Experimental Support That Natural Selection Has Shaped the Latitudinal Distribution of Mitochondrial Haplotypes in Australian *Drosophila melanogaster*

M. Florencia Camus,*[,1,2] Jonci N. Wolff,[1] Carla M. Sgrò,[1] and Damian K. Dowling*[,1]

[1]School of Biological Sciences, Monash University, Clayton, VIC., Australia

[2]Department of Genetics Evolution & Environment, University College London, London, United Kingdom

***Corresponding authors:** E-mails: f.camus@ucl.ac.uk; damian.dowling@monash.edu.

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Abstract

Cellular metabolism is regulated by enzyme complexes within the mitochondrion, the function of which are sensitive to the prevailing temperature. Such thermal sensitivity, coupled with the observation that population frequencies of mitochondrial haplotypes tend to associate with latitude, altitude, or climatic regions across species distributions, led to the hypothesis that thermal selection has played a role in shaping standing variation in the mitochondrial DNA (mtDNA) sequence. This hypothesis, however, remains controversial, and requires evidence that the distribution of haplotypes observed in nature corresponds with the capacity of these haplotypes to confer differences in thermal tolerance. Specifically, haplotypes predominating in tropical climates are predicted to encode increased tolerance to heat stress, but decreased tolerance to cold stress. We present direct evidence for these predictions, using mtDNA haplotypes sampled from the Australian distribution of *Drosophila melanogaster*. We show that the ability of flies to tolerate extreme thermal challenges is affected by sequence variation across mtDNA haplotypes, and that the thermal performance associated with each haplotype corresponds with its latitudinal prevalence. The haplotype that predominates at low (subtropical) latitudes confers greater resilience to heat stress, but lower resilience to cold stress, than haplotypes predominating at higher (temperate) latitudes. We explore molecular mechanisms that might underlie these responses, presenting evidence that the effects are in part regulated by SNPs that do not change the protein sequence. Our findings suggest that standing variation in the mitochondrial genome can be shaped by thermal selection, and could therefore contribute to evolutionary adaptation under climatic stress.

Key words: **mitochondrial DNA, evolution, ecology, thermal adaptation, natural selection, genome evolution.**

Introduction

The mitochondria are essential for eukaryote evolution, taking center-stage in the process of cellular respiration. This process is regulated via a series of finely coordinated interactions between the genes of two obligate genomes—nuclear and mitochondrial (Rand et al. 2004; Wolff et al. 2014). Indeed, because of the strong dependence of cellular respiration on mitochondrial-encoded gene products, biologists traditionally assumed that strong purifying selection would prevent any "function-encoding" genetic variation from accumulating within the mitochondrial DNA (mtDNA) (Ballard and Kreitman 1994; Rand 2001; Dowling et al. 2008). The assumption of selective neutrality has, however, been challenged over the past decade via analyses of polymorphism and divergence data within the mtDNA sequences of metazoans. These analyses have used McDonald–Kreitman or similar tests of selection at the molecular level, to uncover signatures of recurrent adaptive evolution within the mitochondrial genome (Bazin et al. 2006; James et al. 2016). These have been complemented by studies using experimental approaches with the power to partition mitochondrial from nuclear genetic effects, which have demonstrated that the intra-specific genetic variation that exists within the mitochondrial genome commonly affects the expression of phenotypic traits, from morphological, to metabolic, to life-history (Rand 2001; Dowling et al. 2008; Burton et al. 2013; Dobler et al. 2014).

Indeed, several lines of empirical evidence have emerged that support a novel hypothesis, which posits that the standing genetic variation that delineates the mtDNA haplotypes of spatially disjunct populations has been shaped by natural selection imposed by the prevailing thermal climate (Mishmar et al. 2003; Ballard and Whitlock 2004; Ruiz-Pesini et al. 2004; Wallace 2007; Dowling 2014). The first support for this *mitochondrial climatic adaptation* hypothesis was provided by studies of mtDNA variation in humans, where patterns of amino acid variation were observed to align closely to particular climatic regions (Mishmar et al. 2003; Ruiz-Pesini et al. 2004), and where levels of genetic divergence between mtDNA haplotypes of different populations were shown to correlate with temperature differences between these populations (Balloux et al. 2009). These studies on human mtDNA

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sequences are intriguing, but have in some cases proven difficult to replicate with different or larger data sets (Kivisild et al. 2006; Sun et al. 2007).

Additional support for the hypothesis has been provided from studies of other metazoans, some of which have reported signatures of positive selection on mtDNA genes sampled from populations inhabiting particular thermal environments (Foote et al. 2011; Silva et al. 2014; Ma et al. 2015; Morales et al. 2015), and others which have documented variation in mitochondrial gene or haplotype frequencies along clinal gradients associated with climate, such as latitude (Silva et al. 2014; Consuegra et al. 2015), altitude (Fontanillas et al. 2005; Cheviron and Brumfield 2009), or with temperature itself (Quintela et al. 2014). Such clinal patterns are, however, based on correlations between haplotype frequencies and environmental gradients. The possibility remains these correlations could be explained by neutral demographic processes, such as by multiple colonizations from different origins into different locations followed by admixture, sex-specific patterns of dispersal and introgression (given that the mtDNA sequence is maternally inherited), or by recurrent occurrences of secondary contact (Endler 1977; Toews and Brelsford 2012; Adrion et al. 2015; Bergland et al. 2016).

Finally, support has been provided through laboratory experiments in invertebrates, which have reported that the expression of life-history phenotypes (Dowling et al. 2007; Arnqvist et al. 2010; Hoekstra et al. 2013; Wolff et al. 2016), as well as the transmission dynamics (Matsuura et al. 1997; Doi et al. 1999), associated with particular mtDNA haplotypes, or combinations of mitochondrial and nuclear (mito-nuclear) genotype, often depend on the thermal environment in which the study subjects are assayed. These studies have thus indicated that mitochondrial genetic variation is sensitive to thermal selection, at least when measured in the laboratory. However, these studies also have some caveats, given they are based on "between population" (i.e., the mtDNA haplotypes used were drawn from distinct populations), or "between species" (mtDNA from distinct species) experimental designs (Dowling et al. 2007; Arnqvist et al. 2010; Wolff et al. 2016) (Matsuura et al. 1997; Doi et al. 1999; Hoekstra et al. 2013). Inter-population and inter-species designs will presumably maximize the opportunity to detect mitochondrial genetic effects on organismal phenotypes, given that levels of mitochondrial divergence will increase with at inter-population and inter-species scales. Yet, the results obtained via such designs are less straightforward to interpret within the broader context of thermal adaptation, given that the most relevant level at which natural selection acts is on standing variation in mtDNA haplotypes within a population of a given species.

