The Journal of Experimental Biology 213, 1665-1675

doi:10.1242/jeb.040261

Thermal sensitivity of mitochondrial metabolism in two distinct mitotypes of *Drosophila simulans*: evaluation of mitochondrial plasticity

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Accepted 28 January 2010

SUMMARY

The overall aim of this study was to (1) evaluate the adaptive value of mitochondrial DNA by comparing mitochondrial performance in populations possessing different haplotypes and distribution, and to (2) evaluate the sensitivity of different enzymes of the electron transport system (ETS) during temperature-induced changes. We measured the impact of temperature of mitochondrial respiration and several key enzymes of mitochondrial metabolism in two mitotypes (*si*I and *si*III) of *Drosophila simulans*. The temperature dependencies of oxygen consumption for mitochondria isolated from flight muscle was assessed with complex I substrates (pyruvate + malate + proline) and with *sn* glycerol-3-phosphate (to reduce complex III *via* glycerophosphate dehydrogenase) in both coupled and uncoupled states. Activities of citrate synthase, cytochrome *c* oxidase (COX), catalase and aconitase, and the excess capacity of COX at high convergent pathway flux were also measured as a function of temperature. Overall, our results showed that functional differences between the two mitotypes are few. Results suggest that differences between the two mitotypes could hardly explain the temperature-specific differences measured in mitochondria performances. It suggests that some other factor(s) may be driving the maintenance of mitotypes. We also show that the different enzymes of the ETS have different thermal sensitivities. The catalytic capacities of these enzymes vary with temperature changes, and the corresponding involvement of the different steps on mitochondrial regulation probably varies with temperature. For example, the excess COX capacity is low, even non-existent, at high and intermediate temperatures (18°C, 24°C and 28°C) whereas it is quite high at a lower temperature (12°C), suggesting release of respiration control by COX at low temperature.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/10/1665/DC1

Key words: *Drosophila simulans*, metabolism, mitochondrial DNA, mitochondrial respiration, temperature, thermal sensitivity.

INTRODUCTION

Mitochondrial DNA (mtDNA) encodes 13 key peptides of the mitochondrial electron transport system (ETS), and it is therefore suspected to partly evolve to adjust oxidative phosphorylation (OXPHOS) to environmental conditions of the organism (Das, 2006). For example, mtDNA has been suggested to influence the ability to acclimate to different thermal environments (Blier et al., 2001; Fontanillas et al., 2005), and the thermal sensitivity of mitochondrial complexes could therefore be of major significance in the impairment of the functional properties of mitochondria during temperature changes in ectotherms (Blier and Lemieux, 2001). A good strategy to assess the adaptive value of mtDNA is therefore to compare performances of mitochondria from populations characterised by different haplotypes and contrasting ecology or distribution.

Drosophila simulans, an ectothermic and eurythermic species, was recently proposed as a new model to study the adaptive value of mtDNA; it harbours three geographically distinct, subdivided haplogroups (*si*I, *si*II, *si*III) with nearly 3% interhaplogroup divergence but less than 0.06% intrahaplogroup diversity (Ballard, 2000a; Ballard, 2005). Differences in the functional properties of mitochondrial ETS have been detected between these three lines of *Drosophila* with distinct mtDNAs, and one of these (*si*II) has been proposed to have a selective advantage (Ballard, 2005; Katewa et al., 2007). These differences have been considered as strong support of mitochondrial genome adaptation.

In ectothermic species, acclimation or acclimatization to low temperature usually increases the activity of enzymes in the oxidative pathway and the mitochondrial volume density (Guderley, 2004). A metabolic limitation at the level of mitochondrial catalytic capacity is thought to be the cause of these responses (for reviews, see Pörtner, 2002; Guderley, 2004; Blier et al., 2006). Moreover, metabolic rates of *Drosophila melanogaster* and *D. simulans* coming from populations evolving in low-temperature environments are higher than those from populations evolving at higher temperatures (Watada et al., 1986; Berrigan, 1997; Berrigan and Partridge, 1997). This suggests qualitative or quantitative mitochondrial adaptations in these populations, probably resulting from catalytic or regulatory limitations at low temperatures. Chamberlin, in a study on tobacco hornworm (*Manduca sexta*) midgut mitochondria, tried to identify the steps of the OXPHOS process that drive the changes in mitochondrial respiration during temperature variation (Chamberlin, 2004). These results showed that the ‘substrate oxidation system’ (including tricarboxylic acid cycle,

electron transport chain and metabolite transporters), which produces the proton-motive force, confers most of the control over mitochondrial respiration during temperature declines. However, Chamberlin's study was not able to determine what individual reaction within the subsystems exerts this control (Chamberlin, 2004). According to Blier and Lemieux, cytochrome *c* oxidase (COX, complex IV) in trout has a thermal response that is similar to that of mitochondrial respiration (Blier and Lemieux, 2001). COX was, however, generally found in excess in mitochondria and this excess compared with the aerobic mitochondrial capacity is a basis for the high oxygen affinity of the respiration chain (Gnaiger et al., 1998). In this context, thermally induced changes in COX activity would have little effect on the maximal respiration rate while still acting on regulation of respiration mainly through impact upon the redox state of ETS (Blier and Lemieux, 2001).

However, the control of OXPHOS and regulation of mitochondrial respiration is highly complex and other parameters of the system may be influenced by temperature. The oxygen property to diffuse, its availability and its capacity to act as the terminal electron acceptor in the mitochondrial chain for energy production are also affected by temperature; at low temperatures, the aerobic capacity of mitochondria may impose a limit to metabolic capacity whereas at high temperatures, excessive oxygen demand would cause an uncompensated decrease in oxygen levels in body fluids (Pörtner, 2002). At lower temperature, mitochondria probably require a greater availability for ADP to reach a given relative activity than they do at higher temperature because reduced temperatures appeared to decrease mitochondrial sensitivity to control by ADP availability (Blier and Guderley, 1993).

Temperature alterations can also lead to the formation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (Abele et al., 1998). These reactive molecules can oxidise DNA, proteins and lipids (Barja and Herrero, 2000; Das et al., 2001; Bokov et al., 2004), and lead to an oxidative stress when antioxidant enzymes [catalase (CAT), glutathione peroxidase and superoxide dismutase] and other non-enzymatic antioxidant compounds are overwhelmed (Beckman and Ames, 1998). These species arise largely, but not exclusively, from the mitochondria (Abele et al., 2002; Keller et al., 2004), which are known as the major ROS producers (Sastre et al., 2000). Unlike the nuclear DNA, histone proteins do not protect mtDNA and there is evidence that mtDNA exhibits more oxidative damage than nuclear DNA (Wei et al., 1998; Barja and Herrero, 2000) because of its closer proximity to ROS production sites and its limited repair mechanisms (Bogenhagen, 1999).

In *Drosophila*, ROS are mostly generated by complex I (NADH dehydrogenase), complex III (cytochrome *bc*) (both encoded by mtDNA and nuclear DNA) and by glycerol-3-phosphate dehydrogenase (Chen et al., 2003; Miwa et al., 2003; Andreyev et al., 2005). Katewa et al. showed that there were significant differences between two mitotypes of *D. simulans* (*si*II and *si*III) for mitochondrial H_2O_2 production but also for oxygen consumption, COX activity and ATPase activity (Katewa et al., 2007).

