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Evolutionary implications of mitochondrial genetic variation: mitochondrial genetic effects on OXPHOS respiration and mitochondrial quantity change with age and sex in fruit flies

J. N. WOLFF*[1], N. PICHAUD†‡[1], M. F. CAMUS*, G. CÔTÉ‡, P. U. BLIER‡[2] & D. K. DOWLING*[2]

*School of Biological Sciences, Monash University, Clayton, Vic, Australia
†Département de Chimie et Biochimie, Université de Moncton, Moncton, NB, Canada
‡Départment de Biologie, Université du Québec de Rimouski, Rimouski, QC, Canada

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Abstract

The ancient acquisition of the mitochondrion into the ancestor of modern-day eukaryotes is thought to have been pivotal in facilitating the evolution of complex life. Mitochondria retain their own diminutive genome, with mitochondrial genes encoding core subunits involved in oxidative phosphorylation. Traditionally, it was assumed that there was little scope for genetic variation to accumulate and be maintained within the mitochondrial genome. However, in the past decade, mitochondrial genetic variation has been routinely tied to the expression of life-history traits such as fertility, development and longevity. To examine whether these broad-scale effects on life-history trait expression might ultimately find their root in mitochondrially mediated effects on core bioenergetic function, we measured the effects of genetic variation across twelve different mitochondrial haplotypes on respiratory capacity and mitochondrial quantity in the fruit fly, *Drosophila melanogaster*. We used strains of flies that differed only in their mitochondrial haplotype, and tested each sex separately at two different adult ages. Mitochondrial haplotypes affected both respiratory capacity and mitochondrial quantity. However, these effects were highly context-dependent, with the genetic effects contingent on both the sex and the age of the flies. These sex- and age-specific genetic effects are likely to resonate across the entire organismal life-history, providing insights into how mitochondrial genetic variation may contribute to sex-specific trajectories of life-history evolution.

Introduction

The presence of two obligate genomes within eukaryote cells – one mitochondrial (mtDNA) and the other nuclear – represents an enduring legacy of the ancient symbiosis between mitochondria and eukaryotes. Although over the course of eukaryote evolution, many of the original mitochondrial genes have been translocated across to the nuclear genome, curiously a handful of genes (22 transfer RNAs, two ribosomal RNAs and 13 protein-coding genes) remain within the mitochondrial genomes of most animals (Wolstenholme, 1992; Björkholm *et al.*, 2015), and these mtDNA-encoded genes play core roles in maintaining mitochondrial function (Blier *et al.*, 2001; Rand *et al.*, 2004; Wolff *et al.*, 2014).

Owing to the diminutive size and highly conserved gene content of animal mitochondrial genomes, and under the influence of strong purifying selection on the mtDNA (Ruiz-Pesini *et al.*, 2004; Meiklejohn *et al.*,

Correspondence: Jonci N. Wolff, School of Biological Sciences, Monash University, Clayton campus, 3800 Vic, Australia.
Tel.: +61(3)99050737; fax: +61(3)99054007; e-mail: jonci.wolff@gmail.com
and
Nicolas Pichaud, Université de Moncton, Campus de Moncton, Pavillon Léopold-Taillon 18, avenue Antonine-Maillet, Moncton, NB E1A 3E9, Canada.
Tel.: +1(506)8584331; fax: +1(506)8584541; e-mail: pichaud.nicolas@wanadoo.fr

[1]These authors contributed equally to this work
[2]These authors contributed equally to this work

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2007; Stewart *et al.*, 2008; Hill *et al.*, 2014; Ma *et al.*, 2014), it was traditionally assumed that there was little scope for mtDNA polymorphisms to accumulate and affect phenotypic function (Galtier *et al.*, 2009). Contrary to this expectation, numerous experimental studies have demonstrated that the effects of mitochondrial genetic variation on the phenotype are both ubiquitous and sizable in effect (Dobler *et al.*, 2014), with effects traceable to core life-history phenotypes such as fertility, development and longevity (Ballard & Whitlock, 2004; Dowling *et al.*, 2008; Ellison & Burton, 2008; Meiklejohn *et al.*, 2013; Dowling, 2014). Several studies, however, suggest that such mitochondrial genetic effects might also be context-dependent on the environment in which they are measured (Dowling *et al.*, 2007, 2010; Arnqvist *et al.*, 2010; Zhu *et al.*, 2014).

Indeed, recent studies have indicated that mitochondrial genetic effects on the phenotype might often be sex-specific in magnitude (Smith *et al.*, 2010b; Innocenti *et al.*, 2011; Camus *et al.*, 2012, 2015). Such sex specificity can be traced to strict maternal transmission of the mitochondrial genome in most eukaryote taxa (Birky, 1978; Zeh & Zeh, 2005; White *et al.*, 2008). Maternal transmission results in a sex-specific selective sieve (Frank & Hurst, 1996) – sometimes referred to as *Mother's curse* (Gemmell *et al.*, 2004) – which enables the accumulation of male-harming mtDNA mutations, if these same mutations are relatively benign, or even positive, in their effects on females (Zeh & Zeh, 2005; Unckless & Herren, 2009; Beekman *et al.*, 2014). In particular, recent studies in *Drosophila melanogaster* reported male-biased mitochondrial haplotypic variation for fertility and patterns of ageing (Camus *et al.*, 2012, 2015; Yee *et al.*, 2013) and markedly increased sensitivity of the nuclear transcriptome to mitochondrial allelic variance in males relative to females (Innocenti *et al.*, 2011).

