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Damien Roussel, Sonia Janillon, Loïc Teulier, Nicolas Pichaud. Succinate oxidation rescues mitochondrial ATP synthesis at high temperature in *Drosophila melanogaster*. *FEBS Letters*, 2023, 597 (17), pp.2221-2229. 10.1002/1873-3468.14701 . hal-04209706

HAL Id: hal-04209706

<https://hal.science/hal-04209706v1>



Submitted on 22 Feb 2024

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RESEARCH LETTER

Succinate oxidation rescues mitochondrial ATP synthesis at high temperature in *Drosophila melanogaster*

 Damien Roussel¹ , Sonia Janillon², Loïc Teulier¹ and Nicolas Pichaud³ 

1 Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, Villeurbanne, France

2 Univ Lyon, Université Claude Bernard Lyon 1, CNRS, UMR 5558 LBBE, Villeurbanne, France

3 Department of Chemistry and Biochemistry, Université de Moncton, New Brunswick, Canada

Correspondence

 N. Pichaud, Department of Chemistry and Biochemistry, Université de Moncton, Moncton, NB E1A 3E9, Canada
 E-mail: nicolas.pichaud@umoncton.ca

(Received 5 April 2023, revised 5 June 2023, accepted 3 July 2023, available online 27 July 2023)

doi:10.1002/1873-3468.14701

Edited by Aurelio A. Teleman

Decreased NADH-induced and increased reduced FADH₂-induced respiration rates at high temperatures are associated with thermal tolerance in *Drosophila*. Here, we determined whether this change was associated with adjustments of adenosine triphosphate (ATP) production rate and coupling efficiency (ATP/O) in *Drosophila melanogaster*. We show that decreased pyruvate + malate oxidation at 35°C is associated with a collapse of ATP synthesis and a drop in ATP/O ratio. However, adding succinate triggered a full compensation of both oxygen consumption and ATP synthesis rates at this high temperature. Addition of glycerol-3-phosphate (G3P) led to a huge increase in respiration with no further advantage in terms of ATP production. We conclude that succinate is the only alternative substrate able to compensate both oxygen consumption and ATP production rates during oxidative phosphorylation at high temperature, which has important implications for thermal adaptation.

Keywords: ATP synthesis rate; *Drosophila melanogaster*; metabolism; mitochondrial oxygen consumption; oxidative substrate; temperature; thermal tolerance

Temperature is among the most important environmental stressors that can be a major driving force impacting molecular, metabolic, and physiological aspects of phenotype, especially in ectotherms as their body temperature is closely associated with environmental temperature [1,2]. Recently, it has been shown that due to the projected increase in the frequency and intensity of heat waves brought forth by climate warming and the extreme thermal sensitivity of heat failure rates, many ectothermic species will experience severe and disproportionate consequences due to heat

mortality [3]. Thus, the study of the mechanisms underlying upper thermal tolerance has been deemed crucial to understand whether and how these organisms will acclimate and/or adapt to climate changes. Yet, due to the effects of temperatures on all levels of biological organization, from life-history traits to biochemical and molecular processes, the specific mechanism(s) involved in setting upper thermal tolerance has been notoriously difficult to pinpoint.

One of the proximal causes that may explain thermal sensitivity and heat failure in ectotherms is the

Abbreviations

ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATP/O, mitochondrial coupling efficiency; CT_{max}, critical thermal maximum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; FADH₂, reduced flavin adenine dinucleotide; G3P, glycerol-3-phosphate; LEAK, nonphosphorylating respiration rate; Mal, malate; MOPS, 3-(N-morpholino) propanesulfonic acid; mtG3PDH, mitochondrial glycerol-3-phosphate dehydrogenase; NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; OXPHOS, oxidative phosphorylation/phosphorylating respiration rate; Pyr, pyruvate; ROS, reactive oxygen species; Succ, succinate.

effect of temperature on the catalytic capacities and on the stability of metabolic enzymes which control cellular energy fluxes and homeostasis [2,4]. Mitochondria, as the hub of cellular metabolism, are at the forefront of these fluxes, and as metabolic demand increases with temperature, it is crucial that mitochondrial functions adequately respond to maintain cellular homeostasis [5,6]. Hence, it has been suggested that mitochondrial thermal sensitivity plays a key role in organismal adaptations to temperature and is involved in setting thermal tolerance of ectotherms [7–12]. Furthermore, it has been shown in several ectotherms that mitochondrial dysfunctions may explain organismal failure at critically high temperatures [10,13–17]. However, the specific mitochondrial process(es) leading to this failure is still unclear. For example, it can be related to insufficient oxygen supply, mismatched adenosine triphosphate (ATP) demand and supply and/or imbalance of reactive oxygen species (ROS) production and detoxification [5,7].