Our study is aimed at identifying differences in mitochondrial performance and thermal sensitivity associated with haplotype divergences in the *si*II and *si*III mitotypes of *D. simulans*. Different parameters of the mitochondrial metabolism were estimated at 12°C, 18°C, 24°C [24°C is considered as an optimal temperature for *Drosophila* species (David, 1988)] and 28°C. The thermal sensitivity of mitochondrial respiration was measured with pyruvate, malate and L-proline as substrates to reduce complex I and with *sn* glycerol-3-phosphate to reduce complex III *via* glycerol-3-phosphate dehydrogenase. We also determined the thermal sensitivity of an enzyme of the tricarboxylic acid cycle (citrate synthase, CS), as well as that of COX activity. Thermal sensitivity of uncoupled respiration [with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, FCCP] and of OXPHOS (ADP/O ratios) as well as that of the excess capacity of COX at high convergent pathway flux (with pyruvate + malate + proline + *sn* glycerol-3-phosphate) using inhibitor titration experiments were determined in order to compare overall mitochondrial performances at different temperatures of both mitotypes. Finally, enzyme activities of CAT (antioxidant enzyme), aconitase (ACO, tricarboxylic acid cycle) and levels of thiobarbituric reactive substances (TBARS, markers of lipid oxidative damages) were measured to evaluate the antioxidant capacity.

MATERIALS AND METHODS

Fly lines

Two mitotypes of *Drosophila simulans* (Sturtevant 1919) were used during experiments: *si*II and *si*III mitotypes, both from Kenya. In each mitotype, 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 as described previously (Ballard et al., 2007). Within the haplotype groups (four *si*II and four *si*III fly lines) there was low amino acid variation within a 4.5 kb region spanning from position 1450 to position 5983 of the mtDNA genome while significant divergences were noticed (2.04×10^{-2}% nucleotide divergences, 1.21×10^{-2}% amino acid divergences) between the haplotypes (see supplementary material Table S1) (Ballard, 2000b). This region contains three protein-coding loci of complex IV (COI, COII and COIII) and the two of complex V (ATP6 and ATP8) (Katewa et al., 2007). Moreover, many studies have not been able to detect significant nuclear divergences between the two haplotypes (Ballard, 2000a; Ballard et al., 2002; Dean et al., 2003).

Flies were fed with standard cornmeal medium, containing a mixture of 10 g of agar–agar, 12 g of sugar, 54 g of dried yeast and 106 g of cornmeal flour dissolved in 2 l of tap water. Propionic acid (8 ml) and methyl-4-hydroxybenzoate 10% (w/v) in ethanol (32 ml) were added to the mixture to avoid mite and mould contamination. All *Drosophila* lines were maintained at constant density (100 flies for approximately 25 ml of standard cornmeal medium), temperature (24.0±0.1°C), humidity (50% RH) and diurnal cycle (12 h:12 h light:dark). For all experiments, only males were selected because females have a large number of mitochondria in abdominal oocytes, which could bias experiment measurements. After being allowed to spawn in sterile recipients for two days, parents were removed and experimental flies were then sexed on ice two days after hatching and transferred to new recipients for an additional eight days prior to study. To avoid fitness problems due to aging, all chosen parents were less than 14 days old and all experimental male flies were 10 days old. Flies were reared in two incubators (A and B) at the same time to have replicates of experimental lines and avoid the 'incubator' effect. For each recipient of 100 male flies, 40 were used for mitochondrial isolation and others were immediately stored at –80°C for further enzymatic analysis within one month.

mtDNA was determined using allele-specific PCR as described previously in each isofemale line (Dean, 2003), and *Wolbachia* infection was verified using conserved 16S rDNA primers (James and Ballard, 2000). According to Ballard et al. there is only one nonsynonymous change within *si*II lines and one nonsynonymous change within *si*III lines over 5500 bp analysed (Ballard et al., 2007).

PCR assays showed that all isofemale lines belonged to the corresponding mtDNA haplotype and were not infected by *Wolbachia*.

Fly sampling

Due to the low intrahaplogroup variation (Katewa et al., 2007), we have been able to pool flies of each line from the two groups (*si*II and *si*III). The pooling strategy minimises individual differences caused by the nuclear genome of a particular fly line and enables us to detect more general differences that are due to the mtDNA in itself and/or of fixed nuclear differences that interact with each mtDNA haplogroup (Ballard, 2000a; Dean and Ballard, 2003; Katewa et al., 2007). Flies used for mitochondrial isolations were weighed as a pool of the four isofemale lines and from each mitotype while those used for enzymatic analyses were weighed individually with an electrobalance (Mettler-Toledo Inc., Columbus, OH, USA) with a resolution of 0.1 µg. All flies used for this study were reared in the same conditions, and males were weighed at 10 days of age. No mass differences were observed between the different pools of 40 flies (0.754±0.054 mg for *si*II incubator A; 0.751±0.052 mg for *si*II incubator B; 0.748±0.052 mg for *si*III incubator A; 0.751±0.052 mg for *si*III incubator B) or between flies of the two mitotypes weighed individually (0.670–0.831 mg for *si*II both A and B incubators; 0.674–0.839 mg for *si*III both A and B incubators).

Isolation of intact mitochondria

Intact mitochondria were isolated as previously described by Katewa et al. (Katewa et al., 2007). Briefly, flight muscle mitochondria were isolated from 160 thoraxes (40 of each line of each mtDNA type) of 10-day-old male *D. simulans*. All steps were done at 4°C. Thoraxes were separated from abdomens and heads and placed in 400 µl of isolation buffer containing 250 mmol l^{-1} sucrose, 5 mmol l^{-1} Tris, 2 mmol l^{-1} EGTA, 0.1% bovine serum albumin (BSA) (w/v), pH 7.4. Thoraxes were then homogenised with a Kontes polypropylene pellet pestle (Kimbal Kontes, Vineland, NJ, USA). The pestle was pressed firmly to the bottom avoiding any grinding action. Thoraxes were pressed 120 times to produce a smooth, grey homogenate. Homogenates were filtered through gauze pad and the volume was raised to 2.1 ml with isolation buffer. After a first 3 min centrifugation at 300 ***g***, the supernatant was refiltered through gauze (approximately 1.8 ml final volume) and recentrifuged for 10 min at 9000 ***g***. The supernatant was then discarded and the pellet was washed two times in 400 µl of isolation buffer before being resuspended in 120 µl of the same buffer. The mitochondrial preparations were stored on ice and used within 2 h of isolation for mitochondrial oxygen measurements, inhibitor titration experiments and ACO activity. Integrity of mitochondrial preparations was verified in preliminary experiments with cytochrome *c* injection during the respiration state 2 (state 2c). The ratio between state 2 and state 2c measured at 24°C with seven different mitochondrial preparations gave 0.971±0.061, which confirms high quality of preparation.

Mitochondrial respiration

Mitochondrial oxygen consumption was measured at four different temperatures (12°C, 18°C, 24°C and 28°C) using an oxygraph Oxyg 32 (Hansatech Instruments Ltd, Pentney, Norfolk, UK) connected to a circulating water bath. Measurements were done in a final volume of 500 µl at each temperature with 15 µl of mitochondrial preparation incubated in 485 µl of mitochondrial respiration medium containing 120 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} Hepes, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} $MgCl_2$ and 0.2% BSA (w/v), pH 7.2. The polarographic system was calibrated with nanopure water saturated with oxygen at the incubation temperature.

Complex I and III

Pyruvate (10 mmol l^{-1}), L-proline (10 mmol l^{-1}, pH 7.2) and malate (10 mmol l^{-1}, pH 7.2) were added to the chambers to provide substrates for complex I reduction and to achieve state 2 respiration. *Sn* glycerol-3-phosphate (20 mmol l^{-1}, pH 7.2) was used as a substrate to supply electrons to the ubiquinone pool *via* glycerol-3-phosphate dehydrogenase in order to reduce complex III.

