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RESEARCH ARTICLE

Adjustments in the control of mitochondrial respiratory capacity to tolerate temperature fluctuations

Katrina Y. Scott[1], Rebecca Matthew[1], Jennifer Woolcock[1], Maise Silva[1,2] and Hélène Lemieux[1,3,*]

ABSTRACT

As the world's climate changes, life faces an evolving thermal environment. Mitochondrial oxidative phosphorylation (OXPHOS) is critical to ensure sufficient cellular energy production, and it is strongly influenced by temperature. The thermally induced changes to the regulation of specific steps within the OXPHOS process are poorly understood. In our study, we used the eurythermal species of planarian *Dugesia tigrina* to study the thermal sensitivity of the OXPHOS process at 10, 15, 20, 25 and 30°C. We conducted cold acclimation experiments where we measured the adjustment of specific steps in OXPHOS at two assay temperatures (10 and 20°C) following 4 weeks of acclimation under normal (22°C) or low (5°C) temperature conditions. At the low temperature, the contribution of the NADH pathway to the maximal OXPHOS capacity, in a combined pathway (NADH and succinate), was reduced. There was partial compensation by an increased contribution of the succinate pathway. As the temperature decreased, OXPHOS became more limited by the capacity of the phosphorylation system. Acclimation to the low temperature resulted in positive adjustments of the NADH pathway capacity due, at least in part, to an increase in complex I activity. The acclimation also resulted in a better match between OXPHOS and phosphorylation system capacities. Both of these adjustments following acclimation were specific to the low assay temperature. We conclude that there is substantial plasticity in the mitochondrial OXPHOS process following thermal acclimation in *D. tigrina*, and this probably contributes to the wide thermal range of the species.

KEY WORDS: Mitochondrial respiration, Oxidative phosphorylation, Thermal sensitivity, Cold acclimation, Control, Regulation, Complex I, Phosphorylation system, *Dugesia tigrina*

INTRODUCTION

Temperature is an important variable to life processes, affecting the rate of all chemical reactions. For ectothermic organisms, which do not regulate their body temperature, adjusting their biochemical processes to temperature variations is critical for survival (Pörtner, 2002). The molecular response to temperature is complex, and not all enzymes and metabolic pathways are affected in the same order of magnitude. A comprehensive overview is needed to understand how temperature impacts different enzymes and transporters, and how unequal variations of specific steps modify not only the flow through the pathway but also the control and regulation of the pathway. Biologists are challenged with determining the metabolic adjustments essential to preserve function, homeostasis and survival of an organism encountering a thermal challenge. This is becoming increasingly important now, as we are threatened by global warming (Blier et al., 2014; Clark et al., 2013; Franklin et al., 2013).

Mitochondrial oxidative phosphorylation (OXPHOS) is among the most important pathways for survival; it is responsible for a large part of the cellular energy production in eukaryotic organisms. OXPHOS provides the energy to sustain cellular functions and is linked to many other cellular activities and signalling pathways. The tight regulation of the OXPHOS process ensures balanced metabolism and controls the production of reactive oxygen species (ROS). Overproduction of ROS is associated with damage to proteins, lipids and DNA; however, in small quantities, ROS are known to be important signalling molecules playing a role in the regulation of biological processes (Finkel, 2011). Temperature, by changing OXPHOS efficiency and regulation, can affect cell signalling through modification of ROS production (Abele et al., 2002; Jarmuszkiewicz et al., 2015).

Although thermal sensitivity of the electron transport system has been studied in many species, it is still not clear how temperature influences the process (Blier et al., 2014; Norin and Metcalfe, 2019). A recent study examined the thermal sensitivity of OXPHOS pathways in the mouse heart and identified two strong limiting factors at low temperature (Lemieux et al., 2017). The first is the phosphorylation system, the functional unit utilizing the protonmotive force to phosphorylate ADP into ATP (Gnaiger, 2009). The phosphorylation system includes three essential components: ATP synthase, adenine nucleotide translocase and the phosphate carrier (Gnaiger, 2009). When OXPHOS capacity is limited by the phosphorylation system, an increase in respiration is obtained after uncoupling the electron transfer from ATP synthesis (Gnaiger, 2009; Lemieux et al., 2017, 2011; Lemieux and Warren, 2012). The second is the NADH pathway through complex I; more specifically, pyruvate dehydrogenase complex (PDC) was identified as a component responsible for the broad thermal sensitivity of this pathway in the mouse heart (Lemieux et al., 2017). A separate study on the rat heart also showed a very strong effect of temperature on PDC activity compared with multiple other enzymes involved in the OXPHOS process (Lemieux et al., 2010a). In *Drosophila*, increased activity of PDC, due to an increase in calcium activation, was associated with a preference for a cold environment (Takeuchi et al., 2009). However, these studies did not address the adjustment of the phosphorylation system or PDC activity following thermal acclimation. It is unclear whether the controlling steps are modified to cope with temperature variation. Furthermore, there is definitive interspecies variability in steps controlling the OXPHOS pathway. The PDC does not seem to play such a major role in controlling OXPHOS at low temperature in a cold-adapted Atlantic wolffish (*Anarhichas lupus*) (Lemieux et al.,

[1]Faculty Saint-Jean, University of Alberta, Edmonton, AB, Canada, T6C 4G9. [2]Faculdade de Tecnologia e Ciências, Salvador, Bahia, 41741-590, Brazil. [3]Department of Medicine, Women and Children's Health Research Institute, University of Alberta, Edmonton, AB, Canada, T6G 2R7.

*Author for correspondence (helene.lemieux@ualberta.ca)

M.S., 0000-0002-6124-4020; H.L., 0000-0002-8864-6062

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2010b) or in rainbow trout maintained at a temperature of 12°C (Blier and Guderley, 1993). Although it is possible that adjustments occurred in these organisms in order to be able to cope with a cold environment, more knowledge on various species, including the effect of acclimation, is needed to understand the complexity of thermal adaptation.

With a worldwide distribution and abundance that is seemingly controlled by the water temperature (Chandler, 1966; Claussen and Walters, 1982; Hay and Ball, 1979; Kawakatsu, 1965; Lascombe et al., 1975; Reynoldson et al., 1965), the fresh water planarian *Dugesia tigrina* is an ideal animal model to study the thermal effect. Typically found in lakes, ponds and streams in temperate regions, *D. tigrina* is exposed to large daily and seasonal temperature variations. In comparison with other planarian species, *D. tigrina* shows a more eurythermal distribution (Russier and Lascombe, 1970). This species is known for its well-developed acclimation response to quick temperature changes from 5 to 25°C and vice versa (Claussen and Walters, 1982), indicating a pronounced thermal acclimation response. *Dugesia tigrina* can maintain a full activity threshold at temperatures above 13–15°C (Chandler, 1966; Folsom, 1976). It maintains greater body size at temperatures ranging from 10 to 20°C (Folsom, 1976; Russier-Delolme, 1972) and shrinks when the temperature is over 30°C (Folsom, 1976). Planarians are also known as an important animal model to study regeneration and stem cell dynamics (Rink, 2018). Understanding the effect of temperature is directly related to optimizing the planarian culture condition (Vila-Farré and Rink, 2018). They have also been proposed as inexpensive models for ecotoxicology studies (Knakievicz, 2014), and our study extends the scope to include the effect of a harmful substance on mitochondrial metabolism when the animal experiences thermal variation. Furthermore, the small size of the planarians allows the study of the process in the whole animal, rather than in specific tissues; this is essential to understand the overall energy production capacity of the organism facing sudden and long-term changes in temperature.

