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GENETIC ARCHITECTURE OF METABOLIC RATE: ENVIRONMENT SPECIFIC EPISTASIS BETWEEN MITOCHONDRIAL AND NUCLEAR GENES IN AN INSECT

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The extent to which mitochondrial DNA (mtDNA) variation is involved in adaptive evolutionary change is currently being reevaluated. In particular, emerging evidence suggests that mtDNA genes coevolve with the nuclear genes with which they interact to form the energy producing enzyme complexes in the mitochondria. This suggests that intergenomic epistasis between mitochondrial and nuclear genes may affect whole-organism metabolic phenotypes. Here, we use crossed combinations of mitochondrial and nuclear lineages of the seed beetle *Callosobruchus maculatus* and assay metabolic rate under two different temperature regimes. Metabolic rate was affected by an interaction between the mitochondrial and nuclear lineages and the temperature regime. Sequence data suggests that mitochondrial genetic variation has a role in determining the outcome of this interaction. Our genetic dissection of metabolic rate reveals a high level of complexity, encompassing genetic interactions over two genomes, and genotype × genotype × environment interactions. The evolutionary implications of these results are twofold. First, because metabolic rate is at the root of life histories, our results provide insights into the complexity of life-history evolution in general, and thermal adaptation in particular. Second, our results suggest a mechanism that could contribute to the maintenance of nonneutral mtDNA polymorphism.

KEY WORDS: Epistasis, life-history evolution, metabolism, mtDNA, polymorphism, thermal adaptation.

Our understanding of the evolutionary significance of mitochondrial DNA (mtDNA) has recently been fundamentally revised. The traditional view that sequence variation found within the mitochondrial genome is neutral has been challenged by the idea that nonneutral polymorphisms can be readily maintained within the genome by selection (Ballard and Whitlock 2004; Rand et al. 2004; Ballard and Rand 2005; Dowling et al. 2008). Evidence for selection on mitochondrial genes now comes from a variety of sources, ranging from large scale comparative genomic approaches (Bazin et al. 2006) and screening of mtDNA sequences for the hallmarks of selection (Ballard and Kreitman 1994; Meiklejohn et al. 2007) to studies examining enzyme

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function within the mitochondria (Sackton et al. 2003; Ellison and Burton 2006; Ballard et al. 2007) and studies attributing phenotypic variation in life-history traits or fitness to naturally occurring and distinct mtDNA haplotypes (James and Ballard 2003; Dowling et al. 2007a). Selection is especially likely to affect mtDNA via cyto-nuclear epistatic interactions and coadaptation (Rand et al. 2004; Dowling et al. 2008).

We see two particularly important facets of this view. First, it is generally difficult to understand how nonneutral genetic variation in this maternally inherited and nonrecombining haploid genome can be maintained in the face of natural selection. In theory, intergenomic epistatic mitochondrial-nuclear (mitonuclear) interactions can act to maintain mtDNA polymorphism (Rand 2001), but only if the mitonuclear combinations are under negatively frequency-dependent selection (Gregorius and Ross 1984), sexually antagonistic selection (Rand et al. 2001), and/or possibly if they are involved in genotype × environment interactions (Rawson and Burton 2002; Ballard and Whitlock 2004; Dowling et al. 2007a,b). Second, because both mitochondrial and nuclear genes interact closely to form the multisubunit enzyme complexes responsible for energy (i.e., ATP) production in eukaryotes, the possibility exists that the mitochondrial genome will play an important role in life-history evolution (Ballard and Whitlock 2004). That is, if genetic variance in mtDNA is an important determinant of variation in metabolic processes, this will likely have ramifications downstream for the adaptive evolution of basic life-history traits. As such, the peculiarities of the mitochondrial genome (e.g., maternal transmission and very limited, if any, recombination) stand to alter the evolutionary dynamics of such life-history traits (Gemmell et al. 2004; Wade and Brandvain 2009). Although our understanding of the genetic architecture of variation in energy metabolism is incomplete, it is clear that both mitochondrial (King and Attardi 1989; Martin 1995; Ballard and Rand 2005) and nuclear genes (Montooth et al. 2003; Nespolo et al. 2007) may be involved in determining metabolic rate. Thus, epistatic interactions between nuclear and mitochondrial genes are likely to be central to our emerging understanding both of the maintenance of genetic variation in mitochondrial genes (Clark 1984; Rand et al. 2001) and of the effects of selection on life-history traits that are affected by mitochondrial genes (Rand et al. 2004).