ADP (100 µmol l^{-1}, pH 7.2) supplemented with $MgCl_2$ (0.6 mol mol^{-1} ADP to keep $[Mg^{2+}]$ constant during respiration measurement) was added to reach state 3 respiration and state 4 was obtained after exhaustion of ADP. These states were used to calculate ADP/O ratios of complex I and complex III (as an index of mitochondrial efficiency). After first exhaustion, ADP (400 µmol l^{-1}, pH 7.2) was introduced again to achieve maximum active state 3 (state 3m) and state 4o was measured after the addition of oligomycin (2 µg ml^{-1}), which inhibits mitochondrial ATPase and phosphoryl group transfer. According to our preliminary tests, this concentration is higher than required to fully inhibit ATPase in mitochondria that have already reached state 4. Respiratory control ratios (RCRs) were calculated as state 3m/state 4o (as an index of functional integrity of mitochondria). FCCP was then added by steps of 0.1 µmol l^{-1} and uncoupled control ratio (UCR) was measured as state U/state 3m, with state U representing the maximum uncoupled respiration rate (reached between 0.2 µmol l^{-1} and 0.5 µmol l^{-1} of FCCP) to evaluate metabolic flux control by the phosphorylation system over the electron transport capacity.

Azide titration experiment

Following the first experiment, mitochondrial isolations were used to measure oxygen consumption in the presence of pyruvate (10 mmol l^{-1}), L-proline (10 mmol l^{-1}, pH 7.2), malate (10 mmol l^{-1}, pH 7.2) and *sn* glycerol-3-phosphate (20 mmol l^{-1}, pH 7.2) ensuring high metabolic flux through the ETS. After addition of ADP (800 µmol l^{-1}, pH 7.2, this concentration ensured excess ADP during the entire experimental period and the state 3m reached was not different than with 400 µmol l^{-1}), the following steps of azide concentrations were introduced at appropriate intervals to progressively inhibit complex IV (1, 2, 7, 12, 32, 52, 102, 202, 702, 1202, 1702 and 2202 µmol l^{-1}) or until maximum inhibition was achieved. The inhibition constant (K_i) was calculated from transformed data using the Dickson linearisation (Kuznetsov et al., 1996). As azide is a non-competitive inhibitor, the flux control coefficient (C_i) was calculated using the following equation:

$$C_i = -(dJ / J) / (dI / K_i), \quad (1)$$

where J is the respiration flux, dJ is the decrement of respiration flux caused by increment of inhibitor addition dI (between 0 µmol l^{-1} and 80 µmol l^{-1}) and K_i is the inhibition constant for sodium azide (Kuznetsov et al., 1996).

These steps were repeated for each mitochondrial preparation. Extraction and mitochondrial preparations were performed on five different pools of flies for each incubator, at each temperature and for each mitotype (total of 80 mitochondrial isolations). Measurements were averaged and respiration rates are expressed in nmol of oxygen consumed per minute per mg of mitochondrial protein.

Enzymatic analyses and oxidative stress markers

ACO, CS, CAT activities and protein content were measured using a UV/VIS spectrophotometer (Perkin Elmer, Lambda 11, Foster City, CA, USA), and TBARS levels were measured using a fluorescence spectrophotometer (F-2500 from Hitachi High Technologies America, Shaumburg, IL, USA), both equipped with a thermostated cell holder and a circulating refrigerated water bath. COX activity was measured polarographically with the same oxygraph as for mitochondrial oxygen consumption. Each measurement was done in duplicate, and for each incubator, at each temperature and for each mitotype, five different analyses were averaged.

ACO was measured on fresh mitochondria. The remaining mitochondria were then frozen at –80°C for further CS analysis and protein content measurement.

For CAT and protein analysis, 12 flies (three from each isofemale line of each mitotype) were homogenised in 600 µl of ice-cold buffer [100 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EDTA, 0.1% (v/v) Triton X-100, pH 7.5]. In separate preparations, 16 flies (four from each isofemale line of each mitotype) were homogenised in 400 µl of the same ice-cold buffer for malondialdehyde (MDA) assessment and in 400 µl of imidazole buffer [50 mmol l^{-1} imidazole, 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} EDTA, 0.09% (v/v) Triton X-100, pH 7.4] for COX activity measurement.

CS

Mitochondria (1 µl) were transferred in 100 mmol l^{-1} imidazole-HCl (pH 8), 0.1 mmol l^{-1} 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 mmol l^{-1} acetyl CoA and 0.15 mmol l^{-1} oxaloacetate. Activities were measured at the four different temperatures by following the increase in absorbance due to the reduction of DTNB at 412 nm (ε_{412}=13.6 ml cm^{-1} µmol^{-1}) (Thibeault et al., 1997).

ACO

Mitochondria (1 µl) were transferred in 50 mmol l^{-1} Tris-HCl, 0.1% Triton X-100 (v/v), 0.6 mmol l^{-1} $MnCl_2$, 5 mmol l^{-1} sodium citrate, 0.2 mmol l^{-1} NADP, 0.4 U ml^{-1} isocitrate dehydrogenase, pH 7.4. Activity was measured at the four temperatures by following the appearance of NADPH at 340 nm (ε_{340}=6.22 ml cm^{-1} µmol^{-1}) (Miwa and Brand, 2005).

ACO activity was normalised with CS activity and results are expressed in U (units) of ACO activity per U of CS activity in order to express the activity of ACO according to a marker of mitochondria quantity.

COX

The reaction medium for COX activity contained 130 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes, 10 mmol l^{-1} KH_2PO_4, 11 mmol l^{-1} $MgCl_2$ hexahydrate, 20 mmol l^{-1} glucose, 10 mmol l^{-1} ascorbic acid and 5% (w/v) BSA (Blier and Lemieux, 2001). After homogenates were centrifuged at 750 ***g*** for 5 min, 5 µl of the supernatant were incubated in respiration chambers with 995 µl of reactive medium. After addition of cytochrome *c* (150 µmol l^{-1}), azide was introduced step-by-step at concentrations described previously (see Azide titration experiment). This method was compared with the classic method of complex IV titration by azide using the same protein concentration in the chambers (Letellier et al., 1994; Villani and Attardi, 1997) with N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 1 mmol l^{-1}) and ascorbate (5 mmol l^{-1}) as substrates after inhibition of complex I and III with rotenone (0.5 µmol l^{-1}) and antimycin A (2.5 µmol l^{-1}), respectively, and activation by ADP (800 µmol l^{-1}) and cytochrome *c* (150 µmol l^{-1}) addition. Oxidation rates due to autoxidation of TMPD, ascorbate and cytochrome *c* were subtracted from the activity (Gnaiger and Kuznetsov, 2002). Results showed that there were no significant differences for K_i, for metabolic flux control C_i (Table 1), as well as for maximum velocity (V_{max}) (12.14±4.14 nmol min^{-1} ml^{-1} and 14.16±2.73 nmol min^{-1} ml^{-1} for *si*II and *si*III, respectively, for our method; 14.50±5.94 and 14.48±4.00 for *si*II and *si*III, respectively, for the TMPD/ascorbate method) between the two methods at 24°C when using the same protein concentration of homogenates or isolations, which allowed us to validate our protocol (*N*=5 in duplicate for both methods, Fig. 1A,B). We chose the first method because there were no differences between mitotypes in the weights of flies, it did not need any mitochondrial isolation, it required a lower number of flies and it allowed us to freeze the remaining flies. There were no significant differences between the two methods, and consequently we can consider that the membrane solubilisation using Triton X-100 for homogenates preparations was not different from using TMPD. COX activity was measured for the four temperatures and expressed as nmol of oxygen consumed per minute per mg protein.

