $\alpha = 0.05$) the deviance of the model; starting with the highest order interactions, and using log-likelihood ratio tests to assess the change in deviance in the reduced model relative to the previous model ³⁷.

4.4 Acknowledgements

We would like to thank Fiona Cockerell, Allannah Clemson, Winston Yee and Belinda Williams for assistance with the heat tolerance and chill-coma recovery assays. We thank Vanessa Kellerman and Winston Yee for fly collection. This research was supported by funding from the Hermon Slade Foundation, Australian Research Council (DP1092897) and Monash University Research Fellowship to DKD. CMS was funded by an ARC fellowship and the Science and Industry Endowment Fund.

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4.6 Figure Legends

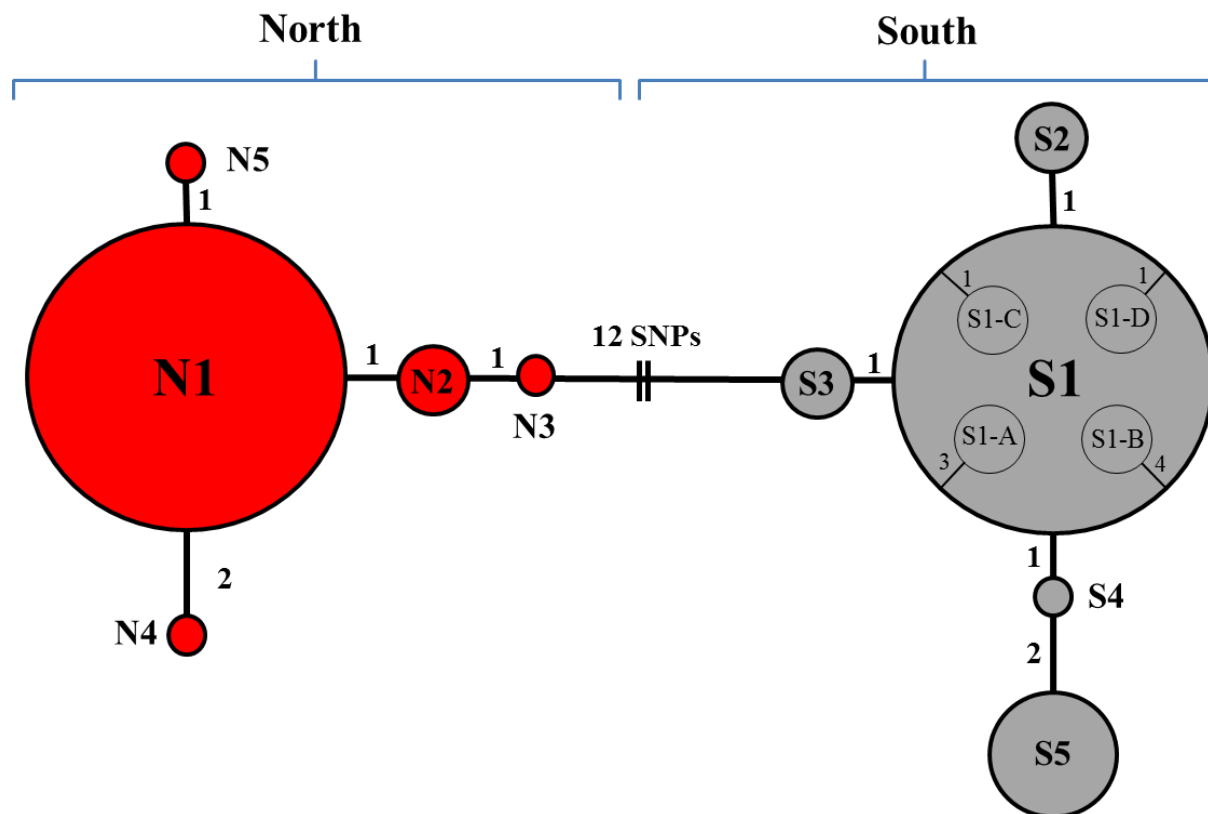


Figure 3: Haplotype network for mitochondrial protein-coding regions derived from 15 SNP genotyping. Circles indicate unique haplotype with size proportional to haplotype frequency. The two different colours correspond to the two haplogroups; with red corresponding to northern haplotypes and grey to southern haplotypes. Further resequencing of N1 and S1 haplotypes reveal that the S1 haplotype is comprised of at least 4 sub-haplotypes (S1-A, S1-B, S1-C, S1-D). Sub-haplotypes all share the same diagnostic 15 SNPs that delineate the S1 from the N1 haplotype, however contain 1 to 4 extra SNPs scattered throughout the genome (see Table S3).

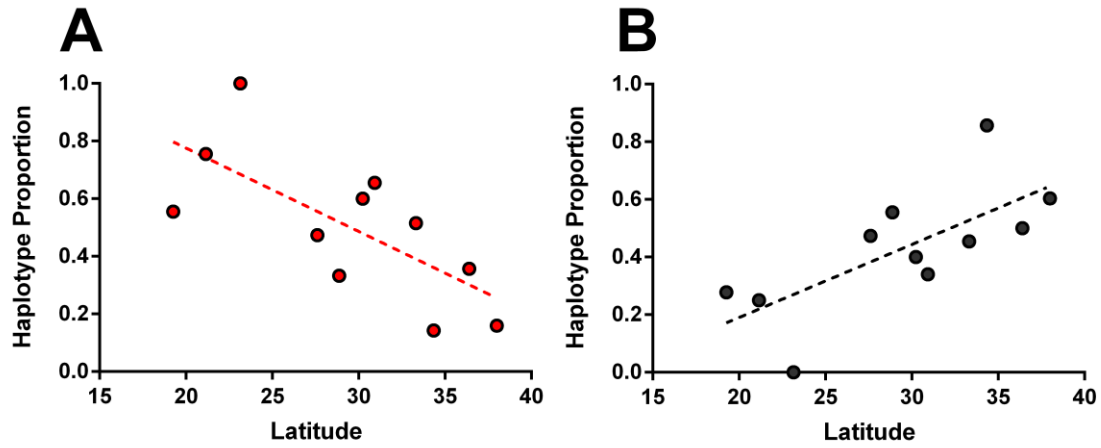


Figure 4: Haplotype abundance across the Australian eastern coast. **A)** Haplotype N1 (red) is predominately found in the north of the Australia, decreasing in frequency as the latitude increases ($R^2 = 0.4924$). **B)** Haplotype S1 (grey) is more common in the south, decreasing in frequency as the latitude decreases ($R^2 = 0.5009$).