Our study addresses important questions in *D. tigrina*. First, we questioned how temperature variations affect the OXPHOS pathway and steps, and how these specific modifications affect the regulation of the process. The answers to these questions are critical for a more comprehensive understanding of the effect of temperature on animal metabolism and adaptation. Second, with our animal model, we can follow-up with simple experiments to study the same pathways and steps after a period of thermal acclimation and see whether the adjustments occur at the level of the controlling steps. As hypothesized, the phosphorylation system and the NADH pathway are severely compromised at low temperatures and are adjusted following cold acclimation. When exposed to a cold environment for an extended period of time, the planarians showed a strong plasticity and adjusted the capacity of specific steps affected by the cold: complex I and the capacity of the phosphorylation system relative to the electron transport system capacity.

MATERIALS AND METHODS

Animals

Dugesia tigrina (Girard 1850) were obtained from Ward's Natural Science Canada (St Catharines, ON, Canada) and maintained at room temperature in a modified zooplankton medium (Lynch et al., 1986) containing (concentrations in mmol l^{-1}): 0.671 KCl, 0.162 $MgSO_4$, 0.180 $CaCl_2$, 0.034 K_2HPO_4, 0.044 KH_2PO_4, 0.588 $NaNO_3$, 0.070 $NaSiO_3$ and 0.004 $FeCl_3$, and preconditioned water from a goldfish tank (100 ml l^{-1}). The animals were kept in dark conditions except during periodic checks, water change and collection of animals. For the thermal sensitivity study, the animals were kept at room temperature. For the thermal acclimation study, data loggers were used to record the temperature for the normothermic group (at room temperature, 22.2±1.2°C) and the cold-acclimated group (at 5.1±0.5°C), kept in a versatile environmental test chamber (Panasonic, MLR-352H). Three 10 gallon tanks were maintained for each treatment. Water was aerated using an air stone connected to an air pump. The animals were fed twice a week with beef liver, and the water was changed after feeding.

High-resolution respirometry on permeabilized animals

The planarians were rinsed twice with 0.5 ml of ice-cold relaxing solution (BIOPS) containing the following (concentrations in mmol l^{-1}): 2.77 CaK_2EGTA, 7.23 K_2EGTA, 20 imidazole, 20 taurine, 6.56 $MgCl_2{\cdot}6H_2O$, 5.77 ATP, 15 phosphocreatine, 0.5 dithiothreitol and 50 K-MES (pH 7.1 at 0°C). The animals were permeabilized using sharp forceps; holes were pierced into the entire body while keeping the body intact. Then, the animals were agitated for 30 min on ice in BIOPS supplemented with 50 µg ml^{-1} saponin (Lemieux and Warren, 2012). Permeabilized animals were immediately transferred to individual respiration chambers (Oxygraph-2k, Oroboros Instruments Inc., Innsbruck, Austria) containing 2 ml of respiration medium [Mir05: 110 mmol l^{-1} sucrose, 60 mmol l^{-1} potassium lactobionate, 0.5 mmol l^{-1} EGTA, 1 g l^{-1} fatty acid-free BSA, 3 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} potassium-Hepes (pH 7.1); Gnaiger et al., 2000]. Respiration was measured at 10, 15, 20, 25 or 30°C for the thermal sensitivity experiments and at 10 and 20°C for the thermal acclimation experiments; 10°C is the minimum temperature at which stable measurements in these animals can be obtained. Datlab software (Oroboros) was used for data acquisition and analysis.

The two protocols for evaluating mitochondrial function included three coupling states: LEAK (without ADP), OXPHOS (coupled with saturating ADP) and ET (electron transfer, non-coupled). The first protocol comprised the following steps: pyruvate (5 mmol l^{-1}) and malate (5 mmol l^{-1}) without adenylate (LEAK state), ADP (2.5 mmol l^{-1}; OXPHOS state for the NADH pathway through complex I), cytochrome *c* (10 µmol l^{-1}; test for integrity of outer mitochondrial membrane), succinate (10 mmol l^{-1}; OXPHOS state for the combined NADH and succinate pathways, NS pathway), uncoupler titration (dinitrophenol, DNP, up to 80–180 µmol l^{-1}; ET state for the NS pathway), rotenone (0.5 µmol l^{-1}; ET state for the succinate pathway through complex II), antimycin A (2.5 µmol l^{-1}; non-mitochondrial residual oxygen consumption, ROX, after inhibition of complex III), ascorbate (2 mmol l^{-1}), and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD; 0.5 mmol l^{-1}; complex IV activity) and azide (100 mmol l^{-1}; chemical background after inhibition of complex IV). The second protocol was similar but replaced pyruvate with glutamate (10 mmol l^{-1}) for one of the substrates feeding the NADH pathway through complex I. Mitochondrial respiration was corrected for oxygen flux, due to instrumental background, and for ROX. For complex IV activity, the chemical background measured in the presence of azide was subtracted. Respiratory flux in planarians is expressed in pmol O_2 per second per milligram wet mass or as the flux control ratio (FCR) normalized to ET capacity in the presence of substrates feeding electrons into the NS pathway.

Apparent complex IV excess capacity was determined by azide titration of both maximum complex IV activity and combined NS pathway flux (ET state). The excess was measured at the two

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extreme low temperatures (10 and 15°C) and the two extreme high temperatures (25 and 30°C). Threshold plots of relative respiration rate against the fraction of inhibited complex IV activity at the same respective azide concentrations were made as previously described (Lemieux et al., 2017; Letellier et al., 1994; Villani and Attardi, 1997). Azide titrations were performed at optimum uncoupler concentrations supporting maximum flux, preventing any effect due to ATP synthase inhibition by azide (Bowler et al., 2006), and eliminating any contribution of the phosphorylation system to flux control. The following azide concentrations were used (in mmol l^{-1}): 0.01, 0.02, 0.04, 0.09, 0.29, 0.49, 2.49, 4.49, 8.49 and 28.49.

Enzyme assays

For determination of enzyme activity, samples of *D. tigrina* were taken after the acclimation period and stored at −80°C until further analysis. The planarians (3–10 animals) were homogenized on ice in a Potter–Elvehjem tissue grinder. Samples were passaged manually 10 times with 9 volumes (wet mass) of Tris-HCl buffer 0.5 mol l^{-1}, pH 8.1, supplemented with 13.3 mmol l^{-1} $MgCl_2$. Enzymatic activity is expressed in IU per milligram of sample per citrate synthase (CS) activity, where IU is one µmole of substrate transformed per minute. CS activity was used as a marker of mitochondrial content and measured at 20°C, as previously described (Srere, 1969). Complex I activity was measured at 10°C, as previously described (Spinazzi et al., 2012).

