JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12565

Within-population genetic effects of mtDNA on metabolic rate in *Drosophila subobscura*

Z. KURBALIJA NOVIČIĆ*, E. IMMONEN†, M. JELIĆ‡, M. ANĐELKOVIĆ*‡§, M. STAMENKOVIĆ-RADAK*‡ & G. ARNQVIST†

*Institute for Biological Research, University of Belgrade, Belgrade, Serbia
†Animal Ecology, Department of Ecology and Genetics, Uppsala University, Uppsala, Sweden
‡Faculty of Biology, University of Belgrade, Belgrade, Serbia
§Serbian Academy of Sciences and Arts, Belgrade, Serbia

Keywords:

metabolic rate;
mitochondria;
mitonuclear epistasis;
mtDNA;
polymorphism;
selection.

Abstract

A growing body of research supports the view that within-species sequence variation in the mitochondrial genome (mtDNA) is functional, in the sense that it has important phenotypic effects. However, most of this empirical foundation is based on comparisons across populations, and few studies have addressed the functional significance of mtDNA polymorphism within populations. Here, using mitonuclear introgression lines, we assess differences in whole-organism metabolic rate of adult *Drosophila subobscura* fruit flies carrying either of three different sympatric mtDNA haplotypes. We document sizeable, up to 20%, differences in metabolic rate across these mtDNA haplotypes. Further, these mtDNA effects are to some extent sex specific. We found no significant nuclear or mitonuclear genetic effects on metabolic rate, consistent with a low degree of linkage disequilibrium between mitochondrial and nuclear genes within populations. The fact that mtDNA haplotype variation within a natural population affects metabolic rate, which is a key physiological trait with important effects on life-history traits, adds weight to the emergent view that mtDNA haplotype variation is under natural selection and it revitalizes the question as to what processes act to maintain functional mtDNA polymorphism within populations.

Introduction

Sequence variation in mitochondrial DNA (mtDNA) has traditionally been considered to be selectively neutral (Ballard & Kreitman, 1995), but this view is being replaced by one where mtDNA is under natural selection (Rand, 2001; Ballard & Whitlock, 2004; Ballard & Rand, 2005; Meiklejohn *et al.*, 2007; Christie *et al.*, 2011; Kazancioglu & Arnqvist, 2014). For example, comparative genomic data indicate a role for positive selection in shaping the mitochondrial genome (Bazin *et al.*, 2006; Meiklejohn *et al.*, 2007; Breen *et al.*, 2012; Frankham, 2012), and several experimental studies have documented important phenotypic effects of between-species or between-population mtDNA variation (Macrae & Anderson, 1988; Fos *et al.*, 1990; Rand *et al.*, 2001, 2006; James & Ballard, 2003; Montooth *et al.*, 2003; Ellison & Burton, 2006; Dowling *et al.*, 2007; Arnqvist *et al.*, 2010). However, very few studies have explored phenotypic effects of sympatrically occurring mtDNA variants, which is unfortunate given that natural selection primarily acts within populations. In *Drosophila simulans*, sympatric mtDNA haplotypes do show differences in *in vitro* assays of metabolic function (Katewa & Ballard, 2007; Pichaud *et al.*, 2012). Similarly, Dowling *et al.* (2007) and Maklakov *et al.* (2006) presented evidence suggesting the presence of mtDNA effects on female longevity and fitness within a laboratory population of *Drosophila melanogaster*, although these effects were to some extent contingent upon the nuclear genetic background.

There are good reasons to predict that sequence variation in the mitochondrial genome should have fundamental effects on reproductive traits (Frank & Hurst,

Correspondence: Zorana Kurbalija Novičić, Institute for Biological Research, University of Belgrade, Despot Stefan Blvd. 142, 11000 Belgrade, Serbia.
Tel.: +381 112 078 328; fax: +381 11 2761 433; e-mail: kurbalija@ibiss.bg.ac.rs

1996; Gemmell *et al.*, 2004) and other life-history traits (Ballard & Whitlock, 2004; Rand *et al.*, 2004; Arnqvist *et al.*, 2010), because energy metabolism in eukaryotes is determined by the intimate coordination of the mitochondrial and the nuclear genomes. Whereas genes encoding mitochondrial proteins essential for transcription and DNA replication are encoded by the nuclear genome, important elements of the mitochondrial translational machinery and the genes producing the actual building blocks of the energy-producing oxidative phosphorylation electron transport chain (OXPHOS) are encoded by both mtDNA and nDNA. Considering the central role of mtDNA in this basal metabolic pathway, we expect mitonuclear genetic effects on metabolic parameters and therefore, indirectly, on all upstream life-history traits (Ballard *et al.*, 2007). Many *in vitro* studies of mitochondrial bioenergetics have confirmed that different naturally occurring mtDNA haplotypes show important differences in metabolic function in both vertebrates and invertebrates (Ballard *et al.*, 2007) and a few between-population studies have shown that whole-organism metabolic rate is affected by mtDNA haplotype in both birds (Tieleman *et al.*, 2009) and insects (Arnqvist *et al.*, 2010).

Mitochondrial DNA typically exhibits sizeable levels of genetic variation in natural populations (Clark, 1984; Moritz *et al.*, 1987; Nachman *et al.*, 1996). This general observation is puzzling, to the extent that mtDNA variation is functional. The reason is simply that positive or negative selection in females should rapidly exhaust within-population mtDNA variation, because the mitochondrial genome shows maternal inheritance, has a relatively low effective population size, is haploid and generally does not recombine (Frank & Hurst, 1996; Rand *et al.*, 2001). This is verified by theory, showing that positive selection can only act to maintain mtDNA polymorphism within populations under special and very restrictive conditions (Clark, 1984; Babcock & Asmussen, 1996; Rand *et al.*, 2001; Liu & Asmussen, 2007). In contrast, negative frequency-dependent selection could in theory maintain functional mtDNA polymorphism (Gregorius & Ross, 1984), a possibility that recently received experimental support in replicated laboratory populations of the seed beetle *Callosobruchus maculatus* (Kazancioglu & Arnqvist, 2014) where the relative fitness of mtDNA haplotypes over 10 generations of experimental evolution was inversely proportional to their starting frequency. However, a first step towards an understanding of the maintenance of mtDNA polymorphism must be to assess phenotypic effects of mitochondrial genetic variation across sympatric mtDNA haplotypes. Here, metabolic phenotypes are prime suspects, both because of the key role that mtDNA plays in metabolic processes (Ballard *et al.*, 2007) but also because metabolism is ultimately the process by which resources are converted to fitness (Brown *et al.*, 2004). Because mtDNA does not respond to selection in males, there are reasons to believe that mitochondrial genetic effects may be sex specific (Frank & Hurst, 1996; Gemmell *et al.*, 2004).