Cytonuclear epistasis has now been established in several taxa (Dowling et al. 2008), by demonstrations of phenotypic differences across particular mtDNA × nuclear genetic combinations. Here, crossed expression of distinct mtDNA haplotypes in different nuclear genetic backgrounds has been successfully employed to quantify mitonuclear interactions for key phenotypic traits such as mitochondrial function (Ellison and Burton 2006) and fitness (Rand et al. 2001; James and Ballard 2003; Dowling et al. 2007a). Yet, the fact that mitochondrial function is environment specific, in particular with regard to temperature (Pörtner et al. 2007), suggests that mitonuclear interactions will not accurately describe the full complexity of the phenotypic effects of genetic variation in mitochondrial genes. It is likely that more complex three-way environment × nuclear × mtDNA interactions are important. In accordance with this, two previous studies have revealed sizeable environmental effects on epistatic interactions between mitochondrial and nuclear genes for development (Dowling et al. 2007b) and viability (Willett and Burton 2003). Although these results suggest that mitonuclear effects on metabolic rate may be influenced by the environment, this has never been demonstrated. Here, we assess this possibility by first using repeated introgressive backcrossing to create fully crossed combinations of distinct cytoplasmic genetic types (cytotypes) and nuclear genotypes in the seed beetle *Callosobruchus maculatus* (Bruchidae). We then assay whole-organism metabolic rate in all cytonuclear lines at two different temperatures using flow-through respirometry. Previous signs of selection on mtDNA in seed beetles come from studies using introgressive backcrossing in the laboratory (Dowling et al. 2007b,c,d) but also from findings of nonrandom distribution of mtDNA haplotypes in the field (Tuda et al. 2004).

Methods

CYTONUCLEAR LINES

For a full description of the methods used to construct the cytonuclear introgression lines used, we refer to Dowling et al. (2007b,c,d). Briefly, outbred stocks of five *C. maculatus* populations were used to generate 25 lines fixed for fully crossed combinations of distinct cytoplasmic and nuclear lineages (Brazil [BR], California [CA], Yemen [YE], Lossa [LO], and Oyo [OY]). A single virgin female from each of the five stocks was first mated to a male from the same stock. These five females were effectively mitochondrial "Eves." Groups of full-sib virgin daughters were subsequently placed with males from one of the five stock populations, in each of the 25 possible crossed combinations. In each subsequent generation, virgin females from each of the 25 lines were backcrossed to outbred males from the same stock population as their fathers. This repeated backcrossing was used to disassociate each of the sampled cytoplasmic genomes from the nuclear genome with which it was originally associated, replacing it with a new complement of nuclear genes (derived from one of the five stocks). In theory, after 15 generations of backcrossing, >99.9% of the original nuclear genome of each line had been replaced (assuming no strong selection for the maintenance of particular cytonuclear combinations within lines during introgression [James and Ballard 2003]), resulting in each of the cytoplasmic genomes being expressed in five distinct and controlled nuclear backgrounds. Differential infection with cytoplasmatically inherited bacteria, such as *Wolbachia*, may confound genetic effects in

this design. Although many *C. maculatus* populations (including those used here) have been screened for *Wolbachia* using diagnostic PCR, they have not been detected in *C. maculatus* (Tuda et al. 2006). We nevertheless treated all lines with tetracycline hydrochloride at generation 9 to eliminate any maternally inherited bacterial infections potentially present. Following generation 15, cytonuclear lines were maintained as separate populations on black-eyed beans, *Vigna unguiculata*, at large population sizes (>100 individuals) at 30°C, 50% relative humidity (RH), without food or water, on a 12:12 h light : dark cycle and a 26- to 28-day discrete generation cycle. To avoid the possibility of line specific adaptation, backcrossing to the outbred stock populations was again conducted in generations 18 and 29, 39–42, 47, and in two consecutive generations immediately preceding the respirometry assays described subsequently (i.e., generations 53 and 54).