Data analysis

Statistical analyses were performed using SigmaPlot 13 (Aspire Software International, Ashburn, VA, USA), and graphics were produced using GraphPad Prism 7.01 (GraphPad Software Inc., San Diego, CA, USA). The criteria of normal distribution and the homogeneity of variance were tested for each variable with Shapiro–Wilk and Brown–Forsythe tests, respectively. For the thermal sensitivity experiments, one-way ANOVA was used to test for differences between assay temperatures, followed by a pairwise comparison with Tukey test. For variables that did not meet the criteria, differences among assay temperatures were tested with Kruskal–Wallis tests followed by *a posteriori* Dunn comparison. Thermal sensitivity of each pathway and states was also compared on the same scale, using the flux relative to 10°C, with a one-way ANOVA followed by a pairwise comparison with Tukey test. For the thermal acclimation experiment, two-way ANOVA followed by *a posteriori* Tukey multiple comparison test was used. The two factors for the ANOVA test were the assay temperature and the acclimation temperature. Data are presented as median (minimum–maximum) without transformation, where N is the number of animal pools included. Significance was set at $P<0.05$.

RESULTS

Cold temperature is associated with loss of NADH pathway capacity and increased limitation of OXPHOS-linked respiration by the phosphorylation system

The thermal sensitivity of the various pathways of electron entry into the electron transfer system is shown in Fig. 1 (raw data for flux per mass are included in Fig. S1). The pathways examined included the NADH pathway, where electrons are transferred from pyruvate and malate into complex I; the succinate pathway, where electrons are transferred from succinate into complex II; and the NS pathway, where there is simultaneous entry of electrons into complexes I and II. The pathways were examined under either the OXPHOS state (coupled to ATP production) or the maximal non-coupled ET state (after uncoupling with DNP). Fig. 1 shows the thermal sensitivity of each pathway relative to the median flux at 10°C. The observed trend for each temperature range was a drop in thermal sensitivity in the ET state compared with the OXPHOS state (see Fig. 1A–D), suggesting a possible involvement of the phosphorylation system in OXPHOS limitation at low temperature. Furthermore, after removing the limitation due to the ADP phosphorylation system in the ET state, the thermal sensitivity was significantly lower for either the succinate pathway or complex IV compared with the NS pathway. This suggests that the NADH pathway makes a larger contribution to the loss of OXPHOS capacity associated with decreasing temperature. An alternative substrate combination was used to distinguish between the contribution of PDC or pyruvate transporter (only needed when pyruvate and malate are the substrates) to the contribution of complex I (needed with both substrate combinations: pyruvate and malate or glutamate and malate). In the planarians, the values of respiration relative to 10°C [median (minimum–maximum)] showed the same increase with temperature with glutamate and malate [1.56 (1.26–1.68) at 15°C, and 1.93 (1.55–2.31) at 20°C] as with pyruvate and malate [1.56 (1.41–1.84) at 15°C (n=4), and 2.08 (193–2.53) at 20°C (n=4)] (P=0.832 at 15°C and P=0.259 at 20°C). These results suggest that the thermal sensitivity was at least partially due to a change in complex I capacity; it is possible that the thermal sensitivity of complex I matches the thermal sensitivity of PDC activity and/or pyruvate carrier.

Mitochondrial function, expressed as FCR, was normalized to the flux at maximal ET capacity with substrates feeding electrons into the NS pathway. The FCR indicates qualitative changes of mitochondrial respiratory control rather than quantitative changes associated with mitochondrial mass (Gnaiger, 2014; Pesta et al., 2011). When the temperature decreased, the FCR showed a reduced contribution of the NADH pathway (Fig. 2A) and an increased contribution of the succinate pathway to the maximal electron flow (Fig. 2B). The succinate pathway alone can provide close to 100% of the NS pathway capacity at 10°C, about 80% in the temperature range 15–25°C, and only 50% at 30°C (Fig. 2B). Fig. 2 presents the results of the NADH pathway in the presence of pyruvate and malate. But the same results were obtained in the presence of glutamate and malate: the FCRs at 10°C were 0.44 (0.39–0.47) for the NADH pathway and 1.00 (0.99–1.00) for the succinate pathway. These results further implicate complex I as a major contributor to the loss of respiration in *D. tigrina* at low temperature.

Changes in OXPHOS limitation by the phosphorylation system (i.e. adenine nucleotide translocase, phosphate carrier and ATP synthase) with temperature were measured using OXPHOS over the ET capacity for the NS pathway (Fig. 2C). This ratio ranged from 0.0, indicating a full limitation of OXPHOS by the phosphorylation system, to 1.0, indicating no limitation of OXPHOS by the phosphorylation system (Gnaiger, 2014). Fig. 2C shows that OXPHOS limitation by the phosphorylation system increased gradually as temperature decreased, suggesting a strong control of OXPHOS by this system at low temperature. Coupling of ET with phosphorylation could potentially affect the limitation. Mitochondrial coupling efficiency was evaluated using the LEAK respiration measured in the presence of pyruvate and malate, before the addition of ADP, and expressed as the OXPHOS coupling efficiency [1−(LEAK/OXPHOS)] (Fig. 2D). A value of 1.00 indicates a fully coupled system, whereas a value of 0.00 indicates a fully uncoupled system (Gnaiger, 2014). Our data showed a preservation of coupling in the temperature range 10–25°C, with similar coupling of mitochondria to that obtained for other permeabilized invertebrates (Kake-Guena et al., 2015, 2017;

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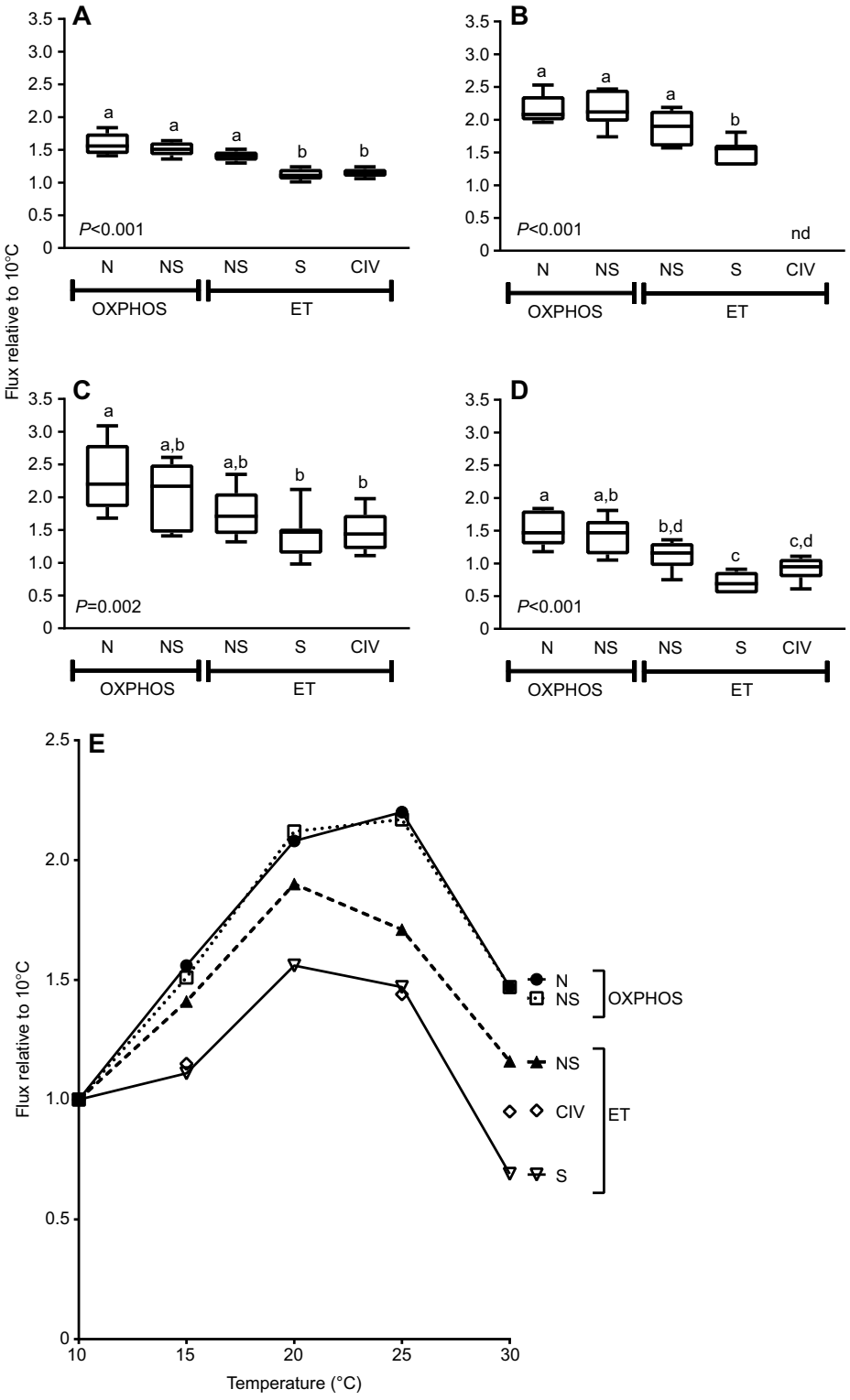