Drosophila subobscura provides a very interesting model system for the study of within-population variation in mtDNA, for three reasons. First, the mitochondrial genome of *D. subobscura* displays sequence divergence as revealed by extensive restriction site polymorphism (RSP; Latorre *et al.*, 1986; Pinto *et al.*, 1997; García-Martinez *et al.*, 1998; Castro *et al.*, 1999; Jelic *et al.*, 2012a). Second, the geographic pattern of RSP is intriguing. Within most populations, the two dominating mtDNA haplotypes (named I and II) occur at almost equal frequencies, whereas a number of population specific haplotypes are very rare (Castro *et al.*, 1999; Christie *et al.*, 2010; Jelic *et al.*, 2012a). This pattern of mtDNA haplotype polymorphism is confirmed when analysing nucleotide sequence data from the mitochondrial *ND5* gene rather than RSP (Castro *et al.*, 2010). Third, *D. subobscura* is a classic model system for studying how selection acts to maintain autosomal polymorphism (Balanyà *et al.*, 2009), and several previous studies collectively suggest that selection plays a role in the maintenance of mtDNA polymorphism in this species. This is in part because there are consistent trends of seasonal changes in frequency of the two dominant mtDNA haplotypes in natural populations that cannot be accounted for by genetic drift (Latorre *et al.*, 1992; González *et al.*, 1994; Christie *et al.*, 2010). In addition, previous laboratory experiments have suggested that flies carrying the two dominant haplotypes show differences in some life-history traits and in behaviour (Castro *et al.*, 2003; Christie *et al.*, 2004). However, these studies did not control for nuclear genetic background and, when expressed in the same uniform nuclear background, phenotypic differences between mtDNA haplotypes seem much less pronounced (Christie *et al.*, 2011). This implies that mitonuclear epistasis may be important in *D. subobscura*, and other studies have provided evidence supporting the existence of mitonuclear epistatic interactions between mtDNA and nuclear markers (Fos *et al.*, 1990; García-Martinez *et al.*, 1998; Castro *et al.*, 1999) which is further supported by within-population linkage disequilibrium between mtDNA haplotypes and nuclear genetic markers (Oliver *et al.*, 2002). Other studies suggest, however, that mitonuclear epistatic interactions are complex and may show temporal and spatial variation (Jelic *et al.*, 2012a, b).

Here, we use mitonuclear introgression lines in conjunction with whole-organism microrespirometry to disentangle the effects of mitochondrial and nuclear genetic variation within a single natural population on metabolic phenotypes in *D. subobscura*. We estimated genetic effects across three sympatric mtDNA haplotypes (I, II and D) to test the hypotheses that (i) sympatric mtDNA haplotypes differ in metabolic rate, (ii)

nuclear genetic background modulates the genetic effects of mtDNA on metabolism and (iii) the metabolic effects of mtDNA are to some extent sex specific.

Material and methods

Construction of mitonuclear introgression lines

Associations between specific mtDNA haplotypes and phenotypic traits across individuals are complicated by the fact that mtDNA may be in linkage disequilibrium with nuclear genes within populations. We overcame this inferential hurdle by setting up a series of mitonuclear introgression lines, which allows disentanglement of the independent effects of mtDNA and nDNA. Flies were genotyped for their mtDNA haplotype using the methods described in Jelic *et al.* (2012a). Our experiments were performed using a series of isofemale (IF) lines collected from a single *D. subobscura* population in the Sicevo Gorge, Serbia (S 43°19′55.58″ N, 22°08′37.98″ E), each IF line deriving from a single inseminated female collected in the wild using fermented fruit traps. The IF lines were maintained under standard laboratory conditions approximately 10 months prior to the backcrossing procedure described below. The founding IF lines carried either of three different mtDNA haplotypes: I (N = 10 IF lines), II (N = 10 IF lines) and D (N = 1 IF lines). The single D IF line was expanded in the laboratory into 10 descendant IF lines. We note here that the D haplotype is interesting as it shows the same restriction sites as haplotype I but is much longer (Jelic *et al.*, 2012a). This is the result of additional sequences in that A + T-rich control region, an additional srRNA gene copy, additional genes for three tRNAs, a hymeric sequence of a large ribosomal RNA (lrRNA) and a duplication of *ND2*. In total, the entire mtDNA of the D haplotype is 2.7 kb longer (17% of total the mtDNA) than any other naturally occurring haplotype in *D. subobscura*, but it is not known whether any of these duplications/insertions are expressed and functional. Below, we denote the nuclear genome associated with a particular mtDNA haplotype after the mtDNA haplotype with which it was co-expressed in the field (i.e. 'nDNA I' represents a nuclear genome from an IF line that originally carried the mtDNA haplotype I).

