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RESEARCH ARTICLE

Mitochondrial thermo-sensitivity in invasive and native freshwater mussels

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ABSTRACT

Climate change is impacting many, if not all, forms of life. Increases in extreme temperature fluctuations and average temperatures can cause stress, particularly in aquatic sessile ectotherms such as freshwater mussels. However, some species seem to thrive more than others in face of temperature-related stressors. Thermal tolerance may, for example, explain the success of invasive species. It is also known that mitochondria can play a key role in setting an ectothermic species' thermal tolerance. In this study, we aimed to characterize the mitochondrial thermo-tolerance in invasive and endemic freshwater mussels. With the use of high-resolution respirometry, we analyzed the mitochondrial respiration of two freshwater bivalve species exposed to a broad range of temperatures. We noticed that the invasive dreissenid *Dreissena bugensis* possessed a less thermo-tolerant mitochondrial metabolism than the endemic unionid *Elliptio complanata*. This lack of tolerance was linked with a more noticeable aerobic metabolic depression at elevated temperatures. This decrease in mitochondrial metabolic activity was also linked with an increase in leak oxygen consumption as well as a stable maintenance of the activity of cytochrome *c* oxidase in both species. These findings may be associated both with the species' life history characteristics, as *D. bugensis* is more adapted to unstable habitats, in which selection pressures for resistance adaptations are reduced. Our findings add to the growing body of literature characterizing the mitochondrial metabolism of many aquatic ectotherms in our changing world.

KEY WORDS: Mitochondria, Climate change, Thermal sensitivity, Invasive species, Aquatic ectotherms

INTRODUCTION

One of the greatest challenges faced by organisms in our time is climate change. Predictive models have estimated that by the end of the 21st century, average temperatures will be 1.5–5°C higher than present (Rogelj et al., 2012; Bush and Lemmen, 2019). Alongside the increase in average temperatures, climate change is expected to result in an increase in extreme temperature fluctuations (Vasseur et al., 2014). Aquatic ectotherms are particularly susceptible to these changes, as their metabolic processes are intimately related to environmental temperatures, and many ectotherms already live close to their upper thermal limit (Nguyen et al., 2011). In comparison with motile animals such as fish, which can attempt to mitigate the negative effects of elevated temperatures by migrating into cooler areas (Buisson et al., 2008a,b), sessile ectotherms, such as bivalves, are at the whims of their local habitat.

Susceptibility of aquatic ectotherms to climate change has been suggested to be inherently linked to mitochondria (Blier and Lemieux, 2001), as these small organelles, present in most eukaryotic cells, are sensitive to environmental temperatures and are key regulators of cellular energy metabolism. Energy in the form of ATP is indeed generated mainly in mitochondria through a process known as oxidative phosphorylation (OXPHOS). As such, disruption of metabolic processes by elevated temperatures has often been associated with failure of multiple functions and often, in more extreme cases, death (Schulte, 2015). While it is generally known that mitochondrial thermo-sensitivity mediates metabolic failure at high temperatures, the exact way in which this occurs is less understood because of the complex metabolic synergies and interactions found in organisms (Schulte, 2015). Temperature sensitivity of various metabolic processes varies wildly from species to species (Blier and Lemieux, 2001). Consequently, while some species may suffer greatly from climate change, others may not be affected as much and may even thrive under these new conditions owing to a decrease in competition from more thermo-sensitive species. Mitochondrial thermo-sensitivity becomes particularly relevant in the context of invasive species and climate change, as some of these may profit from the newfound conditions. Invasive success has already been associated with an elevated resistance to high temperatures in some aquatic ectotherms such as fish and marine bivalves (Lockwood et al., 2010, 2011; Bates et al., 2013), and more thermo-stable mitochondria in particular have been shown to confer an advantage to invasive crab species (Iftikar et al., 2010). However, no such studies have been conducted on invasive and endemic freshwater mussels.

North American freshwater mussels (Bivalvia: Unionida) are already greatly imperiled, with ∼70% of the nearly 300 species being of special concern, threatened, endangered or even extinct (Williams et al., 1993; Lydeard et al., 2004). As these provide many ecological functions and services (Lopes-Lima et al., 2017), the health and maintenance of established populations is critical to a healthy aquatic environment. Studies suggest that unionid mussels are imperilled owing to their limited dispersal and mobility, habitat destruction and pollution, a complex life cycle involving a fish host, and their susceptibility to the effects of introduced/invasive species (Strayer et al., 2004; Ganser et al., 2015). Research on their thermal tolerances also suggests that many species may already be living close to their upper thermal limits (Pandolfo et al., 2010; Ganser et al., 2015), although some studies have indicated that some North American native freshwater mussels may be more robust to elevated temperature than previously reported (Galbraith et al., 2012; Martin,

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2016). For example, Gallardo and Aldridge (2013) modelled the combined effect of climate change and invasive species (including the zebra mussel, *Dreissena polymorpha*) on European endemic unionid species (such as *Pseudanodonta complanata*) and predicted that, by 2050, the zebra mussel would gain up to 15–20% territory while the endemic unionid mussel would suffer territorial losses of up to 14–36%.

Several studies have shown the effects of elevated water temperatures on various physiological processes in freshwater mussels, including oxygen consumption, ammonium excretion, heart rate and enzymatic activities (e.g. Doucet-Beaupré et al., 2010; Ganser et al., 2015). In North American native species, for example, oxygen consumption and ammonium excretion were shown to increase with temperature to levels that led Ganser et al. (2015) to suggest that these alterations could decrease the amount of energy available for key biological processes, such as survival and reproduction. To our knowledge, however, research on mitochondrial thermo-sensitivity of invasive and endemic freshwater mussel species is very limited. In the present study, we investigated the mitochondrial metabolic response of North American invasive and endemic species exposed to elevated temperatures to assess whether mitochondrial thermo-sensitivity might play a role in determining invasion success in freshwater mussels. We hypothesize that if a successful invasion is partly explained by thermal tolerance of mitochondrial function, we can expect that mitochondria will be more robust at elevated temperatures in invasive species.

