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NATURALLY OCCURRING MITOCHONDRIAL DNA HAPLOTYPES EXHIBIT METABOLIC DIFFERENCES: INSIGHT INTO FUNCTIONAL PROPERTIES OF MITOCHONDRIA

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Linking the mitochondrial genotype and the organismal phenotype is of paramount importance in evolution of mitochondria. In this study, we determined the differences in catalytic properties of mitochondria dictated by divergences in the *si*I and *si*III haplogroups of *Drosophila simulans* using introgressions of *si*I mtDNA type into the *si*III nuclear background. We used a novel in situ method (permeabilized fibers) that allowed us to accurately measure the consumption of oxygen by mitochondria in constructed *si*I-introgressed flies and in *si*III-control flies. Our results showed that the catalytic capacity of the electron transport system is not impaired by introgressions, suggesting that the functional properties of mitochondria are tightly related to the mtDNA haplogroup and not to the nuclear DNA or to the mito-nuclear interactions. This is the first study, to our knowledge, that demonstrates a naturally occurring haplogroup can confer specific functional differences in aspects of mitochondrial metabolism. This study illustrates the importance of mtDNA changes on organelle evolution and highlights the potential bioenergetic and metabolic impacts that divergent mitochondrial haplogroups may have upon a wide variety of species including humans.

KEY WORDS: **Catalytic capacity, electron transport system, haplogroup, introgression, mitochondrial DNA.**

It is now well-known that nuclear and mitochondrial genomes evolve at dissimilar rates due to differences in modes of inheritance, recombination, and patterns of mutation (Rand et al. 2004; Burton et al. 2007; Wallace 2007). Specifically, mitochondrial DNA (mtDNA) genes are most often maternally inherited, rarely recombine, and undergo higher rates of nucleotide substitution compared with nuclear DNA (Avise and Vrijenhoek 1987; Avise 1991; Pesole et al. 1999; Blier et al. 2001; Johnson et al. 2003; Burton et al. 2007). These properties have placed the mtDNA at the center of a broad array of evolutionary studies and have led to the suggestion that selection on mtDNA might lead to haplotype-specific adaptation to specific environments (Blier et al. 2001; Fontanillas et al. 2005; Wallace 2007). However, the role of natural selection on the evolution of mtDNA remains elusive,

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principally because the specific relationships between mtDNA gene variation and potentially adapted function can be extremely complex to establish with traditional tools of molecular evolution. In this context, the direct study of functional properties of mitochondria with different mitochondrial haplotypes may help us to provide a robust link between the genotype and the phenotype.

We can predict that a subset of mtDNA mutations influence the functional properties of mitochondria in terms of maximal catalytic capacities of electron transport system (ETS, complexes I-IV) enzymes. Studies on humans have demonstrated that specific mtDNA mutations are associated with increased longevity (Niemi et al. 2003; Ross et al. 2003; Dato et al. 2004), neurodegenerative disease susceptibility (Wallace 1994; Torroni et al. 1997; Ross et al. 2003; van der Walt et al. 2003), sperm motility (Ruiz-Pesini et al. 2000; Montiel-Sosa et al. 2006), sprint performance (Niemi and Majamaa 2005), and possibly climate adaptation (Ruiz-Pesini et al. 2004; Wallace 2005; 2010; Amo and Brand 2007). Moreover, multiple naturally occurring genetic mtDNA variants in different species that have been discovered over the last few years are associated with susceptibility to certain complex disorders, or with tissue-specific alteration in mitochondrial activity (Moreno-Loshuertos et al. 2006). In combination, these data suggest that functional mtDNA variation within a species may influence ATP production efficiency, reactive oxygen species production, and heat generation.

Drosophila simulans could be an appropriate model to study functional specificity of mtDNA. This species exhibits three different mitochondrial haplotypes (*si*I, *si*II, and *si*III) with nearly 3% interhaplogroup divergence (Ballard 2000a,b, 2005) but lacks subdivision at any nuclear-encoded loci tested to date (Ballard 2000a; Katewa and Ballard 2007; Melvin et al. 2008). The *si*II and *si*III populations live in sympatry in Kenya where the frequency of the *si*III haplogroup is about 40% (Ballard 2004), suggesting that there is the potential for random nuclear gene flow. In four wild-caught *si*II and four *si*III fly lines, low amino acid variation was observed within haplogroups in a 4.5-kb region spanning from position 1450 to position 5983 of the mtDNA genome. In contrast, large divergences were noticed between the haplogroups (0.02% nucleotide divergence, 0.012% amino acid divergence in the same region) (Ballard et al. 2007).