To disentangle the phenotypic effects of mitochondrial and nuclear genetic variation, as well as their interaction, we constructed lines that carry distinct mtDNA haplotypes introgressed into potentially distinct nuclear genetic backgrounds using a standard introgressive backcrossing scheme. Briefly, we generated seven crossed combinations of distinct cytoplasmic and nuclear lineages (see Fig. 1). Each such combination was replicated 10 times, such that we constructed 70 distinct mitonuclear introgression (MNI) lines in total. Each MNI line derived from two particular IF lines, which carried the desired mtDNA and nDNA genotypes. Each MNI line was founded by 10 virgin females from a specific IF line carrying desired mtDNA haplotype which were mated to 20 males from an IF line carrying the desired nDNA background. Within a particular mtDNA × nDNA cross-type, the 10 replicate MNI lines were founded by different IF lines. In each subsequent generation, 10 virgin females from a given MNI line were then backcrossed to 20 males from the founding paternal (i.e. nDNA) IF line. We performed 12 generations of backcrossing, thus disassociating the maternal mtDNA genome from the nuclear genome that it was originally co-expressed with, at which point more than 99.9% of the original nuclear genome was replaced with the nuclear genome of the paternal IF line (this number assumes a lack of strong selection on specific mitonuclear allelic combinations during the introgression procedure). To avoid the possibility of MNI line specific adaptation, we conducted additional backcrossing to the nDNA source populations during the two consecutive generations that preceded the respirometry assays described below. The mtDNA integrity of all MNI lines was validated at the 5th, 8th, 10th and 12th generation by genotyping a sample of flies from each MNI line. Our crossing design was constructed to allow comparisons of (A) mtDNA haplotype I and D when expressed in I or D nDNA backgrounds, (B) mtDNA haplotype II and D when expressed in II or D nDNA backgrounds and (C) mtDNA haplotype I and II in a common non-native nDNA background (see 'Statistical Modelling' below).

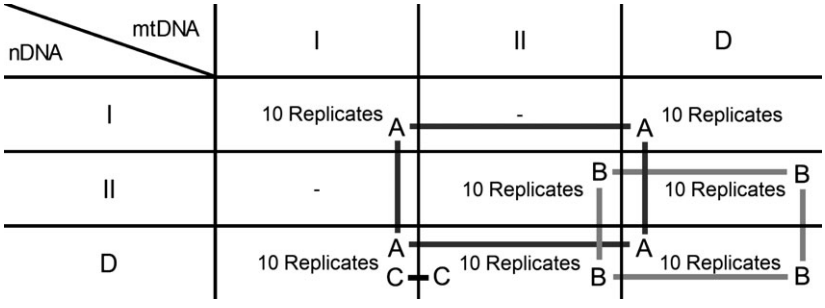

Fig. 1 The crossing design used in the mitonuclear introgression experiment. Indicated are also the three crossed modules used for inferential modelling (A–C).

Many insects are infected by maternally inherited, cytoplasmic bacteria (i.e. *Wolbachia*), and this can potentially confound the results of experiments that aim to investigate mitochondrial genetic effects. We thus screened for the presence of *Wolbachia* in all MNI lines, by a PCR assay using 16S rDNA *Wolbachia*-specific primers (O'Neill *et al.* 1992) according to the method detailed in García-Martinez *et al.* (1998). We used two different *Drosophila* strains containing *Wolbachia* as positive controls (*D. melanogaster* stock no. 5, Bloomington Stock Centre, *D. simulans*, Riverside strain). These PCR assays were negative for all of our MNI lines.

All lines were maintained and all experiments performed under constant laboratory conditions, at 19 °C, at approximately 60% relative humidity, light of 300 lx, and at a photoperiod of 12-h light : 12-h dark. All flies were fed standard *Drosophila* cornmeal medium.

Metabolic rate measurement

Metabolic rate was measured as CO_2 production (Terblanche & Chown, 2007) using a Sable Systems (Las Vegas, NV, USA) microrespirometry system (Lighton, 2008). Briefly, this system pumps air scrubbed of CO_2 and water (using ascarite and drierite, respectively) at a regulated flow rate through a respirometry chamber containing the focal flies. Downstream gas analysers are then used to measure the amount of CO_2 produced by the flies, and this measure provide a direct estimate of metabolic rate. We used an infrared absorption analyser to measure CO_2 (Li-7000; Li-Cor Biosciences, Lincoln, NE, USA), a dual fuel cell differential oxygen analyser (Oxzilla II; Sable Systems) to measure O_2 and a RH-300 water vapour pressure meter (Sable Systems) to measure water vapour. Inflowing air was pumped using a SS-4 (Sable Systems) and flow rate was regulated to 100 ml min^{-1} using a Model 840 mass flow control valve (Sierra Instruments, Monterey, CA, USA). Airflow was alternately allocated using 2 × 8-channel multiplexers (RM8; Sable Systems) to each of 16 respirometry chambers (RC-M, ∅ = 2 cm, length = 4 cm; Sable Systems) that were housed inside a Sanyo MIR-153 incubator, in darkness and at a constant temperature of 19 °C.

The respirometry system was set up in stop-flow mode (Lighton, 2008), in which each chamber was sealed for a period of 50 min and then flushed for a period of 200 s in a cyclical pattern. We ran five consecutive cycles (through all chambers) in each session. This resulted in five readings for each individual chamber, of which the first two were discarded as a burn-in. Each respirometry chamber was placed in an activity detector (AD-2; Sable Systems) connected to a data acquisition interface (Quick-DAQ; National Instruments, Coleman Technologies, Newton Square, PA, USA), which uses reflective infrared light technology to provide a precise and continuous measure of locomotor activity of the subjects in each chamber during the entire session. One of the 16 chambers was left empty and was used as a baseline to control for any drift of the gas analysers during each session. Thus, each single observation (i.e. a cycle) essentially consisted of the amount of CO_2 produced during 53.33 min by the flies in a given chamber and the total amount of activity performed by these flies during this time.

The system was calibrated once every day prior to the experimental runs. The oxygen analyser was calibrated using outside air scrubbed of CO_2 and water vapour with ascarite and drierite, and the carbon dioxide analyser was span-calibrated with pure N_2 and air of a known concentration (1000 p.p.m.) of CO_2 in N_2. Upstream of the CO_2 analyser, but downstream of the water vapour pressure meter, the outflowing air from the chambers was scrubbed of water vapour with magnesium perchlorate Sigma- Aldrich Sweden AB (Stockholm, Sweden). All analogue input gas data were acquired at 1 Hz via a UI2 analogue–digital interface (Sable Systems). Data acquisition and data analyses were performed in ExpeData Pro 1.5.6 (Sable Systems).