Recently, it has been shown that at temperatures below or close to the critical thermal maximum (CT_{max}), complex I mitochondrial oxygen consumption induced by reduced nicotinamide adenine dinucleotide (NADH)-linked substrates (such as pyruvate (Pyr) and malate (Mal)) drastically decreased in a comparative model of *Drosophila* displaying different CT_{max} [17]. Interestingly, this decrease was associated with increased oxidation rates of reduced flavin adenine dinucleotide ($FADH_2$)-linked substrates such as succinate (Succ) for complex II and glycerol-3-phosphate (G3P) for the mitochondrial G3P dehydrogenase (mtG3PDH) [17]. Moreover, this substrate switch at high temperature seems to be conserved but was not necessarily associated with CT_{max} in other insect species such as honeybees and Colorado potato beetles [15]. Considering that NADH oxidation through complex I allows the direct pumping of protons from the matrix to the inner mitochondrial membrane while oxidation of $FADH_2$ does not, this metabolic flexibility at high temperature should entail important functional consequences at the cellular and organismal levels as the nature of the reducing equivalent that is oxidized should diminish the net amount of ATP produced per amount of oxygen consumed by the oxidative phosphorylation (OXPHOS) process.

The aim of this study was to examine the relationship between the mitochondrial substrate switch observed at high temperature in *Drosophila* and the efficiency of mitochondria to produce ATP. Specifically, we used *Drosophila melanogaster* (acclimated to 22.5 °C) to investigate the mitochondrial oxygen consumption induced by NADH- and $FADH_2$ -linked substrates at

assay temperatures of 25 °C (control) and 35 °C (stressful) in mitochondria isolated from thoraces and evaluated the amount of ATP subsequently produced by oxidation of these specific substrates. We hypothesized that the decreased oxidation of NADH-linked substrates observed at high temperatures significantly reduces the amount of ATP produced which is not compensated by increased oxidation of $FADH_2$ -linked substrates, resulting in an uncoupling of mitochondria. This uncoupling will result in a metabolic imbalance between anabolic and catabolic fluxes, which eventually leads to heat mortality.

Materials and methods

Drosophila maintenance and collection

Drosophila melanogaster used in this study were derived from a wild population collected in Gotheron, France (44.98° N, 4.93° E) during summer 2014 [18]. They were raised at 22.5 ± 1 °C and 65% humidity (light/dark: 12/12 h) on a standard cornmeal fly medium [18.2 g.L⁻¹ Agar, 150 g.L⁻¹ cornmeal, 156.8 g.L⁻¹ yeast, 8.2 g.L⁻¹ Nipagin, 11.4% (v/v) ethanol]. Only males of 7 to 14 days old were used for all the experiments.

Mitochondrial isolation

Flight muscle mitochondria were isolated from 30 thoraces as previously described by [19]. Thoraxes were separated from abdomens and heads and rapidly homogenized in 200 µL of isolation buffer (250 mM sucrose, 5 mM Tris-base, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1% bovine serum albumin, pH 7.4). Homogenates were filtered through gauze pad, the volume raised to 825 µL with isolation buffer and the homogenates centrifuged at $300 \times g$ for 3 min. The resulting supernatant was refiltered through gauze and centrifuged at $9,000 \times g$ for 10 min. The pellet was washed, resuspended in 150 µL of isolation buffer without bovine serum albumin, and centrifuged at $9,000 \times g$ for 10 min. This last step was repeated one more time. All steps were carried out at 4 °C. The mitochondrial suspension was then freshly used to measure bioenergetics parameters. The protein content of the mitochondrial preparation was assayed using the biuret method, with bovine serum albumin as a standard.

Mitochondrial oxidative phosphorylation activity and efficiency

Mitochondrial oxygen consumption and ATP synthesis were determined at 25 and 35 °C. These temperatures were chosen to represent normal temperature (25 °C, close to