Furthermore, evolutionary and mechanistic theories of ageing both suggest that the genetic variation underpinning mitochondrial genomes might increase in magnitude with advancing age (Medawar, 1952; Williams, 1957; Harman, 1972). From the evolutionary standpoint, the force of natural selection inevitably declines with age, and this should enable mutations that exert late life deleterious effects in the mtDNA to accumulate (Medawar, 1952; Williams, 1957; Dowling *et al.*, 2009). There is now compelling evidence that mutations in the mtDNA cause early onset of ageing (Trifunovic *et al.*, 2004). Furthermore, naturally occurring mitochondrial haplotypes are commonly associated with differences in longevity, with evidence coming from both experimental studies in invertebrates (James & Ballard, 2003; Maklakov *et al.*, 2006; Rand *et al.*, 2006; Clancy, 2008; Dowling *et al.*, 2010; Zhu *et al.*, 2014) and correlative associations in humans (De Benedictis *et al.*, 1999; Raule *et al.*, 2014). Mechanistically, the *Mitochondrial theory of ageing* (Harman, 1972) predicts that under recurrent exposure to reactive oxygen species (ROS) production, mitochondrial genomes will accumulate somatic mutations with increasing age (Dai *et al.*, 2014). It is plausible that these somatic mutational dynamics will in part be dictated by the nature of germ-line polymorphisms that delineate mtDNA haplotypes. That is, different mtDNA haplotypes could be associated with different rates of somatic mutation accumulation in the mtDNA throughout life, resulting in age-specific mitochondrial genetic effects on respiration, and expression of life-history phenotypes (Ross *et al.*, 2013, 2014).

Ultimately, although it is clear that mitochondrial genetic effects on expression of life-history and morphological traits are pervasive across taxa (Dobler *et al.*, 2014), the underlying physiological mechanisms that might underpin these broad-scale effects on the phenotype remain largely elusive and have been subject to scant experimental attention (Pichaud *et al.*, 2011, 2012; Wolff & Gemmell, 2013). Furthermore, the level of context dependency of mitochondrial genetic effects on trait expression – both at the scale of life-history trait and at the scale of the intermediary physiological level, is poorly understood. Firstly, the core and evolutionary-conserved mitochondrial proteome consists of around 400 conserved proteins (Lotz *et al.*, 2014), which greatly outnumber the 13 that are mtDNA-encoded. Whether or not a small level of genetic variation at a small number of mtDNA-encoded genes can exert effects on mitochondrial physiology that are sizable enough to translate into the pervasive effects that have been observed on life-history trait expression remains an open question. Most studies conducted to date have examined life-history phenotypes (Dobler *et al.*, 2014), rather than those related to mitochondrial physiology (Pichaud *et al.*, 2011, 2012). Secondly, mitochondrial genetic effects on life-history trait expression have been reported to be commonly sex-specific in their magnitude – indeed typically male-biased consistent with *Mother's curse* theory – and such context dependency might well also exist across age classes, although this possibility remains empirically untested. Of particular note, it is currently unknown whether signatures of sex specificity across mitochondrial genotypes are traceable to the level of the intermediary physiological phenotypes that may modulate the link between mitochondrial genotype and phenotype. Yet, the existence of sex- or age-mediated context dependency of mitochondrial genetic effects at the physiological level would plausibly carry important evolutionary implications, given that traits such as mitochondrial respiration produce the ATP, as well as ROS, each of which have been implicated as the currency underpinning the evolution of life-history trade-offs (Sheldon & Verhulst, 1996; Dowling & Simmons, 2009).

Here, we address these open questions, probing for context dependency of mitochondrial genetic variance

underpinning mitochondrial physiological trait expression in *D. melanogaster*. We harnessed 12 mitochondrial strains, each with a distinct mtDNA haplotype expressed alongside a standard nuclear genetic background. We used these strains to estimate the effects of mitochondrial genetic variance on mitochondrial respiratory capacity and mitochondrial quantity, separately in each sex, and at two age classes. We also searched for candidate SNPs in the mtDNA that are associated with these physiological effects. We uncovered large effects of mitochondrial haplotype identity on each trait, but also a remarkable level of context dependency, in terms of the sex- and age specificity of these mitochondrial effects. Our results provide new insights into physiological mechanisms that might link mitochondrial genetics to the dynamic effects often observed at the level of the life-history phenotype, and implicate mtDNA-mediated effects on mitochondrial metabolism as key mediators of population evolutionary trajectories.

Materials and methods

Mitochondrial strains – breeding

We used strains of *D. melanogaster*, each of which harboured a distinct mitochondrial haplotype, sourced from a distinct global locality, which had been translocated into the isogenic nuclear background w^{1118} (Clancy, 2008). Strains are named according to sampling locations and abbreviated with the first three letters of the sampling location (Alstonville, Australia; Barcelona, Spain; Brownsville, USA; Dahomey, Benin; Madang, Papua New Guinea; Mysore, India; Hawai'i, USA; Israel; Japan; Oregon, USA; Puerto Montt, Chile; Sweden). Strains were collected between 1970 and 2002 and have since been maintained as laboratory stocks (for details, see Clancy, 2008; Wolff *et al.*, 2015). We obtained these mitochondrial strains in 2007 and split each into two duplicates, which were thereafter propagated as separate entities, with virgin daughters of each strain duplicate back-crossed to males of the w^{1118} strain for a further 60 generations to ensure that all four chromosomes in the w^{1118} nuclear background were isogenic across all mitochondrial strains. The w^{1118} strain was itself maintained via crosses between one full-sibling pair per generation, to prevent genetic variation from accumulating within this strain. The duplication of each mitochondrial strain enabled us to ensure that the physiological and biochemical differences detected between strains were indeed attributable to mitochondrial genetic variation, and not to cryptic genetic variation that might have accumulated within the nuclear background during the time in which the strains were being constructed, and subsequently maintained. All strains were carefully density-controlled throughout the breeding process, maintained on a potato–dextrose–agar medium at constant temperature (25.0 ± 0.1 °C) and diurnal cycle (12-h : 12-h light/dark). Parental and great-parental generations of all fly strains were age-controlled. Replicate vials were maintained for each strain, and these were staggered temporally, to ensure constant availability of age-controlled flies per genotype, over the course of the experiment (experiments were conducted over consecutive 'sampling days' per age class). All strains were free of *Wolbachia* infection. All mitochondrial strains are routinely genotyped to confirm strain identities.