In a previous study, we used an in situ approach to evaluate the mitochondrial respiration of *D. simulans si*II and *si*III in permeabilized fibers (Pichaud et al. 2011). We measured higher catalytic capacities of ETS at 24°C for the *si*II haplotype when compared to the *si*III haplotype. However, we could not conclude that the observed difference resided entirely at the level of mtDNA as mito-nuclear interactions may also be involved. Indeed, different components of the ETS, either encoded by nuclear genome or mtDNA, should be coadapted to efficiently fulfill their task (Blier et al. 2001; Rand et al., 2004). To investigate if divergences in mtDNA are associated with differences in mitochondrial properties, we employ backcrossing of two populations harboring divergent mitochondrial haplotypes but sharing the same nuclear environment.

In this study, we compared mitochondrial functions between *D. simulans* harboring two mitochondrial haplogroups in the same nuclear background. If the mtDNA dictates the metabolic differences between the two sets of fly lines, we should observe the same set differences in the functional properties of mitochondria when *si*II-introgressed and *si*III-controls are compared with native *si*II and native *si*III flies. Specifically, we predict higher catalytic capacities of ETS for the *si*II-harboring flies (Pichaud et al. 2011). If, on the other hand, the *si*II and *si*III mtDNA are specifically coadapted with their native nuclear genomes, we should detect a disruption of the catalytic capacities in *si*II-introgressed flies due to alteration of mito-nuclear coadaptations.

Methods

FLY LINES

Eight native lines of *D. simulans* (having *si*II and *si*III haplogroups) collected in Kenya were used for experiments. For each haplogroup, four isofemale lines (namely 2KY0412, 2KY0415, 2KY0418, and 2KY0421 for *si*II; 3KY0410, 3KY0412, 3KY0414, and 3KY0420 for *si*III) were reared from flies collected in Nairobi (Kenya) during November 2004 (Ballard et al. 2007). No nuclear subdivision was detected at any nuclear-encoded loci tested among these lines (Ballard 2000a; Dean et al. 2003; Katewa and Ballard 2007; Melvin et al. 2008).

This study aims to determine whether the previously observed differences in mitochondrial functions between *D. simulans* harboring two mitochondrial haplogroups result from differences in the mtDNA (*si*II > *si*III) or specific mito-nuclear interactions (Pichaud et al. 2011). The goal of the genetic crosses was to place *si*II and *si*III mtDNA in the nuclear genetic background from flies that natively harbored *si*III mtDNA. This strategy was considered conservative because it would disrupt any coevolved interactions between *si*II mtDNA and its wild-type nuclear genetic background should they exist (*si*II-introgressed). It would not, however, disturb coevolved genetic interactions between *si*III mtDNA and its native nuclear genetic background (*si*III-control). To examine the possible influence of haplogroup on mitochondrial metabolism, four crossing pairs (CP1, CP2, CP3, and CP4) were constructed in parallel (Fig. 1). Each pair includes a *si*II-introgressed line and a *si*III-control line. To decrease the potential for interindividual variation between the nuclear DNA of the different isofemale lines, F8 individuals resulting from the four independently disrupted *si*II-introgressed lines and F'8 flies from the four *si*III-controls were pooled for subsequent experiments (Fig. 1).