Fig. 1. Thermal sensitivity of mitochondrial function in permeabilized *Dugesia tigrina*. Mitochondrial respiration was measured in two states: oxidative phosphorylation (OXPHOS; coupled, with saturating ADP) or electron transfer (ET; non-coupled). The pathways and steps include the NADH pathway through complex I (N; pyruvate and malate), the combined NADH and succinate pathways with simultaneous entry of electrons into complex I and II (NS; pyruvate, malate and succinate), the succinate pathway alone (S; succinate and rotenone), and complex IV as a single step (CIV; extrapolated from threshold plots; see Fig. 3). (A–D) Respiration was measured at 15°C (A), 20°C (B), 25°C (C) and 30°C (D) and is expressed relative to the median value at 10°C. (E) Comparison of the data at each temperature. Box plots indicate minimum, 25th percentile, median, 75th percentile and maximum. Within a specific panel, boxes without a letter in common are significantly different (P<0.05; ANOVA). N=5 at 10 and 15°C; N=7 at 20, 25 and 30°C.

Lemieux and Warren, 2012). However, a significant increase in uncoupling was detected at 30°C (Fig. 2D).

Apparent complex IV excess capacity is small and is only detected at 10°C

Apparent excess capacity of complex IV was measured over the combined ET pathway flux, at maximum convergent flux through the electron transport system, by eliminating any contribution of the phosphorylation system to flux control (Lemieux et al., 2017), and by preventing the potential impact of inhibition of ATP synthase by azide (Bowler et al., 2006). The apparent excess was measured at four different temperatures (10, 15, 25 and 30°C). Azide titration resulted in hyperbolic inhibition of complex IV (Fig. 3A–D). Threshold plots display the relative pathway flux as a function of relative complex IV inhibition (Fig. 3E–H). The intercept value represents the apparent excess capacity of complex IV over the

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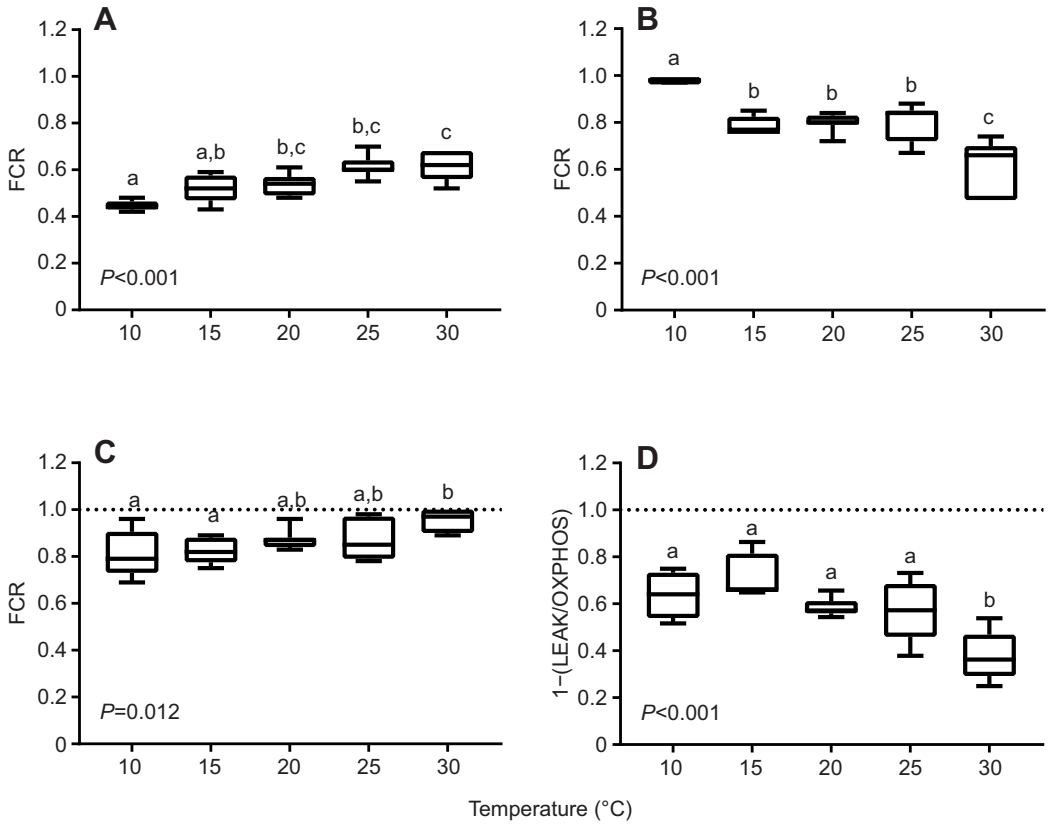

Fig. 2. Mitochondrial respiratory capacity at five different temperatures in permeabilized *D. tigrina*. Mitochondrial respiration was measured in OXPHOS (coupled, with saturating ADP) or ET state (non-coupled). Data are expressed as the flux control ratio (FCR; normalized to maximal ET capacity with combined NADH and succinate pathways: NS). (A) OXPHOS state in the presence of substrates feeding electrons into the NADH pathway through complex I (N; pyruvate and malate). (B) ET state in the presence of substrate feeding electrons into the succinate pathway through complex II. (C) OXPHOS capacity with simultaneous entry of electrons into the NS pathway. The dotted line indicates no control of OXPHOS by the phosphorylation system, while a value of zero indicates full control of OXPHOS by the phosphorylation system. (D) OXPHOS coupling efficiency [1−(LEAK/OXPHOS)] for the NADH pathway (pyruvate and malate; LEAK respiration was measured in the presence of pyruvate and malate, but without adenylates, while OXPHOS state was measured with the same substrates and saturating ADP). The dotted line indicates full coupling, while a zero value is associated with fully uncoupled mitochondria. Box plots indicate minimum, 25th percentile, median, 75th percentile and maximum. Within a specific panel, boxes without a letter in common are significantly different (P<0.05; ANOVA). N=5 at 10 and 15°C; N=7 at 20, 25 and 30°C.

pathway flux. The results showed no or very low excess capacity of complex IV in the temperature range 15–30°C, with intercept values of 1.03 at 15°C, 1.06 at 25°C, and 1.03 at 30°C (Fig. 3F–H). At 10°C, the inhibition of 26% of complex IV activity had a minor effect on NS pathway capacity, resulting in a small excess capacity of complex IV (1.26; Fig. 3E).