We measured metabolic rate of 5-day-old adult virgin flies, placed in a group of five same-sexed flies (i.e. a quintet) inside a respirometry chamber. For each MNI line, we measured metabolic rate in three replicate quintets of each sex ($70 \times 3 \times 2 \times 5 = 2100$ flies assayed in total) and, as stated above, data from each quintet consisted of three repeated observations. Following our metabolic rate measurements, we recorded the body mass of each quintet to the nearest 0.00001 g (Sartorius® Genius ME 235P).

Statistical modelling

Our experimental design represents a partial orthogonal design, and we evaluated our results using three different main inferential modules (see Fig. 1), each representing a distinct subset of the data that is internally orthogonal and crossed. To avoid inflation of the denominator degrees of freedom in our inferential models, each MNI line was regarded as our replicated experimental unit. Each module was thus analysed by a within-subjects ANOVA, where replicate MNI line was treated as a random effects subject, mtDNA and nDNA as between-subjects factors and sex as a within-subjects factor. The first model (A) focussed on differences in metabolic rate between haplotypes I and D, the second (B) on differences between II and D and the third (C) on differences between I and II. These three models represent our main inferential models. For all three models, residual distributions were well behaved and in no case did variance ratio tests reveal potential problems with heteroscedasticity ($P > 0.07$ in all cases).

To provide focused within-subjects ANOVAS of the effects of mtDNA haplotype on variance in standard

metabolic rate, however, we first removed variance in metabolic rate that was due to variation in activity and body weight in initial global general linear models, in the form of quadratic ANCOVAs with sex included. The effect of sex was included here, and hence, the main effect of sex was diminished in the downstream within-subjects ANOVAs, as the slope and intercept of the relationship between metabolic rate and weight/activity differed between the sexes, whereas the shape of this relationship did not (i.e. the sex × activity × activity and the sex × weight × weight interactions were small and nonsignificant; $P > 0.05$ in all cases). These models used raw CO_2 production as the response variable and included sex, activity and weight as covariates (zero-centred data), the quadratic terms of the two covariates and the two 2-way interactions between sex and the covariates (modelled separately for the three modules). Mean residual CO_2 production across cycles and quintets per replicate MNI line and sex from these models was then used as the response variable in our inferential models. Prior to calculating these mean residuals, however, we omitted a few outliers from our data. These were observations showing an absolute value of the standardized residuals larger than 2.57 (corresponding to $Z_{0.995}$) in our global ANCOVAs, likely representing observations from channels with leaky connections to the respirometry chambers (< 2% of all observations).

Results

Overall, mass-specific and activity-adjusted metabolic rate (henceforth, MR) differed across the seven mitonuclear cross-types in both sexes (females: $F_{6,63} = 2.46$, $P = 0.033$; males: $F_{6,63} = 2.89$, $P = 0.015$) and was higher in adult males compared to females (Fig. 2). Males and females differed in MR by some 22% (paired *t*-test: $t = 9.05$, d.f. = 6, $P < 0.001$). Mean male and female MR was positively associated, but the correlation was moderate both across mitonuclear cross-types ($r = 0.67$, $n = 7$, $P = 0.099$) and across replicate lines ($r = 0.29$, $n = 70$, $P = 0.015$).

Our within-subjects ANOVAs of residual MR revealed three main findings (Table 1; Fig. 2). First, there was a lack of significant effects of nuclear genetic background, or of mitonuclear epistatic interactions, on MR. Second, and more interestingly, mtDNA haplotype significantly affected MR. This was most apparent when comparing haplotypes I with D (module A), where flies carrying haplotype I showed a markedly and significantly higher MR compared to those carrying haplotype D. Here, the difference in MR across haplotypes was approximately 13% in males and 23% in females. A similar pattern occurred when comparing haplotype II and D (module B) (Fig. 2), but this was not statistically significant (Table 1). Here, the difference in MR across haplotypes was approximately 18% in males but only 10% in females. Third, flies carrying haplotype I differed in MR from those carrying haplotype II, but this effect was contingent upon sex (module C): whereas males carrying haplotype I showed a 5% reduction in MR compared to haplotype II, females carrying haplotype I instead showed a 10% increase in MR compared to haplotype II (Fig. 2). The significant sex × mtDNA interaction seen (Table 1) provides evidence for sex specificity of mitochondrial genetic effects in *D. subobscura*.

Discussion

Several *in vitro* studies have shown that OXPHOS enzyme activity differs across mtDNA haplotypes, or is affected by mtDNA mutations, suggesting a causal link between mitochondrial genetic effects, metabolic rate

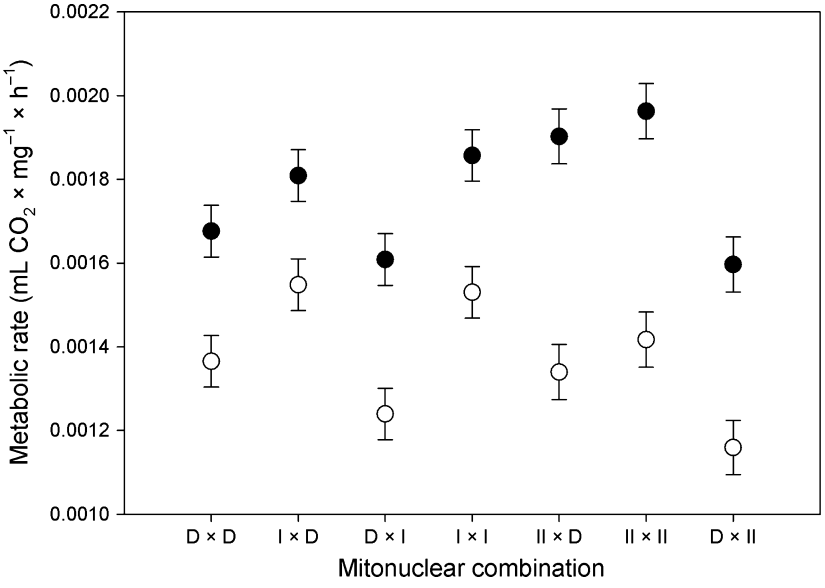

Fig. 2 Mean (± SE) metabolic rate in males (filled circles) and females (open circles) across all mitonuclear cross-types employed (denoted as mtDNA × nDNA). Given are mean amount of CO_2 produced per milligram per hour, after adjusting for the effects of activity (predicted mass-specific resting metabolic rate).