Mitochondrial strains – molecular divergence across haplotypes

Genetic differences between mitochondrial strains were revealed by pair-wise comparisons between the 12 mitochondrial haplotypes (Genbank: KP843842-KP843853; Wolff *et al.*, 2015) using *Geneious* (Kearse *et al.*, 2012). This comparison revealed 354 single nucleotide polymorphisms (SNPs) between the 12 haplotypes: 80 SNPs affecting the protein-coding region (62 synonymous, 18 nonsynonymous), two SNPs were located within intergenic regions, one SNP each in tRNA(Glu) and tRNA(SER), seven SNPs in small and large ribosomal RNAs, 263 within the hypervariable A+T-rich region, and with an average of 130 SNPs separating single haplotypes (genetic divergences between all mitochondrial strains are summarized in Tables S1–S2, and the resulting phylogeny is illustrated in Fig. S1).

Pathogenicity scores for all observed nonsynonymous changes were determined using the *MutPred* algorithm (Li *et al.*, 2009). This analysis identified four nonsynonymous mutations as potentially pathogenic (Table S3): G58S causes the loss of a catalytic residue in mt:COII in (BAR), N177K causes the gain of an additional ubiquitination site in mt:ATP6 (ORE), P180S causes the gain of a catalytic residue in mt:ATP6 (BAR, ISR, ORE, PUE), and Q133P causes the gain of a glycosylation site in mt:ND4 (ISR). The remaining 14 nonsynonymous SNPs are not predicted to alter structural or biochemical parameters of affected proteins. Sequence polymorphisms in structural RNAs among all mitochondrial strains were analysed using *tRNAScan* (Schattner *et al.*, 2005) for tRNAs and *ExpaRNA* (Smith *et al.*, 2010a) for small and large ribosomal subunits. rRNA sequence analyses revealed three distinct srRNA genotypes, four lrRNA haplotypes and one SNP in $tRNA^{Glu}$. None of the variants was predicted to alter structural characteristics or the anticodon of the affected tRNA.

High-resolution respirometry

Experiments were conducted on both males and females at two age classes (15 and 25 days old) and across all 12 mitochondrial haplotypes. For each

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experimental unit (Haplotype × Sex × Age), individual thoraces were dissected and immediately processed for high-resolution respirometry, as previously described for *Drosophila* (Pichaud *et al.*, 2011). Briefly, thorax muscles were permeabilized at 4 °C using BIOPS relaxing solution complemented with saponin, and subsequently blot-dried, weighed and transferred into the respiration chamber of an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria), calibrated with air-saturated respiration medium (115 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 3 mM HEPES, 1 mM EGTA, 0.2% BSA, pH 7.2) at 25 °C. Prior to the transfer of fibres, pyruvate, L-proline and malate (10 mM each) were added to the respiration medium, and oxygen was injected into respiration chambers to facilitate oxygen diffusion throughout muscle fibres. Measurements are expressed as means of respiration rates in pmol of oxygen consumed per second and per mg of permeabilized fibres ± SEM. All measurements carry additional labelling denoting the abbreviation(s) of the complex(es) targeted specifically through inhibiting and uncoupling substrates added throughout measurements, followed by the state of respiration (complex STATE) as previously described (Pichaud *et al.*, 2013). After monitoring CI-LEAK (or CI-L) in the presence of pyruvate, L-proline and malate, sequential injections of the following compounds were performed: excess ADP (5 mM) to measure CI-OXPHOS (or CI-Ox); cytochrome c (15 μM) allowing the evaluation of the functional integrity of the outer mitochondrial membrane (CIc-OXPHOS or CIc-Ox; samples with reduced integrity are discarded); *sn* glycerol-3-phosphate (20 mM) to monitor maximum OXPHOS with the contribution of complex I and glycerol-3-phosphate dehydrogenase (CIc+G3Pdh-OXPHOS or CI+G3Pdh-Ox); FCCP (optimum concentration reached between 0.75 and 1.25 μM) to stimulate uncoupled respiration for complex I and glycerol-3-phosphate dehydrogenase as a measure of ETS capacity (CI+G3Pdh-ETS or CI+G3Pdh-E); rotenone (1 μM) to inhibit complex I and to measure the oxygen flux attributable to the electron input through G3Pdh in the uncoupled state (G3Pdh-ETS or G3Pdh-E); antimycin A (2.5 μM) as inhibitor of complex III to measure residual oxygen consumption (via subtraction of above mentioned oxygen fluxes); and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and ascorbate (0.5 μM and 2 mM, respectively) to measure complex IV activity (Cox) corrected for chemical background after complete inhibition of Cox by sodium azide. At the end of each experiment, respiration media containing the permeabilized fibres were removed from respiration chambers, homogenized with a 10-mL Teflon potter homogenizer and immediately placed at −80 °C until measurement of citrate synthase (CS) activity.