MATERIALS AND METHODS

Experimental strategy

The strategy used to achieve our objectives consisted of collecting bivalves from natural ecosystems and conducting mitochondrial respiration assays to assess whether mitochondria from an invasive species are more robust at elevated temperatures relative to a native species. Specifically, two freshwater bivalves, the North American endemic unionid species *Elliptio complanata* (Lightfoot 1786) (eastern elliptio) and the invasive quagga mussel, *Dreissena bugensis* Andrusov 1897, were exposed to elevated temperatures to determine whether mitochondrial thermo-sensitivity might play a role in the invasive success of the quagga mussel. The eastern elliptio is a well-established species in the Canadian province of Quebec (Paquet et al., 2005; Picard and Houle, 2011), and is actually considered stable throughout most of its range (eastern Canada and United States; Cummings and Cordeiro, 2011). Previous studies suggested that the eastern elliptio is rather thermo-tolerant, with critical thermal maximum for adults reaching 38–40°C (Galbraith et al., 2012; Martin, 2016). The quagga mussel is an invasive species originally from Eastern Europe (Mills et al., 1993) that has prospered and colonized many aquatic environments in Quebec, much to the detriment of native species (Sala et al., 2000). Similar to *E. complanata*, the quagga mussel can tolerate high temperatures (up to 39°C) for hours (Choi et al., 2013). However, although the thermal tolerance of both species has been studied at the whole organism level, no comparable data are available for both species at the cellular level. For example, if the invasive success of *D. bugensis* is partly explained by thermal tolerance of mitochondrial function, we can expect that its mitochondria will be more robust at elevated temperatures relative to *E. complanata*. This could partially explain the predictions obtained by Gallardo and Aldridge (2013) on the combined effect of climate change and invasive species on endemic unionid species (i.e. a loss in territory of the close unionid cousin *P. complanata* and a gain of territory of the closely related invasive zebra mussel).

Experimental animals

Adult specimens of both *E. complanata* and *D. bugensis* were collected from the St Lawrence River basin, in Lake Hertel (45.542454°, –73.154006°) and Pointe-des-Cascades (45.331500°, –73.968033°), respectively, under conditions specified in provincial permit numbers 2018-04-16-2398-06-16-S-P and 2018-5-15-2439-16-05-S-P. *Elliptio complanata* were simply removed by hand from the sediment where they were partly buried, whereas *D. bugensis* were gently cut off from the substrates to which they were attached using a scraper. Mussels were acclimated for a minimum of 6 weeks at the Environment and Climate Change Canada facilities, where they were maintained at 15±0.5°C under a 16 h:8 h light:dark cycle in aquaria filled with dechlorinated and UV-treated tap water (City of Montréal, QC, Canada) under constant aeration. Water was renewed every 2 weeks and mussels were fed three times per week with concentrates of phytoplankton (Phytoplex, Kent Marine, WI, USA) and *Pseudokirchneriella subcapitata* algal preparations until being transferred to the University of Montreal laboratories, where the mitochondrial respiration assays were performed. There, the mussels were placed in an aerated aquarium with dechlorinated water kept at 15±0.5°C and feeding was suspended. Experiments ensued 24 h after arrival.

Mitochondrial respiration

Elliptio complanata (n=12 with n representing one individual's gills) and *D. bugensis* (n=9) were put on ice prior to dissection. Once on ice, the mussels were opened with a sterilized shucker knife and the gills were dissected and immediately placed on ice in an Eppendorf tube containing Biopsy Preservation Solution [BIOPS: CaK_2 EGTA (2.77 mmol l^{-1}), K_2EGTA (7.23 mmol l^{-1}), $MgCl_2{\cdot}6H_2O$ (6.56 mmol l^{-1}), taurine (20 mmol l^{-1}), Na_2phosphocreatine (15 mmol l^{-1}), imidazole (20 mmol l^{-1}), dithiothreitol (0.5 mmol l^{-1}), MES hydrate (50 mmol l^{-1}) and Na_2ATP (5.77 mmol l^{-1}), at pH 7.10] (Gnaiger, 2014). Before experimentation, the gills were removed from the Eppendorf tube and mechanically permeabilized with fine tweezers (Kuznetsov et al., 2002) and further chemically permeabilized with saponin (50 µg ml^{-1} BIOPS) as described by Lemieux et al. (2017). Each sample was then rinsed for 15 min in a mitochondrial respiration medium, weighed (approximately 15–30 mg) and transferred into 2 ml chambers containing O_2-saturated respiration medium [110 mmol l^{-1} D-sucrose, 60 mmol l^{-1} lactobionic acid, 20 mmol l^{-1} taurine, 20 mmol l^{-1} HEPES, 10 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} EGTA, 1 g l^{-1} bovine serum albumin (BSA); Gnaiger et al., 2000] at 15, 25 and 35°C in an Oroboros Oxygraph-2k™ respirometer (Oroboros Instruments, Innsbruck, Austria). Mitochondrial respiration was measured as volume-specific oxygen flux (pmol O_2 s^{-1} ml^{-1}) using DatLab Software V 5.2.1.51 (Oroboros Instruments, Innsbruck, Austria). Mitochondrial respiration was assessed using the substrate–inhibitor titration (SUIT) protocol proposed by Kake-Guena et al. (2015, 2017), with the following modifications: glutamate was added to the protocol and a mitochondrial uncoupler (FCCP) was introduced following succinate addition (Fig. 1). Specifically, the titration protocol consisted of the mitochondrial NADH dehydrogenase (complex I, or CI) substrates glutamate (24 mmol l^{-1}), pyruvate (10 mmol l^{-1}) and malate (2 mmol l^{-1}) to measure state 2′ respiration through CI in the absence of ADP (denoted 'leak state') (Gnaiger, 2014). Excess ADP (5 mmol l^{-1}) was added to stimulate oxidative phosphorylation (Oxphos-I) or state 3 respiration. Cytochrome c (10 µmol l^{-1}) was added to calculate the flux control factor for cytochrome c (FCFc) to determine mitochondrial membrane