Table 1 Within-subjects analyses of variance of residual metabolic rate across mitonuclear introgression lines of *Drosophila subobscura*.

Module	Source	SS × 10^{-6}	d.f.	*F*	*P*
A					
Between subjects	nDNA	2.144	1	3.48	0.070
	mtDNA	2.582	1	4.19	**0.048**
	mtDNA × nDNA	1.984	1	3.22	0.081
	Error	22.186	36		
Within subjects	Sex	0.004	1	0.01	0.937
	Sex × nDNA	0.034	1	0.05	0.816
	Sex × mtDNA	0.590	1	0.96	0.334
	Sex × nDNA × mtDNA	0.710	1	1.15	0.289
	Error	22.151	36		
B					
Between subjects	nDNA	0.894	1	1.08	0.306
	mtDNA	0.967	1	1.16	0.288
	mtDNA × nDNA	0.585	1	0.70	0.407
	Error	29.880	36		
Within subjects	Sex	0.096	1	0.23	0.632
	Sex × nDNA	0.000	1	0.00	0.975
	Sex × mtDNA	0.168	1	0.41	0.526
	Sex × nDNA × mtDNA	0.000	1	0.00	0.995
	Error	14.751	36		
C					
Between subjects	mtDNA	0.856	1	0.01	0.330
	Error	15.342	18		
Within subjects	Sex	0.318	1	0.70	0.414
	Sex × mtDNA	2.534	1	5.58	**0.030**
	Error	8.172	18		

The significant values are in bold.

and life-history phenotypes (Ballard *et al.*, 2007). Yet, very few studies have assessed the effects of mtDNA variation on whole-organism metabolic rate and those that have compared allopatric mtDNA haplotypes (Tieleman *et al.*, 2009; Arnqvist *et al.*, 2010). To our knowledge, our study is the first to document differences in whole-organism metabolic rate across sympatric mtDNA haplotypes in any taxa. Our results have important implications for our understanding of (i) the phenotypic effects of mitochondrial genetic variation and (ii) the maintenance of mitochondrial genetic variation. We discuss both of these issues below.

Drosophila subobscura represents a very interesting model system for microevolutionary studies of mtDNA variation, both because of the extensive data sets on mtDNA variability collected in nature and because of the interesting pattern of mtDNA haplotype polymorphism (see Introduction). It is, however, premature to even try to associate phenotypic effects with particular mtDNA sequence variants: less than a handful of haplotypes have been studied in terms of their phenotypic effects and the mtDNA genome has only been partially sequenced (Beckenbach *et al.*, 1993; Moya *et al.*, 1993; Stenico & Nigro, 1998; Brehm *et al.*, 2004; Christie *et al.*, 2004; Castro *et al.*, 2010; Christie *et al.*, 2011; Herrig *et al.*, 2014). Although these efforts have revealed variation at a very large number of both synonymous and nonsynonymous sites in several genes, it is unclear which genetic variants are associated with phenotypic effects. The best-studied variants thus far are mtDNA haplotypes I and II, for which Christie *et al.* (2004, 2011) documented several differences in life-history phenotypes. To the extent that metabolism provides a link between mtDNA and upstream life-history traits (Ballard *et al.*, 2007; Arnqvist *et al.*, 2010), our study provides a causal explanation for these findings: because metabolic processes are ultimately the manner in which acquired resources are converted to fitness (Brown *et al.*, 2004), the documented effects of mtDNA haplotypes on metabolic rate in *D. subobscura* that we document here are likely to be reflected in differences in life-history traits.

Our results showed that the mtDNA effects were, to some extent, sex specific, such that the effects of mtDNA haplotype differed in males and females. Sex-specific genetic effects of mitonuclear variation are of particular interest, considering the fact that mtDNA is maternally transmitted and hence not under selection in males (Frank & Hurst, 1996). The lack of selection on mitochondrial genes in males can generate a male-specific genetic load, known as the mother's curse (Gemmell *et al.*, 2004). It has been predicted that one should thus observe larger phenotypic main effects of mtDNA variation in males compared to females (Camus *et al.*, 2012). However, this was apparently not the case in the current study, where variation in male and female MR across introgression lines was comparable (variance ratio test: $F_{6,6} = 1.05$, $P = 0.95$) (Fig. 2). We note that there is evidence that sex-specific selection in males on those nuclear metabolic genes that interact with mitochondrial genes may provide at least partial release from the mother's curse, for example by gene duplication and evolution of sex-specific expression of paralogs involved in metabolic pathways in males (Gallach *et al.*, 2010; Gallach & Betrán, 2011). It is therefore possible that the evolution of sex-specific expression of nuclear genes has nullified sex differences in the effects of mtDNA genetic variation on general metabolic processes. This is perhaps most likely in cases where particular mtDNA variants are maintained for extended periods in a polymorphic state, which seems to be the case for haplotype I and II in *D. subobscura* (Latorre *et al.*, 1992).