To confirm that differences in mitochondrial respiration between mitochondrial haplotypes are driven by mitochondrial genetic variation, and not by cryptic nuclear genetic variation that may have accumulated between strains (despite the methodological safeguards described above), we first assessed distinct parameters (CI-LEAK, CI-OXPHOS, CIc-OXPHOS, CIc+G3Pdh-OXPHOX, CIc+G3Pdh-ETS, G3Pdh-ETS, Cox and CS) across both duplicates and sexes of six randomly selected mitochondrial strains at day 15 (per duplicate: BRO females, $n = 3$; HAW males, $n = 5$; and females, $n = 5$; ISR males, $n = 4$; and females, $n = 3$; JAP males, $n = 5$; MAD males, $n = 3$; and females, $n = 5$; SWE females, $n = 5$).

Indices of respiration rate and mitochondrial quantity

Respiratory rate

Because expression across the respiratory parameters was highly correlated, we used principal component analysis (PCA) to transform these variables into linearly uncorrelated principal components (normalized for sample weight; Fig. 1, see Statistical Analysis). Respiratory parameters measured at the level of complex I loaded on both PC1 and PC2, but were more

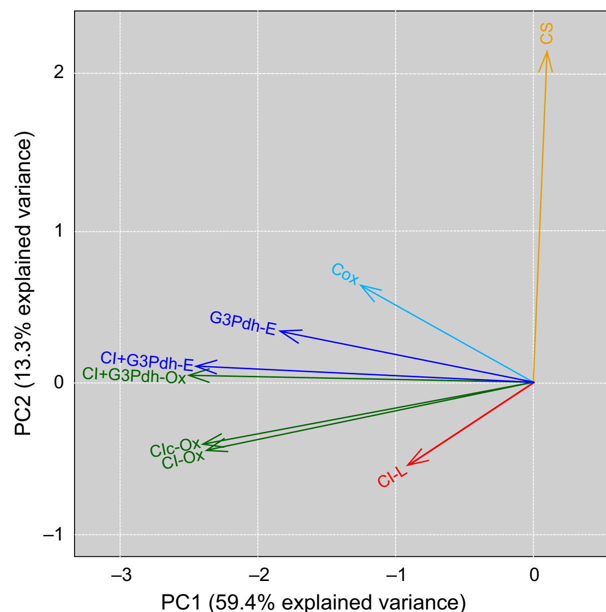

Fig. 1 Principal component analysis (PCA) of respiratory parameters across 12 mitochondrial strains of *Drosophila melanogaster*. The first principal component (PCA axis 1; PC1) accounted for 59.4% of the variance and was mostly driven by respiratory parameters, whereas the second principal component (PCA axis 2; PC2) accounted for 13.3% and was mainly driven by citrate synthase (CS) activity (yellow: CS activity; light blue: complex IV activity; dark blue: uncoupling [ETS] states; green: OXPHOS states; dark red: LEAK state, respiratory parameters: (CI-LEAK or CI-L, CI-OXPHOS or CI-Ox, CIc-OXPHOS or CIc-Ox, CIc+G3Pdh-OXPHOS or CI+G3Pdh-Ox, CIc+G3Pdh-ETS or CI+G3Pdh-Ox, G3Pdh-ETS or G3Pdh-E, Cox and CS).

pronounced on PC1 during phosphorylation states (−0.18 and −0.24 during CI-LEAK, −0.42 and −0.13 during CI-OXPHOS, and −0.43 and −0.12 during CIc-OXPHOS for PC1 and PC2, respectively; PC1 values were inversely related to respiration levels; Fig. 1). When ETS electron flux was increased through the provision of G3P, respiration loaded strongly on PC1, both during the OXPHOS and during the uncoupling (ETS) states (−0,45 and 0.03 during CI+G3Pdh-OXPHOS, and −0.44 and 0.04 during CI+G3Pdh-ETS for PC1 and PC2, respectively; Fig. 1). The same pattern was observed when complex I was inhibited, that is when electron provision was limited to G3Pdh (−0.37 and −0.14 during G3Pdh-ETS for PC1 and PC2, respectively). PC1 thus served as index of respiratory activity. Variance in complex IV (Cox) activity was equally explained by both axes (−0.26 and −0.28 for PC1 and PC2, respectively; Fig. 1).

Mitochondrial quantity

We conducted a CS activity assay to evaluate mitochondrial quantity. CS is a key enzyme of the tricarboxylic acid cycle located in the mitochondrial matrix and as such serves as biomarker of mitochondrial quantity (Larsen *et al.*, 2012). Sample homogenates were freeze-thawed twice to ensure that the entirety of the enzyme was released from the mitochondrial matrix before incubation with a 100 mM imidazole HCl buffer complemented with 0.1 mM of the substrate 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 mM acetylCoA (pH 8) and 0.15 mM oxaloacetate. Absorption of this colorimetric assay was measured at 25 °C at a wavelength of 412 nm for 4 min (DTNB ε_{412} = 13.6 mL cm^{-1} μmol^{-1}) using a microplate reader, and reading intervals of 10 s. CS activities were expressed as U mg^{-1} of permeabilized fibres where U is 1 μmol of substrate transformed per minute, and were not correlated to single respiration parameters or PC1. PCAs showed that CS loaded strongly on PC2 (0.01 and 0.89 for PC1 and PC2, respectively; Fig. 1). PC2 thus served as an index of mitochondrial content.