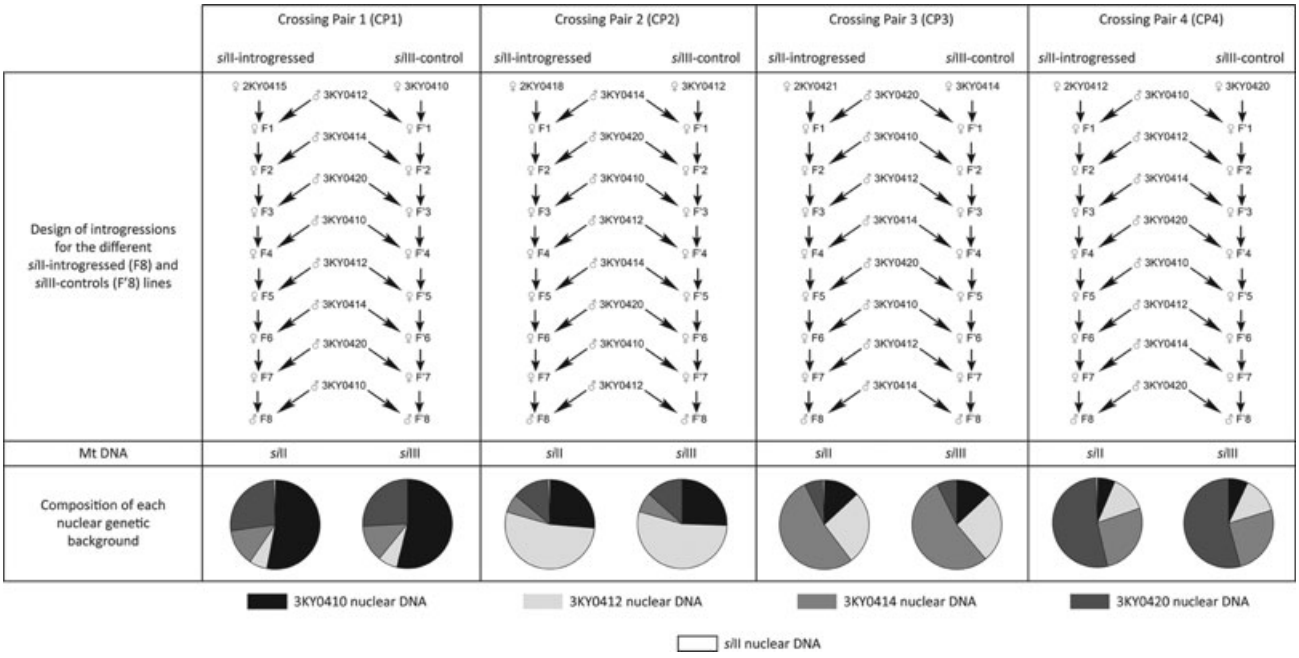

Figure 1. Crossing scheme of the four pairs (CP1, CP2, CP3, and CP4) of *si*II-introgressed and *si*III-control lines. Theoretically, each introgressed line (F8) has a mix of four different *si*III nuclear DNAs (3KY0410, 3KY0412, 3KY0414, and 3KY0420) with different proportions (53.1%, 26.6%, 13.3%, and 6.6%) and a small amount of *si*II nuclear DNA (0.4%). Each control line (F'8) has a mix of four different *si*III nuclear DNAs (3KY0410, 3KY0412, 3KY0414, and 3KY0420) with different proportions (53.9%, 25.8%, 13.3%, and 7%). For each experiment, equal number of flies from each *si*II-introgressed and *si*III-control lines were pooled. This crossing strategy reintroduces the nuclear DNA of the parental female in F'8 and may reconstruct coevolved genetic interactions between *si*III mtDNA and its native nuclear background.

The crossing strategy was performed to minimize inbreeding depression. Within a crossing pair, *si*II-introgressed lines were constructed by mating 15 *si*II virgin females of the four isofemale lines (2KY0415, 2KY0418, 2KY0421, and 2KY0412 for CP1, CP2, CP3, and CP4, respectively) with 30 *si*III males for each of the four isofemale lines (3KY0412, 3KY0414, 3KY0420, and 3KY0410 for CP1, CP2, CP3, and CP4, respectively). After three days, adult flies were removed and virgin female progeny (F1) was backcrossed with *si*III males (Fig. 1). This was repeated for eight generations using an alternate paternal line at each generation (each of the four isofemale line was used twice), and F8 males were collected for experiments. This backcrossing allows maintenance of maternally inherited mtDNA while increasing the proportion of paternal nuclear DNA. Theoretically, this procedure enables a replacement of more than 99.6% of nuclear DNA to obtain *si*II mtDNA within *si*III nuclear DNA environment. Moreover, it decreases the possible inbreeding effect while minimizing the line-specific effect. The *si*III-control lines were developed using a parallel procedure (Fig. 1). A limitation of the crossing strategy is that it reintroduced the nuclear DNA of the parental *si*III female in F'8 and may bias the functional properties of mitochondria in favor of *si*III phenotype. Again, this crossing strategy is conservative as our hypothesis is the properties of mitochondria harboring *si*II mtDNA are divergent from that harboring *si*III mtDNA.

To avoid fitness problems associated with aging, each backcross was performed with flies less than 14 days old. All lines were maintained at 24°C in two different incubators (A and B) to allow replicates of experimental lines and to avoid any "incubator" effect. The pools of *si*II-introgressed and of *si*III-control individuals were weighted before fiber permeabilizations.