the acclimation temperature) and a stressfully high temperature (35 °C), which was however below the known CT_{max} for *D. melanogaster* [20]. Isolated mitochondria were transferred in 500 μ L of respiratory buffer (120 mM KCl, 5 mM KH_2PO_4 , 1 mM $MgCl_2$, 10 μ M cytochrome c, 20 mM glucose, 2 U/mL hexokinase, 0.2% essentially free-fatty acid bovine serum albumin (w/v), and 3 mM Hepes, pH 7.4) for mitochondrial oxygen consumption in a glass cell fitted with a Clark oxygen electrode (Rank Brothers Ltd, Cambridge, UK) with temperature set at either 25 or 35 °C and calibrated with air-saturated respiratory buffer at each temperature. Mitochondria were then energized with three different combinations of respiratory substrates (10 mM Pyr + 2.5 mM Mal \pm 10 mM Succ \pm 10 mM G3P), and the rates of basal nonphosphorylating respiration (LEAK) were recorded. Mitochondrial ATP synthesis was initiated by the addition of 1.25 mM adenosine diphosphate (ADP), and the rates of phosphorylating respiration (OXPHOS) were recorded for 5–7 min; then, four 100 μ L aliquots of mitochondrial suspension were withdrawn every 1 min and immediately quenched in ice-cold 100 μ L perchloric acid solution [10% $HClO_4$, 25 mM ethylenediaminetetraacetic acid (EDTA)]. The denatured proteins were centrifuged at $20,000 \times g$ for 5 min (4 °C), and 180 μ L of the resulting supernatants was neutralized with KOH solution [2 M KOH, 0.3 M 3-(N-morpholino)propanesulfonic acid (MOPS)]. After centrifugation of the neutralized samples ($20,000 \times g$ for 5 min at 4 °C), ATP production was determined from the glucose-6-phosphate content of the resulting supernatants. Glucose-6-phosphate content was determined by spectrophotometry at 340 nm in an assay medium (7.5 mM $MgCl_2$, 3.75 mM EDTA, 50 mM triethanolamine-HCl, pH 7.4) supplemented with 0.5 mM oxidized nicotinamide adenine dinucleotide (NAD) and 0.5 U glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, at room temperature. Mitochondrial respiration rates are expressed as nmol O/min.mg protein and ATP synthesis rates are expressed as nmol ATP/min.mg protein.

Oligomycin-insensitive ATP synthesis activity

In order to make sure that ATP synthesis rates were specific of the mitochondrial ATP synthase, oxygen consumption and ATP synthesis were also assayed in the presence of 2 μ g.mL⁻¹ oligomycin. In the present study, an oligomycin-insensitive ATP synthesis activity was measurable (Table 1). Values for this non-mitochondrial phosphorylating activity were independent of the respiratory substrate combinations used, and slightly higher at 35 °C (+30%) than at 25 °C (Table 1). Overall, the rate of oligomycin-insensitive ATP synthesis contributed to 12% on average of the total ATP production at both assay temperatures regardless of the respiratory substrate combinations. However, it is important to note that there is one

exception for complex-I-supported mitochondrial activity, for which the non-mitochondrial ATP synthesis rate contributed to 46% of the total ATP production at 35 °C. The proportion of these oligomycin-insensitive ATP synthesis activities were taken into account to calculate the rate of mitochondrial ATP synthesis.

Calculation of ATP/O ratio

The mitochondrial oxygen consumption rates during OXPHOS (in the presence of the different substrate combinations and ADP) and the specific mitochondrial ATP synthesis rate (corrected with the oligomycin-insensitive ATP synthesis activity) were used to calculate the mitochondrial efficiency (ATP/O). Specifically, the rate of mitochondrial ATP synthesis was divided by the corresponding rate of phosphorylating respiration (OXPHOS) obtained for the same mitochondrial isolation at each specific temperature (25 and 35 °C) and for each substrate combination (Pyr + Mal, Pyr + Mal + Succ, and Pyr + Mal + Succ + G3P).

P-L control efficiency and substrate contribution ratio

For the oxygen consumption induced by each substrate combination, the P-L control efficiency, which is an indicator of the coupling between the transport of electrons and oxidative phosphorylation, was calculated as $1 - (LEAK/OXPHOS)$. In order to estimate the relative contribution of each substrate contribution (Succ, and G3P) to change in mitochondrial respiration and ATP synthesis, the substrate contribution ratio was calculated as the $(flux_2 - flux_1)/flux_1$, where flux1 represents the oxygen consumption rate or the ATP synthesis rate prior to the supplemental addition of the substrate and flux2 corresponds to the oxygen consumption rate or the ATP synthesis rate recorded with the new substrate. For example, the succ contribution ratio was calculated as $(flux_{Pyr + Mal + Succ} - flux_{Pyr + Mal})/flux_{Pyr + Mal}$. A value close to 0 indicates no additional effect of the added substrate to the mitochondrial fluxes, while a value of 1 or 2 indicates a doubling (100%) or a tripling (200%) change in fluxes, respectively, and so forth.