The differences we document in MR across sympatric mtDNA haplotypes in *D. subobscura* were sizeable, up to 20% or so, and previously reported differences in several fitness components across these mtDNA haplotypes are of similar magnitudes (Christie *et al.*, 2004, 2011). This implies that natural selection upon mtDNA haplotypes must, at least in some environments and at certain times, be strong. This, in turn, motivates the question as to what mechanisms might maintain mtDNA polymorphism in this species. This is particularly intriguing considering the fact that the mtDNA

haplotypes I and II studied here occur in roughly equal frequencies within most investigated Old and New World populations and dominate throughout the distributional range of *D. subobscura* (Latorre *et al.*, 1992; García-Martínez *et al.*, 1998). Although mitonuclear epistasis might help promote genetic variation in mtDNA within populations (Fos *et al.*, 1990; García-Martinez *et al.*, 1998; Castro *et al.*, 1999), theory shows that the conditions for this to be true are actually very restrictive (Clark, 1984; Rand *et al.*, 2001). Further, the absence of within-population mitonuclear epistasis seen in our current experiments is not consistent with this hypothesis, and the lack of stable linkage disequilibrium between nuclear and mitochondrial genes within populations (Jelic *et al.*, 2012a, b) is also not supportive. Instead, we suggest that negative frequency-dependent selection may act to maintain mtDNA polymorphism in *D. subobscura*. Negative frequency-dependent selection on mtDNA haplotypes would arise if, in essence, a given mtDNA haplotype is its own worst competitor (Lewontin, 1974; Maynard Smith, 1982). This scenario has been suggested to maintain mtDNA polymorphism in other insects (Kazancioglu & Arnqvist, 2014) where different mtDNA haplotypes encode for different metabolic syndromes (Dowling *et al.*, 2007; Arnqvist *et al.*, 2010). Negative frequency-dependent selection would then promote mtDNA polymorphism, much like it can act to maintain individual variation in 'slow' and 'fast' lifestyles (Wolf & McNamara, 2012; Løvlie *et al.*, 2014). Three facts provide support for this hypothesis in *D. subobscura*. First, the observation that haplotypes I and II occur at roughly equal frequencies over a wide distributional range, over which both the environment and the nuclear genome show much variation (Latorre *et al.*, 1992; Huey *et al.*, 2000; Noor *et al.*, 2000), is compatible with widespread negative frequency-dependent selection. Second, a recent study provided unequivocal experimental evidence for negative frequency-dependent selection on mtDNA haplotypes within populations in another insect species (Kazancioglu & Arnqvist, 2014). Third, the microevolutionary frequency dynamics of haplotype I and II in replicated laboratory populations of *D. subobscura*, reported by Oliver *et al.* (2005), seemingly match those expected under negative frequency-dependent selection with an equilibrium frequency of about 50% of each haplotype.

In conclusion, our study adds to a recent body of experimental research documenting functional differences between mtDNA haplotypes. Importantly, we show that within-population variation in mtDNA haplotypes in *D. subobscura* is associated with sizeable and partly sex-specific differences in whole-organism metabolic rate, a key life-history trait. We suggest that future efforts in this system should focus on identifying the selective processes that maintain sympatric mtDNA variation.

Acknowledgments

We thank to Bojan Kenig and Marija Tanaskovic for assistance during the construction of introgression lines. David Berger and Johanna Rönn assisted with the respirometry. This study was funded by the Ministry of Education Science and Technological Development (No. 173012 to ZKN, MJ, MSR and MA), European Science Foundation (Exchange grant, LESC FroSpects No. 4008 to ZKN), the European Research Council (GENCON AdG-294333 to GA) and the Swedish Research Council (621-2010-5266 to GA).

References

Arnqvist, G., Dowling, D.K., Eady, P., Gay, L., Tregenza, T., Tuda, M. *et al.* 2010. Genetic architecture of metabolic rate: environment specific epistasis between mitochondrial and nuclear genes in an insect. *Evolution* **64**: 3354–3363.

Babcock, C.S. & Asmussen, M.A. 1996. Effects of differential selection in the sexes on cytonuclear polymorphism and disequilibria. *Genetics* **144**: 839–853.

Balanyà, J., Huey, R.B., Gilchrist, G.W. & Serra, L. 2009. The chromosomal polymorphism of *Drosophila subobscura*: a microevolutionary weapon to monitor global change. *Heredity* **103**: 364–367.

Ballard, J.W.O. & Kreitman, M. 1995. Is mitochondrial DNA a strictly neutral marker? *Trends Ecol. Evol.* **10**: 485–488.

Ballard, J.W.O. & Rand, D.M. 2005. The population biology of mitochondrial DNA and its phylogenetic implications. *Annu. Rev. Ecol. Evol. Syst.* **36**: 621–642.

Ballard, J.W.O. & Whitlock, M.C. 2004. The incomplete natural history of mitochondria. *Mol. Ecol.* **13**: 729–744.

Ballard, J.W.O., Melvin, R.G., Katewa, S.D. & Maas, K. 2007. Mitochondrial DNA variation is associated with measurable differences in life-history traits and mitochondrial metabolism in *Drosophila simulans*. *Evolution* **61**: 1735–1747.

Bazin, E., Glemin, S. & Galtier, N. 2006. Population size does not influence mitochondrial genetic diversity in animals. *Science* **312**: 570–572.

Beckenbach, A.T., Wei, Y.W. & Liu, H. 1993. Relationships in the *Drosophila obscura* species group, inferred from mitochondrial cytochrome oxidase II sequences. *Mol. Biol. Evol.* **10**: 619–634.

Breen, M.S., Kemena, C., Vlasov, P.K., Notredame, C. & Kondrashov, F.A. 2012. Epistasis as the primary factor in molecular evolution. *Nature* **490**: 535–538.

Brehm, A., Harris, D.J., Hernández, M., Perez, J.A., Larruga, J.M., Pinto, F.M. *et al.* 2004. Phylogeography of *Drosophila subobscura* from north Atlantic islands inferred from mtDNA A+T rich region sequences. *Mol. Phylogenet. Evol.* **30**: 829–834.

Brown, J.H., Gillooly, J.F., Allen, A.P., Savage, V.M. & West, G.B. 2004. Towards a metabolic theory of ecology. *Ecology* **85**: 1771–1789.

Camus, M.F., David, J.C. & Dowling, D.K. 2012. Mitochondria, Maternal Inheritance, and Male Aging. *Curr. Biol.* **22**: 1717–1721.