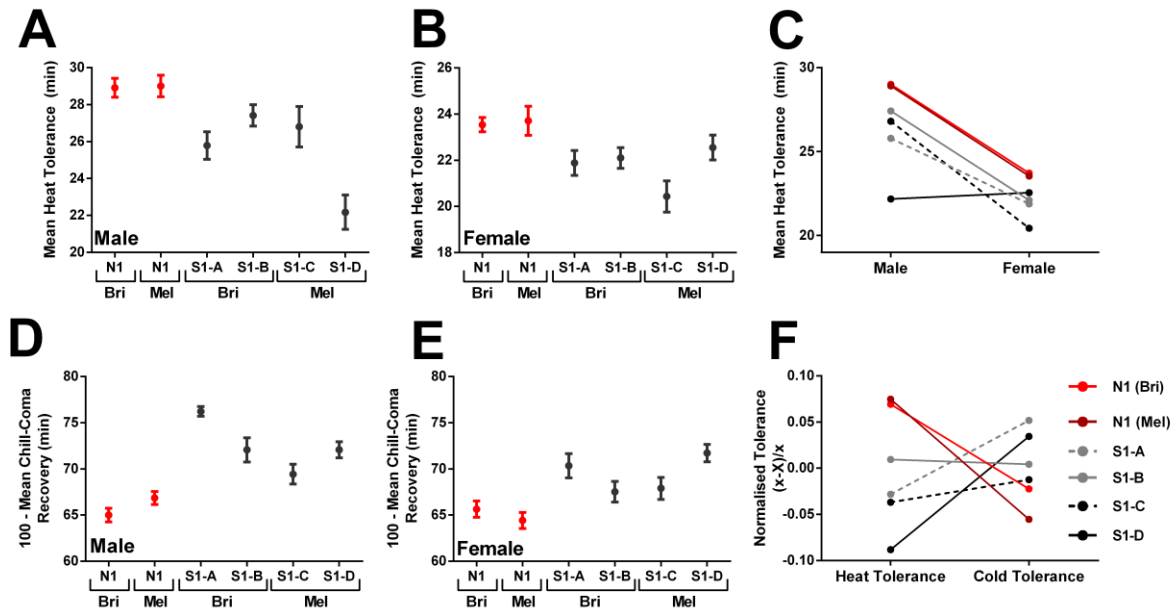


Figure 3: **A)** Mean heat resistance (± 1 S.E) between the north (red) and south (grey) haplotypes / sub-haplotypes for males. Means of each haplotypes are shown separately according to population of origin; Bri refers to Brisbane, Mel refers to Melbourne; **B)** Mean heat resistance (± 1 S.E) between the north (red) and south (grey) haplotypes / sub-haplotypes for females. **C)** Mean heat tolerance values differences between male and females, across mitochondrial haplotypes. **D)** Mean chill-coma recovery (± 1 S.E) between the north (red) and south (grey) haplotypes / sub-haplotypes for males. **E)** Mean chill-coma recovery (± 1 S.E) between the north (red) and south (grey) haplotypes / sub-haplotypes for females. **F)** Mean heat tolerance values (centred on a mean of zero and standard deviation of 1) used to test for differences between thermal traits (heat & cold tolerance), across mitochondrial haplotypes. Normalisation values were calculated as $(x - X)/X$, where x is the trait-specific mean of a mitochondrial line, and X is the overall mean for that trait.

4.7 Supplementary Information

Table S2: Collection sites for populations of *Drosophila melanogaster* from the east coast of Australia

Population	Latitude (°S)	Longitude(°E)
Townsville	19.26	146.79
Rockhampton	23.15	150.72
Brisbane	27.61	153.30
Ballina	28.87	153.44
Coffs Harbour	30.23	153.15
Port Macquarie	30.93	152.90
Wollongong	34.34	150.91
Narooma	36.25	150.14
Gosford	33.31	151.20
Bermagui	36.40	150.06
Melbourne	37.99	145.27

Table S2: Location of all SNPs identified via next-generation sequencing for the 11 mass-bred populations. For each SNP site, we identified the northern and southern major haplotypes. Here we list the location (site) of the SNP, the affected gene, and the codon position. Additionally, for each north and south polymorphism, we list the nucleotide (nt), the codon, amino acid (AA) and the usage bias for the codon.

Site	Gene	Position	North				South			
			nt	Codon	AA	Usage Bias	nt	Codon	AA	Usage Bias
1154	ND2	3	C	AAC	N	2.6	T	AAU	N	48.2
2661	COX1	3	C	CCC	P	1.7	T	CCU	P	23.1
3583	COX2	3	T	GCU	A	3	A	GCA	A	13
4247	ATPase6	3	C	GGC	G	0.1	T	GGU	G	12.7
5396	COX3	1	C	CUA	L	7.1	T	UUA	L	134.5
6299	tRNAE		C				A			
6980	ND5	3	G	UAC	Y	7.1	A	UAU	Y	35.6
7862	ND5	3	G	UUC	F	5.6	A	UUU	F	85
8866	ND4	1	A	UUA	L	134.5	G	CUA	L	7.1
8972	ND4	3	C	UUG	L	4.3	T	UUA	L	134.5
10215	ND6	1	C	CUA	L	7.1	T	UUA	L	134.5
10671	CYTB	1	T	UUA	L	134.5	C	CUA	L	7.1
12121	ND1	3	C	AUG	M	2.8	T	AUA	M	51.2
12334	ND1	1	C	GGG	G	4.1	A	GGU	G	12.7
14665	srRNA		C				T			