Accordingly, in this study we sought to directly test the *mitochondrial climatic adaptation* hypothesis, within the Australian distribution of the vinegar fly, *Drosophila melanogaster*. This species invaded Australia over a century ago (Hoffmann and Weeks 2007), and it is thought the Australian population was established from multiple introductions of flies from two origins; Africa and Eurasia (David and Capy 1988; Singh and Long 1992). A recent study of nuclear genome-wide allele frequencies from Australian populations concurs with this conclusion, with flies sampled from high latitudes closely related to cold-adapted European populations, and those from low latitudes more closely related to African populations (Bergland et al. 2016). This study therefore provides a cautionary note, by indicating that colonization history might well contribute to the existence of any latitudinal patterns in mtDNA haplotype frequencies that occur within Australia, rather than thermal selection acting on standing variation in mtDNA haplotypes (Adrion et al. 2015; Bergland et al. 2016).

Direct experimental evidence for the mitochondrial climatic adaptation hypothesis therefore requires a two-step approach: First, evidence of shifts in the frequencies of mtDNA haplotypes along a gradient that aligns closely to the environment (e.g., latitude); and second, experimental evidence that links thermal sensitivities of these haplotypes when measured under controlled conditions in the lab, to their spatial distributions in the field. This has never previously been achieved for the genetic variation that resides within the mitochondrial genome. Indeed, when it comes to the evolutionary significance of clinal variation in general, there are surprisingly few examples in which latitudinal variation in allele frequencies has been linked clearly to variation in fitness (Adrion et al. 2015).

Results and Discussion

We collected field-inseminated female flies from 11 populations along an eastern Australian latitudinal cline (supplementary table S1, Supplementary Material online), and used these flies to initiate isofemale lines (lines initiated by a solitary gravid female), and ultimately mass-bred populations per latitudinal location (with each population kept in independent duplicate). Previous research has shown linear associations between the expression of thermal tolerance phenotypes, and allele frequencies of underlying candidate nuclear genes, along this latitudinal cline (Hoffmann et al. 2002; Weeks et al. 2002; Hoffmann and Weeks 2007), thus uncovering signatures of thermal adaptation. To gauge levels of mtDNA sequence variation across these populations, we used the cline end populations (Melbourne and Townsville), and estimated F_{st} values for each mtDNA SNP between these populations. We identified 15 SNPs in the mitochondrial genome exhibiting high (and significant) F_{st} values; the rest of the genome was highly conserved (table 1, supplementary table S1*B*, Supplementary Material online). To probe levels of haplotype diversity, and estimate the frequencies of each haplotype within each of the source populations, we designed a custom-genotyping assay based on these 15 SNPs, and used this assay to genotype the field-collected isofemale lines ($N = 312$). We identified a total of ten unique haplotypes. All haplotypes fell into one of two main haplogroups, with a total of 12 SNPs delineating the two groups (fig. 1). Both haplogroups were found to segregate across most of the 11 populations, but as a whole one haplogroup (haplogroup A) predominated in the northern sub-tropical populations while the other (haplogroup B) predominated in southern

Table 1. Location of All SNPs Identified via Next-Generation Sequencing of the 11 Mass-Bred Populations.

				A Haplogroup				B Haplogroup			
Site	Gene	Position	F_{st}	nt	Codon	AA	Usage Bias	nt	Codon	AA	Usage Bias
1154	*mt:ND2*	3	0.23	C	AAC	N	2.6	T	AAU	N	48.2
2661	*mt:COX1*	3	0.2	C	CCC	P	1.7	T	CCU	P	23.1
3583	*mt:COX2*	3	0.04	T	GCU	A	3	A	GCA	A	13
4247	*mt:ATPase6*	3	0.17	C	GGC	G	0.1	T	GGU	G	12.7
5396	*mt:COX3*	1	0.1	C	CUA	L	7.1	T	UUA	L	134.5
6299	*mt:tRNAE*		0.38	C				A			
6980	*mt:ND5*	3	0.17	G	UAC	Y	7.1	A	UAU	Y	35.6
7862	*mt:ND5*	3	0.19	G	UUC	F	5.6	A	UUU	F	85
8866	*mt:ND4*	1	0.09	A	UUA	L	134.5	G	CUA	L	7.1
8972	*mt:ND4*	3	0.1	C	UUG	L	4.3	T	UUA	L	134.5
10215	*mt:ND6*	1	0.19	C	CUA	L	7.1	T	UUA	L	134.5
10671	*mt:CYTB*	1	0.14	T	UUA	L	134.5	C	CUA	L	7.1
12121	*mt:ND1*	3	0.09	C	AUG	M	2.8	T	AUA	M	51.2
12334	*mt:ND1*	1	0.19	C	GGG	G	4.1	A	GGU	G	12.7
14665	*mt:srRNA*		0.4	C				T			

NOTE.—For each SNP site, we identified nucleotides that were diagnostic of the northern and southern major haplotypes. Here we list the location (Site) of the SNP, the affected gene (Gene), and the codon position (Position). Additionally, for each north and south polymorphism, we list the nucleotide (nt), the codon, amino acid (AA), and the usage bias for the specific codon. Furthermore, we provide the F_{st} values obtained from comparing the most northern and southern populations (Melbourne and Townsvile).

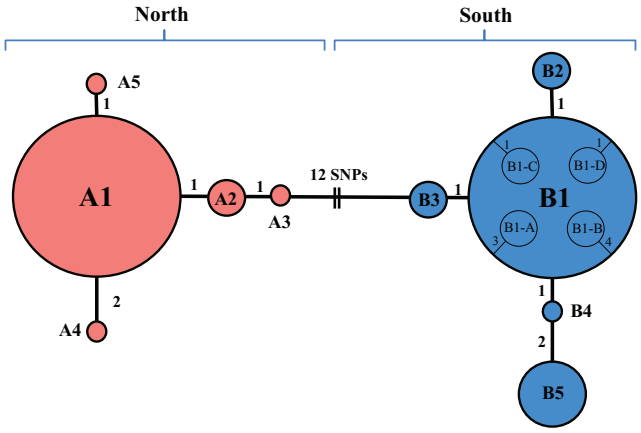

FIG. 1. Haplotype network for mitochondrial protein-coding regions derived from genotyping of 15 SNPs. Circles indicate unique haplotypes with circle size proportional to haplotype frequency. The two different colors correspond to the two haplogroups. The A1 and B1 haplotypes were the only haplotypes present in each of the sampled populations, and are the main drivers of latitudinal association patterns (supplementary table S1*B*, Supplementary Material online). A1 and B1 thus contributed heavily to the frequency of each "haplogroup" per latitudinal location. In this figure, the color red corresponds to the group of haplotypes that, when pooled together into the level of the haplogroup, is more predominant in the north of Australia (termed "North" in the figure), and blue corresponds to the pool of haplotypes that, when pooled together into the level of the haplogroup, is more predominant in the south of Australian (termed "South" in the figure; see supplementary fig. S1, Supplementary Material online). Further re-sequencing of A1 and B1 haplotypes revealed that the B1 haplotype is comprised of at least four subhaplotypes (B1-A, B1-B, B1-C, and B1-D). Subhaplotypes all share the same diagnostic 15 SNPs that delineate the B1 from the A1 haplotype, however contain 1–4 additional SNPs scattered throughout the mitochondrial genome (table 2).

temperate populations (supplementary fig. S1, Supplementary Material online). Furthermore, each of the A and B haplogroups was dominated by one major haplotype (A1 accounting for 93.3% of A haplotypes; and B1 accounting for 77.2% of B haplotypes, fig. 1). The A1 haplotype appears more closely related to other haplotypes of African origin. The ancestral origin of B1 haplotypes is, however, less clear given they are most closely related to haplotypes from other New World populations, but also a haplotype from Japan (supplementary fig. S2, Supplementary Material online). Neither of these haplotypes has been previously studied in the context of thermal selection; and indeed, no study has previously taken a clinal or intra-population approach to studying the thermal sensitivity of variation in the mtDNA genome in *D. melanogaster*. The frequency of the A1 haplotype was negatively associated with the latitude of its source population ($R^2 = 0.4847$, $\beta = -0.02881$, $P = 0.0273$, fig. 2*A*), while the frequency of B1 exhibited a positive association ($R^2 = 0.5137$, $\beta = 0.02718$ $P = 0.0131$, fig. 2*B*). Thus, a latitudinal cline exists for the frequencies of the A1 and B1 haplotypes along the east coast of Australia.