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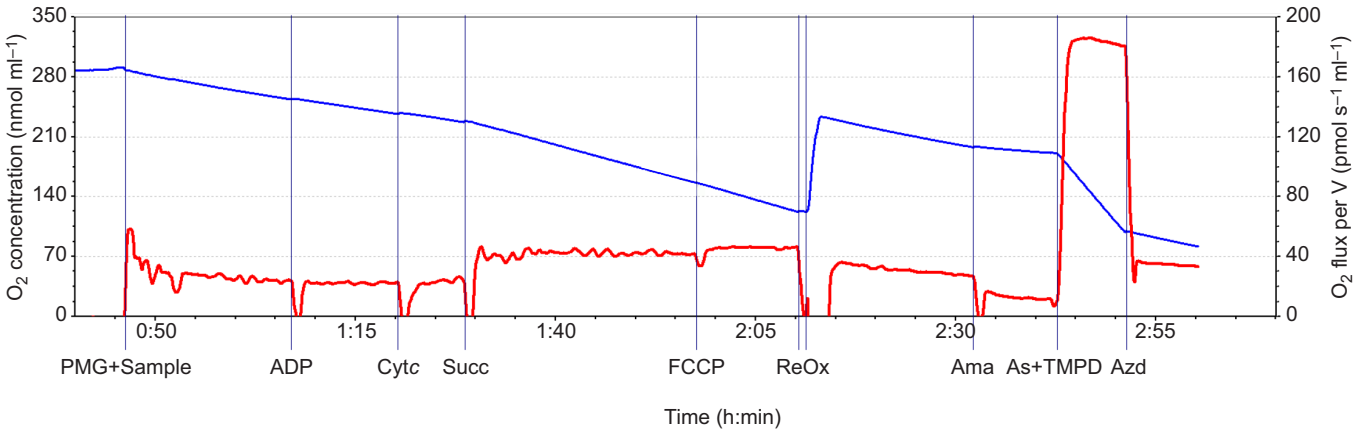

Fig. 1. Oroboros substrate–inhibitor titration (SUIT) protocol used in our experimental design. The addition of pyruvate, malate and glutamate (P, M and G, respectively) allows us to measure the leak state. Adding ADP afterwards gives us complex I (CI)-sustained respiration rate. Cytochrome *c* (Cytc) addition tests for membrane integrity. Addition of succinate (Succ) allows for combined measurement respiratory rates sustained by both CI- and CII-linked substrates. FCCP (FCCP) uncouples the OXPHOS and allows maximal electron transport system (ETS) respiration rates. Rotenone (Rot) inhibits CI for CII measurement. Antimycin (Ama) inhibits CIII and all upstream complexes. Addition of ascorbate (As) and TMPD allows us to feed CIV independently of the rest of the ETS and measure CIV rates. The use of sodium azide (Azd) inhibited CIV. The blue line indicates O_2 concentration (left *y*-axis) and the red line indicates O_2 flux (right y-axis).

integrity (an increase in rate following cytochrome *c* addition is indicative of outer mitochondrial membrane damage) (Gnaiger, 2014). Specifically, values close to 0 represent an undamaged mitochondrial membrane, while values nearing 1 represent a fully damaged mitochondrial membrane (Kake-Guena et al., 2017). Then, succinate (10 mmol l^{-1}) was added to stimulate succinate dehydrogenase (complex II, or CII) activity. This allowed the measurement of respiration sustained by CI- and CII-linked substrates together (Oxphos-I–II). To test for limitation by the phosphorylation system and to measure the maximal flux of the electron transport system (ETS), mitochondrial respiration was uncoupled using carbonyl cyanide-p-(trifluoromethyl) phenylhydrazone (FCCP, 0.5 µmol l^{-1}) addition. The uncoupled respiration sustained by CII was determined through addition of the CI inhibitor rotenone (1 µmol l^{-1}). The residual oxygen consumption was further determined through antimycin A addition (2.5 µmol l^{-1}), an inhibitor of complex III (CIII). Cytochrome *c* oxidase activity was measured by the addition of ascorbate (2 mmol l^{-1}) and TMPD (0.5 mmol l^{-1}). Sodium azide (100 mmol l^{-1}) was then added to inhibit complex IV (CIV) activity and measure auto-oxydation of TMPD. The chamber contents were then recuperated, homogenized for 3×30 s with a PT 1200 homogenizer (Polytron, Kinematica) at maximal velocity and stored at −80°C for further enzymatic analyses.

Protein content

To normalize our mitochondrial respiration data, the protein concentration (mg ml^{-1}) of our homogenized chamber contents containing the mitochondrial respiration samples was determined with a bicinchoninic acid (BCA) assay kit (Sigma-Aldrich BCA1-1KT), which used a BSA-based standard curve. The absorbance was measured at a wavelength of 560 nm using a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbad, Germany).