At the beginning and at the end of the study, the mtDNA type of each constructed line was determined using allele-specific PCR (Dean et al. 2003). The males used for each backcross were also verified for mtDNA type during the eight generations. The absence of *Wolbachia* was verified using conserved 16S rDNA primers (James and Ballard 2000).

PREPARATION OF PERMEABILIZED MUSCLE FIBERS

Fiber permeabilizations were conducted at 4°C with flight muscles from four thoraxes of 10-day-old males *D. simulans* as previously described (Pichaud et al. 2011). Briefly, fibers were permeabilized using a BIOPS-relaxing solution (Veksler et al. 1987; Letellier et al. 1992) complemented with 81.25 $\mu g.mL^{-1}$ saponin (Pichaud et al. 2011) and were transferred into a respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) filled with air-saturated respiration medium.

HIGH-RESOLUTION RESPIROMETRY

Respiration was measured at 24°C, and data acquisition and analysis were performed using the software DatLab (Oroboros Instruments). State 2 was reached after the transfer of the fibers bundles into the respiration chambers filled with respiration medium in presence of pyruvate (10 mM), malate (10 mM), and L-proline (10 mM). The following substrates or uncouplers were then sequentially added to the chamber: ADP (5 mM, to achieve state 3 respiration for complex I, CI), cytochrome *c* from equine heart (10 μM, as an index of functional integrity of the outer mitochondrial membrane, CIc), succinate (10 mM, to reach state 3 respiration for complex I + complex II, CIc + CII), *sn* glycerol-3-phosphate (20 mM, to achieve state 3 respiration for complex I + complex II + glycerol-3-phosphate dehydrogenase, Cic + CII + G3Pdh), 2,4-dinitrophenol (uncoupler, optimum concentration between 5 to 15 μM to reach maximum respiration rate, CIc + CII + G3Pdh + u), rotenone (0.5 μM, inhibitor of complex I, CII + G3Pdh + u), malonate (5 mM, inhibitor of complex II, G3Pdh + u), antimycin A (2.5 μM, inhibitor of complex III, residual oxygen consumption reached after inhibition of complexes I, II, and III), TMPD + ascorbate (0.5 mM and 2 mM, respectively, to measure complex IV activity, COX). This protocol allowed us to calculate the respiratory control ratios (RCR = CI/state 2) for complex I, the cytochrome *c* effect (CIc/CI), as well as the uncoupling control ratios (UCR = CIc + CII + G3Pdh + u/CIc + CII + G3Pdh) with seven fibers preparations for *si*II-introgressed from each incubator and five for *si*III-controls from each incubator. We also measured the respiration rates for CI, CIc, CIc + CII, CIc + CII + G3Pdh, CIc + CII + G3Pdh + u, CII + G3Pdh + u, G3Pdh + u, and for COX with four fibers preparations for *si*II-introgressed from each incubator and three for *si*III-controls from each incubator (Pichaud et al. 2011). Data were expressed as mean respiration rates in pmol of oxygen consumed per second per mg proteins ± SEM corrected with residual oxygen consumption (when complexes I, II, and III were inhibited).

PROTEIN CONTENT

At the end of mitochondrial respiration measurements, fiber bundles were removed and homogenized with a Tekmar homogenizer (Tekmar Company, Cincinnati, OH) and the homogenates were immediately frozen at –80°C for further analyses. Total protein content was determined for homogenates from fiber bundles in duplicate by the bicinchoninic acid method (Smith et al. 1985). Due to addition of cytochrome *c* during experiments as well as the presence of bovine serum albumin in the ice-cold buffer and in the respiration medium, the protein content of the buffer was subtracted from the fibers preparations.

CHEMICALS

Chemicals were purchased from Sigma-Aldrich (St Louis, MO).

Table 1. Values of respiratory control ratio, uncoupling control ratio, and cytochrome *c* effect in permeabilized fibers of *Drosophila simulans* (± SEM).

	*si*II-introgressed	*si*III-controls
Respiratory control ratio	4.97±0.58	5.04±1.02
Uncoupling control ratio	1.03±0.01	1.01±0.01
Cytochrome *c* effect	1.03±0.01	1.05±0.01

STATISTICAL ANALYSES

Statistical analyses were performed with SAS software (9.1.3, SAS Institute, Cary, IN). O'Brien's test was used to verify homogeneity of data. Analyses of variance (ANOVAs) with two independent variables (haplogroup, incubator) and their interaction were performed using a general linear model (GLM) procedure with the Least Square Means method. When an effect was detected, multiple comparisons (post-hoc Tukey's HSD) test was performed. Significance was defined at $P < 0.05$.