We next sought to experimentally assess whether the clinal associations of the A1 and B1 haplotypes are consistent with the hypothesis that these associations have been shaped by thermal selection. Mapping each of the mtDNA haplotypes to components of thermal tolerance is key to the study, given that such clinal associations could alternatively be mediated by the history of colonization and nonadaptive demographic factors, or by other environmental variables that are likely to associate with latitudinal variation, such as humidity or dietary resources. To address this question, it was first necessary to disentangle effects attributable to mitochondrial genetic variation from those caused by segregating nuclear allelic

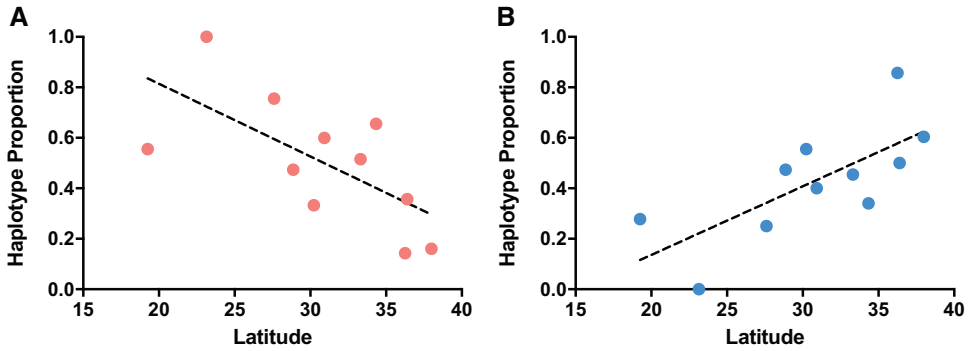

FIG. 2. Haplotype abundance along the Australian eastern coast. (*A*) Haplotype A1 (red) is predominantly found in the north of Australia, decreasing in frequency as latitude increases ($R^2 = 0.4847$). (*B*) Haplotype B1 (blue) is more common in the south, decreasing in frequency as latitude decreases ($R^2 = 0.5137$).

variation, or other sources of environmental variance (Dowling et al. 2008). We thus created eight genetic strains of flies, in which four of the strains harbored the A1 haplotype, and the other four the B1 haplotype, in an otherwise isogenic nuclear background derived from a distinct southern latitudinal population (Puerto Montt [PUE], Chile, South America). We also ensured that all strains were free of *Wolbachia* infection, a maternally-inherited endosymbiotic bacterium, because variation in infection with different strains of *Wolbachia* would confound our capacity to map phenotypic effects to the mtDNA sequence (see "Materials and Methods" for more details on antibiotic treatment and *Wolbachia*-screening). Furthermore, we created these eight strains such that each haplotype was replicated across two levels (intra- and inter-latitudinal replication per haplotype), which therefore enabled us to statistically partition effects attributable to the mitochondrial haplotype from effects attributable to other sources of variation. Specifically, the haplotypes that sourced the strains were collected from each of two geographically disjunct populations—Melbourne (37.99°S) and Brisbane (27.61°S). Because each mass-bred population was kept in independent duplicate, we ensured each duplicate contributed one A1 haplotype and one B1 haplotype to the strains (4 duplicates × 2 haplotypes = 8 strains, thus creating replication within and between latitudes, supplementary fig. S3*A* and *B*, Supplementary Material online).

Once created, full protein-coding re-sequencing of the mitochondrial genomes of each strain revealed that those harboring the A1 haplotype were indeed all homogeneous; characterized by a single haplotype. The strains harboring the B1 haplotype were, however, heterogeneous (fig. 1), and could be further partitioned into four unique "subhaplotypes" (B-1, B-2, B-3, and B-4). Each B subhaplotype was delineated by 1–4 SNPs, but all shared the same pool of 12 SNPs that delineate them from the A haplogroup (fig. 1, table 2, supplementary fig. S4, Supplementary Material online). This enabled us to partition mitochondrial genetic effects over two levels—at the level of the haplotype, and the subhaplotype. The genetic variation differentiating the A1 and B1 haplotypes was comprised of 15 synonymous SNPs in the protein-coding genes. Synonymous SNPs have traditionally been considered to be functionally silent because they do not change the amino acid sequence. However, a growing body of empirical evidence suggests that synonymous polymorphisms might routinely modify the phenotype and thus be of functional and evolutionary significance (Kimchi-Sarfaty et al. 2007; Hurst 2011). On the other hand, the SNPs delineating the "subhaplotypes" hubbed within the B1 haplotype, consisted of a mixture of synonymous and nonsynonymous SNPs (table 2).

Flies harboring the A1 haplotype, which predominates in the sub-tropics, exhibited greater tolerance to an extreme heat challenge than flies harboring the B1 haplotype (haplotype, $\chi^2 = 6.04$, $P = 0.014$, supplementary table S2, Supplementary Material online). However, the magnitude of these effects changed across the sexes (haplotype × sex, $\chi^2 = 24.7$, $P < 0{,}001$, fig. 3*A* and *B*, supplementary table S2, Supplementary Material online). We also uncovered sex-specific effects that mapped specifically to the level of the mtDNA subhaplotype (sex × subhaplotype[haplotype], $\chi^2 = 25.04$, $P = <0.001$, fig. 3*C*). This interaction was primarily attributable to the B1-D subhaplotype, which conferred inferior heat tolerance in males, but high heat tolerance in females, relative to the other subhaplotypes. Only one synonymous SNP, located in the *mt:ND4* gene, delineates the protein-coding region of this subhaplotype from the other B1 subhaplotypes (table 2). This polymorphism is therefore a candidate SNP in conferring sex-specific outcomes in heat tolerance, although we cannot rule out the possibility that further variation within the noncoding region of the mtDNA sequence and regulatory elements (which we did not sequence) contributed to this effect. Nonetheless, the observed pattern associated with this subhaplotype is striking in the context of a hypothesis proposed by Frank and Hurst (1996), often called *Mother's Curse*, which proposes that maternal inheritance of the mitochondria will facilitate the accumulation of mtDNA mutations that are deleterious to males, but benign or only slightly deleterious to females (Frank and Hurst 1996; Gemmell et al. 2004; Beekman et al. 2014). However, while this haplotype harbors variation that causes a male-limited reduction in heat tolerance, it did not confer a detrimental effect on male capacity to tolerate cold stress (supplementary table S3, Supplementary Material online, fig. 3*D*). Thus, further studies are required to determine

whether the male-specificity of the B1-D subhaplotype on heat tolerance effect extends to pleiotropic effects on other life-history traits such as reproduction and longevity (Camus et al. 2015), or whether this effect is sensitive to genotype-by-environment interactions that mediate the severity of effect in males (Mossman et al. 2016; Wolff et al. 2016).