Table S3: Location of all SNPs identified via next-generation sequencing for each sub-haplotype within haplotype. Below is the list comprising the location (site) of the SNP, and which gene it affects. Additionally for each polymorphism we list the nucleotide (nt) and amino acid change (AA).

nu-origin	dup	h.type	sub-h.type	Gene	Syn?	nt change	position	AA change
MEL	1	N1	N1					
MEL	2	N1	N1					
BRIS	1	N1	N1					
BRIS	2	N1	N1					
MEL	1	S1	S1-C	COXII	N	C → T	3359	P → S
MEL	2	S1	S1-D	ND4	Y	C → T	8033	
BRIS	1	S1	S1-A	tRNA-ASP		A → C	3892	
				COXIII	Y	T → C	4954	
				ND5	Y	A → G	7877	
BRIS	2	S1	S1-B	COXI	Y	G → A	2262	
				COXII	Y	C → T	3385	
				COXIII	N	G → A	5162	V → I/M
				ND4-L	Y	A → T	9341	

Table S4: Multilevel model examining the effects of sex and haplotype (N1, S1) and sub-haplotype nested within haplotype on heat resistance. Sex, haplotype and sub-haplotype were modelled as fixed effects. Duplicate nested within population was modelled as a random effect alongside Trial ID.

	Chisq	df	Pr (> Chisq)	
(Intercept)	1276.141	1	<0.001	***
Haplotype	6.0396	1	0.01399	*
Sex	109.3203	1	<0.001	***
Haplotype × Sex	24.7332	1	<0.001	***
Sub-haplotype[Haplotype]	0.9866	3	0.80449	
Sub-haplotype[Haplotype] × Sex	25.0445	3	<0.001	***
	St.Dev			
Duplicate[Population]	1.109			
Population	0.000			
Trial ID	0.857			

Table S5: Multilevel model examining the effects of sex and haplotype (N1, S1) and sub-haplotype nested within haplotype on chill-coma recovery. Sex haplotype and sub-haplotype were modelled as fixed effects. Duplicate nested within population was modelled as a random effect alongside scorer (person recording the data).

	Chisq	Df	Pr(>Chisq)	
(Intercept)	739.776	1	< 0.001	***
Haplotype	34.3162	1	< 0.001	***
Sex	21.0568	1	< 0.001	***
Sub-haplotype[Haplotype]	3.8459	3	0.07498	
Haplotype × Sex	3.0658	1	0.08000	
Sub-haplotype[Haplotype] × Sex	7.5048	3	0.057436	
	St.Dev			
Duplicate[Population]	0.2549			
Population	1.5592			
Scorer	0.5850			

Table S6: Multilevel model examining the effects of sex and haplotype (N1, S1) and gene on mitochondrial gene expression. Copy number was added as a fixed covariate in the analysis in order to account for mitochondrial DNA variability. Duplicate nested within population was modelled as a random effect.

	Chisq	Df	Pr(>Chisq)	
(Intercept)	50.3807	1	<0.001	***
Haplotype	0.5319	1	0.46581	
Gene	2579.5876	4	<0.001	***
Copy Number	2.7067	1	0.09993	
Haplotype × Gene	32.9293	4	<0.001	***
	St.Dev			
Duplicate[Population]	8.009e-09			
Population	0.1288			

Table S7: 2×2 contingency table for AT and GC substitutions between the north and south haplotypes. A Fishers exact test was used to test for departures from the expectation of equal numbers in the two classes. $P = 0.002814$ indicating that the proportion of nucleotide sites with A-T is higher in the south haplogroup than the north haplogroup.

	A-T	C-G
North	3	12
South	12	3

Table S8: 2×2 contingency table for preferred and unpreferred codons between the north and south haplotypes. A Fishers exact test was used to test for departures from the expectation of equal numbers in the two classes. $P = 0.001203$, indicating that the proportion of preferred codons is higher in the south haplogroup than the north haplogroup.

	Preferred	Unpreferred
North	2	11
South	11	2

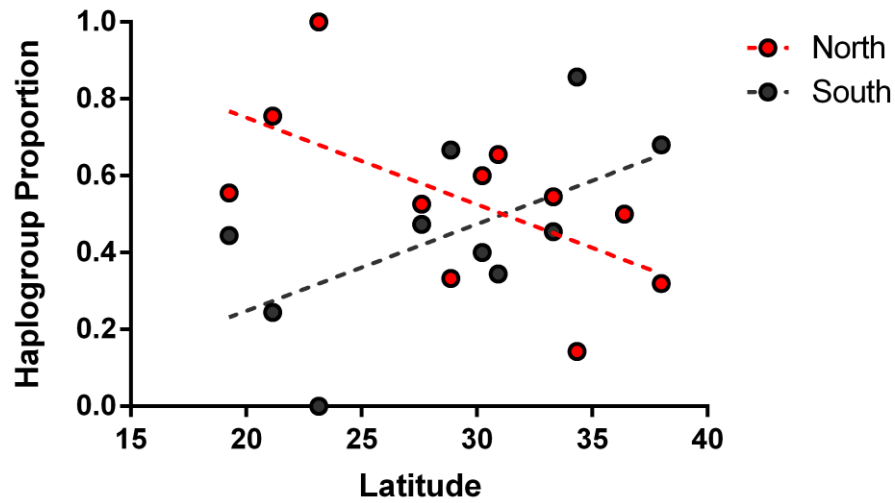


Figure S1: Proportion of northern and southern haplogroups (north = red, south = grey) found across each population latitude ($R^2 = 0.3679$, $p \leq 0.05$ for both haplogroups).