Statistical analysis

Principal component analysis and linear modelling: statistical analyses were performed using the R platform (R_Core_Team, 2013). As stated above, we used PCA to reduce all of the respiratory parameters and CS activity to two linearly uncorrelated components. To achieve this, continuous data (CI-LEAK, CI-OXPHOS, CIc-OXPHOS, CIc+G3Pdh-OXPHOX, CIc+G3Pdh-ETS, G3Pdh-ETS, Cox and CS) were ln-transformed, centred and standardized prior to analysis using the ‘prcomp’ function. Analyses were conducted on two principal components (PCs) as suggested by the elbow point of the Scree plot and because respiratory parameters and CS activity loaded heavily on PC1 and PC2, respectively.

We fitted PC1 and PC2 to separate linear mixed-effects models, using the ‘lme’ function of the ‘NLME’ package (Pinheiro *et al.*, 2014) and treating haplotype, sex and age as fixed effects. The sampling day of the experiment and identity of the reaction chamber (three different respirometers with two reaction chambers each were used: chambers A and B for the first, C and D for the second, and E and F for the third respirometer) were treated as random effects. Residual maximum likelihood was used to estimate the variance of the model parameters. The significance of the fixed effects was estimated using a type III model, and maximum likelihood, to assess the change in deviance – using Wald chi-square (χ^2) tests – associated with progressively simplified models that excluded the nonsignificant effects, one at a time.

To validate that variation in PC2 accurately reflected variation in CS, we also modelled the effects of haplotype, sex and age on CS directly. We modelled ln-transformed raw CS activity with weight as covariate (there was no correlation between raw CS and weight) and fitted results again to a Type III linear mixed model. This model confirmed effects that we found for PC2 (Table S4; Fig. S2). Results of this model were thus qualitatively identical to the modelling of PC2, as confirmed by linear correlation between the ‘least squares’ means generated by the two models (Pearson’s coefficient = 0.49, $P < 0.001$). Overall, these additional analyses reinforced our findings that the majority of the variance explained by PC2 is indeed attributable to differences in CS activity.

Finally, we used all variable sites for a mitochondrial genomewide association study (GWAS) to probe for potential correlations between specific SNPs and mitochondrial respiratory parameters. However, because of the reliance on large sample sizes, and high linkage disequilibrium between polymorphic sites for mitochondrial DNA, the power associated with the GWAS was limited. We also conducted a Mantel test to examine whether increased molecular divergence between mitochondrial haplotypes was associated with increased phenotypic divergence in PC1 or PC2. To achieve this, we compared a matrix of differences in the number of synonymous and nonsynonymous SNPs, to matrices of phenotypic differences in PC1 and PC2, across all pairwise combinations of mitochondrial haplotypes (all methodological detail can be found in the supplemental material).

Results

High-resolution respirometry

First, to confirm that differences in mitochondrial respiratory capacity were attributable to mitochondrial

haplotypic effects, and not to effects of cryptic and residual nuclear allelic variation that might accumulated across the mitochondrial strains (despite our safeguards to prevent this from occurring) during the breeding process, we measured respiratory parameters in both sexes across mitochondrial strain *duplicates* of six randomly selected mitochondrial strains at day 15. In no instance did we detect differences in the respiratory parameters across the two duplicates of a mitochondrial strain (CI-LEAK, CI-OXPHOS, CIc-OXPHOS, CIc+G3Pdh-OXPHOS, CIc+G3Pdh-ETS, G3Pdh-ETS, Cox and CS; supplementary Fig. S3, supplementary Table S5), and thus, all ensuing effects of mitochondrial strains on metabolic function could be confidently attributed to effects of mitochondrial genetic variation *per se* rather than to other confounding effects. Following this analysis, flies from duplicates of each mitochondrial strain were pooled in the subsequent experiments, and respiratory and enzymatic measurements were conducted on a minimum of six individual and randomly chosen flies per experimental unit [each unit denoted by a particular Haplotype (12 haplotypes) × Sex (males, females) × Age (15, 25 days) combination; $n = 325$ individuals in total].

Expression across the respiratory parameters was generally highly correlated, and thus, we used PCA to transform these variables into linearly uncorrelated principal components. The first component (PCA axis 1; PC1) accounted for 59.4% of the variance in the data set, with respiratory parameters (CI-LEAK, CI-OXPHOS, CIc-OXPHOS, CIc+G3Pdh-OXPHOX, CIc+G3Pdh-ETS, G3Pdh-ETS, Cox) loading heavily onto this axis (Fig. 1). The second component (PCA axis 2; PC2) accounted for 13.3% of the variance, with CS activity, a biomarker for mitochondrial quantity (Larsen *et al.*, 2012), loading heavily onto this axis (Fig. 1).

PC1, our index of respiratory activity, was affected by the mtDNA haplotype ($\chi^2_{(11,\ n = 325)} = 62.85$, $P < 0.001$; Table 1, Fig. 2). However, these mitochondrial haplotypic effects were contingent on complex interactions involving the sex and age of the flies (Haplotype × Sex × Age; $\chi^2_{(11,\ n = 325)} = 50.62$, $P < 0.001$, Table 1, Fig. 2). Notably, there was generally more variation across mtDNA haplotypes for respiratory activity in younger flies than in older flies (Haplotype × Age: $\chi^2_{(11,\ n = 325)} = 32.50$, $P < 0.001$, Table 1, Fig. 2). Furthermore, some haplotypes conferred higher respiratory efficiencies (i.e. more negative PC1 values) in males than females, whereas others conferred higher efficiencies in females, and the magnitude and direction of these sex-specific effects themselves changed across the age classes (Fig. 2).