Flies harboring the B1 haplotype were superior at withstanding an extreme cold challenge, relative to their A1 counterparts ($\chi^2 = 34.31$, $P < 0.001$, fig. 3D and E, supplementary table S3, Supplementary Material online), but there was no significant effect traceable to the level of the subhaplotype (supplementary table S3, Supplementary Material online). Importantly, the effects of mitochondrial haplotype on both thermal tolerance phenotypes was robust to the source of origin of the haplotypes (i.e., whether they were sourced from Brisbane or Melbourne), providing clear evidence that the phenotypic effects are directly tied to the mtDNA sequence (fig. 3). Furthermore, all B1 subhaplotypes exhibited decreased heat tolerance and increased cold tolerance when compared to the A1 haplotype, providing support for the suggestion that the differences in thermal tolerance observed between northern and southern haplogroups are mapped to the 15 SNPs that delineate the A1 and B1 haplotypes (or to cryptic variation in the noncoding region that we did not genotype), rather than the SNPs that delineate the different subhaplotypes hubbed within B1 (supplementary tables S2 and S3, Supplementary Material online, and fig. 3*F*). Alternatively, it is possible that the unique SNPs that delineate the B1 subhaplotypes drive the bulk of the differences in thermal response between the A1 and B1 haplotypes, and

Table 2. Location of All SNPs Identified via Next-Generation Resequencing of the Mitochondrial Genomes of Each Genetic Strain, Revealing that the B1 Haplotype Can be Further Partitioned into Four Unique "Sub-Haplotypes."

Origin	dup	h. type	sub-h.type	Gene	SNP	nt change	Site	AA change
MEL	1	A1	A1					
MEL	2	A1	A1					
BRIS	1	A1	A1					
BRIS	2	A1	A1					
MEL	1	B1	B1-C	*mt:COXII*	N	C → T	3359	P → S
MEL	2	B1	B1-D	*mt:ND4*	S	C → T	8033	
BRIS	1	B1	B1-A	*tRNA-ASP*		A → C	3892	
				mt:COXIII	S	T → C	4954	
				mt:ND5	S	A → G	7877	
BRIS	2	B1	B1-B	*mt:COXI*	S	G → A	2262	
				mt:COXII	S	C → T	3385	
				mt:COXIII	N	G → A	5162	V → I/M
				mt:ND4-L	S	A → T	9341	

NOTE.—Above is the list comprising the origin from which each genetic strain was originally sourced (Origin), the identity of the duplicate of each population (Dup), the haplotype associated with each strain (h.type), the subhaplotype (sub-h.type), the affected gene (Gene), whether the SNP is synonymous (S) or nonsynonymous (N), the nucleotide change (nt change), the location (Site) of the SNP, and amino acid change (AA change).

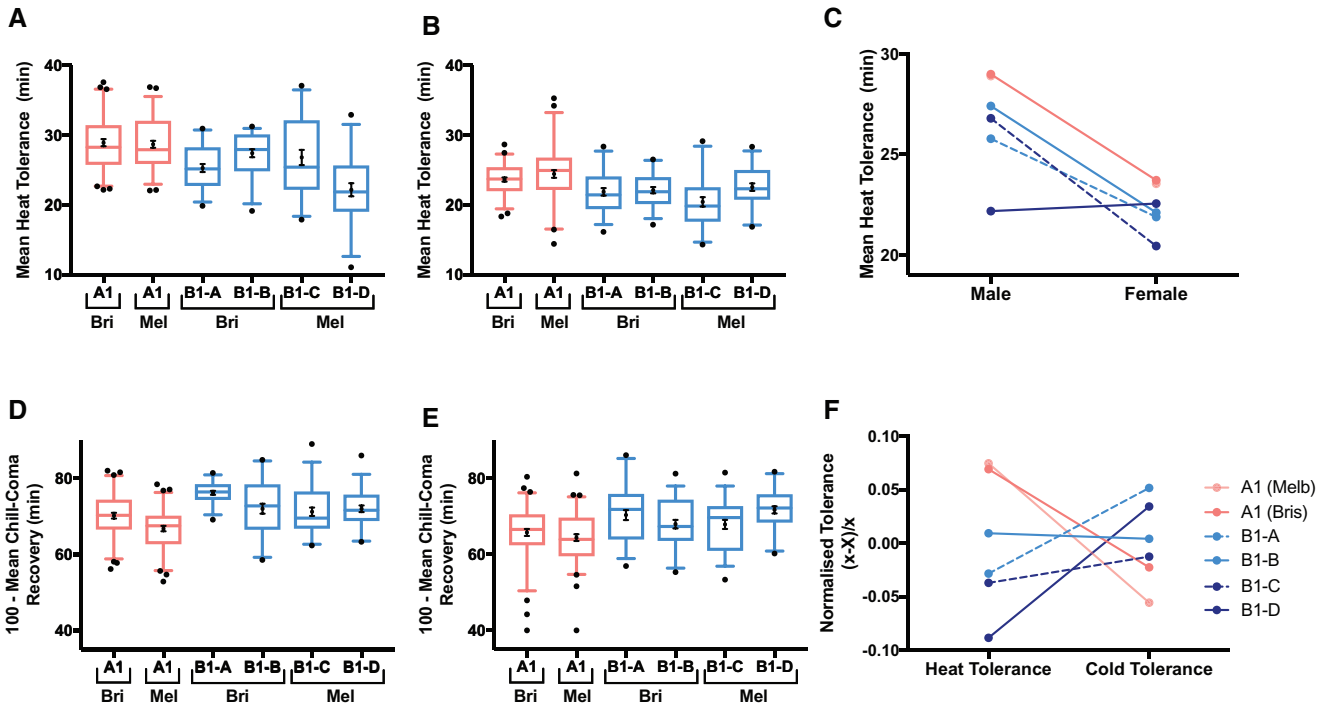

FIG. 3. (*A*) Heat tolerance (mean heat "knockdown" time ±1 S.E) of males carrying the A1 (red) and B1 (blue) haplotypes/subhaplotypes. Means for each haplotype are shown separately according to population of origin; Bri refers to Brisbane, Mel refers to Melbourne. (*B*) Heat tolerance (mean heat "knockdown" time ± 1 S.E) of females carrying the A1 (red) and B1 (blue) haplotypes/subhaplotypes. (*C*) Differences in male and female heat tolerance means across mitochondrial haplotypes. (*D*) Cold tolerance (100 − mean chill-coma recovery time ± 1 S.E) of males carrying the A1 (red) and B1 (blue) haplotypes/subhaplotypes. (*E*) Cold tolerance (100 − mean chill-coma recovery time ± 1 S.E) of females carrying the A1 (red) and B1 (blue) haplotypes/subhaplotypes. (*F*) Heat and cold tolerance (centered on a mean of zero and standard deviation of 1) across mitochondrial haplotypes.

represent cases of parallel evolution for thermal tolerance brought about by different underlying SNPs (Arendt and Reznick 2008). While we cannot definitively disentangle these possibilities, we note that the polymorphisms that delineate the A1 from the B1 subhaplotypes only include nonsynonymous SNPs in two of four cases. Thus, while we are unable to definitively ascertain whether differences in the A1 and B1 thermal responses are underpinned primarily by the 15 shared SNPs that separate all B1 from A1 haplotypes, by other cryptic regulatory variation in the noncoding region, or by the unique SNPs that delineate the B1 subhaplotypes, our results suggest that SNPs that do not change the amino acid sequence are likely to be responsible for this thermal divergence in at least two cases.