Castro, J.A., Ramon, M., Picornell, A. & Moya, A. 1999. The genetic structure of *Drosophila subobscura* populations form the islands of Majorca and Minorca (Balearic Islands, Spain)

based on allozymes and mitochondrial DNA. *Heredity* **83**: 271–279.

Castro, J.A., Oliver, P., Christie, J.S., Picornell, A., Ramon, M. & Moya, A. 2003. Assortative mating and fertility in two *Drosophila subobscura* strains with different mitochondrial DNA haplotypes. *Genetica* **119**: 295–301.

Castro, J.A., Barrio, E., González, A., Picornell, A., Ramon, M.M. & Moya, A. 2010. Nucleotide diversity of a ND5 fragment confirms that population expansion is the most suitable explanation for the mtDNA haplotype polymorphism of *Drosophila subobscura*. *Genetica* **138**: 819–829.

Christie, J.S., Castro, J.A., Oliver, P., Picornell, A., Ramon, M.M. & Moya, A. 2004. Fitness and life-history traits of the two major mitochondrial DNA haplotypes of *Drosophila subobscura*. *Heredity* **93**: 371–378.

Christie, J.S., Picornell, A., Moya, A., Ramon, M.M. & Castro, J.A. 2010. Dynamics of the mtDNA haplotype variability in a *Drosophila subobscura* population over a two-year period. *Open Evol. J.* **4**: 23–30.

Christie, J.S., Picornell, A., Moya, A., Ramon, M.M. & Castro, J.A. 2011. Mitochondrial DNA effects on fitness in *Drosophila subobscura*. *Heredity* **107**: 239–245.

Clark, A.G. 1984. Natural selection with nuclear and cytoplasmic transmission. 1. A deterministic model. *Genetics* **107**: 679–701.

Dowling, D.K., Abiega, K.C. & Arnqvist, G. 2007. Temperature-specific outcomes of cytoplasmic-nuclear interactions on egg-to-adult development time in seed beetles. *Evolution* **61**: 194–201.

Ellison, C.K. & Burton, R.S. 2006. Disruption of mitochondrial function in interpopulation hybrids of *Tigriopus californicus*. *Evolution* **60**: 1382–1391.

Fos, M., Dominguez, M.A., Latorre, A. & Moya, A. 1990. Mitochondrial DNA evolution in experimental populations of *Drosophila subobscura*. *Proc. Natl. Acad. Sci.* **87**: 4198–4201.

Frank, S.A. & Hurst, L.D. 1996. Mitochondria and male disease. *Nature* **383**: 224.

Frankham, R. 2012. How closely does genetic diversity in finite populations conform to predictions of neutral theory? Large deficits in regions of low recombination. *Heredity* **108**: 167–178.

Gallach, M. & Betrán, E. 2011. Intralocus sexual conflict resolved through gene duplication. *Trends Ecol. Evol.* **26**: 222–228.

Gallach, M., Chandrasekaran, C. & Betrán, E. 2010. Analyses of nuclearly encoded mitochondrial genes suggest gene duplication as a mechanism for resolving intralocus sexually antagonistic conflict in Drosophila. *Genome Biol. Evol.* **2**: 835–850.

García-Martinez, J., Castro, J.A., Ramón, M., Latorre, A. & Moya, A. 1998. Mitochondrial DNA haplotype frequency in natural and experimental populations of *Drosophila subobscura*. *Genetics* **149**: 1377–1382.

Gemmell, N.J., Metcalf, V.J. & Allendorf, F.W. 2004. Mother's curse: the effect of mtDNA on individual fitness and population viability. *Trends Ecol. Evol.* **19**: 238–244.

González, A., Carrió, R., Fernández-Pedrosa, V. & Moya, A. 1994. Lack of seasonal changes in mitochondrial DNA variability of a *Drosophila subobscura* population. *J. Evol. Biol.* **7**: 29–38.

Gregorius, H.R. & Ross, M.D. 1984. Selection with gene-cytoplasm interactions. I. Maintenance of cytoplasm polymorphisms. *Genetics* **107**: 165–178.

Herrig, D.K., Modrick, A.J., Brud, E. & Llopart, A. 2014. Introgression in the *Drosophila subobscura*. Madeirensis sister species: evidence of gene flow in nuclear genes despite mitochondrial differentiation. *Evolution* **68**: 705–719.

Huey, R.B., Gilchrist, Q.W., Carlson, M.L., Berrigan, D. & Serra, L. 2000. Rapid Evolution of a Geographic Cline in Size in an Introduced Fly. *Science* **287**: 308–309.

James, A.C. & Ballard, J.W.O. 2003. Mitochondrial genotype affects fitness in *Drosophila simulans*. *Genetics* **164**: 187–194.

Jelic, M., Castro, J.A., Kurbalija Novičic, Z., Kenig, B., Dimitrijevic, D., Savic Veselinovic, M. *et al.* 2012a. Absence of linkage disequilibria between chromosomal arrangements and mtDNA haplotypes in natural populations of *Drosophila subobscura* from the Balkan Peninsula. *Genome* **55**: 214–221.

Jelic, M., Kenig, B., Tanaskovic, M., Stamenković-Radak, M. & Andjelković, M. 2012b. Relationship between chromosomal and mitochondrial DNA variability of *Drosophila subobscura* population from the Lazar's River Canyon. *Genetika* **44**: 409–417.

Katewa, S.D. & Ballard, J.W.O. 2007. Sympatric *Drosophila simulans* flies with distinct mtDNA show age related differences in mitochondrial metabolism. *Insect Biochem. Mol. Biol.* **37**: 923–932.

Kazancioglu, E. & Arnqvist, G. 2014. The maintenance of mitochondrial genetic variation by negative frequency-dependent selection. *Ecol. Lett.* **17**: 22–27.

Latorre, A., Moya, A. & Ayala, F.J. 1986. Evolution of mitochondrial DNA in *Drosophila subobscura*. *Proc. Natl. Acad. Sci.* **83**: 8649–8653.