Citrate synthase activity

Citrate synthase (CS) activity was measured because it is an important player in the tricarboxylic acid (TCA) cycle that allows the estimation of the mitochondrial content in our tissue samples (Breton et al., 2009). CS activity was assessed in triplicate with a Mithras LB940 microplate reader at each of the three respiration assay temperatures (15, 25 and 35°C), and the data were analyzed using the MikroWin 2010V 5.15 software (Labsis Laborsysteme, Neunkirchen-Seelscheid, Germany). Specifically, homogenized chamber contents containing the mitochondrial respiration samples (125 µl) were transferred to 100 mmol l^{-1} imidazole-HCl, pH 8, 0.1 mmol l^{-1} 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) alongside 0.1 mmol l^{-1} acetyl-CoA and 0.15 mmol l^{-1} oxaloacetate. CS activity was quantified by observing the increase in absorbance at a wavelength of 405 nm. Our data were expressed in mIU ml^{-1}, where IU refers to 1 µmol of substrate transformed per minute.

Chemicals

All chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Data analysis

Mitochondrial respiratory rates were expressed in pmol O_2 consumed s^{-1} mg^{-1} protein (means±s.e.m.). Flux control ratios (FCRs) were obtained by normalizing respiratory rates for an internal parameter: the maximal ETS capacity (U). This was achieved after FCCP-mediated uncoupling (Gnaiger, 2014) with parallel electron flow coming from CI+CII. This allowed us to qualify the relative capacity of each measured complex (CI, CII and CIV) over the maximal respiratory capacity of the mitochondria, a character dictated by mitochondrial properties instead of mitochondrial content (Gnaiger, 2014; Lemieux et al., 2017). CIV apparent excess capacity (j_{ExCIV}), which allows us to quantify cytochrome *c* oxidase activity exceeding the maximum ETS capacity, was calculated as (CIV U^{-1})−1. CS activity was expressed in mIU s^{-1} mg^{-1} protein. Temperature coefficient values [Q_{10}=(rate$_2$/rate$_1$)$^{10/(t_2-t_1)}$], which represent the change in respiratory rate following an increase in temperature of 10°C, were calculated for 15–25°C as well as 25–35°C. While a value of 1 indicates thermal independence of the specific reaction, higher or lower values indicate positive or negative thermal dependence, respectively (Pisek et al., 1973). Individual Q_{10} values were calculated and then averaged to calculate the mean±s.e.m. at each temperature for each species.

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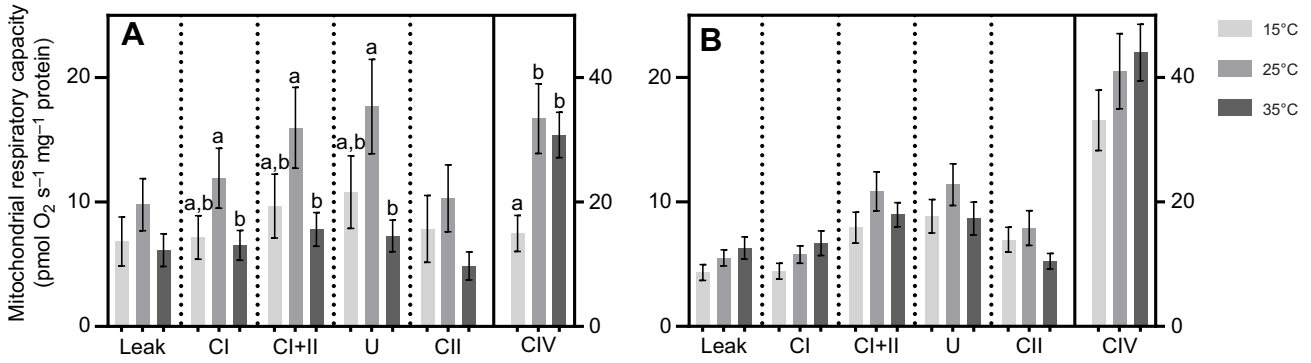

Fig. 2. Mitochondrial respiratory capacity measured in permeabilized gills at three different temperatures in the two study species. (A) *Dreissena bugensis* and (B) *Elliptio complanata*. Data, normalized to mg of protein, are represented as means±s.e.m. (n=9 for *D. bugensis*, n=12 for *E. complanata*). Respiratory parameters: Leak, presence of CI-linked substrates without ADP; CI, presence of CI-linked substrates with ADP; CI+II, presence of CI/CII-linked substrates with ADP; U, presence of CI/CII-linked substrates with ADP and added uncoupler (FCCP); CII, presence of CI/CII-linked substrates with ADP and added uncoupler (FCCP) as well as CI-linked inhibitor; CIV, presence of CI/CIII-linked inhibitors with ascorbate/TMPD for sustained activity. ANOVA was performed followed by a *post hoc* pairwise *t*-test with correction for multiple comparisons using the Holm method. Significance was set as $P \leq 0.05$; different letters denote significant differences between temperatures within a parameter. All parameters are associated with the left *y*-axis except for CIV, which is associated with the right *y*-axis.

Statistical analysis

All statistical analyses were performed with RStudio software (https://rstudio.com/). Normality of data was verified using the Shapiro–Wilk test. Levene's test was used to verify homogeneity of variance. One independent factor was considered: 'temperature' (three levels). For each species, the effect of temperature was ascertained using a one-way repeated-measures ANOVA (with each individual's data paired at all temperatures) followed by a *post hoc* pairwise *t*-test with correction for multiple comparisons using the Holm method. A $P \leq 0.05$ was considered statistically significant. Figures were produced with Graphpad Prism software (GraphPad Prism version 8.0.1 for Windows, GraphPad Software, La Jolla, CA, USA).