Results

First, we tested whether the flies from the two groups (*si*II-introgressed and *si*III-controls) differed in mass or whether there was any incubator effect. The resulting pools were not different for means mass ± SD between haplogroup or between incubators (0.67 ± 0.1 mg for *si*II-introgressed incubator A; 0.66 ± 0.08 mg for *si*II-introgressed incubator B; 0.65 ± 0.09 mg for *si*III-controls incubator A; 0.65 ± 0.08 mg for *si*III-controls incubator B).

RCRs from 14 and 10 different preparations for *si*II-introgressed and *si*III-control, respectively (seven and five preparations per incubator), were used as an index of functional integrity of mitochondria and showed well-coupled respiration (Table 1). Nested ANOVA showed that neither haplogroup ($F_{1,20} = 0.02$, $P = 0.90$) nor incubator ($F_{1,20} = 0.03$, $P = 0.86$) or their interaction ($F_{1,20} = 0.25$, $P = 0.62$; for haplogroup × incubator) influenced the RCR. No significant differences between haplogroups or between incubators were detected with the Least Square Means method.

To assess mitochondrial integrity, O_2 flux was compared before and after cytochrome *c* injection. Consistent with functional integrity of outer mitochondrial membrane, ratios of CIc/CI showed little effect of cytochrome *c* addition on the mitochondrial respiration (Table 1).

O_2 fluxes were also compared before and after the addition of an uncoupler to see if the ATP synthase and the adenine nucleotide translocator (ANT) exerted a limitation on mitochondrial respiration. As previously reported (Pichaud et al 2011), all UCRs were close to 1.0 (Table 1) showing that ATP synthesis and ANT capacity can support the maximum respiration rates induced by experimental substrates. This result suggests that