Statistical analyses

Statistical analyses were performed in R version 4.2.3 [21]. For all parameters measured, two-way ANOVAs were performed with respiratory substrates and assay temperatures as fixed factors, followed by a Tukey's *post hoc* test using the emmeans function (estimated marginal means) to estimate specific differences when an interaction between respiratory substrates and assay temperatures was detected. For the control efficiency ($1 - (LEAK/OXPHOS)$), the interaction between fixed factors was not significant, and data were analyzed independently with a one-way ANOVA and a

Table 1. Mitochondrial oxygen consumption during LEAK respiration (with oxidative substrates but no ADP) and oligomycin-insensitive ATP synthesis rates (with oligomycin) measured at two different temperatures (25 and 35 °C) in thorax mitochondria of *Drosophila melanogaster*. For statistical analysis, *F*-values (with df values) and *P*-values for two-way ANOVAs are reported.

	Mitochondrial fluxes					
	Oxygen consumption rate during LEAK respiration (nmol O/min.mg protein)		Oligomycin-insensitive ATP synthesis rate (nmol ATP/min.mg protein)			
	25 °C	35 °C	25 °C	35 °C		
Substrates						
Pyr + Mal	23.97 ± 2.51 ^a	N.S	31.87 ± 5.31 ^A	76.48 ± 8.34	N.S	97.54 ± 15.04
Pyr + Mal + Succ	43.87 ± 2.20 ^b	**	107.26 ± 12.95 ^B	69.39 ± 10.06	N.S	92.90 ± 4.85
Pyr + Mal + Succ + G3P	303.14 ± 35.22 ^c	***	659.53 ± 69.04 ^C	78.52 ± 7.39	N.S	98.54 ± 22.32
Statistical analysis						
Substrates	$F_{2,48} = 272.09; P < 0.0001$		$F_{2,17} = 0.15; P = 0.864$			
Temperature	$F_{1,48} = 30.04; P < 0.0001$		$F_{1,17} = 3.36; P = 0.084$			
Interaction	$F_{2,48} = 4.03; P = 0.024$		$F_{2,17} = 0.006; P = 0.994$			

Asterisks represent differences between assay temperatures for each substrate combination tested with ** $p < 0.01$ and *** $p < 0.001$. Different letters represent significant differences between substrate combinations at either 25 (lower case letters) or 35 °C (capital letters).

Table 2. Mitochondrial oxygen consumption and ATP synthesis rates measured during OXPHOS respiration (with oxidative substrates and ADP) at two different temperatures (25 and 35 °C) in thorax mitochondria of *Drosophila melanogaster*. For statistical analysis, *F*-values (with df values) and *P*-values for two-way ANOVAs are reported.

	Mitochondrial fluxes					
	Oxygen consumption rate during OXPHOS respiration (nmol O/min.mg protein)		Specific mitochondrial ATP synthesis rate during OXPHOS respiration (nmol ATP/min.mg protein)			
	25 °C	35 °C	25 °C	35 °C		
Substrates						
Pyr + Mal	168.82 ± 18.69 ^a	***	78.68 ± 8.54 ^A	487.84 ± 37.73	***	101.65 ± 9.68 ^A
Pyr + Mal + Succ	277.07 ± 31.82 ^b	N.S	299.17 ± 20.03 ^B	633.34 ± 48.65	N.S	585.05 ± 92.51 ^B
Pyr + Mal + Succ + G3P	677.21 ± 80.96 ^c	*	1104.72 ± 93.14 ^C	677.94 ± 52.07	*	429.59 ± 72.05 ^B
Statistical analysis						
Substrates	$F_{2,48} = 186.37; P < 0.0001$		$F_{2,48} = 50.57; P < 0.0001$			
Temperature	$F_{1,48} = 1.69; P = 0.200$		$F_{1,48} = 89.80; P < 0.0001$			
Interaction	$F_{2,48} = 19.80; P < 0.0001$		$F_{2,48} = 23.40; P < 0.0001$			

Asterisks represent differences between assay temperatures for each substrate combination tested with * $p < 0.05$ and *** $p < 0.001$. Different letters represent significant differences between substrate combinations at either 25 (lower case letters) or 35 °C (capital letters).

Student's *t*-test for respiratory substrates and assay temperatures, respectively. Normality was verified with visualization of the residuals, and homogeneity of variances was verified using the Levene's test (or an *F*-test for homogeneity of variance for coupling efficiency considering assay temperatures), and data were transformed when required to meet the assumptions.