METABOLIC RATE

Because activity among adult beetles precludes accurate measures of resting or standard metabolic rate, we measured metabolic rates using beetle pupae (which are entirely immobile). Pupae reared at 30°C were carefully extracted from beans approximately 20 days after egg laying (mean age of pupae = 20.16 days, range 17–24 days) and weighed to the nearest milligram using a microbalance (Mettler Toledo UMX2). Standard metabolic rate (SMR) of the pupae was then measured as CO_2 production (Terblanche and Chown 2007) using flow-through respirometry, a method that provides precise and dynamic measures of whole organism metabolic rate (Lighton 2008). Briefly, a LiCor LI-7000 infra-red gas analyzer (Lincoln, Nebraska, USA) was attached to a Sable Systems (Las Vegas, NV) RM8 eight-channel multiplexer housed inside a Sanyo MIR 553 incubator temperature controlled at either 23 or 30°C. Compressed zero air (a 21%O_2, 79%N_2 mix) was scrubbed of any residual CO_2 and water (with ascarite and drierite, respectively) and fed through the multiplexer and into 125 mL cuvettes housing the beetle pupae at 100 mL/min (controlled at ±1% with a mass-flow control meter: Sierra Instruments, Monterey, CA). The multiplexer regulates the flow to individual pupae, and one channel was used as a blank for initial and final base-line readings to control for drift. The mass-flow meter regulated airflow that was switched sequentially through each chamber for 20 m at a time, with sampling every second. A thermistor probe was placed next to the cuvettes in the incubator to monitor the thermal environment. CO_2 production and temperature data were acquired at 1 Hz via a Sable Systems UI2 analog-digital interface connected to a PC running Sable Systems Expedata software. All measures were made during the day under weak florescent light. After respirometry, pupae were returned to their standard housing conditions and were checked daily for their final metamorphosis. This was recorded, as was the sex of all pupae reaching the adult stage. We note, however, that hatching success of pupae was quite low when removed from their natal bean. Expedata was used (blind to beetle identity) to calculate metabolic rate. Only data from the last 10 m of each recording were used to allow for CO_2 washout. We chose the most stable part of the recording (minimum 300 readings) (Chown and Nicolson 2004) to calculate mean CO_2 production per pupa. Due to logistic constraints, replicates were performed during generations 55–57. To account for nonfocal variation in metabolic rate across the three sets of replicates, generation was included as a "blocking" factor in all subsequent analyses. Our design was fully crossed, such that data for each line and temperature regime was recorded for at least one individual in each generation. In total, data on metabolic rate was secured for 159 individual pupae.

GENETIC VARIATION ACROSS CYTOTYPES

We sequenced 1005 bp of cytochrome oxidase subunit I (COI) and 473 bp of cytochrome b (Cyt-b) (see Simon et al. [1994], Kergoat et al. [2004], and Tuda et al. [2006] for a description of primers and PCR conditions) from several (≥3) individuals per line and used DnaSP version 5.10.00 (Librado and Rozas 2009) and Phylip version 3.68 (Felsenstein 2005) for analysis of our sequence data. These mitochondrial genes are main subunits of the enzyme complexes III and IV in the respiratory electron transport chain of mitochondria, which is located in the mitochondrial membrane. The goal with our sequencing effort was twofold. First, we wished to determine whether the mtDNA haplotypes of our five cytoplasmic lineages were indeed distinct. Second, we wished to ordinate haplotypes according to relative sequence divergence. Because mtDNA is maternally inherited, haploid, and generally nonrecombining, selection should be less efficient at removing even deleterious mutations in this genome compared to the nuclear genome (Lynch 1997), and mitochondrial haplotypes will accumulate both synonymous and nonsynonymous substitutions over time. Nonsynonymous and synonymous substitution rates are typically correlated across species in mitochondrial (e.g., Nabholz et al. 2008; Montooth et al. 2009), and also in nuclear (e.g., Alvarez-Valin et al. 1998; Comeron and Kreitman 1998), genomes. Several studies show that this is the case also across mitochondrial haplotypes within species (e.g., Ballard 2000; Bachtrog et al. 2006; Burton et al. 2007; Howell et al. 2007). Therefore, total sequence divergence in the sequenced genes should, to some extent, predict mitochondrial genome wide divergence at nonsynonymous sites across haplotypes and we use sequence divergence as an approximate index of mtDNA haplotype wide genetic divergence.

Results

Variance in metabolic rate was analyzed in a mixed model analysis of covariance (type III SS), treating nuclear background,

Table 1. Mixed model analysis of covariance of variation in metabolic rate in *C. maculatus*.