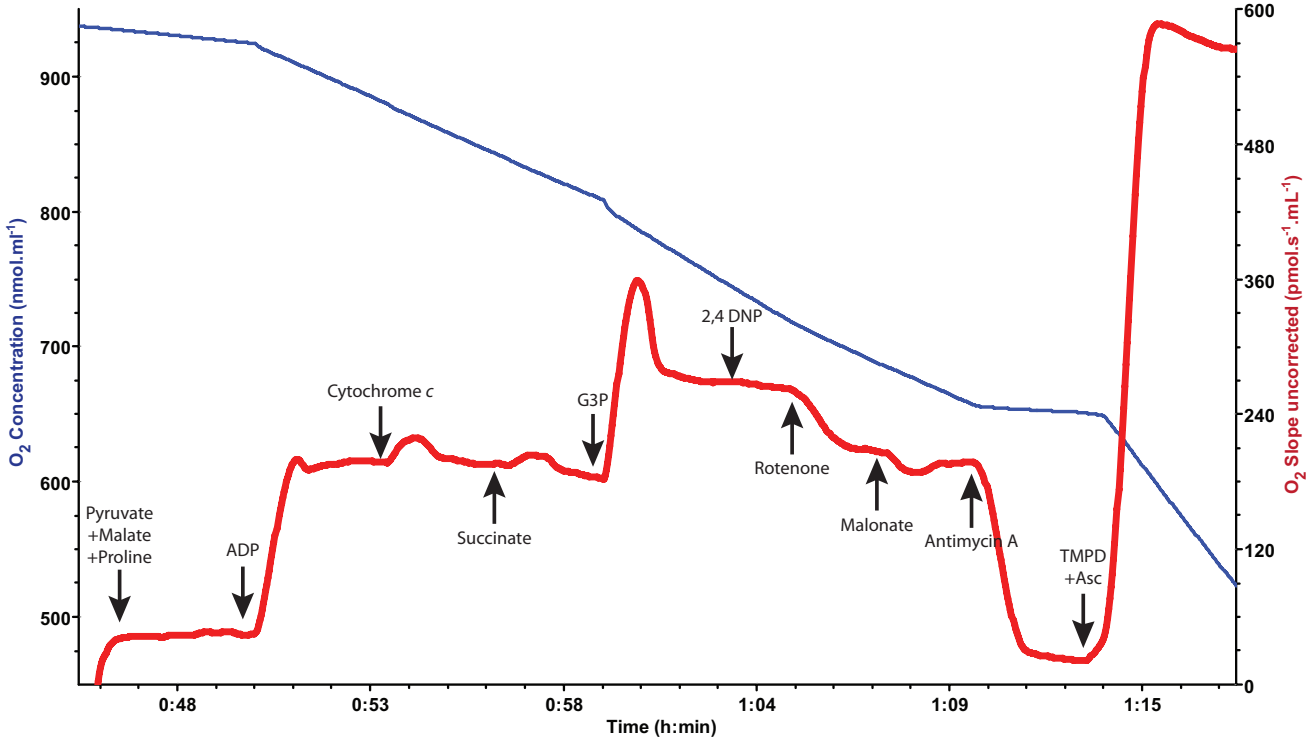

Figure 2. Oxygen concentration in nmol.mL^{-1} (blue) and oxygen consumption in pmol.sec^{-1}.mL^{-1} (red) measured at 24°C. Arrows denote injection of substrates for assessment of state 2 (Pyruvate + Malate + Proline), state 3 at the level of complex I (+ADP; CI), cytochrome *c* effect (+Cytochrome *c*; CIc), state 3 at the level of complex I + complex II (+Succinate; Cic + CII), state 3 at the level of complex I + complex II + glycerol-3-phosphate dehydrogenase (+Glycerol-3-phosphate; Cic + CII + G3Pdh), uncoupled respiration (+2,4-dinitrophenol; Cic + CII + G3Pdh + u), and sequential inhibition of complex I (+Rotenone; CII + G3Pdh + u), complex II (+Malonate; G3Pdh + u), complex III (+Antimycin A; residual oxygen consumption), and state 3 at the level of complex IV (+TMPD + Ascorbate; COX).

Table 2. Analyses of variance results—*F* ratios.

	Error df	Haplogroup df=1	Incubator df=1	Haplogroup× incubator df=1
Respiration rates				
CI	10	0.47	0.44	0.05
CIc	10	0.46	0.28	0.00
Cic+CII	10	5.26*	1.33	0.08
Cic+CII+G3Pdh	10	5.07*	0.60	0.89
Cic+CII+G3Pdh+u	10	6.10*	1.34	1.52
CII+G3Pdh+u	10	4.92	1.22	2.85
G3Pdh+u	10	4.92	1.60	3.51
COX	10	3.34	1.27	2.34

*$P < 0.05$.

CI and CIc, complex I (without and with cytochrome *c*, respectively); CII, complex II; G3Pdh: glycerol-3-phosphate dehydrogenase; u: uncoupled respiration; COX, cytochrome *c* oxidase.

when the ETS is "nearly saturated" with electrons from the three branches (CI, CII, and G3Pdh), both ATP synthesis and ANT capacity can support the maximum electron flux through ETS and consequently that the phosphorylation of ADP and the transport of ADP and/or ATP do not appear to be limiting steps.

A typical graph of O_2 concentration and uncorrected O_2 consumption (in pmol.sec^{-1}.mL^{-1}) measurements is presented in Figure 2. ANOVAs results showing *F* ratios are presented in Table 2. No parameter for the O_2 fluxes measured at the different steps of the ETS (CI, CIc, CIc + CII, CIc + CII + G3Pdh, CIc + CII + G3Pdh + u, CII + G3Pdh + u, G3Pdh + u, and COX) was influenced by incubator or by the interaction haplogroup × incubator (Table 2). However, the variable haplogroup influenced CIc + CII, CIc + CII + G3Pdh, and CIc + CII + G3Pdh + u (Table 2). Multiple comparisons (post-hoc Tukey's HSD test with eight and six different preparations for *si*II-introgressed and *si*III-controls, respectively) showed significant differences between haplogroups for CIc + CII, CIc + CII + G3Pdh, and CIc + CII + G3Pdh + u ($P = 0.04$, $P = 0.04$, and $P = 0.03$, respectively), with *si*II-introgressed having higher oxygen consumption than *si*III-controls (Fig. 3B).