Results

Mitochondrial oxidative phosphorylation activity and efficiency

The activity of complex-I-(Pyr + Mal)-supported oxidative phosphorylation was significantly inhibited at

35 °C compared with values at 25 °C, substantiated by significant differences detected for both oxygen consumption and ATP synthesis rates that were decreased at high temperature by ~50% and 80%, respectively (Table 2). This asymmetric inhibition of mitochondrial fluxes led to a significant 50% decrease in coupling efficiency (ATP/O ratio) at 35 °C (Fig. 1A). In contrast, there was no significant effect of high temperature (35 °C) on the rates of ATP synthesis and phosphorylating oxygen consumption and on the coupling efficiency when mitochondria were energized with Pyr + Mal + Succ (Table 2 and Fig. 1B). It is worth noting that there are two very high values of ATP synthesis rate at 35 °C, which increased variability and mean value of this mitochondrial flux (Table 2).

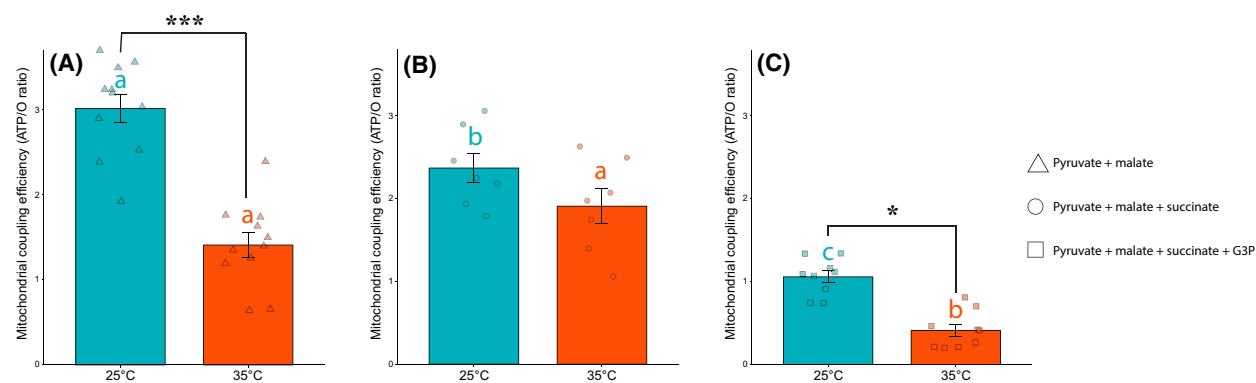


Fig. 1. Mitochondrial coupling efficiency (ATP/O) estimated by ATP/O ratios measured at two different temperatures (25 and 35 °C) in thorax mitochondria of *Drosophila melanogaster*. The rates of ATP synthesis and corresponding oxygen consumption were measured during maximum phosphorylating state in the presence of 1.25 mM ADP, at either 25 (blue symbols) or 35 °C (red symbols). The ATP/O ratio was calculated by dividing the rate of mitochondrial ATP synthesis by the corresponding rate of phosphorylating respiration (OXPHOS) obtained for the same mitochondrial isolation. Mitochondria were energized with either (A) Pyr + Mal (triangles; $n = 11$); (B) Pyr + Mal + Succ (circles; $n = 7$); or (C) Pyr + Mal + Succ + G3P (squares; $n = 9$). See [Materials and Methods](#) for details. Values are means \pm S.E.M. from independent mitochondrial preparations. Asterisks represent differences between assay temperatures for each substrate combination tested with $*p < 0.05$ and $***p < 0.001$. Different letters represent significant differences between substrate combinations at either 25 (blue letters) or 35 °C (red letters).

When these two values are omitted, the rate of ATP synthesis and resulting coupling efficiency decreased, becoming on average 30% lower at 35 °C than at 25 °C. Moreover, the addition of Succ at 25 °C induces a decrease in the ATP/O from 3.01 to 2.37 (Fig. 1A,B). The supplemental addition of G3P led to a drastic increase in oxygen consumption rate at 25 °C, which was 14-fold and nearly 4-fold higher than oxidative activity recorded with complex-I and complexes-I,II respiratory substrates, respectively (Table 2). Interestingly, while the rate of oxygen consumption doubled at 35 °C, the rate of ATP synthesis in the presence of G3P dropped by 40% compared with value at 25 °C (Table 2). Yet, the ATP synthesis remained four-fold higher with the full combination of respiratory substrates compared with complex-I-supported mitochondrial ATP production at 35 °C (Table 2). Consequently, the supplemental addition of G3P led to a further decrease in coupling efficiency at 35 °C compared with values at 25 °C (Fig. 1C).