Source	SS	*df*	*F*	*P*
Temperature	592.53	1	154.18	0.013
Nuclear genotype	17.52	4	1.14	0.505
Cytotype	2.43	4	0.22	0.905
Generation[1]	43.11	2	10.23	<0.001
Nuclear genotype × Cytotype	55.40	16	0.84	0.632
Temperature × Nuclear genotype	18.04	4	1.11	0.387
Temperature × Cytotype	13.73	4	0.84	0.522
Temperature × Nuclear genotype × Cytotype	65.68	16	1.95	0.023
Pupal weight[1]	148.35	1	70.39	<0.001
Residual	223.41	106		

[1]Interactions involving generation ($F_{66,40} = 0.97$, $P = 0.556$) and pupal weight ($F_{11,95} = 0.59$, $P = 0.835$) did not improve model fit to data and so were not included in the inferential model.

cytotype, and generation as random effects factors, temperature as a fixed effect factor, and pupal weight as a continuous covariate. The residual distribution of this main inferential model did not differ from normality (Kolmogorov–Smirnov test, $P = 0.509$) and variance in metabolic rate was homogeneous across factor levels (Bartlett's test for homogeneity of variance: $P > 0.264$ for all factors). Overall metabolic rate differed across generations (Table 1), but inspection of interaction plots showed that the effects of other factors on metabolic rate did not vary markedly across generations. Further, male and female pupae did not differ significantly in metabolic rate ($F_{1,54} = 0.04$, $P = 0.839$) and the age of the pupa had no significant effect on metabolic rate ($F_{1,105} = 0.10$, $P = 0.756$), as tested by the addition of these variables to our inferential model.

As expected, metabolic rate increased with pupal weight and was much higher at 30°C than at 23°C (Table 1, Fig. 1). There were no overall differences in metabolic rate across nuclear genotypes or cytotypes (Table 1, Fig. 2). However, the phenotypic effects of cytotype were contingent upon the nuclear genetic background in which it was expressed but the pattern of this epistatic interaction was temperature dependent (Fig. 2), as revealed by the significant temperature × nuclear genotype × cytotype interaction (Table 1). When restricting the analysis to either of the two temperatures, the nuclear genotype × cytotype interaction was significant at 30°C ($F_{16,52} = 2.03$, $P = 0.028$) but not at 23°C ($F_{16,51} = 1.50$, $P = 0.135$). A closer inspection of the three-way interaction (Fig. 2) generated two insights. First, despite the nonsignificant nuclear genotype × cytotype interaction at 23°C, the three-way interaction did not seem to be due to a nuclear genotype × cytotype interaction simply being present at 30°C and completely absent at 23°C. In contrast, there was extensive crossing-over of reaction norms: cytonuclear types that showed a relatively high metabolic rate at one temperature frequently showed a relatively low metabolic rate at the other temperature, and vice versa (Fig. 1B). Hence, epistatic effects in one environment were very different from those in the other. Second, this effect was not due to introgressed lines (i.e., those with disrupted cytonuclear combinations) being different from those with reconstituted cytonuclear combinations (i.e., those in which the cytoplasmic lineage had potentially coevolved with its nuclear genetic background [e.g., BR mtDNA within a BR nuclear background]). The metabolic rates of the five reconstituted lines were all nested within the other lines, with the associated performance of these reconstituted lines seemingly dispersed randomly throughout the interaction plot (see Fig. 1) (see also Dowling et al. 2007b). That introgression per se had no dramatic effect on metabolic rate under the thermal regimes used was confirmed in a mixed model analysis of covariance, including generation as a random effects factor, temperature and introgression (introgressed or reconstituted) as fixed effect factors, their interactions and finally pupal weight as a continuous covariate (test for the effect of introgression: $F_{1,146} = 2.15$, $P = 0.274$).

We found substantial genetic variation in mtDNA across the five cytotypes. In the sequenced segment of COI, 28 sites were polymorphic across cytotypes (2.8%) and 6 sites were polymorphic in Cyt-b (1.3%). None of the five cytotypes shared an identical mtDNA sequence (i.e., the five cytotypes had distinct mtDNA haplotypes). In COI, one of the 335 amino acids encoded by the sequenced segment was polymorphic (valine in California and Oyo and isoleucine in the other cytotypes) and one of 157 was polymorphic in Cyt-b (serine in Lossa and Yemen and asparagine in the other cytotypes). Nucleotide diversity, π, across the five haplotypes was 0.015 for COI and 0.007 for Cyt-b, which is comparable to estimates of within species nucleotide diversity in these genes in other insects (for COI, for example, *Drosophila simulans* [$\pi = 0.02$; Ballard 2000], *Aglais urticae* [$\pi = 0.01$; Vandewoestijne et al. 2004] and *Melitaea cinxia* [$\pi = 0.03$; Wahlberg and Saccheri 2007]).