Adjustments occurring after 4 weeks of cold acclimation specifically target the limiting steps at low assay temperature

Mitochondrial function was then measured at two assay temperatures (10 and 20°C) and compared between a group of *D. tigrina* acclimated under normothermic condition (22°C) or cold acclimated (5°C). After 4 weeks, the planarian mitochondria exhibited changes in the pattern of respiratory control. At low assay temperature, the NADH pathway capacity (expressed per mg of tissue) showed a trend for an increase in the cold-acclimated animals compared with the normothermic animals (Fig. 4A). When expressed as FCR, the increase in cold-acclimated animals becomes significant (Fig. 4B). Furthermore, the thermal sensitivity (expressed as the flux at 20°C relative to the flux at 10°C) of the NADH pathway was reduced following cold acclimation (Fig. 4C). In order to determine whether the low-temperature-induced change in NADH pathway capacity was due to a modification of complex I, an enzymatic assay of complex I was performed at 10°C. The results confirmed that the adjustment of the NADH pathway following cold acclimation was caused, at least in part, by an increase in complex I activity at 10°C (Fig. 4D). At the low assay temperature following cold acclimation, the increase in complex I activity was observed with a decrease in succinate pathway capacity through complex II, reaching significance when expressed either in flux per mass (Fig. 4E) or in FCR (Fig. 4F). This led to a significant increase in thermal sensitivity of the succinate pathway in the cold-acclimated animals (Fig. 4G). In contrast, at an assay temperature of 20°C, the capacity of both NADH and succinate pathways, expressed as flux per mass, was reduced in the cold-acclimated animals (Fig. 4A,E), without any change in the contribution of each specific pathway to maximal ET capacity (Fig. 4B,F). Complex IV capacity showed no adjustment following thermal acclimation at an assay temperature of 10°C (Fig. 4H,I). However, at an assay temperature of 20°C, the FCR for complex IV was higher in the cold-acclimated group than in the normothermic group (Fig. 4I). The thermal sensitivity of complex IV (Fig. 4J) and the mitochondrial content, evaluated by CS activity (Fig. 4K), did not change following cold acclimation.

The phosphorylation system is known to control OXPHOS in planarians (Lemieux and Warren, 2012), but this control increases

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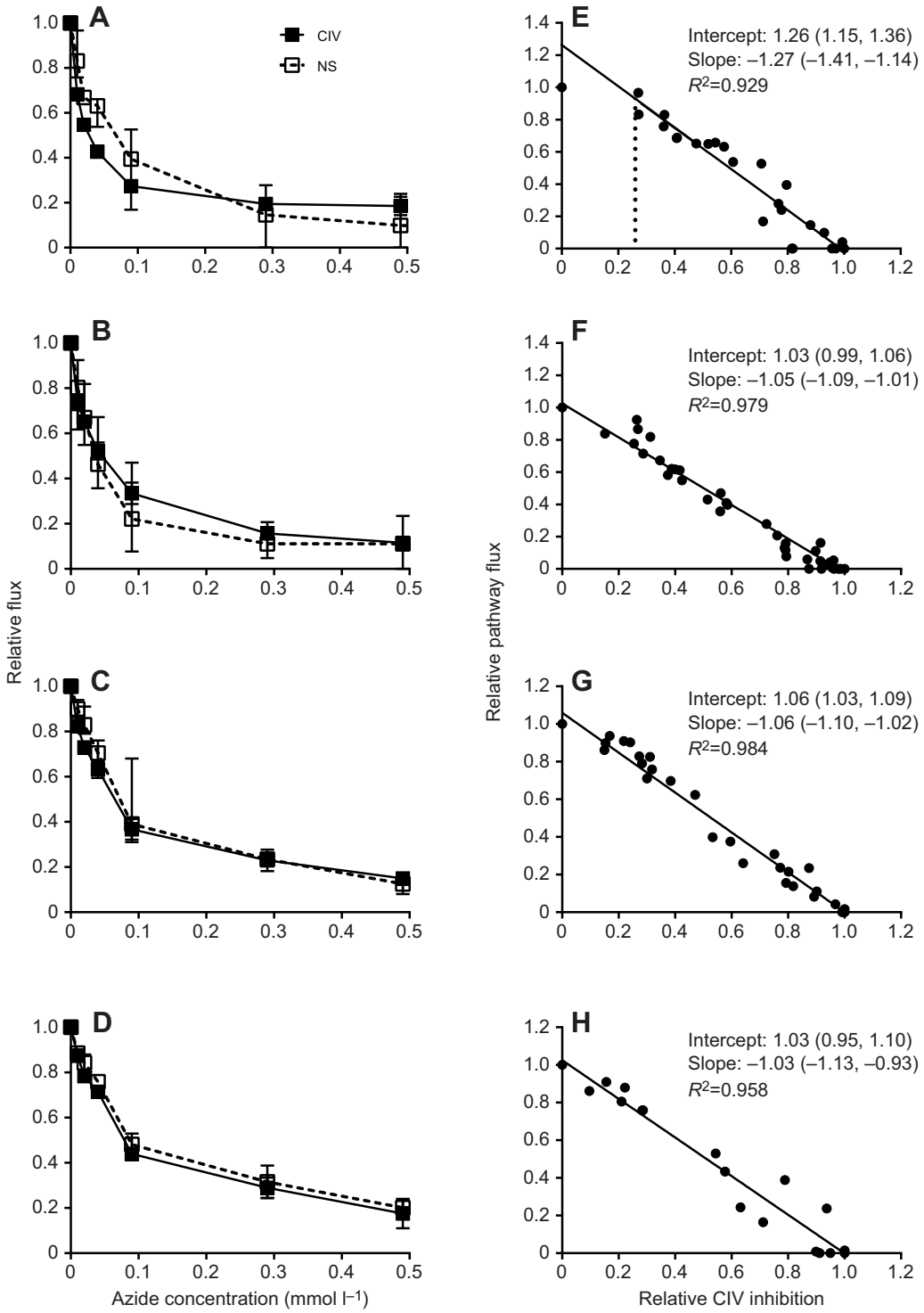