Mitochondrial LEAK respiration and P-L control efficiency

At both 25 and 35 °C, the LEAK respiration significantly increased at each supplemental addition of respiratory substrate, being the lowest in the presence of Pyr + Mal and the highest for the full combination of respiratory substrates, that is, Pyr + Mal + Succ + G3P (Table 1). LEAK respiration rates were significantly higher at 35 °C than at 25°C when Succ and

G3P were present. In the presence of Pyr + Mal, it was slightly increased by 33%, but did not reach statistical significance.

At 25°C, the P-L control efficiency of complex-I-(Pyr + Mal) respiratory substrates was high and not significantly different from the value calculated with complexes I,II-(Pyr + Mal + Succ) respiratory substrates (Fig. 2). In contrast, the P-L control efficiency significantly dropped when G3P was present for both assay temperatures (Fig. 2). The elevation of temperature to 35 °C significantly decreased P-L control efficiency values for all the three respiratory substrate combinations (Fig. 2).

Substrate contribution to oxygen consumption and ATP synthesis rates

Fig. 3 reports substrate contribution ratios to change in mitochondrial fluxes. At 25 °C, the addition of succ stimulates the mitochondrial oxidative (+60%, Fig. 3A) and phosphorylation (+25%, Fig. 3B) rates slightly above those measured with only Pyr + Mal. At the same temperature, the presence of G3P doubled the rate of oxygen consumption (Fig. 3A), with no stimulation of the ATP synthesis compared with fluxes measured with Pyr + Mal + Succ (Fig. 3B). At high temperature (35 °C), substrate contribution to change in the rate of oxygen consumption was very high for both Succ and G3P compared with those calculated at 25 °C but only significantly for Succ (Fig. 3A). Interestingly, the Succ contribution to change in the rate of

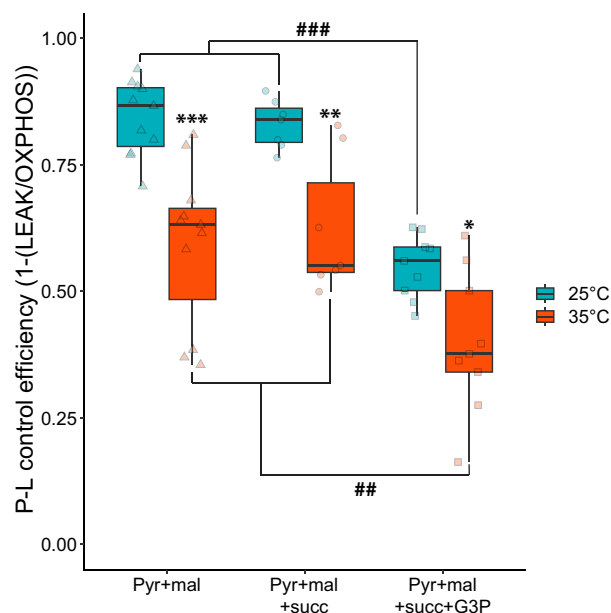


Fig. 2. Mitochondrial P-L control efficiency calculated from mitochondrial respiration rates measured at two different temperatures (25 and 35 °C) in thorax mitochondria of *Drosophila melanogaster*. P-L control efficiency was calculated with the LEAK (with oxidative substrates but no ADP) and OXPPOS (with oxidative substrates and ADP) respiration rates measured at 25 °C (blue symbols) and 35 °C (red symbols) for each substrate combination (Pyr + Mal, triangles, $n = 11$; pyr + mal + succ, circles, $n = 7$; and pyr + mal + succ + glycerol-3-phosphate (G3P), squares, $n = 9$) as $1-(LEAK/OXPPOS)$. Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values $<1.5 \times IQR$ (Tukey-style). *** $p < 0.001$ and * $p < 0.05$, significantly different between assay temperatures for each substrate combination. Within the same assay temperature, ### $p < 0.001$ and ## $p < 0.01$, significantly different between substrate combinations.

ATP synthesis was also very high and significantly different at 35 °C, while the value for G3P was close to zero (no additional effect, Fig. 3B). Altogether, these values indicate that the rates of oxygen consumption and ATP synthesis were markedly stimulated by the presence of Succ at 35 °C, whereas the supplemental presence of G3P only slightly stimulated the oxidative activity of mitochondria.