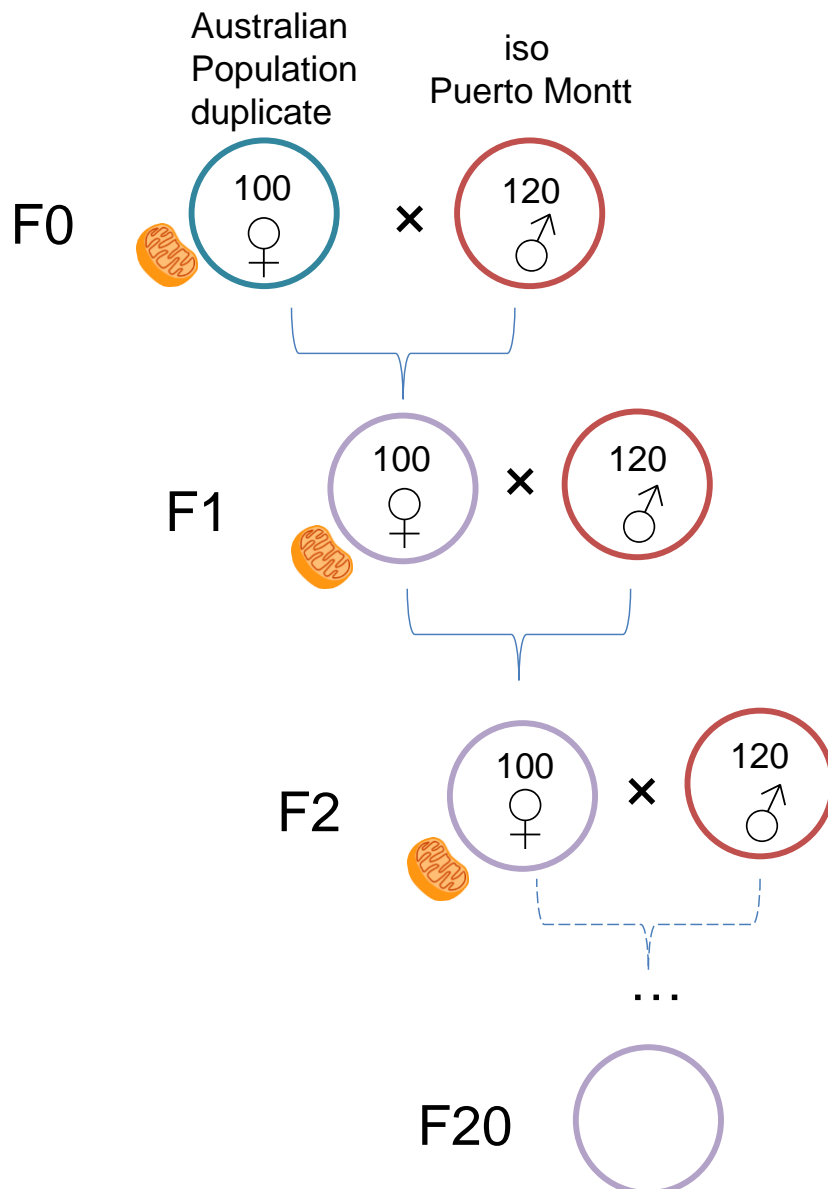


Figure S2: Schematic of experimental setup (part 1).

Eleven populations were collected from along the Australian east coast, and two independent duplicates from each location were kept as massbred populations (11 populations \times 2 duplicates = 22 experimental units). This figure represents the introgression regime for one experimental unit (one duplicate within a mass-bred population). Females (N=100) from each duplicated population (Australian population duplicate) were backcrossed to males (N=120) from an isogenic strain derived from Puerto Montt (iso Puerto Montt), for 20 sequential generations.