RESULTS

Mitochondrial respiration

Comparison of mitochondrial respiration rates (normalized per milligram of protein) for *D. bugensis* at the three temperatures (Fig. 2A) showed that they were almost always lower at 15°C than at 25°C, but the only statistical significance was denoted for CIV (P=0.03). Respiration rates for *D. bugensis* were also significantly lower for most complexes at 35°C than at 25°C. A significant difference was denoted for CI (P=0.036), CI+CII (P=0.031) and U (P=0.028). For leak and CII, a trend was discernable, but the differences were not statistically significant. For CIV, respiration rates were maintained at approximately unchanged levels. When comparing 15°C with 35°C, the only significant difference was found for CIV, where mitochondrial respiration was much higher at 35°C than at 15°C (P=0.03). In *E. complanata*, comparison of respiration rates (Fig. 2B) yielded no significant differences in all parameters. However, the trends in mitochondrial respiration were similar to those of *D. bugensis*. Respiration values at 25°C were higher than those at 15°C. Respiration rates at 35°C were similar to those at 15°C except for CIV, which was maintained between 25°C and 35°C. Respiratory rates were also normalized per milligram of tissue (Fig. S1) and CS activity (Fig. S2), and similar trends were noted (increase at 25°C compared with 15°C followed by a noted decrease at 35°C).

Our results were also expressed as FCRs in order to assess qualitative (instead of quantitative) changes in mitochondria. In *D. bugensis*, comparison of FCRs (Fig. 3A) showed no significant changes between 15°C and 25°C in all parameters. At 35°C, for leak, CI, CI+CII and CIV, a significant increase in the FCRs was detected compared with 25°C (P=0.011, P=0.024, P=0.001 and P=0.002, respectively) as well as when compared with 15°C (P=0.039, P=0.028, P=0.0053 and P=0.0002, respectively). For

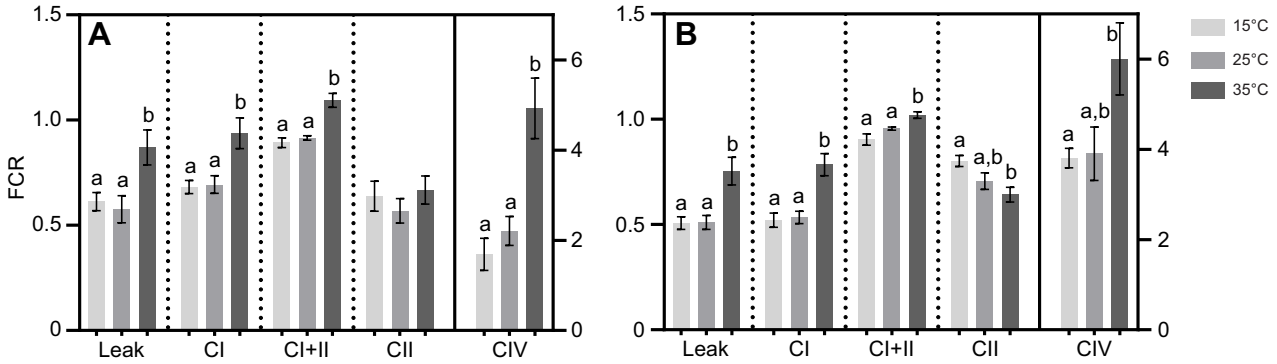

Fig. 3. Flux control ratios (FCRs) calculated in permeabilized gills at three different temperatures in the two study species. (A) *Dreissena bugensis* and (B) *Elliptio complanata*. Data are represented as means±s.e.m. (n=9 for *D. bugensis*, n=12 for *E. complanata*). ANOVA was performed followed by a *post hoc* pairwise *t*-test with correction for multiple comparisons using the Holm method. Significance was set as $P \leq 0.05$; different letters denote significant differences between temperatures within a parameter. All parameters are associated with the left *y*-axis except for CIV, which is associated with the right *y*-axis.

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Table 1. Q_{10} values for mitochondrial respiratory capacity in *Dreissena bugensis* and *Elliptio complanata*

Temperature range	Leak	CI+CII	U	CIV
Dreissena bugensis				
15–25°C	2.12±0.68	2.33±0.62	2.23±0.61	3.44±1.03
25–35°C	0.73±0.16	0.57±0.11	0.49±0.11	1.03±0.13
Elliptio complanata				
15–25°C	1.55±0.23	1.69±0.30	1.61±0.29	1.74±0.42
25–35°C	1.31±0.23	1.05±0.19	1.00±0.24	1.32±0.21

Values are presented as means±s.e.m. with n=12 for *E. complanata* and n=9 for *D. bugensis*.

CII, FCRs remained constant across all temperatures with no significant differences. In *E. complanata*, comparison of FCRs (Fig. 3B) showed no significant difference across all parameters when comparing values at 25°C with those at 15°C. When comparing 35°C with 25°C, a significant difference was denoted for leak, CI and CI+CII (P=0.0094, P=0.00045 and P=0.0113, respectively), whereas no significant differences were found for CII and CIV, although a noticeable increase was observed for CIV. When comparing 15°C with 35°C, a significant increase was found for leak, CI, CI+CII and CIV (P=0.0094, P=0.00036, P=0.0043 and P=0.039, respectively), whereas a significant decrease was found for CII (P=0.031).

Temperature coefficients

Results for *D. bugensis* (Table 1) denote a significant change in thermal sensitivity depending on the thermal range accounted, 15–25°C or 25–35°C. Overall, all respiratory rates experienced a decrease of Q_{10} values, passing from a positive (15–25°C) toward a negative thermal dependence (25–35°C). That was the case of the respiratory parameters CI–CII (passing from 2.33 to 0.57; P=0.026), U (passing from 2.23 to 0.49; P=0.02) and CIV (passing from 3.44 to 1.03; P=0.04). Although not significant, a trend of decreasing Q_{10} values was also denoted for leak (decreasing from 2.12 to 0.73). In *E. complanata* (Table 1), a decrease in thermal sensitivity was noted from 15–25°C to 25–35°C across all parameters (with values ≥1), but they were not statistically significant.

FCF*c* and mitochondrial membrane integrity

In both species, at all three temperatures, the FCF*c* were all close to 0 and were not significantly different at different temperatures (Fig. S3A).