The results we obtained in this study are broadly consistent with a previous study (Pichaud et al. 2011) performed at the same temperature on native *si*II and *si*III haplogroups (Fig. 3A). We therefore compared the results from the present study with those from the study on native haplogroups (Pichaud et al. 2011) using the Least Square Means method (Table S1). No significant

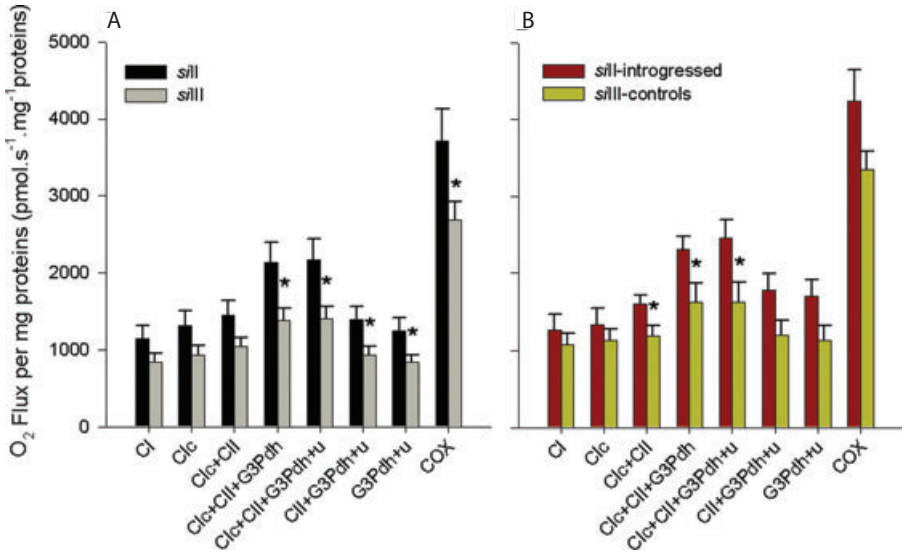

Figure 3. Mitochondrial functions measured at 24°C. (A) From the two native haplogroups *si*I and *si*III (Pichaud et al. 2011) and (B) from the two constructed lines *si*I-introgressed and *si*III-controls. O_2 fluxes are expressed in pmol.sec^{-1}.mg^{-1} proteins with Pyruvate + Malate + Proline + ADP (CI), +Cytochrome *c* (CIc), +Succinate (Cic + CII), +Glycerol-3-phosphate (Cic + CII + G3Pdh), +2,4-dinitrophenol (Cic + CII + G3Pdh + u), +Rotenone (CII + G3Pdh + u), +Malonate (G3Pdh + u), +Antimycin A (Rox) + TMPD + ascorbate (COX) and were corrected for residual oxygen consumption. Results are means ± SEM for 13 and 16 different fiber preparations for native *si*I and *si*III flies, respectively, and for eight and six fibers preparations for *si*I-introgressed and *si*III-controls flies, respectively. Significance was set as $P < 0.05$; *Differences between haplogroups.

differences were denoted between *si*III and *si*III-controls for all the respiration rates, and only G3Pdh + u was different between *si*II and *si*II-introgressed ($P = 0.049$).

Discussion

It is well established that mtDNA mutations may cause disease in humans (review in Taylor and Turnbull 2005). It is less well established how naturally occurring mutations could influence the phenotype and the biochemistry of mitochondrial bioenergetics. In this study, we investigated the influence of naturally occurring mutations in sympatric populations of *D. simulans* to shed light on the role of natural selection on the evolution of mtDNA. Globally, we observed similar patterns in the functional properties of mitochondria when *si*II-introgressed and *si*III-controls are compared with native *si*II and native *si*III flies. We did not detect any evidence that the disruption of coevolved mito-nuclear genotypes impaired the catalytic capacities of mitochondria in the experimental flies, when measured in the conditions described in the present study. Therefore, the most parsimonious explanation is that the measured mitochondrial functions divergences are mainly conferred by the mtDNA and not by the nuclear DNA or mito-nuclear interactions. To our knowledge, this is the first study that tests the effect of mtDNA differences on the catalytic capacities of the distinct complexes of the ETS with introgressed and wild-type mitochondrial genomes from closely related populations of the same species.

In this study, we used an in situ approach (on permeabilized fibers) to quantify O_2 consumption in thorax tissue (Pichaud et al. 2011). O_2 fluxes were measured at maximal state 3 with saturating concentrations of substrates to first identify the locus of divergences in terms of catalytic capacities of ETS enzymes in *si*II-introgressed and *si*III-controls populations. Significant differences in O_2 fluxes for CIc + CII, CIc + CII + G3Pdh, and CIc + CII + G3Pdh + u (as well as a clear overall pattern for the other parameters) were detected between introgressed and control organisms, with *si*II-introgressed having higher catalytic capacities than *si*III-controls (Fig. 3B). In a previous study, we detected similar differences between native *si*II and *si*III haplogroups (Pichaud et al. 2011) for CIc + CII + G3Pdh, CIc + CII + G3Pdh + u, CII + G3Pdh + u, G3Pdh + u, and COX (Fig. 3A).