We then examined whether the thermal tolerance phenotypes might be mediated by patterns of differential gene expression of protein-coding mtDNA genes, copy number variation in mtDNA, or codon usage bias across the A1 and B1 haplotypes. While we did not detect differences in mtDNA copy number between A1 and B1 haplotypes (supplementary table S5, Supplementary Material online), we did detect differences in mitochondrial gene expression. Specifically, we extracted RNA of females of the A1 and B1 haplotypes, and examined expression patterns in five genes involved in complex I and complex IV of the electron transport chain (complex I: *mt:ND4*, *mt:ND5*, complex IV: *mt:COXI*, *mt:COXII*, *mt:COXIII*). Emerging evidence suggests that genetic variation within complex I genes (both mitochondrial and nuclear) might contribute disproportionately to trajectories of mito-nuclear, and ultimately, life history evolution (Camus et al. 2015; Garvin et al. 2015; Morales et al. 2015). Complex IV, on the other hand, harbours genes with the lowest levels of *dN/dS*, indicative of greater selective constraints on these mitochondrial genes across taxonomically diverse organisms (Nabholz et al. 2013). Accordingly, we found that strains harbouring the B1 haplotype exhibited higher gene expression for the complex I genes *mt:ND4* and *mt:ND5*, which belong to the same transcriptional unit (Torres et al. 2009), than strains with the A1 haplotype (haplotype × gene < 0.001, fig. 4, supplementary table S4, Supplementary Material online). We note we conducted these analyses in females only, since a study of mitochondrial gene expression across a global sample of mtDNA haplotypes had previously indicated that mtDNA haplotypes affect the expression of protein-coding mtDNA genes, but that these haplotype-specific effects are consistent across the sexes (Camus et al 2015). Future work could, however, examine whether these patterns of gene expression across the A1 and B1 haplotypes are upheld in males, and whether the differences in *mt:ND4* and *mt:ND5* expression observed across haplotypes, extend further to differences at the level of the individual B1 subhaplotypes. We note that all of the SNPs located in the *mt:ND4* and *mt:ND5* genes, which delineate A1 from B1 haplotypes, are synonymous (table 1). This observation is interesting in light of a recent report that found that patterns of expression of *mt:ND5* and *mt:CYTB* genes in *D. melanogaster* mapped to candidate SNPs that lay directly within the affected genes, and which presumably exerted their effects via post-transcriptional modification of RNA, potentially altering the stability of the transcripts (Camus et al. 2015). This is also consistent with reports showing that coding variants in both the nuclear and mitochondrial genome affect gene expression patterns in humans (Birnbaum et al. 2012; Cohen et al. 2016), and recent evidence demonstrating that transcription regulators specifically bind to the human mtDNA coding region to regulate transcription (She et al. 2011; Blumberg et al. 2014). In combination, these studies suggest that the synonymous SNPs delineating the A1 and B1 haplotypes could be involved in regulating transcription of these genes, via mito-nuclear interactions involving nuclear-encoded transcription regulators.

Evidence is also mounting that variation in patterns of genomic DNA base composition (GC content [Šmarda et al. 2014]), as well as variation in codon usage bias across DNA sequences (Sharp et al. 1995) can be shaped under natural selection. For example, in bacteria and metazoans, higher levels of GC base pairs have been associated with the thermal environment, with the GC base pair associated with higher thermal stability (Bernardi 2007). In bacteria, this correlates with greater tolerance of higher temperatures (Musto et al. 2004). In the green alga *Chlamydomonas*, experimental alteration of mitochondrial codons drastically changes translational efficiency, suggesting that mitochondrial codon usage has been optimized for translation of mitochondrial products (Salinas et al. 2012). In our study, the A1 and B1 haplotypes differ by 15 synonymous SNPs that are evenly distributed across the mitochondrial protein-coding region, with most protein-coding genes harboring at least one SNP site. SNPs of the A1 haplotype show a high GC bias, with 80% of the SNPs represented by a guanine or cytosine, and conversely those of B1 reveal a GC content of only 20% (supplementary table S6*A*, Supplementary Material online, Fishers exact test, $P = 0.001$). Thus, the A1 haplotype, which confers higher tolerance to an extreme heat challenge, has a higher GC content; concordant with previous observations in bacteria and metazoans suggesting higher thermal stability of the GC base pair relative to AT (Bernardi 2007). Additionally, the SNPs delineating the A1 haplotype change the codon bias and produce rarer codons (supplementary table S6*B*, Supplementary Material online, Fishers exact test $P = 0.002$). These findings suggest that GC content and codon bias may play a role in the observed haplotype effects on gene expression of *mt:ND4* and *mt:ND5*, with ultimate upstream effects on thermal tolerance.

By harnessing an experimental genomic approach applied to the mitochondrial genome, within a clinal framework, we have documented latitudinal patterns in standing mtDNA haplotypes, and provided experimental evidence that these patterns are linked to the capacity of these haplotypes to tolerate thermal stress. While these results are consistent with the suggestion that the clinal patterns of mtDNA variation are likely to have been shaped at least in part by thermal selection, it is difficult to fully resolve the relative influence of thermal selection from history of colonization and other demographic factors, given that the Australian east coast is thought to have been subjected to recurrent colonization

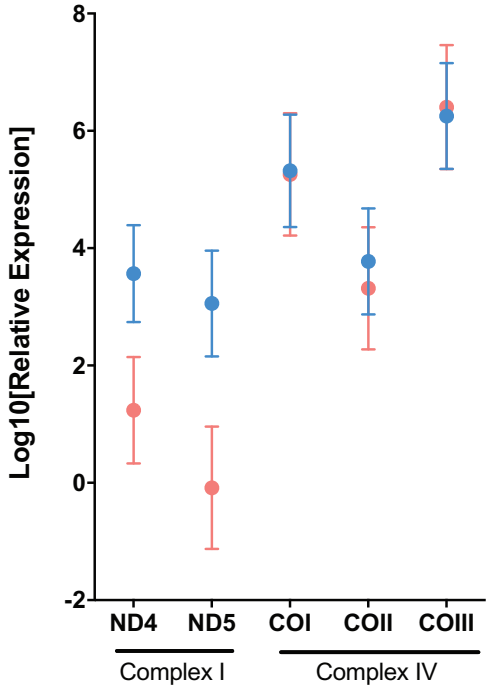

Fig. 4. Least-squares means (±1 S.E.) of female gene expression across A1 (red) and B1 A–D combined (blue) haplotypes for the *mt:ND4* and *mt:ND5* genes (OXPHOS complex 1) and *mt:COI*, *mt:COII*, *mt:COII* genes (OXPHOS complex IV). *mt:COI*, *mt:COII*, *mt:COII* all belong to one transcriptional unit and encode subunits of complex IV, whilst *mt:ND4* and *mt:ND5* are members of a second transcriptional unit and encode subunits of complex 1 of the mitochondrial electron transport chain. Least-square means for all plots were derived from the multilevel models, which take into account mtDNA copy number as a covariate (supplementary table S4, Supplementary Material online).