Complex IV apparent excess capacity

We found no statistically significant difference in apparent CIV excess capacity for *D. bugensis* and *E. complanata* between 15°C and 25°C (Fig. 4A). However, there was a sharp increase at 35°C, reaching up to 390% in *D. bugensis* (P=0.0026) and upwards of 500% in *E. complanata*, albeit without statistical significance in comparison with 25°C. A very significant difference was noted when comparing 15°C with 35°C in both species (P=0.0002 for *D. bugensis* and P=0.032 for *E. complanata*).

Citrate synthase activity

Comparison of CS activity (Fig. 4B) in both species showed no significant changes across all three temperatures, although a trend similar to the one obtained for respiratory rates was observed (an increase from 15°C to 25°C followed by a decrease at 35°C).

DISCUSSION

Metabolic depression at elevated temperatures

We investigated the thermal sensitivity of the mitochondrial metabolism of two freshwater bivalves, the North American endemic unionid species *E. complanata* (eastern elliptio) and the invasive quagga mussel, *D. bugensis*, to determine whether it might play a role in the invasive success of the quagga mussel in North America. The metabolic rate increased gradually in both species from 15 to 25°C (Fig. 2), temperatures that are within their survival range (Galbraith et al., 2012; Choi et al., 2013; Martin, 2016; McMahon, 1996), and this increase was also reflected by the Q_{10} values. It is generally accepted that the normal range of Q_{10} values is between 2.0 and 3.0 in aquatic ectotherms (Pisek et al., 1973; Reyes et al., 2008). Q_{10} values measured for OXPHOS parameters in the quagga mussel (Table 1) seem to be consistent with this. For example, McMahon (1996) and Fanslow et al. (2001) denoted Q_{10} values ranging from 1.7 to 2.3 for temperature between 15°C and 25°C for oxygen consumption rates and ETS activity in *Dreissena polymorpha*, a closely related species of the quagga mussel. Fanslow et al. (2001) also reported respiration and ETS Q_{10} values for 10–20°C of 1.6 to 2.5. Our Q_{10} values (15–25°C) were 2.12 (leak), 2.33 (CI+CII) and 2.23 (U), which falls well into the range described above. However, Q_{10} values for CIV activity were well above at 3.44. This suggests that, in the quagga mussel, cytochrome *c* oxidase activity is the most thermally sensitive OXPHOS parameter in this temperature range, with its activity increasing much more quickly than that of other respiratory parameters. Overall, our results reflected a positive

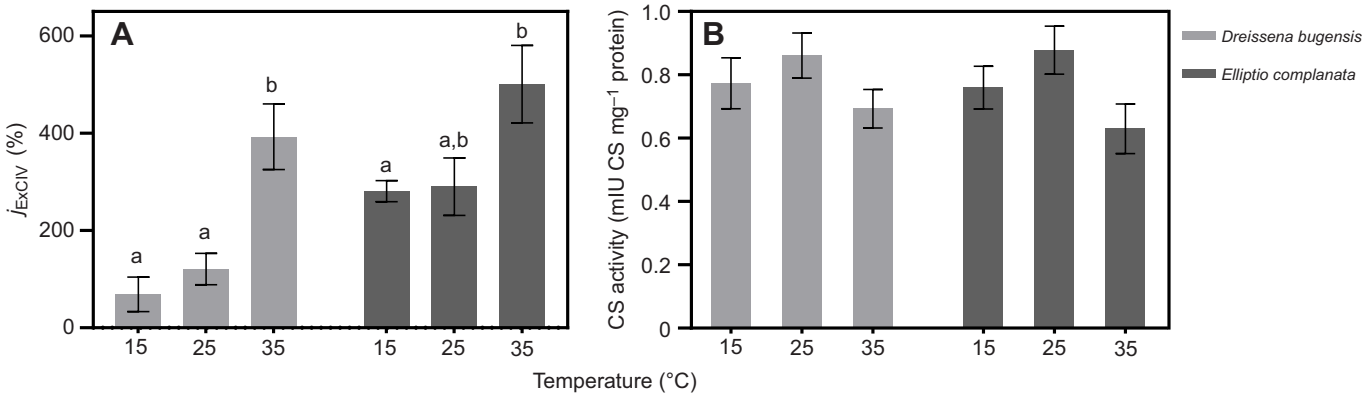

Fig. 4. Complex IV apparent excess capacity (j_{ExCIV}) and citrate synthase (CS) activity in both species at the three measured temperatures. (A) j_{ExCIV}; (B) citrate synthase activity (mIU mg^{-1} protein). Data are means±s.e.m. (n=9 for *D. bugensis*, n=12 for *E. complanata*). ANOVA was performed followed by a *post hoc* pairwise *t*-test with correction for multiple comparisons using the Holm method. Significance was set as P≤0.05; letters denote differences between temperatures within a parameter.

thermal dependence of metabolic reactions. Because ectotherm metabolic needs are severely dependent on ambient temperature (Clarke and Fraser, 2004), an increase in temperature (and, therefore, ATP requirements) without an increase in metabolic activity would hinder an individual more than aid it.