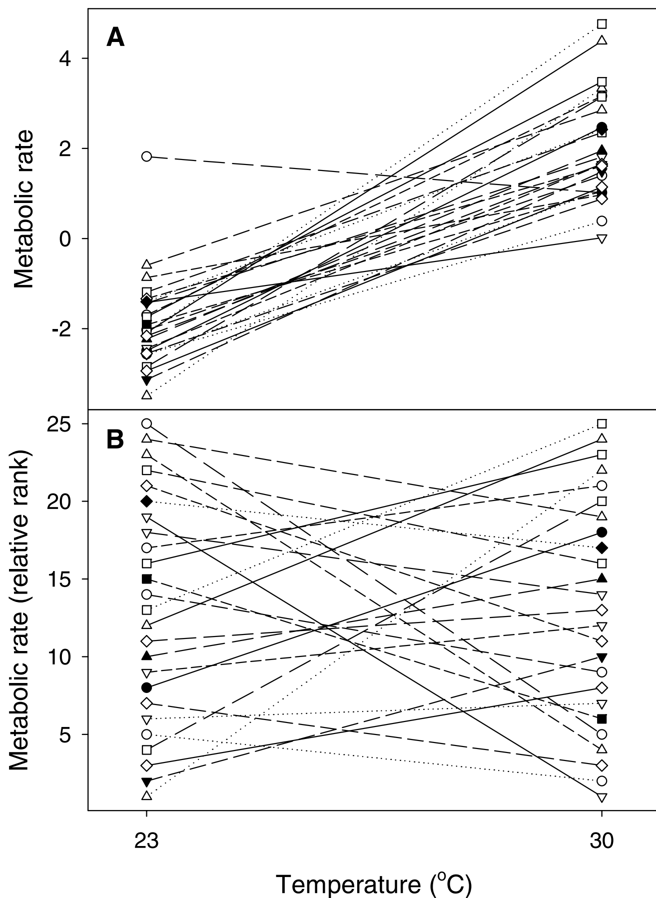

Figure 1. Metabolic rate at two temperatures across the 25 different cytonuclear lines. Here, cytoplasmic types are grouped by different lines and nuclear backgrounds are grouped by different symbols. The five reconstituted combinations in which each cytoplasmic type is expressed in its original nuclear genetic background are indicated by filled symbols. Panels show (A) mean residual standard metabolic rate (mL CO^2/min × 10^2), accounting for variation due to pupal weight and generation, and (B) the rank of residual metabolic rate within each temperature regime exhibited by the 25 cytonuclear lines.