events from flies of disparate origins over the past 150 years. This, of course, is a caveat that is not unique to our study on mtDNA variation, but extends to all studies of clinal variation of New World populations of *D. melanogaster*. We thus point out that these haplotypes might have been pre-adapted to tropical and temperate conditions of Africa and Eurasia prior to their introductions into Australia, and that the relevant mitochondrial variation under selection along the Australian cline is likely to have already existed upon the arrival of these haplotypes into Australia. Accordingly, the Australian distribution of mtDNA haplotypes is likely to have been shaped both by the history of colonization, followed by the subsequent and ongoing action of thermal selection in spatially sorting the haplotypes along the latitudinal cline. Our study suggests that further research into the mitochondrial climatic adaptation hypothesis is warranted. In particular, we suggest our conclusions can be tested by future studies that utilize other established latitudinal clines, in *D. melanogaster* and in other species, to determine whether mtDNA haplotypes show similar associations to latitude as revealed in the Australian cline, and to determine whether the mtDNA haplotypes involved exhibit thermal sensitivities that concord with the clinal patterns.

In conclusion, our results provide support for the hypothesis that standing genetic variation within the mitochondrial genome has been shaped, in part, by natural selection imposed by thermal stress. We also presented evidence that SNPs found within the mtDNA, and which do not change the amino acid sequence, contribute to the regulation of phenotypic responses to thermal stress in *D. melanogaster*. This thus suggests a role for a set of SNPs that were traditionally thought to evolve under neutrality (Kimchi-Sarfaty et al. 2007; Hurst 2011), within a genome that was likewise traditionally thought to be devoid of phenotype-changing genetic variation (Ballard and Rand 2005; Dowling et al. 2008), in the dynamics of thermal adaptation. Second, and more broadly, our results add to an emerging body of research in *Drosophila* (Sorensen et al. 2007; Chen et al. 2012; Lavington et al. 2014; Cogni et al. 2015) and other metazoans (Porcelli et al. 2015), which highlights metabolic genes (including those targeted to the mitochondria) as important substrates on which thermal selection is likely to act to shape adaptive evolutionary responses. Several studies have now reported variation in allele frequencies, or expression patterns, of nuclear-encoded metabolic genes along latitudinal clines (Chen et al. 2012; Lavington et al. 2014; Cogni et al. 2015), or across replicated laboratory populations that have evolved under differing thermal regimes (Sorensen et al. 2007), in *Drosophila melanogaster*. These studies, however, did not screen for involvement of the evolutionary-conserved mitochondrial genes. The function of key metabolic enzymes, however, relies on close coordination between mitochondrial and nuclear genomes (Rand et al. 2004; Levin et al. 2014; Wolff et al. 2014; Quiros et al. 2016). This point, when reconciled with the emerging studies, would suggest that genetic interactions between the mitochondrial and nuclear genomes could represent key mediators of evolutionary adaptation of the metabolic machinery under thermal stress.

Materials and Methods

Field Collection, Isofemale Line Establishment, and Maintenance

Populations of *Drosophila melanogaster* were sampled from the east coast of Australia during March–April 2012 from 11 locations. The population names (latitude and longitude) are: Townsville (19.26, 146.79), Rockhampton (23.15, 150.72), Brisbane (27.61, 153.30), Ballina (28.87, 153.44), Coffs Harbor (30.23, 153.15), Port Macquarie (30.93, 152.90), Wollongong (34.34, 150.91), Narooma (36.25, 150.14), Gosford (33.31, 151.20), Bermagui (36.40, 150.06), and Melbourne (37.99, 145.27). Samples were collected as close to sea level as possible to avoid altitudinal differences between the populations (Collinge et al. 2006). Individual field-inseminated females were isolated into individual vials in the laboratory to initiate independent isofemale lines. At least 20 isofemale lines were generated for each population. Each line was treated with tetracycline to eliminate cytoplasmic endosymbionts, such as *Wolbachia* (Clancy and Hoffmann 1998), and tested using *Wolbachia*-specific primers (O'Neill et al. 1992). We further verified infection status when analyzing next-generation

sequencing data by confirming that none of our obtained reads mapped to the *Wolbachia* genome (NC_002978).

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

Next Generation Sequencing and SNP Genotyping

To identify regions of variation between the 11 populations, we first used pooled samples of 100 individuals (both males and females) from each population and used next generation sequencing to obtain full mitochondrial genomes. DNA samples were enriched for mitochondrial DNA to obtain the best coverage possible. This process was achieved by using *Wizard SV* Miniprep Purification Kit (Promega, Madison, WI) for DNA extraction, which captures circular DNA. Enriched DNA samples were made into 200 bp paired-end libraries and sequenced using the Illumina GAIIx platform (Micromon, Monash University, Australia). Reads were aligned to the *Drosophila melanogaster* mitochondrial reference genome (NCBI reference sequence: NC_001709.1) using *Geneious* (Kearse et al. 2012), generating mitochondrial protein coding regions for each of the 11 latitudinal locations. Given the high A-T richness of the mitochondrial genome of *Drosophila melanogaster* it is extremely difficult to map reads to the control region (D-loop). The D-loop is a 5 kb repetitive region with A-T richness of over 90%, making this region extremely difficult to accurately map reads (Tsujino et al. 2002). Although our level of coverage was more than sufficient to examine the protein-coding region, we were not able to accurately map sufficient reads to the D-loop.

We first aimed to identify SNPs in the mitochondrial genome that had high levels of genetic differentiation between locally extreme populations. For this, we used poolSeq data from the cline extremes (Melbourne and Townsville) and calculated F_{ST} values for individual SNPs of the mitochondrial genome using *Popoolation2* (Kofler et al. 2011). To obtain allele frequencies from each population, SNP sites with high F_{st} were used as markers. DNA from each isofemale line was extracted using the *Gentera Puregene Cell and Tissue Kit* (Qiagen, Hilden, Germany). Even though each mass-bred population was created using 20 randomly chosen isofemale lines, we genotyped all isofemale lines collected from each latitudinal location. A custom SNP genotyping assay was developed (Geneworks, Thebarton, Australia) for the 15 SNPs identified via mass sequencing, and genotyping was performed by Geneworks (Thebarton, Australia) on a SEQUENOM MassARRAY platform (Agena Bioscience, San Diego, CA). This genotyping revealed the presence of northern-predominant (i.e., predominating in northern latitude populations) and southern-predominant (i.e., predominating in southern latitude populations) haplogroups, with each haplogroup characterized by one major haplotype (A1 and B1).