Fig. 3. Azide titration and complex IV threshold in permeabilized *D. tigrina* at different temperatures. (A–D) Effect of azide concentration on relative combined NADH and succinate pathways (NS) under ET state (non-coupled) and velocity of the single enzyme cytochrome *c* oxidase (complex IV; CIV). Three points over an azide concentration of 0.5 mmol l^{-1} were omitted from the graphs in A–D to improve visualization of the curves. (E–H) Threshold plots of relative NS pathway flux as a function of relative inhibition of complex IV at identical azide concentrations (see Materials and Methods). The intercept values (median with lower and upper value of the confidence interval 95%) represent the apparent excess capacity of complex IV. The dotted vertical line at 10°C in E illustrates the complex IV inhibition (26%) needed to inhibit the NS pathway flux and the percentage was calculated from the linear regression intercept. A,E: 10°C; B,F: 15°C; C,G: 25°C; and D,H: 30°C.

as temperature decreases (Fig. 2C). Thus, this important system constitutes a major target point of regulation to be adjusted during cold acclimation. Following acclimation, there was no significant change in maximal OXPHOS (Fig. 5A) or ET capacity (Fig. 5B) at the low assay temperature. But the ratio of maximal OXPHOS/ET increased significantly at an assay temperature of 10°C, indicating a

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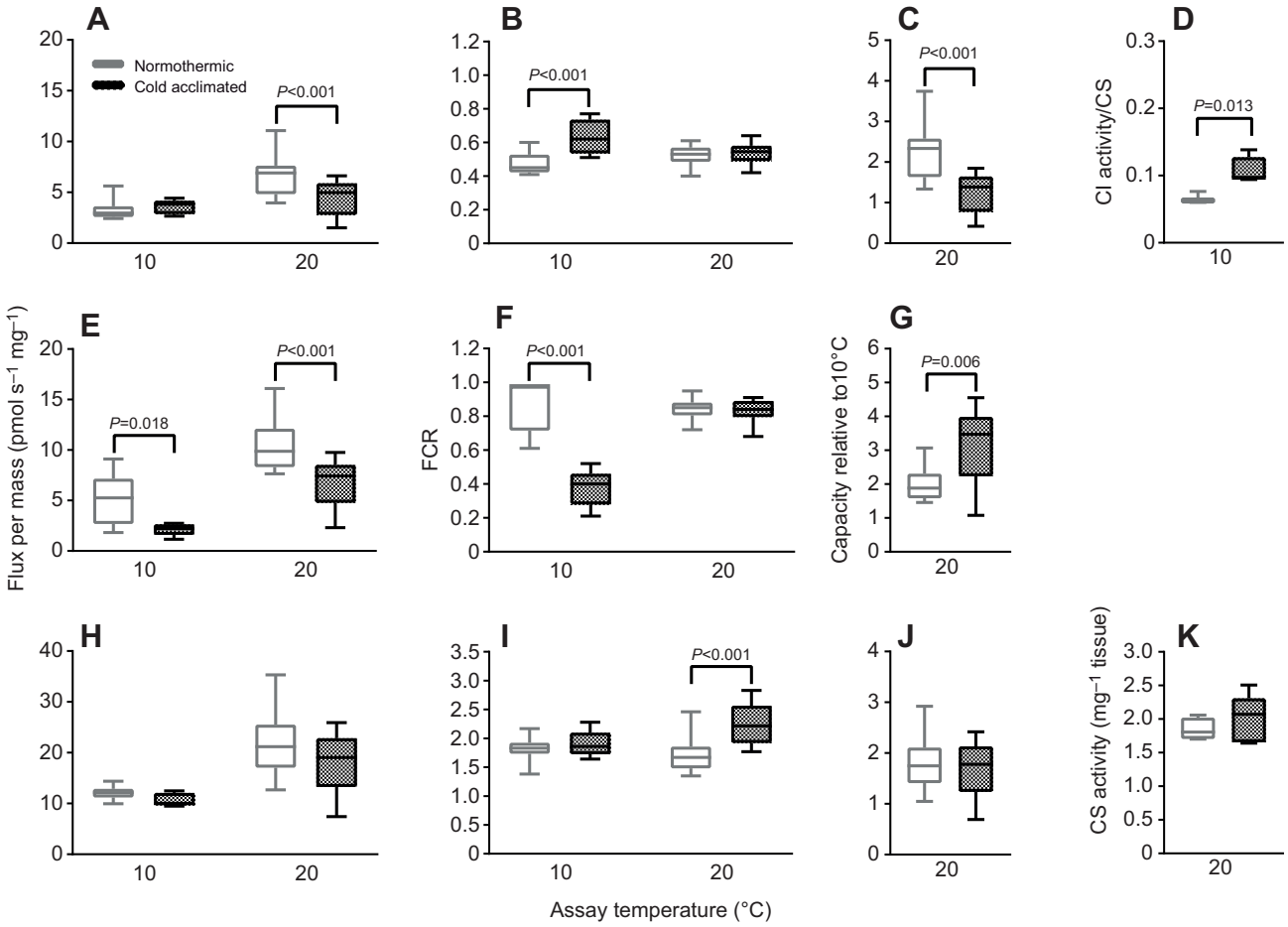

Fig. 4. Effect of thermal acclimation on the capacity of separate OXPHOS pathways and steps in *D. tigrina*. Animals were either maintained under normothermic conditions (22°C) or cold acclimated (5°C) for 4 weeks. Mitochondrial respiration rates in permeabilized animals were measured at 10 and 20°C, and expressed as flux per mass (A,E,H) or FCR (over the maximal electron transport capacity when feeding electrons into complexes I and II simultaneously; B,F,I). The thermal sensitivity of each pathway is expressed as the value relative to the median of respiration at 10°C (C,G,J). Data are for the NADH pathway capacity in the OXPHOS state (A–C), complex I (CI) activity expressed per unit of citrate synthase (CS) activity (D), the succinate pathway capacity in the ET state (E,F,G), complex IV capacity in the ET state (H,I,J), and citrate synthase (CS) activity (K). Box plots indicate minimum, 25th percentile, median, 75th percentile and maximum. Significant differences between acclimation temperature within an assay temperature are indicated ($P<0.05$; ANOVA). For mitochondrial respiration, the number of assays was 12 and 5 at 10°C, and 21 and 10 at 20°C for normothermic versus cold-acclimated animals. For enzyme activity, the number of assays on pools of 3–10 animals was 3 and 5 for complex I, and 4 and 6 for CS for normothermic versus cold-acclimated animals. The FCR for complex IV was square-root transformed to meet the assumptions.

decreased limitation of OXPHOS by the phosphorylation system following cold acclimation (Fig. 5C). This might be partially explained by a trend for a lower ET capacity at the low assay temperature (Fig. 5B; $P=0.077$) in the cold-acclimated animals, which needed less phosphorylation capacity to support OXPHOS. The change in control by the phosphorylation system after cold acclimation could also be related to the coupling status; partial uncoupling would reduce the need for ADP phosphorylation. Our data showed no significant change in OXPHOS coupling efficiency following cold acclimation (Fig. 5D). In contrast, at an assay temperature of 20°C, both maximal OXPHOS (Fig. 5A) and ET capacity (Fig. 5B) decreased following cold acclimation, and there was no change in the OXPHOS/ET ratio (Fig. 5C).