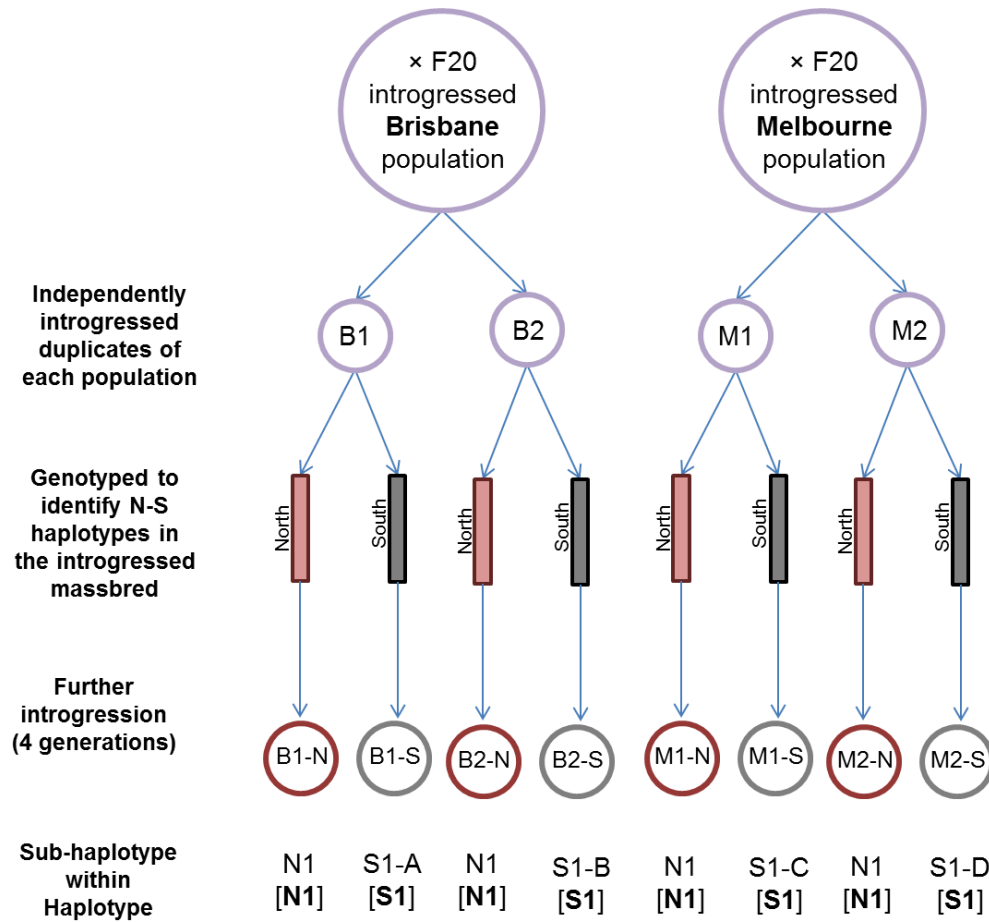


Figure S3: Schematic of experimental setup (part 2):

This figure illustrates the creation of eight new mitochondrial strains from the introgressed massbred populations (see Figure S2). Following 20 generations of introgression into the Puerto Montt nuclear background, we sourced N1 and S1 haplotypes from each of the population replicates of each of two introgressed populations; Melbourne and Brisbane. To initiate these strains, one female with an N1 haplotype, and 1 with an S1 haplotype was taken per population duplicate and crossed to one male of PM. One daughter of each of these crosses was then backcrossed to 1 PM male, each generation for three generations. Backcrossing was done in triplicate in case one of the crosses did not produce offspring. Next generation sequencing of these eight strains identified four unique sub-haplotypes within the main S1 haplotype, whereas the N1 haplotype was isogenic across all four strains.

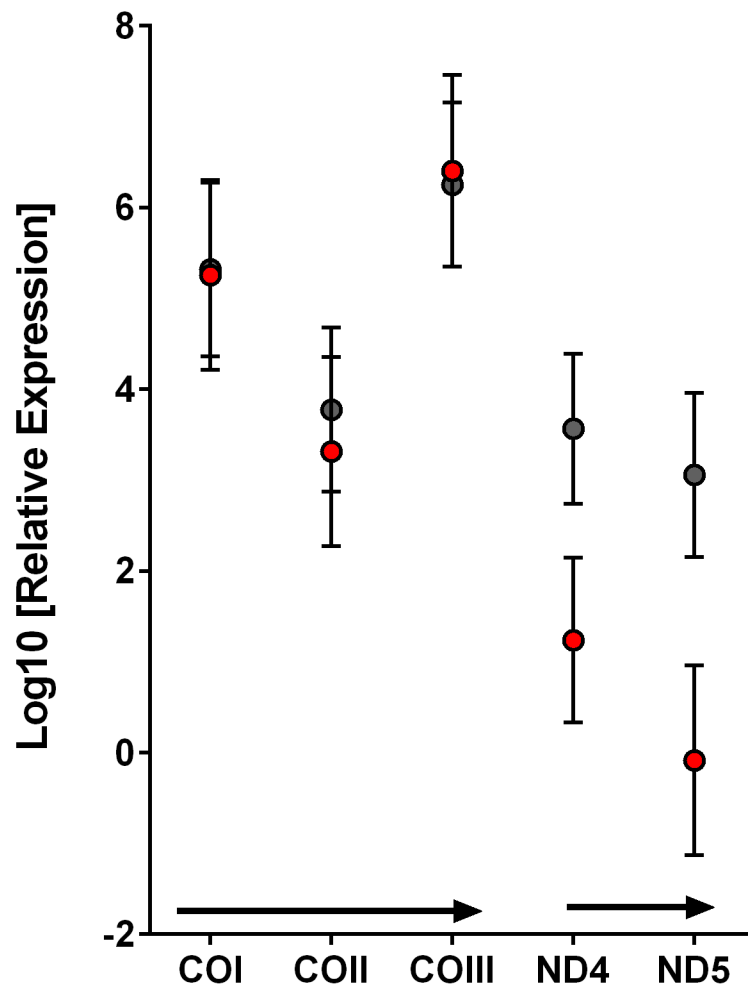


Figure S4: Least-squares means (± 1 S.E.) of gene expression across N1 (red) and S1 (grey, S1A-D combined) haplotypes for the *COI*, *COII*, *COIII*, *ND4* and *ND5* genes. Black arrows at the bottom of the figure denote the transcriptional units that genes correspond to. *COI*, *COII*, *COIII* all belong to one transcriptional unit and encode subunits of complex IV, whilst *ND4* and *ND5* are members of a second transcriptional unit and encode subunits of Complex 1 of the mitochondrial electron transport chain. Least-square means for all plots are derived from the multilevel models, which take into account mitochondrial abundance as a covariate (Table S6)

CHAPTER 5

Discussion

Overview and Future Directions

The mitochondrial genome is thought to have derived from the nuclear genome of an α -proteobacterium that was consumed by an ancient eukaryotic cell (Gray 1992). The end-product of this endosymbiotic event resulted in two co-existing genomes; mitochondria (derived from the α -proteobacteria) and nuclear (derived from the ancient eukaryotic cell). These two genomes have been co-evolving for millions of years, and form one of the most important and enduring of all symbioses involving eukaryotes (Andersson et al. 2003). Through evolutionary time, the mitochondrial genome has lost most of the ancestral genes derived from the α -proteobacteria either being lost or relocated to the nucleus of the eukaryotic cell (Timmis et al. 2004). Moreover, the mtDNA sequence that remains is characterised by a high mutation rate relative to most nuclear genomic regions, and the effective population size (N_e) of the mitochondrial genome is also thought to be low relative to its nuclear counterpart, due to the combined effects of haploidy of the genome and maternal inheritance (Ballard and Kreitman 1994; Rand 2001). The effect of a reduced N_e , in theory, is a reduced capacity for selection to shape the evolutionary trajectories of the mtDNA sequence.