Latorre, A., Hernandez, C., Martinez, D., Castro, J.A., Ramon, M.M. & Moya, A. 1992. Population structure and mitochondrial DNA gene flow in Old World populations of *Drosophila subobscura*. *Heredity* **68**: 15–24.

Lewontin, R.C. 1974. *The genetic basis of evolutionary change*. Columbia University Press, New York, NY.

Lighton, J.R.B. 2008. *Measuring Metabolic Rates: A Manual for Scientists*. Oxford University Press, USA.

Liu, R. & Asmussen, M.A. 2007. Cytonuclear dynamics in selfing populations under selection. *Theor. Popul. Biol.* **71**: 445–453.

Løvlie, H., Immonen, E., Gustavsson, E., Kazancioglu, E. & Arnqvist, G. 2014. The influence of mitonuclear genetic variation on personality in seed beetles. *Proc. R. Soc. B* **281**: 20141039.

Macrae, A.F. & Anderson, W.W. 1988. Evidence for non-neutrality of mitochondrial DNA haplotypes in *Drosophila pseudoobscura*. *Genetics* **120**: 485–494.

Maklakov, A.A., Friberg, U., Dowling, D. & Arnqvist, G. 2006. Within-population variation in cytoplasmic genes affects female lifespan and ageing in *Drosophila melanogaster*. *Evolution* **60**: 2081–2086.

Maynard Smith, J. 1982. *Evolution and the theory of games*. Cambridge University Press, Cambridge.

Meiklejohn, C.D., Montooth, K.L. & Rand, D.M. 2007. Positive and negative selection on the mitochondrial genome. *Trends Genet.* **23**: 259–263.

Montooth, K.L., Marden, J.H. & Clark, A.G. 2003. Mapping determinants of variation in energy metabolism, respiration and flight in Drosophila. *Genetics* **165**: 623–635.

Moritz, C.T.E., Dowling, D.K. & Brown, W.M. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annu. Rev. Ecol. Syst.* **18**: 269–292.

Moya, A., Barrio, E., Martinez, D., Latorre, A., González-Candelas, F., Ramon, M. *et al.* 1993. Molecular characterization and cytonuclear disequilibria of two *Drosophila subobscura* mitochondrial haplotypes. *Genome* **36**: 890–898.

Nachman, M.W., Brown, W.M., Stoneking, M. & Aquadro, C.F. 1996. Nonneutral mitochondrial DNA variation in humans and chimpanzees. *Genetics* **142**: 953–963.

Oliver, P., Castro, J.A., Picornell, A., Ramon, M.M., Solé, E., Balanyà, J. *et al.* 2002. Linkage disequilibria between mtDNA haplotypes and chromosomal arrangements in a natural population of *Drosophila subobscura*. *Heredity* **89**: 133–138.

Oliver, P., Balanyà, J., Ramon, M.M., Picornell, A., Serra, L., Moya, A. *et al.* 2005. Population dynamics of the 2 major mitochondrial DNA haplotypes in experimental populations of *Drosophila subobscura*. *Genome* **48**: 1010–1018.

O'Neill, S.L., Giordano, R., Colbert, A.M., Karr, T.L. & Robertson, H.M. 1992. 16SrRNA phylogenetic analysis of the bacterial endosymbiont associated with cytoplasmatic incompatibility in insects. *Proc. Natl. Acad. Sci. USA* **89**: 2699–2702.

Noor, M.A.F., Pascual, M. & Smith, K.R. 2000. Genetic variation in the spread of *Drosophila subobscura* from a nonequilibrium population. *Evolution* **54**: 696–703.

Pichaud, N., Ballard, J.W., Tanguay, R.M. & Blier, P.U. 2012. Naturally occurring mitochondrial DNA haplotypes exhibit metabolic differences: insight into functional properties of mitochondria. *Evolution* **66**: 3189–3197.

Pinto, F.M., Brehm, A., Hernandez, M., Larruga, J., González, A.M. & Cabrera, V.M. 1997. Population genetic structure and colonization sequence of *Drosophila subobscura* in the Canaries and Madeira Atlantic islands as inferred by autosomal, sex-linked and mtDNA traits. *J. Hered.* **88**: 108–114.

Rand, D.M. 2001. The units of selection on mitochondrial DNA. *Annu. Rev. Ecol. Syst.* **32**: 415–448.

Rand, D.M., Clark, A.G. & Kann, L.M. 2001. Sexually antagonistic cytonuclear fitness interactions in *Drosophila melanogaster*. *Genetics* **159**: 173–187.

Rand, D.M., Haney, R.A. & Fry, A.J. 2004. Cytonuclear coevolution: the genomics of cooperation. *Trends Ecol. Evol.* **19**: 645–653.

Rand, D.M., Fry, A. & Sheldahl, L. 2006. Nuclear-mitochondrial epistasis and drosophila aging: introgression of *Drosophila simulans* mtDNA modifies longevity in *D. melanogaster* nuclear backgrounds. *Genetics* **172**: 329–341.

Stenico, M. & Nigro, L. 1998. Differences in evolutionary rates among amino acid classes in the mitochondrial genes cytochrome oxidase I and cytochrome oxidase II in Drosophila. *Mol. Biol. Evol.* **15**: 777–778.

Terblanche, J.S. & Chown, S.L. 2007. The effects of temperature, body mass and feeding on metabolic rate in the tsetse fly *Glossina morsitans centralis*. *Physiol. Entomol.* **32**: 175–180.

Tieleman, B.I., Versteegh, M.A., Fries, A., Helm, B., Dingemanse, N.J., Gibbs, H.L. *et al.* 2009. Genetic modulation of energy metabolism in birds through mitochondrial function. *Proc. R. Soc. B* **276**: 1685–1693.

Wolf, M. & McNamara, J.M. 2012. On the evolution of personalities via frequency-dependent selection. *Am. Nat.* **179**: 679–692.

Received 4 November 2014; revised 25 November 2014; accepted 26 November 2014