We also relied on published genomic data sets to screen for signatures of latitudinal variation in mtDNA SNPs within established latitudinal clines of *D. melanogaster* along the east coast of North America, and Africa. These analyses, and interpretations, are presented in the Supplementary Information, Supplementary Material online.

Creation of Mitochondrial Strains from Mass-Bred Populations

We created "introgression strains" from each of the population duplicates (11 latitudes × 2 population duplicates = 11 introgression strains × 2 duplicates), by introgressing the pool of mtDNA variants of each population duplicate into a standard and isogenic nuclear background originally sourced from Puerto Montt (PUE), Chile (41.46°S, 72.93°W) (Calboli et al. 2003), which had been created via 20 generations of full-sibling mattings. We chose this background primarily because it was from a distinct southern-hemisphere, and is very unlikely to have shared a recent co-evolutionary history with either of the A1 and B1 haplotypes (supplementary fig. S4, Supplementary Material online), which might have inadvertently favored one or other of the mtDNA haplotypes in our phenotypic assays of thermal tolerance. To initiate each strain, 100 virgin female flies were sampled from each population duplicate and crossed to 120 males from the PUE strain. Then, for 20 sequential generations, 100 daughters were collected per strain and backcrossed to 120 PUE males. This crossing scheme aimed to maintain the pool of segregating mitochondrial haplotypes within each population, while translocating them alongside that of an isogenic nuclear background, to enable partitioning of mitochondrial genetic effects from cryptic variance tied to the nuclear genome (supplementary fig. S3A, Supplementary Material online). In order to prevent mitochondrial contamination from the Puerto Montt (PUE) line, all lines were tested every five generations during the introgression regime, to ensure there were no instances of contamination of the lines (by rogue females of the PUE strain) by using qRT-PCR melt curve analysis that would detect PUE-specific mtDNA SNPs.

We then created a new set of isofemale lines from each of the introgression strain duplicates, and re-genotyped females of each line using the custom SNP genotyping assay described above (Geneworks, Thebarton, Australia). From the genotyping results, we were able to identify female lineages that carried individual haplotypes (A1 [northern] or B1 [southern]), and using this information we then selected one isofemale line carrying the A1 haplotype and one isofemale line

carrying the B1 haplotype, from each of the two independent population duplicates from two (Brisbane, Melbourne) of the 11 latitudinal locations (supplementary fig. S3*B*, Supplementary Material online). We continued to backcross virgin females of each isofemale line to males of the isogenic PUE line for a further seven generations. We acknowledge that in the presence of strong mito-nuclear coevolution, such a backcrossing approach could in theory fail to disrupt essential allelic pairings spanning mitochondrial and nuclear genotypes, meaning that a few nuclear alleles that are essential to maintaining mito-nuclear compatibility might remain, even following 27 generations of backcrossing. While a theoretical possibility, this seems unlikely from a population genetic perspective (Eyre-Walker 2017); particularly in our study, given that the A1 and B1 haplotypes under introgression here co-occur within the same panmictic populations, and differ only by a small number of SNPs do not change the amino acid sequence (table 1), and given that we have never previously come across combinations of mito-nuclear genotypes in *D. melanogaster* (even at the inter-population scale) that incur complete inviability in females (the sex that transmits the mtDNA) or juveniles. Prior to this step, the PUE line had been propagated via a protocol of mating between one full-sibling pair for five generations, to remove any genetic variation that had accumulated within this nuclear background during the course of the introgressions described above. We chose to use isofemale lines from Brisbane (latitude: 27.61°S) and Melbourne (latitude: 37.99°S) because they are geographically disjunct, and because re-genotyping confirmed that both A1 and B1 haplotypes were segregating in each of the introgression strain duplicates following the 20 generations of introgression. Following this process, each of the A1 and B1 haplotypes was represented across four independent genetic strains each, at two levels of replication; an intra-latitudinal (between the two population duplicates of a given latitude) and an inter-latitudinal (between two latitudes, Brisbane and Melbourne) replicate (supplementary fig. S3*B*, Supplementary Material online).

We then re-sequenced these strains, and obtained full complete mitochondrial genomes for all eight mitochondrial strains, again using the next generation sequencing approach described above. Re-sequencing results revealed that haplotype A1 was isogenic across all four A1 strains, while we found that the B1 strains could be delineated into four unique subhaplotypes that were nested within the B1 haplotype. These four southern subhaplotypes all shared the known SNPs that delineate the north and south haplogroups (and the A1 and B1 haplotypes), however they each carried between one and three additional SNPs (table 2).

Extreme Heat Challenge

Tolerance to an extreme heat challenge was measured for 120 flies of each sex from each mitochondrial strain (Hoffmann et al. 2002). Flies were placed in individual 5 ml water-tight glass vials and subsequently exposed to a 39 °C heat challenge, by immersion of the glass vials in a preheated circulating water bath. Heat "knock-down" time was recorded as the time taken for each individual fly to become immobilized (in a coma-like state) at 39 °C (Williams et al. 2012). This experiment was conducted over two trials within the same generation. Each trial of the experiment consisted of a fully balanced replicate of the experimental units (i.e., equal numbers of flies of each sex × mitochondrial strain), separated in time by 2 h within the same day. The position of flies of each experimental unit was randomized within each trial of the experiment. The assay was conducted blind to the genotype or sex of the fly.

Extreme Cold Challenge

This assay measures the amount of time it takes a fly to regain consciousness and stand on all legs after succumbing to a cold-induced coma (Hoffmann et al. 2002). In each trial of the assay, 40 flies from each experimental unit ($N = 640$) were placed individually in 1.7 ml microtubes. These tubes were then submerged in a water bath set to 0 °C (comprised of water and engine coolant) for 4 h, to place flies into coma. At 4 h, all microtubes were removed from the bath, and laid out on a bench at 25 °C, and the time taken (seconds) for each fly to regain consciousness and stand upright was recorded. The assay was conducted blind to the genotype or sex of the fly.

Statistical Analyses of Thermal Tolerance Data

We used separate multilevel linear mixed models to test the effects of mtDNA haplotype and subhaplotype on responses to each of the heat and cold challenges. The response variable for the heat challenge assay was the time taken to fall into coma, while the response variable for the cold challenge assay was the time taken to wake from coma. Fixed effects were the identity of the mtDNA haplotype (A1, B1), the subhaplotype nested within haplotype (A1, B1-A, B1-B, B1-C, and B1-D), sex and their interactions. This analysis assumes the effect of the SNPs separating the A1 and the B1 haplotypes, and those that separate the B1 subhaplotypes, are hierarchical and can be statistically partitioned (i.e., any significant "haplotype" effects in the model can be mapped to the 15 SNPs that separate the A1 and B1 haplotypes, while significant "subhaplotype" effects are mapped to the unique SNPs that separate the four B1 subhaplotypes). We, however, acknowledge the alternative possibility that the unique SNPs separating B1 subhaplotypes could in theory underpin the differences between the A1 and B1 thermal responses, if such SNPs have accumulated under parallel evolution (Arendt and Reznick 2008). Random effects described the biological structure of the mitochondrial strains; there were two tiers of replication—with each haplotype replicated across two "duplicates" within each of two latitudinal "populations." Thus, duplicate nested within population was included as a random effect, as well as other known and random environmental sources of variance (the trial identity, and the identity of the person scoring the response variable [two people]).