When I set about my PhD research, it was clear that non-neutral genetic variation was typically found within the mitochondrial genome (Dowling et al. 2008). However, the relative contributions of adaptive (selection) and non-adaptive (drift) forces to the accumulation of this non-neutral genetic variation remained elusive. The goal of my PhD was, therefore, to elucidate the contributions of selection and mutation accumulation to the genetic variation found within the mitochondrial genome.

5.1. Phenotypic Consequences of Mitochondrial Maternal Inheritance

The maternal inheritance of the mitochondrial genome will in theory lead to a situation whereby mutations that are beneficial, neutral or even slightly deleterious to female fitness are able to accumulate in the mitochondrial genome, even when these same mutations exert deleterious effects on males (Frank and Hurst 1996; Gemmell et al. 2004). Maternal transmission of mitochondrial genomes can therefore lead to the accumulation of male-biased mutation loads in the mtDNA, which might affect trajectories of life history evolution in males. This evolutionary process has been termed “Mother’s Curse” (Gemmell et al. 2004).

In Chapter 2, I aimed to estimate i) the effects of genetic variation across distinct and naturally-occurring mitochondrial haplotypes on components of reproductive success in both males and females, and to investigate patterns of mitochondrial haplotypic covariation between traits, both within and across the sexes. Intersexual negative pleiotropy would provide evidence that mtDNA haplotypes harbour polymorphisms that are overtly sexually antagonistic – augmenting the reproductive success of one sex at the expense of the other, consistent with evolutionary predictions based on mitochondrial maternal inheritance. I presented experimental evidence that polymorphisms within the mitochondrial genome shaped the expression of both male and female components of reproductive success. These mitochondrial genetic effects were ubiquitous, and apparent when reproductive success was measured either as a “short burst” of reproduction following a single exposure to the opposite sex, or via a “sustained” assay of reproductive stamina, in which the focal flies had continual mating opportunities over a period of time that encompassed approximately twenty percent of the mean life time of a fly.

Strikingly, while signatures of pleiotropy between short-burst and sustained reproductive success were positive within the sexes, I uncovered a pattern of negative covariance between

the sexes for short-burst reproduction. Mitochondrial haplotypes that conferred high ‘short-burst’ fitness in females, conferred lower ‘short-burst’ fitness in males. Thus, I demonstrated that mitochondrial genetic effects on components of reproductive success are pervasive – they affected all reproductive traits measured, and in each of the sexes. Furthermore, my results further substantiate the findings of earlier studies that found sex-biased patterns of mitochondrial genetic effects on genome-wide patterns of transcript expression (Innocenti et al. 2011), and on longevity (Camus et al. 2012). Unlike earlier studies, the effects that I uncovered were not obviously male-biased in their expression, but it is important to note that the male and female traits under study in this chapter were not homologous – female reproductive success is underpinned by a different array of genes and tissues than is the male counterpart (Chandley and Cooke 1994; Ding et al. 2010). As such, one must be wary in applying predictions of Mother’s Curse theory to this data, since it is in theory possible that the mitochondrial genetic polymorphisms that affect components of female reproductive success are different than those that affect male components, and these different sets of polymorphisms might have accumulated under different evolutionary processes. However, that said, I uncovered a strong negative correlation between the sexes for short-burst reproductive success, which would suggest that the polymorphisms involved were the same for each sex. Furthermore, it suggests that these polymorphisms are accumulating under positive selection because they augment some components of female reproductive success, at cost to males. This provides the first experimental evidence to date that the evolution of mitochondrial genomes might follow overtly sexually antagonistic trajectories, since polymorphisms that confer even modest benefits to females will be favoured, even if they incur significant cost to males. The mitochondrial genetic effects on females were of similar magnitude, as gauged by the coefficients of variation, as the effects on males, suggesting the polymorphisms involved exert effects of similar magnitude across the sexes. Currently, it is

unclear whether these effects are underpinned by few mtDNA polymorphisms of major effect, or many of minor effect, but some evidence for the latter comes from an earlier study by Camus et al (2012), which provided evidence that levels of phenotypic divergence for male longevity were positively associated with levels of molecular divergence across haplotypes, supporting the notion that male longevity was affected by numerous mtDNA polymorphisms of minor effect.

Thus, mitochondrial genetic effects on life-history trait expression appear to be pervasive. They affect all measured components of reproductive success in fruit flies, as demonstrated in Chapter 2, and have previously been shown to affect longevity (Maklakov et al. 2006; Camus et al. 2012). This observation leads to an enigmatic question. How can a diminutive genome, whose few genes encode vital biological functions and are evolutionary conserved across species, exert such dynamic and broad-scale effects on the phenotype? In Chapter 3, I set out to explore the candidate molecular mechanisms by which mitochondrial DNA affects life-history trait expression. Previous research has found asymmetry in nuclear gene expression driven by polymorphisms within the mitochondrial genome, with 10% of the nuclear transcripts, in males, being differentially expressed across mtDNA haplotypes (Innocenti et al. 2011). The effect of mtDNA polymorphisms on patterns of gene expression within the mtDNA-encoded mitochondrial transcriptome had not been previously investigated. I hypothesized that patterns of gene expression at mtDNA-encoded genes, and the copy number of those genes, were two possible intermediary pathways by which mtDNA polymorphisms might regulate their effects on the life-history phenotype.