DISCUSSION

Preservation of mitochondrial metabolism during temperature variations is crucial for maintaining activity level and energetic balance, especially in eurythermal species such as *D. tigrina*. Our understanding of the necessary adjustments is still limited. Our study used high-resolution respirometry to measure quantitative and qualitative changes in the OXPHOS process, both with short-term temperature variations (thermal sensitivity of the process) and long-term temperature variations (modifications following 4 weeks of acclimation to low temperature). Our data identified two steps with pronounced thermal sensitivity that are able to effectively increase their capacity at low temperature following cold acclimation: complex I and the phosphorylation system.

As temperature drops, the contribution to the maximal ET capacity by NADH pathway also decreases, and an increased contribution of electrons from the succinate pathway partially compensates for this decrease. This effect is due to a higher thermal sensitivity of the NADH pathway compared with the succinate pathway, agreeing with previous reports on fish (Christen et al., 2018; Mark et al., 2012) and mammals (Lemieux et al., 2017, 2010a). The pathway-dependent thermal sensitivity of OXPHOS may be designed to allow mitochondrial energy production over a broad range of temperatures. While less-thermosensitive pathways (e.g. succinate pathway) attenuate the rapid decline of mitochondrial

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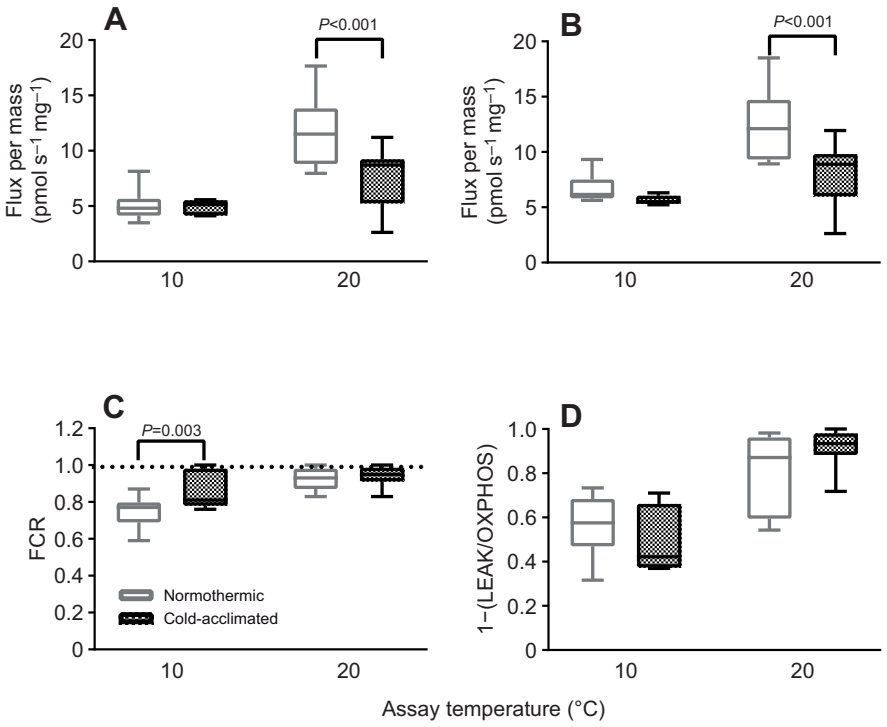

Fig. 5. Effect of thermal acclimation on combined NADH and succinate (NS) pathway capacity and coupling efficiency in *D. tigrina*. Animals were either maintained under normothermic conditions (22°C) or cold acclimated (5°C) for 4 weeks and mitochondrial respiration rate in permeabilized animals was measured at 10 and 20°C. (A,B) Mitochondrial respiration rate expressed as flux per mass under OXPHOS (coupled; A) or ET (non-coupled; B) state. (C) Mitochondrial respiration rate expressed as the ratio of OXPHOS to ET state (FCR) in the presence of pyruvate, malate and succinate, showing the control of OXPHOS by the phosphorylation system, with a value of 1.0 (dotted line) indicating no control. (D) OXPHOS coupling efficiency [1−(LEAK/OXPHOS)] in the presence of pyruvate and malate. Box plots indicate minimum, 25th percentile, median, 75th percentile and maximum. Significant differences between acclimation temperatures within an assay temperature are indicated (P<0.05; ANOVA). The number of assays was 12 and 5 at 10°C, and 21 and 10 at 20°C for normothermic versus cold-acclimated animals. The NS pathway under OXPHOS state was square-root transformed to meet the assumptions. Data are presented without transformation.

ATP generation at low temperatures, more-thermosensitive pathways (e.g. NADH pathway) serve as important energy producers at physiological temperature and minimally contribute to energy production at lower temperatures. However, the energy provided by the succinate pathway at low temperature also means a lower P/O ratio, so more oxygen is consumed for the same amount of ATP formed (Hinkle, 2005). This adjustment is plausible for an aquatic animal as more oxygen is available at low temperature as a result of the increase in oxygen solubility in water. But it might also mean an increase in ROS production; electrons coming through $FADH_2$ have been suggested to increase ROS production (Speijer, 2014; Speijer, 2019).

Our data also demonstrate that following cold acclimation for 4 weeks, the contribution of the NADH pathway to maximal OXPHOS capacity increased, and the rise is specific to low assay temperature. Keeping the NADH pathway active at low temperatures, rather than producing all the energy from the succinate pathway, would avoid an increase of oxygen consumption relative to the energy produced. For the NADH pathway, three proton pumps are active compared with two with $FADH_2$-linked substrates such as succinate. With NADH-linked substrates, more protons can be used to produce energy with the same amount of electron transferred to oxygen. Keeping the NADH pathway more active also ensures a wider range of potential substrates available for energy production. Several studies have suggested a strong importance of adjustment in the NADH pathway for thermal acclimation in animals. A positive effect of cold acclimation on NADH pathway capacity was previously shown in various tissues and species including rainbow trout (*Oncorhynchus mykiss*) red muscle (Kraffe et al., 2007), short-horned sculpin (*Myoxocephalus scorpius*) red muscle (Guderley and Johnston, 1996), killifish (*Fundulus heteroclitus*) liver (Chung and Schulte, 2015), goldfish (*Carassius aurratus*) white skeletal muscle (Dos Santos et al., 2013) and tilapia (*Oreochromis mossambicus*) caudal muscle (Schnell and Seebacher, 2008). Our data indicate that both the thermal sensitivity of the NADH pathway and its adjustment following cold acclimation in *D. tigrina* were at least partly due to an adjustment in complex I activity. And our data on *D. tigrina* are not the only ones pointing toward the importance of complex I adjustment at low temperature. A translocation of the mitochondrial gene encoding the NADH dehydrogenase subunit 6, an indispensable part of complex I, has been shown to be an adaptive and supportive event of cold stenothermy in Antarctic nototheniids (Mark et al., 2012). In contrast, in hearts of homeotherms such as mouse (Lemieux et al., 2017) and rat (Lemieux et al., 2010a), the PDC was identified as the step causing the reduction in NADH pathway capacity at temperatures under the physiological range. This suggests that in species facing a wide range of temperatures or living in cold environments, an adjustment in thermal sensitivity of PDC might occur to reduce the loss of NADH pathway capacity at low temperature. For example, in the Atlantic wolffish (*A. lupus*), a fish with a very low optimal temperature range (5–10°C in captivity; Tveiten and Johnsen, 1999), the thermal sensitivity of the PDC is very low (Lemieux et al., 2010b) compared with that observed in mammals (Blier et al., 2014; Lemieux et al., 2010a).