Discussion

Although this study does not evaluate the long-term effects of temperature (*i.e.*, acclimation) on mitochondrial metabolism, it does provide new understanding on the acute temperature effects on mitochondrial functions and especially the contributions of

alternative dehydrogenases to oxygen consumption and ATP synthesis rate which might have important consequences for thermal tolerance in *D. melanogaster*. It has been previously reported that Pyr + Mal oxidation in fruit flies is sensitive to temperature and cannot sustain mitochondrial oxygen consumption at high temperatures [15,17]. The cause of this failure is not ascribed to a denaturation of complex I catalytic function, since its activity increases with increasing temperature, remaining elevated at the extremely high temperature of 45 °C [17]. In contrast, the activities of Pyr dehydrogenase and citrate synthase have been reported to decrease between 38 and 45 °C, suggesting a failure of these TCA enzymes in providing NADH to complex I [17]. Notwithstanding the underlying mechanisms, the present study further highlights that this failure in complex-I-supported oxygen consumption occurred at 35 °C and is associated with a collapse in the rate of ATP synthesis, resulting in a large decrease in the ATP/O. In insects, the substrate oxidation system exerts most of the control over the flux through the phosphorylation system [22], especially at the level of complex III of the electron transport system [23]. Thus, the huge decrease in complex-I-supported ATP synthesis rate at 35 °C, that is, a 80% decrease in ATP synthesis compared with the 50% decrease in oxidative activity (Table 2) would result from the large decrease in Pyr + Mal oxidation. As previously reported in fruit flies [15,17], the use of alternative substrates offsets the large decrease in complex-I-supported phosphorylating respiration at high temperature (Table 2). Here, we show that at 35 °C, Succ oxidation maintains both the rates of oxygen consumption and ATP synthesis (Table 2 and Fig. 1B). In contrast, G3P oxidation led to a significant increase in respiration but a 40% drop in the phosphorylating activity (Table 2 and Fig. 1C). Yet, Pyr + Mal + Succ + G3P oxidation allowed the rate of ATP synthesis to remain four-fold higher than complex-I-supported ATP synthesis at high temperature (Table 2). Thus, the compensatory oxidation of these two alternative substrates, that is, Succ and G3P, do allow fruit flies mitochondria to maintain a high level of ATP production rate at high temperature. However, the values of substrate contribution ratio further indicate that the addition of G3P adds no further advantage in terms of ATP production, when Succ is already present (Fig. 3B). At 25 °C, the ATP/O declined from 3.01 to 2.37 when Succ was added and decreased to 1.06 when G3P was added (Fig. 1). These declines fall within the range of the expected theoretical values of ATP/O calculated with NADH- and FADH₂-linked substrates due to the number of

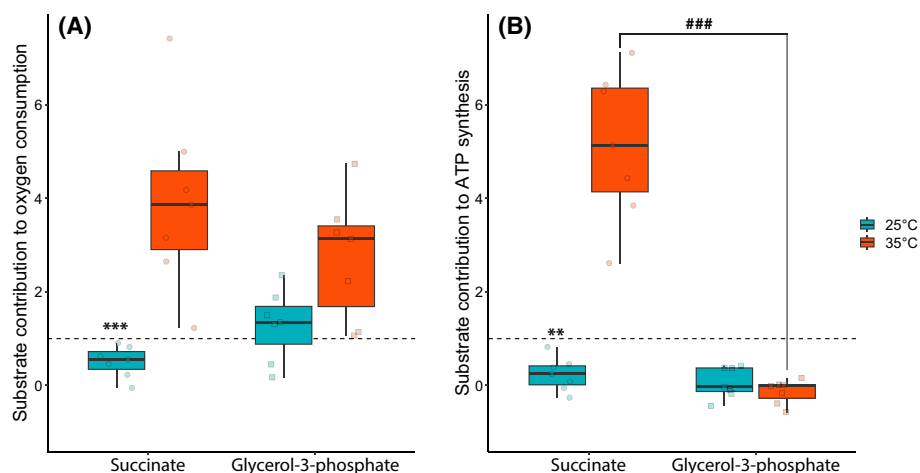


Fig. 3. Mitochondrial substrate contribution ratios calculated from mitochondrial respiration rates measured at two different temperatures (25 and 35 °C) in thorax mitochondria of *Drosophila melanogaster*. (A) Succinate (circles) and glycerol-3-phosphate (G3P) (squares) contribution to mitochondrial oxygen consumption ($n = 7$) measured at 25 (blue symbols) and 35 °C (red symbols); and (B) Succinate (circles) and G3P (squares) contribution to ATP synthesis rates ($n = 7$) measured at 25 (blue symbols) and 35 °C (red symbols). See [Materials and Methods](#) for details. Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values $<1.5 \times$ IQR (Tukey-style). The dashed line represents a 100% increase in substrate contribution, that is, a respiration rate doubled with the addition of substrate. *** $p < 0.001$ and ** $p < 0.01$, significantly different between assay temperatures for each substrate combination. Within the same assay temperature, ### $p < 0.001$ significantly different between substrate combinations.