CAT

After homogenates were centrifuged at 13,000 ***g*** for 3 min at 4°C, the supernatant was incubated with 100 mmol l^{-1} of sodium phosphate and 60 mmol l^{-1} of H_2O_2. Decrease in absorbance, corresponding to the decomposition rate of H_2O_2, was measured at the four temperatures and at a wavelength of 240 nm for 1 min (ε_{240}=43.6 ml cm^{-1} µmol^{-1}) (Orr and Sohal., 1992).

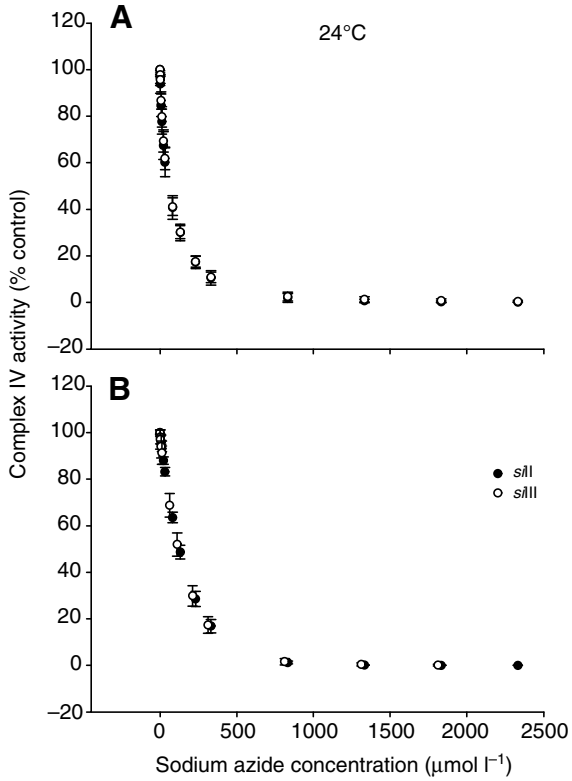

Fig. 1. Sodium azide inhibition of cytochrome *c* oxidase activity at 24°C using (A) the detergent-solubilised cytochrome *c* oxidase (complex IV) (COX) method performed on homogenates from whole flies or (B) the TMPD-Ascorbate method performed on mitochondrial isolations from thoraxes. Results are means ± s.d. for five mitochondrial preparations or crude homogenates.

MDA

MDA, the product of the peroxidation of fatty acids, was measured with a TBARS assay kit from Zeptometrix (Buffalo, NY, USA). This method relies on the fluorometric measurement of pink chromophor produced during the reaction of thiobarbituric acid with MDA. MDA contents were measured with light excitation set at 530 nm, emission set at 550 nm and width of 5 nm, and estimated following the manufacturer's protocol.

Protein content

Total protein content was determined both for mitochondria and homogenates in duplicate by the bicinchoninic acid method (Smith et al., 1985). As isolation buffer contained BSA, the protein due to the buffer was subtracted from the mitochondrial preparations.

Temperature coefficient values (Q_{10})

Q_{10} values for mitochondrial functions (state 3 and state 4 for both complexes I and III, COX activity and ACO/CS ratios) were calculated using the following formula:

$$Q_{10} = (\text{Rate 2} / \text{Rate 1})^{10/(t2-t1)}, \quad (2)$$

where Rate 1 is for the rate of the parameter measured at the temperature $t1$, and Rate 2 is the rate of the parameter measured at the temperature $t2$.

Chemicals

All chemicals were purchased from Sigma-Aldrich (Mississauga, ON, Canada) except for the TBARS determination assay kit from Zeptometrix.

Construction of biochemical threshold plots

An azide titration experiment of elevated pathway flux (with pyruvate, L-proline, malate, *sn* glycerol-3-phosphate and ADP), as well as azide titration experiment of COX activity allowed us to construct plots of relative respiration rate against the percentage of inhibition of COX activity at the same azide concentration (Letellier et al., 1994; Villani and Attardi, 1997). Measurements were done at the four temperatures and COX excess capacity was determined for each temperature as previously described.

Statistical analyses

All statistical analyses were performed with SAS software (9.1.3, SAS Institute, Cary, IN, USA). O'Brien's tests for homogeneity and analysis of variance (ANOVA) with three independent variables (incubator, temperature and mitotype) were performed using a general linear model (GLM) procedure with the least square means method for multiple comparisons tests. This allowed us to determine any interactions between the variables, and the effect of each variable on the different parameters measured. Significance was defined at $P<0.05$. Data are expressed as means of five different samples from each incubator, at each temperature, and for each mitotype measured in duplicate.

RESULTS

No differences between incubators were detected for all the parameters measured, consequently data from incubators A and B were pooled.

Mitochondrial respiration

State 3m

For both complexes, state 3m exhibited the same pattern between the mitotypes for 12°C, 18°C and 24°C (Fig. 2A,B) as well as among the temperatures for the two mitotypes. There were significant differences between the two mitotypes at 18°C ($P=0.0168$ for pyruvate + malate + proline and $P<0.0001$ for *sn* glycerol-3-phopshate), *si*II being higher than *si*III.

*si*II showed a slight increase from 12°C to 24°C, with significant differences between 12°C and 18°C (P-values ≤ 0.0201 for both complexes), and between 18°C and 24°C ($P<0.0001$ for both complexes). We detected a significant decrease when comparing 28°C with 24°C ($P<0.0001$ for both mitotypes). The state 3m of complex III markedly increased from 18°C to 24°C ($P<0.0001$) while staying similar at 24°C and 28°C (*si*II) or slightly increasing for *si*III ($P=0.0426$).

State 4o

*si*II had a significantly higher state 4o than *si*III at 18°C ($P\leq 0.0021$ for both complexes) and 24°C (P-values ≤ 0.0123 for both complexes), while at 12°C and 28°C, it was significantly higher for *si*III (P-values ≤ 0.0497) but only when substrates are provided to complex I (Fig. 2C,D).

We also observed significant differences between 12°C and 18°C (complex I, $P<0.0001$), 12°C and 24°C ($P<0.0001$ for both complexes), and between 18°C and 24°C (P-values ≤ 0.0011 for both complexes) in *si*II. At 28°C, a significant decrease was observed comparatively with 24°C for complex I in *si*II ($P=0.0002$).

In *si*III state 4o of complex I significantly increased from 12°C to 28°C while in complex III it decreased between 12°C and 18°C ($P=0.0106$) and increased between 18°C, 24°C and 28°C (Fig. 2C,D).

ADP/O ratios

When using substrates for complex I (Fig. 2E), there was a statistical difference between the two lines at 12°C, with a higher ADP/O ratio for the *si*II mitotype ($P=0.0003$). Among the temperatures, *si*II and *si*III exhibited significant differences, with a higher ADP/O ratio at 24°C compared with 12°C, 18°C and 28°C (all P-values ≤ 0.0002).

For complex III (Fig. 2F), the only significant difference between mitotypes occurred at 28°C, with *si*III being lower ($P=0.0093$). Among the temperatures, no significant differences were detected for *si*II while *si*III exhibited significant increases at 18°C and 24°C compared with 12°C (P-values ≤ 0.0054) and with 28°C (P-values ≤ 0.0026).

RCRs and UCRs

Results of RCRs are presented in Fig. 3. Comparisons between mitotypes showed that there were significantly higher RCRs for *si*II at 12°C at the complex I level ($P=0.0231$), as well as at 28°C at the complex III level ($P=0.0143$). Surprisingly, the RCR of *si*II was significantly lower than *si*III at 24°C ($P=0.0292$) when the substrates were provided to complex I.