PC2, our index of mitochondrial quantity, was also affected by the mtDNA haplotype, with these effects again contingent on interactions with sex and age (Table 1, Fig. 3). We found greater mitochondrial haplotypic variance for mitochondrial quantity in males, than females, but these male biases in variance were only clear in flies of younger age (Fig. 3a–d).

Despite finding pervasive mtDNA genetic effects on respiratory activity and mitochondrial quantity, *in silico* pathogenicity tests indicated that only four of the 80 SNPs affecting the protein-coding region (62 synonymous, 18 nonsynonymous) are likely to be of functional significance (Table S3). None of the four candidate SNPs that were identified as putatively pathogenic could be tied to the mtDNA-mediated effects on respiration, and indeed, we were unable to associate any particular SNP to PC1 or PC2 expression using mt-GWAS. Neither did we detect any association between genetic and phenotypic divergence using a Mantel test.

Discussion

Our results demonstrate that core respiratory parameters are modulated by the genetic variation located within the mitochondrial genome of *D. melanogaster* and indicate that these mtDNA-mediated effects are context-dependent on both the sex and age of the flies. These findings thus provide mechanistic insights: firstly, into how allelic variation found within the mitochondrial genome may ultimately affect the expression of core life-history traits, such as fertility and ageing, via direct effects on intermediary phenotypes such as respiratory capacity and mitochondrial quantity. Secondly, the sex-specific outcomes of mitochondrially encoded effects on respiratory parameters help to reconcile why similar mtDNA-mediated effects on life-history trait expression have been found to be sex-specific in several cases (Innocenti *et al.*, 2011; Camus *et al.*, 2012, 2015; Yee *et al.*, 2013). Cellular respiration is a trait that arguably stands at the root of the expression of most life-history traits (Wikelski & Ricklefs, 2001), and here, we have shown pervasive and dynamic effects on this trait that are traceable to the mitochondrial haplotype. These findings have important implications, particularly in the context of understanding mitochondrial genetic contributions to the evolution of energy metabolism, life histories and generally mitochondrial disease (Ellison & Burton, 2008; Wallace, 2010; Hwang *et al.*, 2011; Camus *et al.*, 2012; Barreto & Burton, 2013; Moreno-Loshuertos *et al.*, 2013; Yee *et al.*, 2013; Zhu *et al.*, 2014; Liu *et al.*, 2015). They indicate that the effects of naturally occurring polymorphisms, which lie within the mitochondrial genome and which define divergent mitochondrial haplotypes, might well have been routinely underestimated.

The mitochondrial haplotypic effects on both respiratory activity and mitochondrial quantity exhibited a high degree of context dependency and were contingent on both the sex and the age of the flies in which they were expressed. There were no consistent differences between the sexes in levels of mitochondrial

Table 1 Results of separate general linear mixed models, modelling effects of mtDNA haplotype, sex and age on PC1 and PC2, with sampling day and reaction chamber modelled as random effects. χ^2 values are reported, and *P* values based on a Type III ANOVA. Akaike information criterion, Bayesian information criterion and log-likelihood values were 1375.42, 1568.40 and −636.71, respectively, for PC1, and 759.08, 952.05 and −328.54, respectively, for PC2. SD values are listed for random effects day, reaction chamber and residual for PC1 and PC2.

		χ^2	
Fixed effects	Numerator df	PC1	PC2
Haplotype	11	62.85*** ($P < 0.001$)	25.70** ($P = 0.007$)
Sex	1	6.48* ($P = 0.011$)	1.86 ($P = 0.17$)
Age	1	1.20 ($P = 0.27$)	1.84 ($P = 0.17$)
Haplotype × Sex	11	43.62*** ($P < 0.001$)	68.37*** ($P < 0.001$)
Haplotype × Age	11	32.50*** ($P < 0.001$)	26.14** ($P = 0.006$)
Sex × Age	1	0.50 ($P = 0.48$)	1.19 ($P = 0.28$)
Haplotype × Sex × Age	11	50.62*** ($P < 0.001$)	57.29*** ($P < 0.001$)

Random effects (SD)	Day	Reaction chamber	Residual
PC1	0.98	0.73	1.52
PC2	0.65	0.11	0.61

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

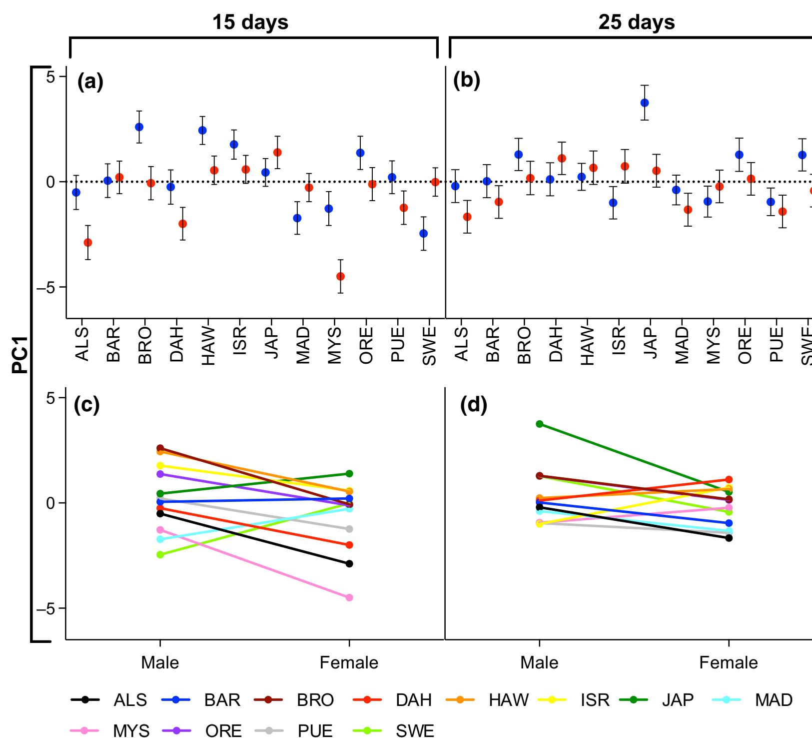

Fig. 2 Effects of mtDNA haplotype, sex and age on respiratory capacity, as represented by PC1. (a–b): least square means (± SEM) of each haplotype, for each sex and age class generated from the general linear mixed model. Males are denoted by blue, and females by red data points. (c–d): Interaction plots comparing male and female LS means per haplotype, separately for each age class.