Once reaching 35°C (i.e. temperature extremes that may occur on an intermittent basis in shallow/turbid ditches and small creeks in both species' habitats; McMahon, 1996), a decrease in respiratory rates was observed for all parameters (except CIV) in the quagga mussel (Fig. 2A), whereas there was no significant effect of temperature on respiration rates among all parameters measured in the eastern elliptio (Fig. 2B). This is in line with a metabolic depression, a phenomenon that may become more frequent with global warming, and that has already been seen in several studies (e.g. Christen et al., 2018). This is again reflected by the Q_{10} values, which indicate a switch toward a negative thermal sensitivity in *D. bugensis* (increasing temperature is associated with decreasing reaction activity, which does not sustain the increased energetic demand). Our results are incongruent with published studies that had shown that both species' critical thermal maximum (temperature at which loss of bodily equilibrium occurs) was around 39–41°C (Galbraith et al., 2012; Choi et al., 2013). Our results are also incongruent with the fact that the southern geographical distribution range for the exotic quagga mussels lies more to the south (in Mexican lakes) than that of the eastern elliptio (northern Florida) (Cummings and Cordeiro, 2011; Wakida-Kusonoki et al., 2015). This incongruence with previous studies could be due to experimental procedures. For example, Galbraith et al. (2012) studied the whole organism while we concentrated on mitochondria from gill tissues. Thus, there may be factors potentiating thermo-tolerance at the organism level that we could not account for with our experimental strategy that aimed to elucidate thermo-tolerance at the mitochondrial level. In *E. complanata*, Q_{10} values hovered between 1.5 and 1.7 for 15–25°C (Table 1), and although these values dipped to 1.0–1.3 at 25–35°C, the differences were not statistically significant for all measured parameters (leak, CI+II, U and CIV). This leads us to surmise that the eastern elliptio possesses a more thermally independent OXPHOS than the quagga mussel, as denoted by the lack of significant differences in respiratory rates and Q_{10} values across the 25–35°C temperature range. This thermal resistance mirrored results noted in a previous study conducted by Ganser et al. (2015), which characterized the eastern elliptio as a very thermally tolerant species by measuring oxygen consumption at temperature ranges similar to the ones we chose (20, 25, 30 and 35°C in Ganser et al., 2015) through the use of whole-animal respirometry.

Interestingly, cytochrome *c* oxidase appeared to be the only complex in both species able to maintain an elevated activity level at 35°C akin to levels at 25°C (Fig. 2 for respirometry and Fig. 3 for FCR). This seems to reflect a greater maintenance of CIV function when compared with other complexes. Similar results have already been observed in other aquatic ectotherms (Blier and Lemieux, 2001; Oellermann et al., 2012; Blier et al., 2013; Kake-Guena et al., 2017). Otherwise, in both species, O_2 flux of the leak state was mirrored almost exactly by CI. This result, which is congruent with previous findings for *E. complanata* (Bettinazzi et al., 2019b), suggests a relatively small input in electron flow provided by NADH dehydrogenase in these two freshwater bivalves. However, we cannot discard the possibility that in one or both species, alternate pathways play an important role in fueling OXPHOS. Many species across the animal kingdom (e.g. hummingbirds, bats, naked mole rats and certain flies) have been found to use alternate substrates such as proline (McDonald et al., 2018). In bivalves, glycerophosphate has already been found to be important for mitochondrial functions (Bettinazzi et al., 2019a), as well as fatty acid oxidation (Moyes et al., 1990). As such, it is presumable that even the bivalves we tested may rely on these alternative fuels as sources of electrons. The total flux of CI+II is matched by uncoupled respiratory rates, suggesting that in both *E. complanata* and *D. bugensis*, there is little to no limitation of OXPHOS on the phosphorylation system. This lack of limitation may reflect little control exerted by the phosphorylation system over OXPHOS capacity in both species (Bettinazzi et al., 2019a).

Thermo-induced OXPHOS impairment

Overall, respiratory rates in both species suggest (to different extents) a negative impact of elevated temperatures on aerobic capacity and this impairment is also reflected by the FCRs. FCRs in both species (Fig. 3) did not vary in all parameters between 15°C and 25°C, suggesting that in this range, the gills preserved their original mitochondrial organization. However, at 35°C, FCRs were significantly higher for all parameters in both species except for CII, reflecting a thermally induced change of OXPHOS organization. In the quagga mussel, the CII FCR value remained unchanged (unlike the observed decrease in the eastern elliptio), which suggests that across all three measured temperatures, CII's proportional contribution to the maximal OXPHOS capacity remained constant. The general increase in FCRs at 35°C is found in both species and can be attributed to the observed sharper decrease in the maximal uncoupled respiration compared with the coupled respiration. This qualitative change could be in line with an increased membrane permeability, and this is also supported by the increase in leak FCR (reflecting the proton leak). These findings are congruent with previous research (Pörtner et al., 1999) that found an unusually elevated thermal sensitivity in proton leakage in *Laternula elliptica*, an Antarctic bivalve species, and linked it with an increase in membrane permeability. Pörtner et al. (1999) also noted that the high thermal sensitivity of proton leakage may cause an excessive rise in mitochondrial oxygen demand and a decrease in efficiency of OXPHOS, thus potentially neutralizing an organism's ability to sustain itself aerobically. The observed increase in the futile proton cycle may be associated with an increase in ROS production (Abele et al., 2002), potentially as a direct consequence of damage and impairment caused by ROS (Abele et al., 2002) or as a putative defense mechanism in order to lessen the detrimental effects of ROS (Brand, 2000). However, whether the increase in proton leakage could be due to thermally induced damaged membranes or the result of activity of uncoupling proteins is still controversial. In fact, FCF*c* values (Fig. S3) in both species across all three temperatures were not significantly different or elevated, and as such, reflected a relatively unchanged and preserved membrane integrity. Moreover, even though uncoupling proteins are widely expressed among bivalve tissues, their putative role in stress-induced mild uncoupling is still controversial (Buttemer et al., 2010), and thus may be inadequate to induce the observable increase in proton leakage. As it remains unclear how mitochondria mediate membrane permeability and the proton cycle under thermal stress in bivalves, further experimentation is required.