Backcrossing has previously been used to study mito-nuclear interactions among different geographically isolated populations of *D. simulans* and of the marine copepod *Tigriopus californicus* (Edmans and Burton 1998; Willett and Burton 2001; Sackton et al. 2003; Willett and Burton 2004; Ellison and Burton 2006, 2007, 2008). Globally, these studies showed that in allopatric populations the disruption of mito-nuclear interactions led to a decrease in COX activity levels (Edmans and Burton 1998; Sackton et al. 2003), as well as in the activity of complexes I and III, and ATP production (Ellison and Burton 2006). In contrast, not only did we not detect any decrease in the activity of the ETS complexes in introgressed *D. simulans* but we measured

higher activity in that population. The *si*II and *si*III isofemale lines used for this study were collected in sympatry from Kenya and it is possible that gene flow between the populations may limit the fixation of nuclear-encoded mutations and prevent the establishment of coadapted mito-nuclear complexes. Another possibility would be that if there is a strong effect of coadaptation, then selection would act to keep the specific nuclear genes with coadapted mitochondria (Ellison and Burton 2010). When Ellison and Burton (2010) made recombinant inbred lines in *Tigriopus*, they found far fewer mismatches between mtDNA and the mitochondrial RNA polymerase than expected by chance, with mtDNA and mitochondrial RNA polymerase from the same line overrepresented in hybrid F_2 populations. In the present work, hitchhiking of nuclear genes with mitochondrial DNA introgression would have lead to the observed phenotype modification in the introgressed population. No evidences of subdivision in nuclear genes has, however, been detected between *si*II and *si*III.

The different isofemale lines used could also have accumulated unknown deleterious nuclear mutations that could influence the functional properties of mitochondria. However, this seems quite unlikely to have influenced our results. Within each pair, the *si*II-introgressed and *si*III-control lines were crossed with the four paternal lines in the same order (Fig. 1). Between pairs, *si*II-introgressed and *si*III-control lines were crossed with paternal lines in a different order such that the composition of the nuclear genetic background in the flies differed (Fig. 1). To balance the nuclear contribution, equal numbers of males from the four F8 *si*II-introgressed lines were pooled and equal numbers of males from the F'8 *si*III-controls were pooled in each experiment (Fig. 1).

The overall higher catalytic capacities of *si*II-introgressed can better be explained by a higher catalytic efficiency of the ETS complexes encoded by *si*II mitochondrial DNA. This could give an advantage to *si*II either in terms of intensity of aerobic activity, endurance, and/or lower production of reactive oxygen species (Pichaud et al. 2011) under laboratory conditions (i.e., when the resources are unlimited and when the temperature, the humidity, and the fly density are controlled). Indeed, it has been shown that *si*II flies outperform *si*III when resources are continuous and abundant whereas *si*III is favored when resources are discontinuous (Ballard et al. 2007), explaining the persistence of these two haplotypes in Kenya. Previously, we have shown that the different parts of ETS are differently affected by temperature change (Pichaud et al. 2010, 2011) supporting divergent functional properties of ETS between *si*II and *si*III. Further studies on the catalytic properties of ETS and mitochondrial metabolism at different temperatures are required to determine if the functional divergences have any adaptive value according to optimal temperature range of the flies.

Drosophila simulans is a well-developed model to study the influence of distinct mtDNA types on mitochondrial bioenergetics. The next development in our understanding of the influence of mtDNA on bioenergetics may be to relate these differences to the specific fitness associated to haplotypes. These studies have the potential to illustrate the adaptive value of mtDNA or of the coadapted mito-nuclear DNA complexes in a wide variety of species including humans.

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Supporting Information

The following supporting information is available for this article:

Table S1. Respiration rates (pmol.min^{-1}.mg^{-1} protein) comparisons between *si*II (Pichaud et al. 2011) and *si*II-introgressed (means ± SEM of 13 and eight different fiber preparations, respectively) and between *si*III (Pichaud et al. 2011) and *si*III-controls (means ± SEM of 16 and six different fiber preparations, respectively).

Supporting Information may be found in the online version of this article.

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