In both mitotypes, RCRs of complex I were not different between 18°C and 28°C but both were significantly higher at 12°C (P-values ≤ 0.0309 for both mitotypes) and 24°C (P-values ≤ 0.0139 for both mitotypes). Moreover, RCR at 12°C was higher than at 24°C for *si*II ($P=0.0238$), while we observed the opposite trend for *si*III ($P=0.0043$). We observed slight but significant increases from 12°C to 28°C at the complex III level. Results of UCRs are shown in Table 2. No significant differences were detected in UCRs between strains or between temperatures.

Enzymatic measurements

No significant differences were detected between mitotypes (Fig. 4A) in ACO activity at any temperature, suggesting no differences in the

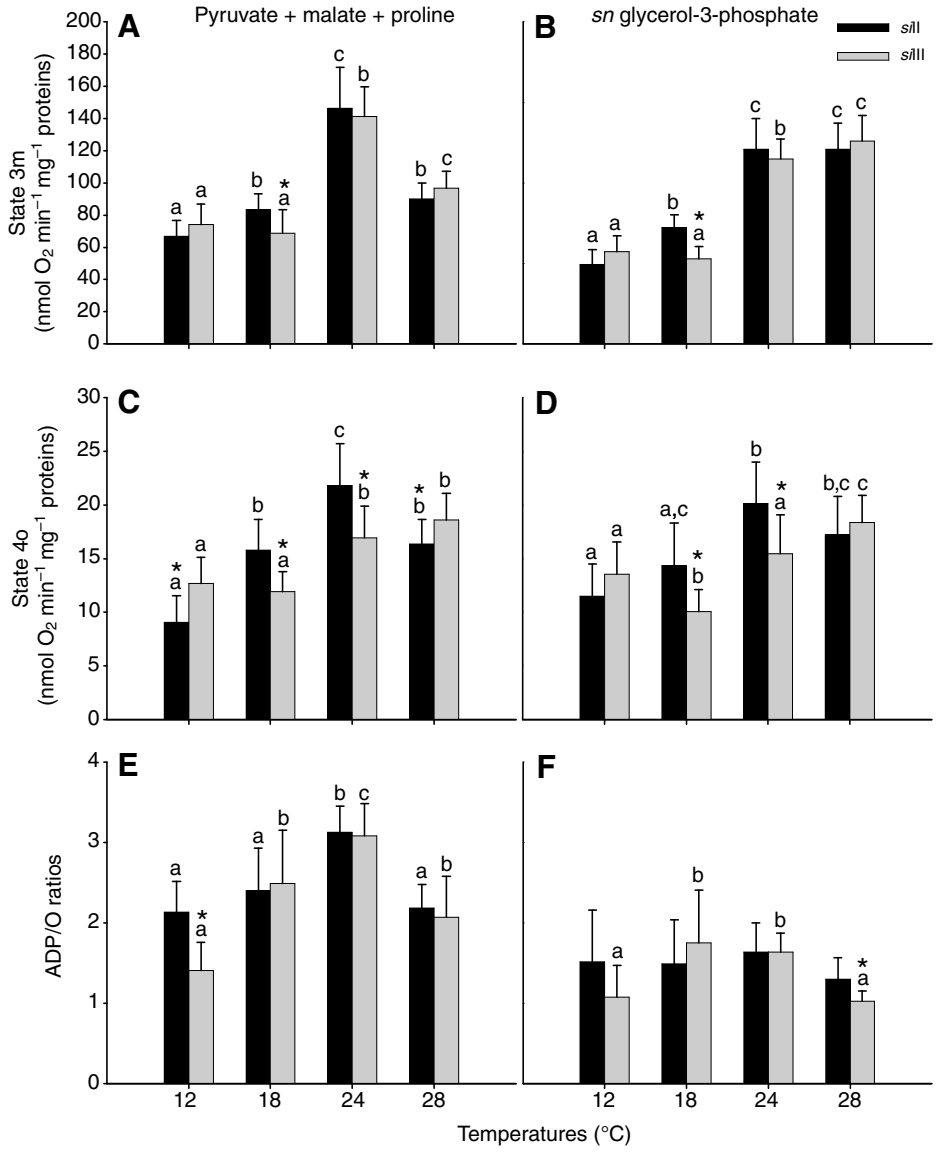

Fig. 2. Mitochondrial functions measured at four different temperatures in isolated mitochondria from the two mitotypes of *Drosophila simulans si*II and *si*III. State 3 respiration with (A) complex I substrates (pyruvate + malate + proline) and (B) *sn* glycerol-3-phosphate; State 4 respiration with inhibitor olygomycin at level of (C) complex I and (D) complex III; ADP/O ratios calculated from (E) complex I and (F) complex III. Results are means ± s.d. for 10 mitochondrial preparations. Significance was set as $P<0.05$; * denotes differences between mitotypes; letters denote differences between temperatures with a statistically different from b and c, b statistically different from c.

oxidative stress supported by each of these lines, at least in mitochondria. This is reflected by absences of significant differences between both mitotypes in MDA levels (data not shown).

COX activity was significantly higher for *si*III only at 12°C ($P<0.0001$; Fig. 4B). Moreover, we observed a slight increase from 12°C to 28°C for both mitotypes with significant differences between 12°C, 18°C, 24°C and 28°C.

At 18°C CAT activity was higher in *si*II compared wih *si*III ($P=0.0116$) whereas at 28°C it was lower ($P=0.0005$) (Fig. 4C).

COX excess capacity and flux metabolic control

Complex IV activity and mitochondrial respiration with pyruvate, malate, L-proline and *sn* glycerol-3-phosphate were inhibited by sodium azide. We examined the apparent excess capacity of COX at high flux through the ETS using a combination of substrates that maximally reduce complexes I and III. Azide titration resulted in hyperbolic inhibition of COX. The threshold plots display pathway flux as a function of COX activity; the threshold for inhibition of COX is defined as the intercept of the initial slope with the linear fit of the final slope (Fig. 5). The apparent excess capacity of COX is the intercept of the extrapolation of the linear regression for the final slope with the axis at zero COX inhibition. We detected a threshold, and consequently, a COX excess capacity at 12°C with no distinctions between the two mitotypes (604%, $R^2=0.9139$ for *si*II and 613%, $R^2=0.9301$ for *si*III).

Surprisingly, with increasing temperatures the COX excess capacity vanished and comparisons were made between the different K_i and C_i. We observed increasing K_i with increasing temperatures but no differences between mitotypes were detected (Table 1).

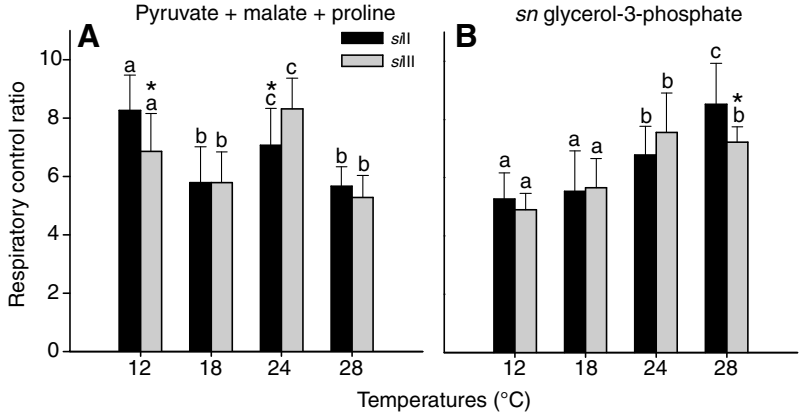

Fig. 3. Effect of temperature on respiratory control ratio (RCR) when mitochondrial preparations from the two mitotypes, *si*II and *si*III, were supplied with pyruvate + malate + proline at the complex I level (A) or with *sn* glycerol-3-phosphate *via* glycerol-3-phosphate dehydrogenase to reduce complex III (B). Results are means ± s.d. for 10 mitochondrial preparations. Significance was set as $P<0.05$; * denotes differences between mitotypes; letters denote differences between temperatures with a statistically different from b and c, b statistically different from c.