It was, however, previously believed that mitochondrial DNA abundance regulation was purely under the control of the nucleus, given that the DNA polymerase γ responsible for

mitochondrial replication is nuclear-encoded (Kelly et al. 2012). Similarly, differential expression of mitochondrially-encoded genes across naturally-occurring mtDNA variants had not been previously documented, and scope for individual mtDNA genes to exhibit gene-specific patterns of expression across haplotypes was hitherto thought to be low, given that many of these genes are transcribed as part of broader transcriptional units (Torres et al. 2009). However, I found that variation in mtDNA copy number was clearly linked to the mitochondrial haplotype, and furthermore I uncovered gene-specific patterns of expression across mtDNA haplotypes. These results thus highlight two plausible molecular mechanisms involved in the regulation of mitochondrial genetic effects on the phenotype. In this chapter, I was able to link mitochondrial genotypes to several different phenotypes including mtDNA gene expression, copy number variation, ageing and fertility. In particular, I found intriguing effects on the expression of two genes – ND5 and CYTB – and was able to trace these effects to particular SNPs within the mitochondrial genome. The candidate SNPs identified were located at non-synonymous sites and nested within the very same genes whose expression they affected, rather than within genomic regions more traditionally associated with regulatory function. These candidate SNPs are therefore likely to exert their effects post-transcriptionally, potentially by altering the stability of transcripts. Furthermore, although the effects of these SNPs on patterns of gene expression were generally of similar magnitude across the sexes, their effects on longevity and fertility were sex-specific. This implicates these mitochondrial SNPs as contributors to the sex gaps often observed in traits such as longevity, in which the females of many animal species generally outlive the males (Dowling 2014a).

My results thus uncover cryptic mechanisms by which single nucleotide changes within the mitochondrial genome can modify the expression of life-history phenotypes, and potentially mediate the outcomes of mitochondrial-nuclear ('mito-nuclear') interactions. Mito-nuclear

interactions are not only critical to the upkeep of fundamental biological processes such as OXPHOS function; but evolutionary theory and empirical evidence suggests they will likely be drivers of key evolutionary processes, such as sexually antagonistic coevolution and speciation (Frank and Hurst 1996; Rand et al. 2004; Dowling et al. 2008; Werren et al. 2010; Dowling 2014b). For example, in the case of speciation, population-specific coadaptation of mitochondrial and nuclear genomes could lead to the evolution of Dobzhansky-Muller incompatibilities (DMI), thus driving reproductive isolation between incipient populations (Burton and Barreto 2012). Mapping of the nuclear loci involved in these interactions in recombinant inbred lines (Corbett-Detig et al. 2013) could help to elucidate the extent to which negative epistatic interactions, involving mitochondrial and nuclear genomes, occur in natural populations of *D. melanogaster*.

By tracing effects of naturally segregating single nucleotides to the expression of protein-coding mitochondrial genes, and then linking these effects to patterns of longevity and fertility, Chapter 3 provided novel insights into the contribution of this diminutive genome to the evolution of sex differences in life-history. However, while I was able to trace genotype to phenotype linkages to the level of individual mtDNA SNPs in some cases, in others the underlying nucleotides driving the observed mitochondrial genetic effects remained obscure. Indeed, at the level of whole haplotypes, the genetic effects exhibited considerable phenotypic plasticity, with expression of mitochondrial copy number and gene expression contingent on both the sex and age of the flies. This plasticity suggests that the links between mitochondrial genotype and phenotype will be difficult to predict in many cases; a finding that helps clarify the complex epidemiology of mitochondrial diseases in humans, where simple links between candidate mtDNA mutations and disease penetrance remain elusive (Dowling 2014b; Wolff et al. 2014).

5.2. Non-Neutral Mitochondrial Genetic Variation Accumulating under Thermal Selection

Previously, it has rarely been acknowledged that polymorphisms with the mitochondrial genome that delineate individual mtDNA haplotypes might have accumulated under natural selection. However, thermal sensitivity of mitochondrial functioning, coupled with the observation that mitochondrial haplotype frequencies tend to associate with latitude or altitude, suggests that thermal selection may play a role in shaping the molecular architecture of the mitochondrial DNA (Shertzer and Cascarano 1972; Cheviron and Brumfield 2009; Silva et al. 2014).

In this chapter I present experimental support for the contention that some of the polymorphisms that delineate distinct mtDNA haplotypes have accumulated because they enhance an individual's thermal tolerance to the prevailing environment. I found two major mitochondrial haplogroups in *Drosophila melanogaster*, which exhibit opposing patterns of clinal variation along the Australian eastern seaboard. The haplogroups are delineated by 12 synonymous SNPs, with one haplogroup predominantly found in the north of Australia, whilst the other in the south. I extracted the major haplotype from each of these haplogroups, from each of two latitudinally-different populations, and placed each haplotype alongside a single isogenic nuclear background. I was then able to phenotype these newly created mitochondrial strains, and hone in on fine-scale levels of mitochondrial genetic variance for key thermal tolerance phenotypes. I found that northern-predominant haplotype confers greater resistance to an extreme heat challenge than its southern counterpart, but the southern haplotype conferred greater tolerance to a cold challenge. Resequencing of these haplotypes revealed a further layer of sequence variation within the southern mtDNA haplotype, which could be partitioned into four distinct sub-haplotypes. Nonetheless, the major differences in

thermal tolerance responses between flies of different mtDNA genotypes could be traced directly to the level of the haplotype (i.e. N1 and S1), which is notable because these haplotypes were only separated by a small number of synonymous polymorphisms that do not change the amino acid sequence. This result suggests that previous molecular analyses testing the capacity for mitochondrial genomes to respond to natural selection might have routinely underestimated this capacity, because they infer histories of genomic evolution based on ratios of nonsynonymous (amino acid changing) mutations to synonymous, assuming the synonymous mutations are neutral to selection. My study challenges this notion, and highlights a role for a set of SNPS within the mtDNA genome that were previously thought to evolve under a neutral equilibrium model.

Genetic polymorphisms that separate the four southern (S1) sub-haplotypes were, however, also associated with the ability of flies to withstand the extreme heat challenge, and one of these sub-haplotypes (S1-D) conferred particularly poor performance in males but not females, relative to the other sub-haplotypes, reinforcing the emerging view that the mitochondrial genome is a repository for polymorphisms exerting sex-specific effects, and which presumably accumulate because maternal inheritance creates a sex-specific selective sieve in mitochondrial genome evolution (Innocenti et al. 2011; Camus et al. 2012).