genetic variance, although there was clearly more mitochondrial haplotypic variance underpinning the expression of PC2 (mitochondrial quantity) in males than females at 15 days of age (Fig. 2e), consistent with the predictions of *Mother's curse* theory (Frank & Hurst, 1996; Gemmell *et al.*, 2004); these effects were not apparent among 25-day-old flies (Fig. 2f). These results indicate that future studies probing the hypothesis that maternal inheritance of mitochondria leads to the accumulation of male-biased mitochondrial genetic variance should test such effects across multiple age classes to ensure the effects are not simply an artefact of the age class sampled. Similarly, levels of mitochondrial haplotypic variance for PC1 in females were higher in

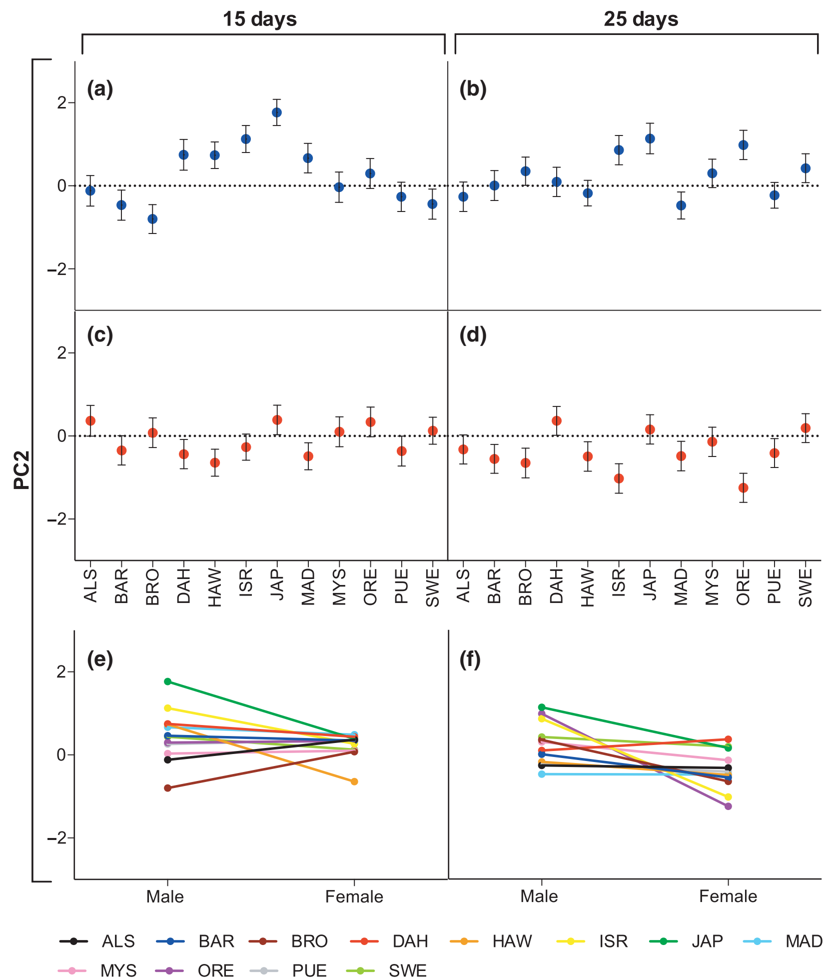

Fig. 3 Effects of mtDNA haplotype, sex and age on mitochondrial quantity, as represented by PC2. (a–d): Least square means (± SEM) of each haplotype, for each sex and age class generated from the general linear mixed model. Males are denoted by blue, and females by red data points. (e–f): Interaction plots comparing male and female LS means per haplotype, separately for each age class. [Correction added on 24 February 2016 after first online publication: Figure 3 has been replaced to reflect the correct order of panels (a-d), and the correct values for panels (e-f).].

younger than older flies. The patterns of age specificity of these effects are not easily reconciled by evolutionary theory, which predicts that levels of genetic variation should generally increase with advancing age due to a decreasing efficiency of selection (Medawar, 1952; Williams, 1957; Dowling *et al.*, 2009).