For C_i comparisons, there were no significant differences between mitotypes but significant increases were detected from 12°C to 28°C (Table 1). All temperature comparisons were significantly different (all P-values ≤0.0005).

Q_{10} values for ACO/CS and state 3m are presented in Table 3.

DISCUSSION

Comprehension of the physiological and biochemical bases of temperature sensitivity of mitochondrial function appears essential to determine the capacity of organisms to cope with changing environmental conditions and constraints they may face under these conditions. In our study, we compared two mitotypes of *D. simulans* in order to (1) evaluate the plasticity of different enzymes of the ETS, and to (2) link mtDNA divergences with mitochondrial bioenergetics at several steps of the ETS. To our knowledge, this study is the first to deal with impact of temperature variations on mitochondrial functions in different mitotypes of the same species.

The two Krebs' cycle enzymes that are fully encoded by the nuclear genome, CS and ACO, showed no differences between mitotypes. This result suggests a weak impact of the nuclear genes on divergences of mitochondrial functions between these two populations. ACO is an enzyme selectively carbonylated and functionally altered in the housefly mitochondrial matrix by ROS (Yan et al., 1997; Yan and Sohal, 1998; Yarian and Sohal, 2005), and can be used as a proxy of the steady-state concentration of $O_2^{\bullet-}$ in the matrix (Gardner, 2002; Miwa and Brand, 2005). CAT activity of *si*II mitotype was higher at 18°C than *si*III. The latter mitotype showed more elevated CAT activity at 28°C. As MDA levels and ACO activities remain constant between mitotypes at all temperatures, we suggest that these different CAT thermal sensitivities may result from expression of different allozymes in both populations, and consequently of different antioxidant activity.

The maximal rates of mitochondrial respiration were reached at 24°C for both mitotypes and when mitochondria were provided *via* glycerophosphate dehydrogenase to reduce complex III these rates were similar to those found at 25°C by Katewa et al. (Katewa et al., 2007). However, when mitochondria were supplied in electrons by pyruvate + malate + proline to reduce the complex I, we obtained higher respiration rates than these authors, which are likely to be due to the addition of malate as substrate in the present study. Katewa et al. found significant differences between mitotypes at 25°C whereas we did not detect any differences between mitotypes except at 18°C where *si*II showed higher catalytic capacity (Katewa et al., 2007). Interestingly, this higher capacity came with higher CAT activity. Maximal state 3m with pyruvate + malate + proline was found at 24°C whereas with *sn* glycerol-3-phosphate it was at 24°C and 28°C.

The decrease of catalytic capacities of mitochondrial state 3m respiration between 24°C and 28°C (Table 3) follow the same pattern as in other ectothermic species as reported in rainbow trout red muscle (Blier and Guderley, 1993; Guderley et al., 1997) and in Arctic char (Blier and Lemieux, 2001), which show a decrease at high temperature. There is however no decrease in the catalytic capacities when electrons are supplied to the ubiquinone pool *via* glycerophosphate dehydrogenase (Table 3). This clearly suggests that the decline in respiration rates at higher temperature when electrons are supplied at the level of complex I is induced by decreasing activities of enzymes upstream complex III.

Interestingly, state 4o follows the same pattern as state 3m with more pronounced differences between mitotypes (for all temperatures using complex I substrates, and for 18°C and 24°C using *sn* glycerol-3-phosphate). At 18°C and 24°C, *si*II exhibits a higher state 4o than *si*III for both complexes whereas at extreme temperatures (12°C and 28°C) the higher state 4o is measured in *si*III but only with complex I substrates and this difference is not maintained for *sn* glycerol-3-phosphate. During state 4, $O_2^{\bullet-}$ and H_2O_2 production are believed to be the highest (Forman and Boveris, 1982). Our results on state 4o are consistent with a previous study by Katewa et al. (Katewa et al., 2007), which showed higher H_2O_2 production in *si*II at 25°C. Our results could also suggest that flies with *si*II mtDNA are better adapted to a large temperature range and specifically to extreme temperatures whereas *si*III has a smaller

Table 1. Values of inhibition constant (K_i) of sodium azide calculated from transformed data using the Dickson linearisation and values of metabolic flux control (C_i) calculated according to the following formula: $C_i=-(dJ/J)/(dI/K_i)$ (see Materials and methods) in the *si*II and *si*III mitotypes at the four different temperatures

	*si*II		*si*III	
Temperatures (°C)	K_i	C_i	K_i	C_i
12	26.3±15.1	0.12±0.07	18.8±9.0	0.09±0.04
18	35.7±13.2	0.32±0.12	41.1±10.0	0.31±0.08
24	60.5±10.8	0.52±0.09	59.3±10.0	0.51±0.08
24 (TMPD+Asc)	66.4±16.1	0.57±0.14	60.1±16.2	0.52±0.14
28	93.8±3.5	0.60±0.02	97.9±9.7	0.66±0.07

Results are means ± s.d. (N=10). TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; Asc, ascorbate.

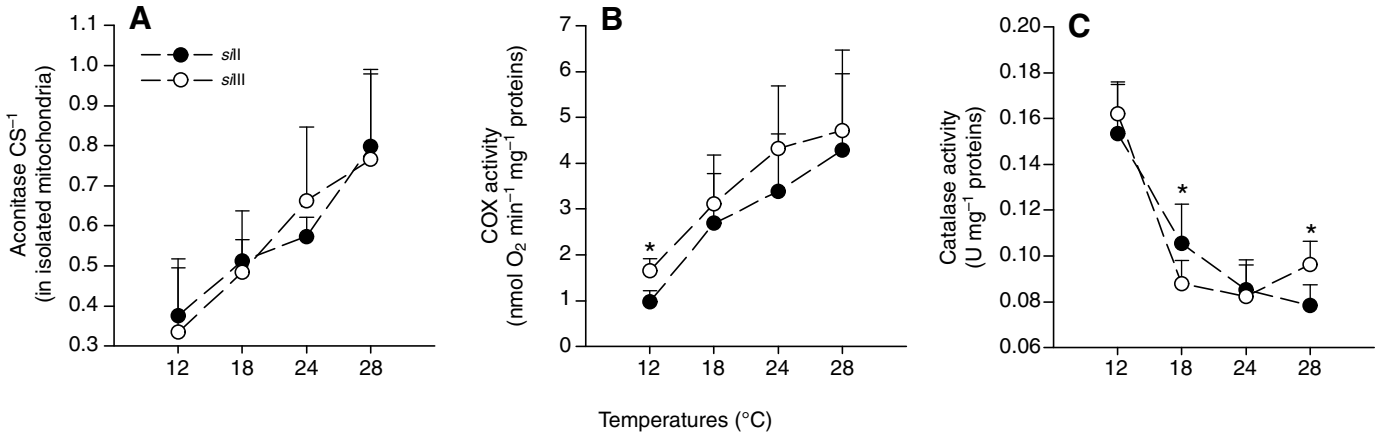

Fig. 4. Thermal sensitivity of different key enzymes of mitochondrial metabolism and the antioxidant system from the two mitotypes, *si*II and *si*III. (A) Ratios of aconitase activity over citrate synthase activity calculated from measurements in isolated mitochondria from the two mitotypes, *si*II and *si*III; (B) cytochrome *c* oxidase (COX) activity and (C) catalase activity measured in crude homogenates from the two mitotypes, *si*II and *si*III. Results are means ± s.d. for 10 mitochondrial preparations or crude homogenates. Significance was set as $P<0.05$; * denotes differences between mitotypes.

thermal range. This would be in line with the lower impact of lower temperature on ADP/O ratio as well as with the geographical distribution of both mitotypes. Flies from the two mitotypes were collected in Kenya where they live in sympatry; however, *si*II has a worldwide distribution whereas *si*III is endemic to continental east Africa, Madagascar and Reunion Island (see supplementary material Fig. S1) (Ballard, 2004). This spatial niche differentiation hypothesis does not however exclude the possible temporal niche differentiation (diurnal or seasonal differences in haplotype performance/abundance). Obviously, at this stage of experimentation we cannot determine if the differences in mtDNA *per se* have any adaptive significance. Indeed, while there are isolated temperature-specific differences between mitotypes, these differences are not consistent across the various functional attributes measured. Consequently, there is little support for a conclusion that one mitotype has a broader temperature range for mitochondrial performance than the other.