I also found differences in the levels of expression of key mtDNA protein-coding genes, ND4 and ND5 across the northern and southern haplotypes and was able to show differences in patterns of codon usage bias across the two haplotypes, thus substantiating the results of Chapter 3 in showing that mitochondrial genetic effects on core life history phenotypes are likely to be regulated by direct effects of mtDNA SNPs on the expression of mtDNA protein-coding genes. Thus, in sum, in this Chapter, I uncovered a new-found role for a set of SNPs

that were previously thought to be selectively neutral, inside a genome that was likewise traditionally considered to be devoid of functional segregating allelic variation. The results provide strong support for the hypothesis that some of the genetic variation that accrues within and across populations is likely to have been shaped directly by natural selection to the prevailing environmental conditions.

5.3 Future Directions

This thesis did not examine the dynamics of mitochondrial-nuclear interactions. Throughout all experimental chapters, I used a powerful experimental design, with mitochondrial haplotypes gathered from around the globe coupled to a standard nuclear background. All haplotypes were independently replicated, giving me a powerful system that allowed me to hone into mitochondrial genetic effects (even for effects that were small in magnitude). This isogenic nuclear background was intended to be foreign to all mitochondrial haplotypes in order to avoid cytoplasmic compatibilities confounding possible results. Of course, some mitochondrial haplotypes might have been better adapted to nuclear background than others, due to sharing a similar sequence to the co-evolved mtDNA of the isogenic nuclear genome. This possibility can be investigated in future experiments, by placing focal mtDNA haplotypes alongside several isogenic strains, to test the context-dependency of the results across nuclear backgrounds. There is a trade-off between number of nuclear backgrounds and mitochondrial haplotypes utilized for the experiments. For this thesis I sought to maximize the diversity of haplotypes, and hence used just a single nuclear background. Although I expect that results reported in this thesis, inside single isogenic nuclear backgrounds, are general in their magnitude and direction, it will be important for future studies to substantiate this by testing the across a variety of different nuclear backgrounds. Indeed, one study has now achieved this, using three of the mitochondrial strains used in Chapters 2 and 3, to screen

the magnitude of mitochondrial genetic effects on components of male reproductive success, across a diverse range of nuclear genomic backgrounds (Dowling et al. 2015). Although mito-nuclear interactions were prevalent, the nuclear backgrounds only affected the magnitude of the mitochondrial genetic effects and not the rank order of haplotypes across nuclear backgrounds. That is, the best performing mtDNA haplotype was generally the best performing haplotype regardless of the nuclear background that it was assayed alongside. Similarly the worst performing haplotype was unanimously the worst performer across all nuclear backgrounds (Dowling et al. 2015).

Furthermore, population cage experiments provide an excellent opportunity for studying consistent changes in mtDNA haplotype frequencies over generations, thereby providing evidence for mitochondrial genomic adaptation under selection. Previous studies have found fluctuations in mitochondrial haplotype frequency, with certain mitochondrial haplotypes dominating in frequency over others within a population (Kambhampati et al. 1992; García-Martínez et al. 1998; Ballard and James 2004; Rand 2011). Kazancıoğlu & Arnqvist (2014) performed such experiments, using seed beetles (*Callosobruchus maculatus*). The authors had set-up population cages with seed beetles carrying different mitochondrial-nuclear combinations at varying frequencies (two haplotypes at these frequencies = 20:80, 50:50 and 80:20). After 10 generations, they re-sampled the mitochondrial frequencies in each population cage and found signatures of negative frequency-dependent selection, meaning that the frequency of a mitochondrial haplotype following ten generations was inversely related to its starting frequency in a population (Kazancıoğlu and Arnqvist 2014). Further studies should utilize this powerful experimental framework to explore the dynamics of mitochondrial-nuclear evolutionary trajectories, and determine the extent to which mtDNA haplotypes respond to selection, and to unravel the predominant types of selection that shape

the mtDNA sequence (e.g. the relative importance of directional selection imposed by the environment versus frequency-dependent selection).

5.4 Conclusions

This PhD expands on our understanding of the evolutionary forces that shape the mitochondrial genome, suggesting that maternal inheritance enables sex-specific, even sexually antagonistic, polymorphisms to accumulate within the genome, and furthermore that the molecular architecture of the genome has been shaped in part by thermal selection. By using the model organism, *Drosophila melanogaster*, I was able to remove confounding effects of segregating nuclear variation in order to examine mitochondrial genetic effects on the expression of both molecular (gene expression and copy number) and life-history traits (reproductive success, thermal tolerance). This approach proved powerful, and enabled me to tie transcriptomic effects to sex-specific effects on life history trait expression, across mtDNA haplotypes. Furthermore, I was able to map these effects backwards to specific phylogenetic branches, and even to specific nucleotides, in the mtDNA sequence. The approach enabled me to screen for patterns of mtDNA-linked pleiotropy within and between the sexes, and to extend upon studies in other species that previously reported clinal associations between mtDNA haplotype frequency and latitude, by reconciling such data with a robust phenotypic assay that could unambiguously tie thermal tolerance responses to the mitochondrial genotype. These advances pave the way for further studies that explore the dynamics and trajectories by which mitochondrial genomes evolve in nature, and the implications – both conceptually, when it comes to the evolution of sex differences in life history, and applied, for example by addressing the capacity of the mitochondrial genome to respond, and indeed cope, with ever increasing levels of environmental stresses that many of our native fauna, here and abroad, are likely to face over the next decades.

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“We are the music makers,
And we are the dreamers of dreams,
Wandering by lone sea-breakers,
And sitting by desolate streams;
World-losers and world-forsakers,
On whom the pale moon gleams:
Yet we are the movers and shakers
Of the world for ever, it seems.”
- *Ode by O'Shaughnessy (1874)*