Although mitochondrial genetic variance has previously been reported for longevity and ageing (Rand *et al.*, 2006; Clancy, 2008; Dowling *et al.*, 2009, 2010; Camus *et al.*, 2012; Zhu *et al.*, 2014), our findings are notable because they indicate that polymorphisms harboured within the mitochondrial genome exert age-specific effects on core physiological function. The classic evolutionary theory of ageing (Medawar, 1952; Williams, 1957; Dowling, 2012) predicts the accumulation of germ-line mutations in the genome that cause ageing. The mechanistic *Mitochondrial theory of ageing* (Harman, 1972) predicts the accumulation of somatic mutations in mtDNA within a lifetime that cause ageing. We hypothesized that these ultimate and proximate theories of ageing are likely to be functionally linked, with the germ-line mutations that delineate mtDNA haplotypes shaping rates of somatic expansion of mtDNA mutations with advancing age. As such, we predicted greater variance across mitochondrial haplotypes, for respiratory activity and mitochondrial quantity, at the older age class (25 days) we sampled. Counter to prediction, however, we generally observed more mitochondrial genetic variation for respiratory activity in the younger (15-days-old) flies, relative to 25-days-old flies in which mitochondrial catalytic capacity of the haplotypes converged towards the same trait values. That is, the polymorphisms involved exerted effects that were seemingly more pronounced at the earlier age class, and exhibiting little evidence of positive pleiotropy between the two age classes (i.e. the better performing haplotypes at 15 day were not generally the better performing at 25 days). Although the mitochondrial mutational profiles underpinning respiratory capacity are seemingly not characterized by mutations of predominantly later-life effect, as would be predicted under mutation accumulation hypotheses of

ageing (Medawar, 1952; Williams, 1957), the age specificity of mitochondrial genetic effects deserves a more detailed study that encompasses a broader span of the adult life span. We sampled only two age classes that putatively represented midlife ages, and lie past the reproductive peak. The ageing trajectories across the strains of *D. melanogaster* used here are, however, characterized by an exponential increase in population-level mortality at around day 30 to 35 in adult life (Camus *et al.*, 2012), and thus, neither of the age classes we used is likely to have captured flies that were experiencing advanced stages of physiological senescence. However, although population-level mortality rates had not begun to increase exponentially by day 25, it is key to note that signatures of physiological senescence generally do manifest within the first 2 weeks of adult life in *Drosophila*, and result in impairment to traits such as locomotive activity and sensitivity to oxidative stress, both of which are entwined with mitochondrial quantity and respiratory function (Grotewiel *et al.*, 2005).

While it is clear from our study that allelic variation within the mitochondrial genome has pervasive effects on core metabolic function, the evolutionary forces that have shaped this variation remain largely enigmatic. Some of the polymorphisms might represent *de novo* deleterious mutations maintained under mutation–selection balance (Lynch, 1997), and some of these, as discussed above, might be sex-specific and reflecting a male-biased mutation load (Frank & Hurst, 1996; Gemmell *et al.*, 2004; Zeh & Zeh, 2005; Innocenti *et al.*, 2011) that has diverted expression of metabolic processes in males away from the phenotypic optima. Other polymorphisms might represent adaptations shaped by selection to the local environment (Dowling *et al.*, 2008) or via coevolution to the population-specific nuclear backgrounds (Wolff *et al.*, 2014). These variants might no longer be adaptive if expressed outside of the environments (Wallace, 2007) or nuclear backgrounds in which they originally evolved. The w^{1118} nuclear background alongside which the twelve mitochondrial genotypes are expressed is not the natural nuclear DNA for any of the strains used in this study. The disruption of mitonuclear interactions could therefore account for some of the functional consequences observed in co-evolved OXPHOS complexes (Burton & Barreto, 2012; Wolff *et al.*, 2014; Hill, 2015). An interesting future avenue would therefore be to extend the approach employed here, by screening the bioenergetic performance of mtDNA haplotypes when placed alongside nuclear backgrounds with which they have coevolved, vs. alongside backgrounds that are evolutionary novel. Such a design could help to elucidate whether mtDNA-mediated effects of respiratory function are primarily underpinned by additive effects that are consistent across nuclear genotypes, or by epistatic interactions between mitochondrial and nuclear genotype (Dobler *et al.*, 2014).

In summary, our study indicates that previously identified links between mitochondrial genotype and life-history phenotype might ultimately be traceable to mtDNA-encoded effects on intermediary metabolic phenotypes. These effects, however, exhibit high degrees of context dependence, across flies of different sexes and ages. We note that these results could have implications for understanding the complex genetics underlying the expression of mitochondrial disease. Research aimed at understanding these diseases is complicated because of the heterogeneous nature, and variability of disease expression with which they are associated (Wallace, 2005, 2010). If similar context dependency in expression of mitochondrial allelic variation, as found here in *Drosophila*, extends to humans, then it could prove far more difficult than previously anticipated to predict health consequences attributable to particular functional mtDNA polymorphisms.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Table S1 Single nucleotide polymorphisms (SNPs) within the protein-coding region, tRNAs, and rRNAs between all twelve mitochondrial haplotypes.
Table S2 Genetic distance matrix. Pair-wise comparison of all twelve mitochondrial haplotypes, indicating the number of single nucleotide polymorphisms that exist between single pairs of mitochondrial lines.
Table S3 Pathogenicity scores (probability to have pathogenic effect) for all observed non-synonymous changes among the twelve mitochondrial haplotypes.
Table S4 Significance values from ANCOVA on raw citrate synthase values (ln transformed) fitted to a linear mixed model by a Type III ANOVA.
Table S5 Replicate control.
Figure S1 Phylogenetic tree inferred from whole mitochondrial genomes (Genbank KP843842- KP843853, Wolff *et al.*, 2015) of twelve mitochondrial haplotypes.

Figure S2 Least square means (± SEM) generated by linear mixed effects model (see text for details) on ln transformed raw citrate synthase activity with sample weight as covariate across mitochondrial haplotypes for males (blue) and females (red) at two ages (15, 25 days).
Figure S3 Duplicate control.

Appendix S1 Mitochondrial Genome-wide Association Study (GWAS) and Genotype-Phenotype correlations.

Data deposited at Dryad: doi: 10.5061/dryad.g56f4

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