The ratio of OXPHOS capacity to leak respiration is used as a coupling index of isolated mitochondria and of the functional integrity of mitochondria (RCR) (Chance and Williams, 1956). RCRs obtained using substrates for complex I and the substrate *sn* glycerol-3-phosphate indicate a well-coupled respiration. *si*II showed more coupled respiration, as well as a higher ADP/O ratio at 12°C when mitochondria were supplied in electrons at complex I level. This result shows that *si*II exhibits a higher efficiency of substrate oxidation and OXPHOS capacity at 12°C. Moreover, low RCRs at 18°C (5.78±1.2 for *si*II and 5.79±1.1 for *si*III) and 28°C (5.67±0.7 for *si*II and 5.27±0.8 for *si*III) suggest that the mixture pyruvate + malate + proline is associated with a lower efficiency of OXPHOS at these temperatures, which is supported by ADP/O ratios calculated at the same temperature. The ADP/O ratios for complex I also show optimal temperature at 24°C. The decrease in ADP/O ratios are probably induced by higher thermal sensitivity (and decrease) in state 3m respiration compared with state 4o as illustrated by RCRs. Surprisingly, high RCR values for both mitotypes at 12°C do not correspond to high ADP/O ratios, even if differences in RCRs between mitotypes were paralleled by differences in ADP/O ratios for this temperature. This result can only be explained by loss of OXPHOS capacity during state 3m at low temperature.

When mitochondria are supplied at the complex III level *via* glycerophosphate dehydrogenase, we observe a slight increase of RCRs from 12°C to 28°C. The only difference between mitotypes for this complex occurs at 28°C where, once again, *si*II exhibits higher RCR. At all temperatures, RCRs as well as ADP/O ratios are more elevated in complex I, showing higher OXPHOS capacity with pyruvate + malate + proline induced by activation of complex I proton pump. The only exception is at 28°C, where the higher RCR with *sn* glycerol-3-phosphate reflects a lower thermal sensitivity of state 3 respiration when ETS is supplied with *sn* glycerol-3-phosphate. However, at this temperature, we do not observe a concomitant increase in the ADP/O ratios, which suggest that OXPHOS capacity is partly dissipated downstream of the ETS during state 3m respiration (at complex IV, or by higher rate of proton leakage). When comparing state 3m respiration of mitochondria supplied either with pyruvate + malate + proline or *sn* glycerol-3-phosphate, the state 3m is higher with electrons provided to complex I, illustrating that complex IV (cytochrome *c* oxidase) can support much higher rate of oxidation than the one

Table 2. Values of uncoupled control ratios (UCRs) measured in mitochondrial isolations

	*si*II		*si*III	
Temperatures (°C)	Pyruvate + malate + proline	*Sn* glycerol-3-phosphate	Pyruvate + malate + proline	*Sn* glycerol-3-phosphate
12	0.98±0.08	0.99±0.07	0.99±0.06	1.00±0.06
18	0.98±0.03	0.98±0.078	0.95±0.06	0.95±0.05
24	0.99±0.07	0.96±0.05	0.98±0.05	0.95±0.05
28	0.94±0.60	0.96±0.04	0.99±0.10	0.98±0.04

UCRs were calculated as state U/state 3m with state U representing the maximum uncoupled respiration rate [reached between 0.2 μmol l^{-1} and 0.5 μmol l^{-1} of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)] and state 3m representing the maximum active state 3 achieved with injection of 400 μmol l^{-1} of ADP. Results are means ± s.d. for 10 mitochondrial preparations.

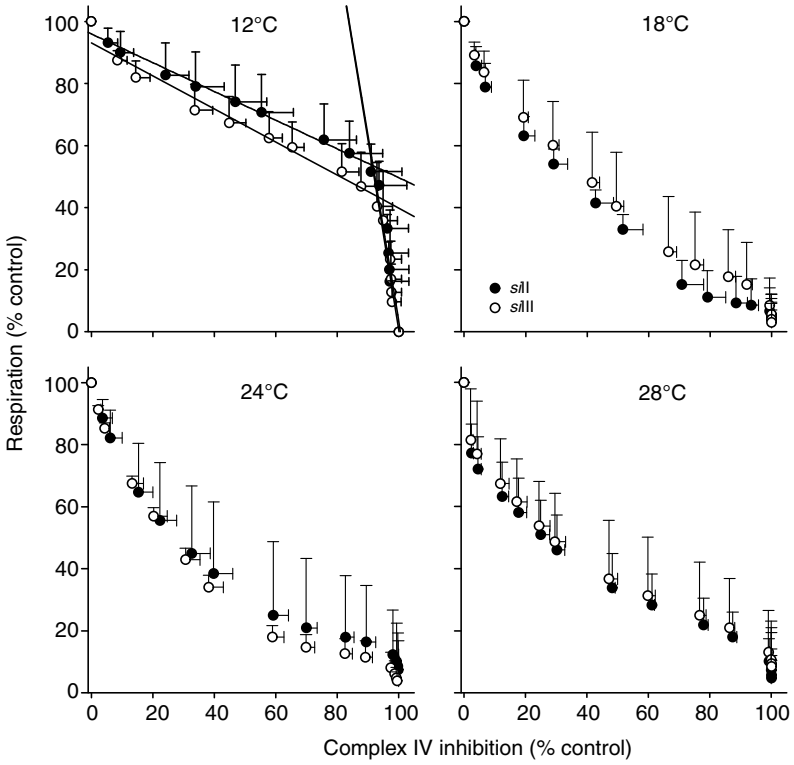

Fig. 5. Complex IV thresholds measured at four different temperatures in the two mitotypes studied, *si*I and *si*III. Respiration was measured in isolated mitochondria fed with pyruvate + proline + malate + *sn* glycerol-3-phosphate as substrates followed by an azide titration of flux through the respiratory system. Complex IV inhibition was measured in crude homogenates following azide titration of the enzyme velocity using the same azide titration steps as for the respiratory system. The cytochrome *c* oxidase (COX) excess capacity is represented by the threshold plot, which shows the relative flux through the electron transport system (ETS) as a function of relative inhibition of COX at similar azide concentrations. At 12°C, two linear regressions were calculated from initial and final slopes, and extrapolated to zero COX inhibition (*si*I, R^2≥0.9840 and R^2≥0.9139, respectively; *si*III, R^2≥0.9651 and R^2≥0.9301, respectively). The intercepts are the COX excess capacity (see text below). Results are means ± s.d. (N=10).

provided when supplying electrons only *via* glycerol-3-phosphate dehydrogenase to reduce complex III (*sn* glycerol-3-phosphate), at least at three temperatures we tested. The important decrease of complex I state 3m respiration between 24°C and 28°C, despite the absence of thermal sensitivity of complex III state 3m respiration at these same temperatures, suggests that the likely limitation of respiration comes upstream of complex III. This is also supported by the low UCR values (below but not significantly different from 1.0, Table 2) at any temperature, suggesting that ATP synthesis capacity (ATP synthase) can support the maximum respiration rates.