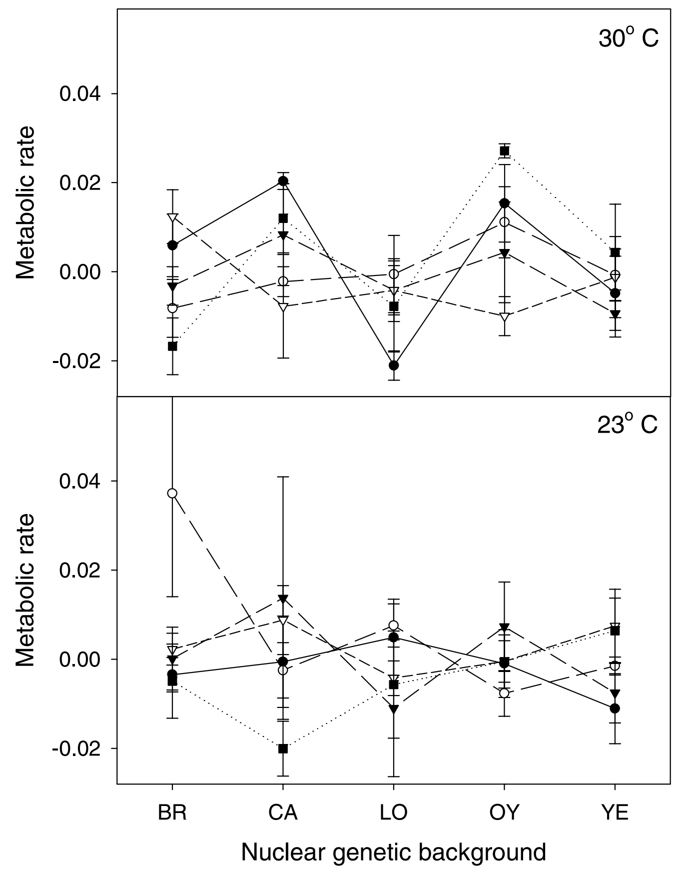

Figure 2. Metabolic rate for all cytonuclear introgression lines across the two temperatures: 30°C (top panel) and 23°C (bottom panel). Nuclear types are given along the abscissa and cytotypes are denoted by different symbols and lines (BR: filled circles and solid lines; CA: open circles and long dashed lines; LO: filled triangles and medium dashed lines; OY: open triangles and short dashed lines; YE: filled squares and dotted lines). Given is mean residual standard metabolic rate (mL CO^2/min × 10^2) (±SE) for each cytotype, accounting for variation due to pupal weight, temperature regime and generation. Note the difference in the pattern of cytonuclear interaction in the two temperature regimes.

To assess whether the complex cyto-nuclear effects on metabolic rate seen above were potentially related to mtDNA haplotype, we tested for covariation between the relevant metabolic phenotype and mtDNA sequence divergence. The genetic distances across the five haplotypes for COI were closely correlated with those for Cyt-b (LogDet genetic distance [Swofford et al. 1996] matrix correlation $r = 0.81$, Mantel test based on 10,000 iterations $P = 0.033$). Because the two genes thus show a very similar pattern of divergence across haplotypes we pooled all sequence data in the analyses described below. In face of the analytical constraints that result from the restricted number of haplotypes, we used the following analytical path. First, sequence data at the 34 polymorphic sites was transformed into binary code, and the dimensionality of the 34 × 5 matrix describing genetic variation was reduced by means of a multiple correspondence analysis. This yielded two derived variables with a corrected eigenvalue larger than 1/K, where K is the number of variables in the original matrix (Greenacre 1993), the first of which (MCA1) explained 66.2% and the second (MCA2) 27.7% of genetic variance across the mtDNA haplotypes. Second, to describe variation in the pattern of the nuclear genotype × temperature interaction across cytotypes, we first constructed a 5 × 5 matrix (in which cytotypes were rows and nuclear type columns) of the mean metabolic rate at 30° minus the mean metabolic rate at 23° for each of the 25 cytonuclear types. We then reduced the dimensionality of this matrix by means of principal component analysis based on the covariance matrix. We retained two derived variables (Jackson 1993), which explained 55% (PC1) and 38% (PC2) of the variance in the

pattern of nuclear genotype × temperature interactions across the five cytotypes. Third, we correlated the two composite measures of mtDNA variation with the two composite measures of phenotypic variation, using canonical correlation analysis. This analysis revealed a tight correlation along the first covariance dimension ($\rho = 0.996$), although this was marginally nonsignificant ($\chi^2_4 = 8.65$, $P = 0.070$) due to the low sample size ($n = 5$). This covariation was caused by a significant correlation between MCA1 and PC2 ($r = 0.93$, $P = 0.039$) and a marginally nonsignificant correlation between MCA2 and PC1 ($r = 0.82$, $P = 0.093$) (using FDR compensation for multiple tests [Storey 2002]). Thus, mtDNA haplotypes that were genetically more distinct were also more dissimilar in terms of their effect on temperature × nuclear interactions for metabolic phenotype. We interpret these analyses as supporting the hypothesis that mtDNA has a causal role in determining metabolic rate at different temperatures and in different nuclear genetic backgrounds.

Discussion

Our results reveal complex genetic effects involving the cytoplasmic haplotype on metabolic phenotypes. Not only do cytoplasmic genetic effects depend on the nuclear genetic background (Rand et al. 2001; James and Ballard 2003; Ellison and Burton 2006), but the pattern of this epistatic interaction varies across environments. Our results strongly suggest that these cytoplasmic effects were due to mitochondrial genetic effects. Below, we restrict our discussion to three implications of our results. First, we suggest that the type of nontrivial and nonadditive genetic variation in metabolic rate documented here might generally affect the evolvability of life-history traits. Second, complex epistasis × environment interactions may play a key role in the maintenance of genetic variation in mitochondrial genes. Third, our findings support the view that coadaptation between the nuclear and mitochondrial genomes may depend on the environment.