Parameter estimates were calculated using restricted maximum likelihood algorithm in the *lme4* package of R (Bates et al. 2012). The fitted model was evaluated by simplifying a full model, by sequentially removing terms that did not change (at $\alpha = 0.05$) the deviance of the model, starting with the highest order interactions, using log-likelihood ratio

tests to assess the change in deviance in the reduced model relative to the previous model (Fox 2002).

Haplotype Network, Divergence, Codon Bias, and RNA Analysis

Relationships among haplotypes were visualized on a median-joining network (Bandelt et al. 1999) and constructed in the software NETWORK version 4.6.1.2 (www.fluxus-engineering.com).

We obtained divergence estimates between A1 and B1 haplotypes using *Geneious* (Kearse et al. 2012) and *MEGA6* (Tamura et al. 2013). Using *Geneious*, divergence was calculated by examining the *%identity function* and subtracted that value from 100 to derive the percentage divergence. In *MEGA6*, we performed a pairwise distance comparison using a maximum composite likelihood model. Both methods gave concordant estimates of divergence (divergence = 0.001%)

We obtained *Drosophila melanogaster* mitochondrial codon usage bias values from the Codon Usage Database (Nakamura et al. 2000). For both haplotypes, each SNP site was given the title "preferred" or "unpreferred" based on the codon usage bias score. Results were then analyzed as a 2×2 contingency table using Fishers exact tests (supplementary table S6*A* and *B*, Supplementary Material online).

Sequence polymorphisms in structural RNAs between the two haplogroups were analyzed using tRNAScan (Schattner et al. 2005) for tRNAE and ExpaRNA (Smith et al. 2010) for the polymorphism present in the small ribosomal subunit. Secondary structures are presented in the supplementary figures S5 and S6, Supplementary Material online.

Total RNA/DNA Extraction and cDNA Synthesis

For RNA extractions, we placed single female flies from each A1 and B1 strain into a microtube. We thus combined source population and duplicate into one sample. Each extraction was performed in triplicate, thus resulting in three microtubes with flies possessing the A1 haplotype and three microtubes with flies harboring the B1 haplotype. In the case of the A1 haplotype all flies harbored the same haplotype (although originating from different rearing vials), whereas for the B1 haplotype each biological replicate was formed by combining a single fly from each subhaplogroup into a microtube.

We then performed a coupled RNA and DNA extraction as per the supplier's protocols using *TRIzol® Reagent* (Thermo Fisher Scientific, Waltham, MA) to first create a phase separation of RNA and DNA from which the total RNA was then purified using a *HighPure RNA extraction kit* (Roche Applied Science, Penzberg, Germany). In this manner, both the DNA and RNA was independently separated and stored from the one sample. The separated nucleic acids (~100 µl of each sample extracted) were quantified by Nanodrop UV/Vis spectrophotometry (Thermo Fisher Scientific, Waltham, MA) and the purity of total RNA was confirmed using the A_{260}/A_{280} ratio with expected values between 1.8 and 2.0. The integrity of both the RNA and DNA was assessed by electrophoresis (1% TBE agarose gel).

The cDNA was synthesized from 1µg of RNA using the *Transcriptor First Strand cDNA Synthesis* Kit (Roche Applied Science, Penzberg, Germany) and a mixture of random hexamers and oligodT primers to capture mitochondrial transcripts both in the transitory polycistronic stage and as individual polyadenylated single transcripts (Clayton 2000).

mtDNA Copy Number Quantification

mtDNA copy number was measured for each DNA extraction performed (see *Total RNA/DNA Extraction and cDNA Synthesis*). MtDNA copy number was calculated relative to a single copy gene in the nuclear genome (Correa et al. 2012). Copy number was determined using quantitative real-time PCR of a 113 bp region of the large ribosomal subunit (CR34094, FBgn0013686). No nuclear copies of this gene are found in the *Drosophila melanogaster* genome. Similarly, nuclear DNA was quantified by amplifying a 135 bp region of the single-copy (Aoyagi and Wassarman 2000) subunit of the RNA polymerase II gene (CG1554, FBgn0003277). The copy number was then determined as the relative abundance of the mtDNA to nuclear DNA ratio and thus reflects the average number of mtDNA copies per cell.

Gene Expression Quantification

Five of the 13 total mitochondrial protein-coding genes were amplified to quantify gene expression levels. Quantified genes were: *mt:COI*, *mt:COII*, *mt:COIII*, *mt:ND4*, and *mt:ND5*. Gene expression of each biological replicate (three biological replicates per haplotype) was measured using quantitative real time (qRT)-PCR (Lightcycler 480—Roche Applied Science, Penzberg, Germany). Reactions were performed in duplicate (technical duplicates) using a *SYBRGreen I Mastermix* (Roche Applied Science, Penzberg, Germany), whereby each well contained 5 µl of SYBR buffer, 4 µl of 2.5 µM primer mix and 1 µl of diluted cDNA. The following amplification regime used was: 90 °C (10 s), 60 °C (10 s), 72 °C (10 s) for 45 cycles, followed by a melt curve analysis to verify the specificity of the primer pair.

The *Bestkeeper©* software (Pfaffl et al. 2004) was used to select nuclear housekeeping genes (HKGs) for quality assessment. Three suitable HKGs were chosen: succinate dehydrogenase A (CG17246), 14-3-3ϵ (CG31196), and an unknown protein-coding gene (CG7277). All three genes had similar expression levels with high correlation coefficients (>0.8) against each other. For each experimental sample, the expression values of the mitochondrial target genes were standardized as follows:

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

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

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

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

Gene expression levels of all five mitochondrial genes were obtained by determining the ΔCt per sample, measured at

the maximum acceleration of fluorescence, using the Second Derivative Maximum Method (Rasmussen 2001) in the *Lightcycler Software V1.5.0* (Roche Applied Science, Penzberg, Germany). When the ΔCt values between two technical duplicates for each sample fell within 0.5 units of each other, then the mean gene expression estimates were pooled to form a single data point (Bustin et al. 2009).

Statistical Analysis of Gene Expression Data and Copy Number Variation

We fitted linear models, in which mitochondrial copy number and gene expression data were modeled separately as response variables. Mitochondrial haplotype (A1, B1), and gene identity were modeled as fixed effects. Mitochondrial copy number values were added as a fixed covariate to the analysis of gene expression, and F statistics and associated probabilities estimated using a Type III sums-of-squares tests in the *car* package (Fox 2002) in *R* (R Core Team, 2016). Mitochondrial copy number variation was modelled with haplotype (A1 and B1) as a factor.

Supplementary Material

Supplementary information is available at *Molecular Biology and Evolution* online.

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