For COX activity, an increase from 12°C to 28°C was observed, as seen in other ectothermic species (Blier and Lemieux, 2001; Lemieux et al., 2009) (H. Lemieux, J.-C. Tardif, J.-D. Dutil and P.U.B., personal communication). We did not detect a loss of activity between 24°C and 28°C (Q_{10}24–28=1.81 for *si*II and Q_{10}24–28=2.66 for *si*III). This result suggests that COX is probably not responsible for the reduction of catalytic capacities at 28°C when mitochondria are supplied in electrons at the complex I level. The decrease of the catalytic capacities of complex I observed at 28°C is therefore likely to be due to upstream ETS (complex I, II or III) or dehydrogenases, which drive the entry of the substrates in the ETS.

COX excess capacity was measured at high pathway flux with both complexes fully fuelled in electrons (pyruvate + malate + proline + glycerol-3-phosphate). An important excess was observed only at 12°C, with an excess of about 604% for *si*II and 613% for *si*III. At this temperature, the COX maximal activity should reach a significantly higher level of inhibition before impairment of mitochondrial respiration (approximately 91.7% for *si*II and 93.0% for *si*III). Excess capacity of COX has been reported in ectotherms (Blier and Lemieux, 2001; Farge et al., 2003) as well as in endotherms (Rossignol et al., 1999). At 12°C, COX catalytic capacities do not exert a strong control over the OXPHOS capacity (C_i=0.12±0.07 for *si*II and C_i=0.09±0.04 for *si*III). From 18°C to 28°C, no excess capacity of COX was detected and C_i increased slightly along with temperature, suggesting important control of OXPHOS capacity by COX. This result also suggests that maximal, uninhibited COX activity could not support higher respiration rates than the maximal rate experimentally measured in the present study, where we used excess concentrations of different substrates. These results may indicate that when the ETS is 'nearly saturated' with electrons from carbon substrates, the system is close to its limit and therefore complex IV does not present any excess. Other studies have found high threshold values for the complex IV in *Drosophila* (Farge et al., 2003), as well as in rat mitochondria (Letellier et al., 1994; Rossignol et al., 1999) but only when the complex I was reduced using glutamate + malate or pyruvate + malate. Particularly, Farge et al. (Farge et al., 2003) found the same type of threshold curve for *Drosophila subobscura* at 28°C

Table 3. Temperature coefficients (Q_{10}) for ACO/CS ratios and for mitochondrial respiratory fluxes in state 3m

	Temperature coefficients (Q_{10})					
	12–18°C		18–24°C		24–28°C	
	*si*I	*si*III	*si*I	*si*III	*si*I	*si*III
ACO/CS	1.68	1.85	1.21	1.68	2.29	1.44
State 3m						
Pyruvate + malate + proline	1.58	0.95*	2.68	3.40	0.35	0.43
Sn glycerol-3-phosphate	2.03	0.92*	2.37	3.74*	1.17	1.31

Q_{10} values for mitochondrial functions were calculated according to the following formula: Q_{10} = (Rate 2 / Rate 1)$^{10/(t2-t1)}$. Results are means of 10 mitochondrial preparations. Significance was set as P<0.05.
* denotes differences between mitotypes with P<0.05.

with glutamate + malate that we did at 12°C with pyruvate + malate + proline + glycerol-3-phosphate. This result suggests that at 12°C, when all substrates were used, there is impairment of electron entry into the ETS, and likely at the level of upstream dehydrogenases.

This highlights the fact that the mitochondrial metabolic enzymes have different thermal sensitivities and that temperature changes should necessarily modify the proportions of catalytic capacities of the different enzymes and therefore, the regulation of the whole pathway. At low temperatures (12°C in our study) regulation at the level of COX was relaxed, likely to be due to much higher thermal sensitivity of other mitochondrial enzymes or processes. From 18°C to 28°C, which covers the optimal temperature conditions, mitochondria are more regulated by COX.

In summary, we have shown that differences between the two mitotypes are observed at low temperature (12°C) where *si*II display higher RCR and ADP/O ratio than *si*III as well as lower state 4o. To investigate the significance of state 4o, the experiment must be repeated with an assessment of membrane potential, because proton leak can only be estimated from state 4o when membrane potential is known or controlled. At this stage, we cannot however conclude about the adaptive value of mtDNA partly because the temperature-specific differences detected are not consistent across the various functional parameters measured and they cannot be linked yet to any fitness divergences at the specific ecological niches of the two populations. We have also shown that different ETS enzymes have different thermal sensitivities which can lead to different distribution of control along the ETS and dehydrogenases process at different temperatures leading to quite low level of respiration control by COX at low temperature. However, important properties of mitochondria may differ *in vivo* and *in vitro* (Kuznetsov et al., 2008). Further *in situ* studies (on permeabilised fibres) among mitotypes of *D. simulans* should reveal characterisation of functional mitochondria in their normal intracellular position and assembly, conserving interactions with other organelles. Moreover, introgressions between mitotypes should highlight the adaptive value of *Drosophila* mitochondria in co-adapted cellular environments.

LIST OF ABBREVIATIONS

ACO	aconitase
BSA	bovine serum albumin
CAT	catalase
C_i	flux control coefficient
COX	cytochrome *c* oxidase (complex IV)
CS	citrate synthase
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
ETS	electron transport system
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
H_2O_2	hydrogen peroxide
K_i	inhibition constant
MDA	malondialdehyde
mtDNA	mitochondrial DNA
$O_2^{\bullet-}$	superoxide
OXPHOS	oxidative phosphorylation
Q_{10}	temperature coefficient
RCR	respiratory control ratio
ROS	reactive oxygen species
TBARS	thiobarbituric reactive substances
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
UCR	uncoupled control ratio

ACKNOWLEDGEMENTS

Many thanks are due to H. Lemieux, S. Breton and R. Vergilino for their help with experimental protocols. This study was supported by research grants from the Natural Sciences and Engineering Research Council (NSERC) to P.U.B.

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Table S1. MtDNA variation in *Drosophila simulans* (*D. sim.*) (*si*I, *si*II, *si*III) and *Drosophila melanogaster* (*D. mel.*)

	Nucleotides		Amino acids	
	Total	%	Total	%
Intrahaplogroup				
*si*I	5.83	3.90×10^{-4}	2.00	5.40×10^{-4}
*si*II	4.78	3.20×10^{-4}	2.29	6.17×10^{-4}
*si*III	0.59	4.00×10^{-5}	0.25	6.80×10^{-4}
Interhaplogroup				
*si*I *vs si*II	392.05	2.60×10^{-2}	47.57	1.28×10^{-2}
*si*I *vs si*III	345.01	2.30×10^{-2}	26.68	7.2×10^{-3}
*si*II *vs si*III	305.26	2.04×10^{-2}	45.11	1.21×10^{-2}
Intraspecific				
D. sim.	236.068	1.58×10^{-2}	26.84	7.25×10^{-3}
D. mel.	53.061	3.5×10^{-3}	10.00	2.70×10^{-3}
Interspecific				
D. sim. vs D. mel.	605.33	4.06×10^{-2}	89.89	2.4×10^{-3}

Data from Ballard (Ballard, 2000b).