Characterizing higher order epistasis and epistasis × environment interactions is fundamental to our understanding of life-history evolution and the maintenance of genetic variation (e.g., Turelli and Barton 2004) and several recent studies have investigated such interactions (e.g., Bergland et al. 2008). Metabolism must lie at the heart of life-history evolution, simply because at some level fitness is built by conversion of resources acquired from the environment into offspring. Although the link between metabolism and life-history traits may be complex (Hayes et al. 1992; Dowling and Simmons 2009), the rate or efficiency with which energy conversion occurs is demonstrably related to the evolution of basic life-history traits such as body size (Brown et al. 1993), brood size (White and Seymour 2004), and lifespan (Ricklefs 1998; Speakman et al. 2002). Yet, the genetic architecture of metabolic rate is not well understood (Mosseau and Roff 1987; Hoffman 2000) and direct data is limited. The level of additive genetic variance in metabolic rate is generally low among both vertebrates (Lacy and Lynch 1979; Dohm et al. 2001; Labocha et al. 2004; Nespolo et al. 2005; Ronning et al. 2007) and invertebrates (Roff and Sokolovska 2004; Nespolo et al. 2007; Ketola and Kotiaho 2009) although there are clearly some exceptions (Sadowska et al. 2005; Tieleman et al. 2009). In combination with the fact that repeatabilities of metabolic rate tend to be high (Nespolo and Franco 2007), this suggests that the genetic architecture of metabolic rate may generally be complex (Montooth et al. 2003; Roff and Sokolovska 2004; Nespolo 2007).

Our results suggest a potentially consequential explanation for the general pattern of genetic variation in metabolic rate: genetic variation in mitochondrial genes may commonly play a significant role in modulating metabolic phenotypes. At a mechanistic level, we suggest that this is reasonable considering the key role that several mitochondrial genes have in metabolic pathways (Martin 1995; Das 2006) although it is contingent upon the maintenance of genetic variation in mtDNA within populations (see subsequently). At least two previous observations support this scenario. First, studies of transmitochondrial cytoplasmic hybrids (cybrids), in which cell cultures are first depleted of mtDNA and then experimentally repopulated with exogenous mitochondria, have shown that in vitro metabolic rate depends not only on mtDNA haplotype but also on an interaction between nuclear and mitochondrial DNA (e.g., King and Attardi 1989; Chomyn et al. 1994). Second, studies of mitochondrial bioenergetics have revealed a role for mtDNA variation and interactions with nuclear genes in determining the rate of mitochondrial activity (Ellison and Burton 2006; Ballard et al. 2007). Our study suggests that genetic polymorphism across mtDNA haplotypes affects whole-organism metabolic rate, and that such effects are contingent upon the nuclear genetic background. Notably, our results show that the outcomes of these intergenomic epistatic interactions are environment specific, such that the relative metabolic rates of particular mitonuclear genetic combinations vary across temperature regimes. This added level of complexity, if common, would contribute to a complex genetic architecture (sensu Hansen 2006) of metabolic rate. We note that the number of mitochondria per cell (i.e., mitochondrial density), which also affects metabolic rates (e.g., Weibel et al. 2004), could potentially add a fourth and interactive genetic dimension to this complexity. We are, however, unaware of any studies exploring genetic variation in mitochondrial density although mitochondrial DNA copy number is heritable and has phenotypic effects in mammals (see Montier et al. 2009).

It is reasonable to expect that selection will ultimately purge (or fix) mtDNA haplotypes associated with low (or high) fitness in populations, and understanding how genetic variation can be maintained within the mitochondrial genome in the face of selection is therefore a challenge (Ballard and Whitlock 2004;

Dowling et al. 2008). Only a handful of studies, restricted to the *Drosophila* model system, have experimentally screened for, and documented, the presence of nonneutral mtDNA polymorphism within populations (Clark and Lyckegaard 1988; Rand et al. 2001; Dowling et al. 2007a). Such experimental results complement descriptive sequence data available for populations of other species. For example, natural populations of seed beetles may host up to nine distinct mtDNA haplotypes and at least some of these differ by nonsynonymous substitutions (Tuda et al. 2004). Theory suggests that epistasis generally promotes the maintenance of genetic variation (Brodie 2000; Turelli and Barton 2004; Hansen et al. 2006). This is true for mitonuclear interactions as well, but only under rather restrictive conditions (Rand et al. 2001). Although there is no theory explicitly addressing the effect of three-way mito × nuclear × environment interactions on the maintenance of mtDNA polymorphism, we suggest that such interactions may promote polymorphism for two principal reasons. First, because mtDNA may commonly be involved in thermal adaptation (see subsequently) and because temperature regimes vary both spatially and temporally in most taxa, selection on mtDNA might fluctuate spatially and temporally. Fluctuating selection will generally be less efficient in eroding genetic variation (Ellner and Hairstone 1994; Mitchell-Olds et al. 2007). Second, in theory, negatively frequency-dependent selection can act to maintain variation in mitochondrial genes (Gregorius and Ross 1984; Anderson et al. 2006), but it is not obvious how frequency-dependent selection on mtDNA could be generated. We suggest that presence of mitonuclear × thermal environment interactions generally makes frequency-dependent selection more likely. If microhabitats differ in temperature regimes or resource conditions, competition within microhabitats will generate frequency dependent selection at the population level (Haldane 1932; Wright 1969), on both mtDNA haplotypes and the nuclear genes that interact with mitochondrial genes, if the optimal mitonuclear combination differs across microhabitats. Under this scenario, fluctuating and frequency-dependent selection may act in concert to maintain sizeable levels of genetic variation in nonneutral mtDNA. Sex specific selection on mitonuclear combinations would add to this effect (Rand et al. 2001).