Our results emphasize the importance of qualitative change, rather than quantitative change, in order to allow the animals to cope with the temperature shifts. The changes in contribution of the NADH and succinate pathway to OXPHOS capacity in *D. tigrina* occurred without a change in mitochondrial content or maximal OXPHOS or ET capacity (in flux per mass) at low temperature. The qualitative adjustments have been suggested to be of prime importance during thermal adaptation to ensure proper kinetic equilibrium among reactions and appropriate regulation of the process (Blier et al., 2014). Preserving the performance at low temperature can also be associated with a loss of overall capacity at temperatures not stressful prior to acclimation, known as the cost of adaptation (Chung and Schulte, 2015). This was shown in our results; the decrease in OXPHOS and ET capacity at 20°C in the cold-acclimated animals, even if the mitochondrial content is preserved, is indicative of a reduced OXPHOS efficiency per

mitochondrial unit. Similarly, in the heart and brain of the killifish (*F. heteroclitus*), a decrease in OXPHOS capacity was measured after cold acclimation, and the difference was more pronounced at a higher assay temperature (Chung et al., 2017). Some fish species avoid this cost by adopting a metabolic suppression or a quiescent state following cold acclimation (Campbell et al., 2008; Costa et al., 2013; Crawshaw, 1984). Surprisingly, even with a large reduction in feeding below 6°C (Cash et al., 1993; Pickavance, 1971), *D. tigrina* does not adopt a metabolic suppression strategy as its energy production capacity is preserved at low temperature. This is even more interesting as starvation in planarians was previously shown to reduce the density of mitochondria (Kotomin and Dontsova, 1980), but it is apparently not the case for reduced feeding linked to cold exposure. However, it is important to note that the reduction in capacity can vary in different organs, as previously reported (Glanville and Seebacher, 2006; Yan and Xie, 2015). By studying the whole animal, we could miss the tissue-specific pattern of metabolic compensation. To survive while facing a thermal shift, planarians only need a few remaining neoblast cells. This is because of the particularity of these organisms, being considered as immortal organisms as a result of their incredible regenerative properties and the highly effective mechanisms that preserve genome stability in the neoblast stem cell population (Sahu et al., 2017).

Another crucial step to consider in thermal sensitivity is the phosphorylation system, a strong regulator of OXPHOS in multiple species of planarians (Lemieux and Warren, 2012). Our data in *D. tigrina* point to increased control of OXPHOS by the phosphorylation system as temperature decreases, implicating this system as a potentially important target for cold adaptation. The increase in control of OXPHOS by the phosphorylation system as temperature decreases has been shown in the heart of mouse (Lemieux et al., 2017), rat (Dufour et al., 1996; Quentin et al., 1994) and triplefin fish, *Forsterygion lapillum* (Khan et al., 2014). However, our data in *D. tigrina* are the first to show an adjustment following cold acclimation, and this adjustment is specific to the low assay temperature. One way to relieve the control of OXPHOS by the phosphorylation system is to partially uncouple the mitochondria; changes in coupling have been suggested as a mechanism to cope with cold acclimation in various species (Bryant et al., 2018; Chicco et al., 2014; Colinet et al., 2017; Talbot et al., 2004). Our data did not detect a significant change in coupling efficiency, but the median value showed lower coupling in the cold-acclimated group. Further investigation is required to determine whether any change of control by the phosphorylation system is due to an adjustment in coupling. The regulation of OXPHOS by the phosphorylation system is a critical and unexplored aspect of temperature adaptation. It will not only affect our understanding of how animals adjust to the climate but also help in human health through a better understanding of the consequences of hypothermia used to reduce ischaemic damages during surgeries and to preserve organs for transplantation.

Finally, complex IV has also been suggested to play an important role in the thermal sensitivity of OXPHOS (for review, see Blier et al., 2014). In *D. trigrina*, the lack of apparent complex IV excess capacity over the combined NS pathway flux, in the temperature range 15–30°C, implicates complex IV as an important regulator of the flux and the redox state of the electron transport system. Data on invertebrate species, such as *Daphnia* (Kake-Guena et al., 2017) or *Drosophila* (Pichaud et al., 2013, 2010), also indicated no apparent excess capacity of complex IV over the combined NS pathway flux in the physiological temperature range. Complex IV is generally less negatively affected by low temperature compared with other steps of the pathway, so the apparent excess typically increases while temperature decreases. The rise in apparent excess capacity can be very pronounced, increasing by 2- to 6-fold at 12°C in *Drosophila* (Pichaud et al., 2013, 2010), and by 7.2-fold at 4°C in the mouse heart (Lemieux et al., 2017). However, in *D. tigrina*, the change in complex IV excess capacity over the combined pathway flux was small at low temperature, increasing only slightly by 1.26-fold at 10°C. Interestingly, data on mammal tissues showed a relatively large complex IV apparent excess capacity over the combined pathway flux even at the physiological temperature: 1.7-fold in the mouse heart (Lemieux et al., 2017) and 1.4-fold in the human skeletal muscle (Kunz et al., 2000). Studies have suggested that the complex IV excess capacity ensures the maintenance of the electron transport system in an oxidized state allowing a sharp thermodynamic gradient even with variation in the conditions: substrate availability, temperature and oxygen availability (Gnaiger et al., 1998; Harrison et al., 2015). Our data on *D. tigrina* might contradict this assumption as this eurythermal species survives in a wide range of temperatures without apparent complex IV excess capacity. Interestingly, the heart of the cold-adapted Atlantic wolfish has a high thermal sensitivity of complex IV compared with that of the NADH pathway, the PDC and complex I (Lemieux et al., 2010b). These data instead suggest that the capacity of the NADH pathway and associated steps at low temperature are potentially important sites of adaptation to upgrade mitochondrial capacity in ectotherm species (Blier et al., 2014). And it occurs at the cost of reducing the apparent excess capacity of complex IV.

Concluding remarks

In summary, we identified complex I and the control exerted by the phosphorylation system as important sites of adjustments necessary for adapting to a cold environment in *D. tigrina*. This is interesting as a recent study on the evolutionary divergence of genomes in the spider mite identified two mitochondrial genes, related to complex I (ND4) and the phosphorylation system (ATP6), as having crucial roles during adaptation to climatic condition (Sun et al., 2018). Our data also showed the plasticity of the eurythermal organism *D. tigrina*; the ability to adjust critical steps at low temperature probably contributes to its ability to tolerate a wide range of temperatures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.L.; Methodology: K.Y.S., M.S., H.L.; Validation: H.L.; Formal analysis: K.Y.S., R.M., J.W., M.S., H.L.; Investigation: K.Y.S., R.M., J.W., M.S., H.L.; Resources: H.L.; Data curation: K.Y.S., H.L.; Writing - original draft: H.L.; Writing - review & editing: K.Y.S., R.M., J.W., M.S., H.L.; Visualization: H.L.; Supervision: H.L.; Project administration: H.L.; Funding acquisition: H.L.

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Data availability

All datasets supporting this manuscript are available from the Dryad Digital Repository (Scott et al., 2019): dryad.727r289

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.207951.supplemental

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