protons per oxygen pumped with these substrates and the capacity of the ATP synthase to process these protons and form ATP [24,25], and similar ATP/O ratios with CI and CII substrates combined have been reported in isolated mitochondria from rat hearts and in permeabilized skeletal muscle myofibers [26,27]. Overall, the present study suggests that Succ is the only alternative substrate able to compensate both sides of the oxidative phosphorylation, that is, the oxygen consumption as well as ATP production rates.

The huge positive impact of G3P oxidation upon mitochondrial respiration reinforces the view that G3P might be a thermogenic substrate for endothermic insects [28,29]. However, due to the low surface-to-volume ratio of *Drosophila*, the thermogenic role of G3P is highly unlikely in these small insects, as the heat produced will be immediately dissipated. An alternative explanation would be that in *Drosophila*, G3P uncouples mitochondrial oxygen consumption from ATP synthesis at high temperature to lower the inevitable increase in ROS [30–32]. The increased oxidation of FADH₂-linked at high temperature is not only linked to the production of ATP as demonstrated here, but should also be connected to ROS production and the redox status of the cell: if high levels of FADH₂ are oxidized compared with NADH, this would translate into a higher Ubiquinol/Ubiquinone ratio for complex I, resulting in reverse electron transport

(RET) and increased ROS generation at the level of complex I [33]. Thus, the uncoupling induced by G3P might be a strategy to avoid a surge of ROS production and prevent associated oxidative damages at high temperatures. Further characterizing the amount of ROS produced at specific mitochondrial sites at high temperatures in *Drosophila* as well as the mitochondrial capacity to buffer these ROS [34] in models with mtG3PDH deficiencies would thus shed light on the potential role of this uncoupling.

The mitochondrial inner membrane fluidity and proton leakage is also well known to be sensitive to change in temperature, increasing at elevated temperature [22,27,35–37]. Since proton leakage competes with ATP synthesis for the same driving force, that is, the electrochemical gradient of proton, any increase in the proton leak activity at high temperature would have a substantial negative impact on the phosphorylation system [22]. In the present study, the LEAK respiration, that is, a measure of the maximal rate of proton leakage, increased at high temperature, being on average 2.35-fold higher at 35 °C than at 25 °C in the presence of Succ and/or G3P (Table 1). The slight nonsignificant increase of the LEAK respiration in the presence of complex-I respiratory substrates is mainly explained by the fact that both substrate oxidation system and proton leak have similar control over non-phosphorylating respiration at high temperature [22].

Thus, the strong inhibition of the Pyr + Mal oxidation at high temperature has mitigated the positive effect that proton leakage should have had on the LEAK respiration. Overall, the acute effect of an elevated temperature on inner mitochondrial proton conductance explains why the coupling efficiency decreases in mitochondria oxidizing the different respiratory substrate combinations.

In conclusion, our study revealed that at high temperatures (35 °C), *Drosophila* mitochondria undergo drastic changes in oxygen consumption rates which is also associated with changes in ATP synthesis rates. Specifically, although Pyr + Mal oxidation and ATP synthesis rates greatly decreased at 35 °C resulting in diminished ATP/O ratio, the oxidation of Succ triggered a full compensation of both oxygen consumption and ATP synthesis rates at this temperature. The addition of G3P however, importantly uncouples oxygen consumption from ATP synthesis, which might be a strategy to lower the unavoidable increased ROS production at high temperatures. This should result in important consequences at the organismal level, as the lack of ATP synthesis might impede metabolic fluxes and hamper cellular processes, eventually leading to heat mortality.

Acknowledgements

NP acknowledges supports from the Natural Sciences and Engineering Research Council of Canada (Discovery grant RGPIN-2017-05100), the Université de Moncton, and the New Brunswick Innovation Foundation.

Author contributions

DR, LT, and NP designed the research. DR, SJ, LT, and NP performed the research. DR and NP analyzed the data and wrote the first draft of the manuscript. All authors participated in the subsequent versions of the manuscript.

Peer review

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/1873-3468.14701>.

Data availability

The data that support the findings of this study are openly available in Mendeley Data at <https://data.mendeley.com/datasets/4kf6pgsp5v/1>, DOI: 10.17632/4kf6pgsp5v.1.

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