Evolutionary modulation of metabolic rate is clearly a key aspect of adaptation to different thermal regimes (Weathers 1979; Bozinovic and Rosenmann 1989; Hosken and Withers 1997; Rezende et al. 2004; Swanson and Garland 2009). Several authors have suggested that mitonuclear coevolution may be an important component of thermal adaptation (Dowling et al. 2008), but although there is some comparative (Mishmar et al. 2003; Dalziel et al. 2006) and experimental support for this tenet via studies showing thermal selection on mito- or mitonuclear types (Rawson and Burton 2002; Willett and Burton 2003; Dowling et al. 2007b), it remains controversial (Kivisild et al. 2006). Models of mitonuclear coadaptation typically envision mtDNA mutations being met by the spread of compensatory nuclear mutations (e.g., Rand et al. 2004) but it is very difficult to assess whether results such as those reported here are consistent with mitonuclear coadaptation or whether they reflect cytonuclear variation that cosegregates for other reasons (Rawson and Burton 2002; Willett and Burton 2003; Dowling et al. 2007b). To distinguish between these possibilities, detailed information on the net fitness effects of phenotypic variation is needed. In our case, for example, such inferences are precluded by the fact that the strength and form (i.e., stabilizing, disruptive or directional) of net phenotypic selection on metabolic rate in the two environments is unknown. In any case, because the presence of mitochondrial × nuclear × environment interactions affects the genetic architecture of metabolic rate it has implications for the ability of natural populations to respond adaptively to global climate change (Fangue et al. 2009). The net effect on the potential rate of thermal adaptation is, however, not intuitive and we are unaware of theory directly addressing this situation. We suggest that three related reasons collectively imply that these interactions may primarily hamper the rate of adaptation. First, theory predicts that the elevated nonadditive genetic variation of metabolic rate that would result from intergenomic epistatic variation and G × E interactions will slow down the phenotypic response to directional selection under many circumstances (Wade 2000; Hansen et al. 2006). Second, as argued above, mitonuclear × thermal environment interactions are likely to generate an element of fluctuating and/or frequency dependent selection on mitonuclear genotypes, which may also tend to decelerate the rate of phenotypic response to directional selection. Third, because mtDNA is generally expected to have a lower effective population size than autosomal nuclear DNA (but see Lynch 2007), we expect the substitution rate in loci under positive selection to be relatively low in mtDNA (Ballard and Whitlock 2004). However, we note that other effects may instead accelerate the rate of thermal adaptation. Most importantly, the fact that the mitochondrial genome generally exhibits a high mutation rate (Moriyama and Powell 1997) suggests that mtDNA may show elevated rates of origin and fixation of novel beneficial mtDNA alleles (Ballard and Whitlock 2004). Whatever the net effect may be of complex G × G × E interactions on the evolvability of thermal phenotypes, our results do suggest that an appreciation of the contribution of mtDNA sequence variation to metabolic phenotypes is important for a complete understanding of thermal adaptation.

The results of our study add weight to the emerging view that genetic variation in the mitochondrial genome encodes sizeable and fitness related phenotypic effects (Fenster and Galloway 2000; Dowling et al. 2008). In particular, our results show that the effects of cytonuclear genetic variation may be contingent upon the environment. Further, the fact that mtDNA sequence

variation was related to whole-organism metabolic rate suggests that mtDNA may play a more fundamental role in life-history evolution in general, and in thermal adaptation in particular, than generally appreciated. Clearly, our understanding of the complex but important phenotypic effects of mtDNA variation is still far from complete.

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