Elevated temperatures also had an impact on the apparent CIV excess capacity in both species (Fig. 4A). This high apparent excess capacity remained stable at 15°C and 25°C, reaching approximately 100% for the quagga mussel and approximately 250% for the eastern elliptio. This may represent a strategy used by both species in non-lethal temperatures to mediate mitochondrial dysfunction to

a certain extent, as an increased j_{ExCIV} has been shown to aid in maintaining proper mitochondrial function in face of metabolic stress (Blier et al., 2017). Such apparent excess capacity has already been found in many bivalve species (Bettinazzi et al., 2019a). At 35°C, the increase in CIV excess capacity compared with 25°C, reaching up to 390% in *D. bugensis* and >500% in *E. complanata*, is a product of decreased OXPHOS activity coupled with maintenance of CIV respiratory rates at the same temperature. There is scientific literature denoting an increased j_{ExCIV} at lower temperatures (Pichaud et al., 2011; Blier et al., 2013). Our results show that at the opposite end of the temperature spectrum, the same phenomenon can be present. It has been suggested that the maintenance of a high CIV excess capacity may aid in offsetting the detrimental effects of ROS (Blier et al., 2017). It has also been proposed to regulate the redox state of upstream ETS complexes and aid in O_2 binding (Blier et al., 2017). As such, our results could possibly reflect a compensatory process involving the lowering of ROS production as well as the facilitation of O_2 binding in hypoxic environments, which are more common at high temperatures. Solely characterizing the aerobic response may not paint the whole picture. Indeed, bivalves are known to possess significant anaerobic capabilities (Müller et al., 2012). Future experimentation should therefore consider measuring the anaerobic response of these species in addition to their aerobic capacity.

CS activity was assayed (Fig. 4B) to determine whether the highlighted metabolic depression observed in our species at 35°C could be reflected in metabolic processes upstream of OXPHOS. Our results, in both species, showed no significant difference in CS activity. These findings allowed us to surmise that CS activity is not directly responsible for temperature-induced metabolic depression in the two studied freshwater mussels. However, the trend we noticed (an increase at 25°C followed by a decrease at 35°C) seemed to mirror activity levels in most OXPHOS parameters. These findings concur with previous research (Dunphy et al., 2006; Doucet-Beaupré et al., 2010) showing that CS did not have a bottleneck effect on aerobic metabolism in multiple ectotherm species. Despite that, previous research (Blier et al., 2013) has shown that CS as well as other enzymes upstream of OXPHOS (e.g. pyruvate dehydrogenase complex, or PDH) were effectively thermally sensitive in the Atlantic wolffish (*Anarhichas lupus*), showing an increased activity in response to increased temperature exposure with no sign of depression. They also found that at low temperatures, PDH was responsible for metabolic depression in the same species. As such, while CS activity did not have a bottleneck effect on metabolic activity in our study, it is possible that other key enzymes (such as PDH) might.

Altogether, our results in both species in terms of respiratory rates, FCRs, CIV apparent excess capacity and Q_{10} values highlighted a temperature-induced metabolic depression suffered at 35°C linked with a mitochondrial impairment potentially caused by the disruption of OXPHOS' complex synergies. This depression was generally more evident in the quagga mussel than in the eastern elliptio.

Thermal tolerance linked with life history and reproductive strategies

Overall, our results demonstrate that the aerobic metabolism of the invasive quagga mussel is less thermo-tolerant than that of the endemic eastern elliptio, and this could be related to both species' life history traits. As argued by McMahon (1996), contrary to unionid freshwater mussels, dreissenids are relatively recent colonizers of freshwater habitats, and they have retained some of the primitive characteristics of their marine ancestors, including planktonic larvae and reduced resistance to environmental stress. Unionids have evolved better resistance adaptations, such as long lifespans, extensive iteroparity, low effective fecundities, large offspring, delayed maturity and reduced growth rates, which make them better suited to survive in stable and extreme environments, but prevent them from rapid habitat recolonization after extirpation by environmental disturbance (McMahon, 1996). By contrast, dreissenids are less competitive in stable and extreme habitats and they have evolved different life history traits such as elevated fecundity, fast growth, early maturity and attenuated lifespans that allow them to rapidly recolonize unstable habitats after extirpation by unpredictable environmental stressors (McMahon, 1996). Selection pressures for resistance adaptations are reduced in unpredictable habitats, and for a species such as *D. bugensis*, metabolic resistance is of little use as it relies on its elevated growth and fecundity to either invade new environments or maintain prior populations (McMahon, 2002). An elevated metabolic resistance would require greater energy investments from an individual, which is much more characteristic of species such as the eastern elliptio. As such, although an elevated temperature is more detrimental to the quagga than it is to the eastern elliptio, on a larger scale, elliptio populations, being long-lived and slower to reproduce, will potentially suffer more from increased thermal instability, not being able to bounce back as easily as the quagga. As suggested by McMahon (1996), adaptations to unstable habitats have allowed *D. bugensis* and its cousin *D. polymorpha* to be extremely successful invaders of North American freshwater habitats.

Conclusions

Our research highlights a clear effect of elevated temperatures on the mitochondrial metabolism of both studied species, i.e. at temperatures close to the upper limit, mitochondrial functions are compromised. In both species tested, the metabolic depression induced by exposure to high temperatures links with a similar impairment of the OXPHOS machinery, characterized by a lower coupling and an increased futile proton cycle. Our study provides a key comparative analysis of mitochondrial OXPHOS activity in two North American freshwater bivalves and emphasizes the essential role played by mitochondria in setting thermal tolerance in aquatic ectotherms, highlighting how mitochondrial thermo-sensitivity may indeed determine the upper thermal limit and affect the survival and distribution of animal species. However, a more comprehensive study outlying thermal tolerance of the multiple components of the mitochondrial machinery (e.g. glycolysis, TCA cycle, OXPHOS, potential alternative pathways) is required in order to better understand how metabolic thermo-sensitivity plays into the global equation underlying the decline, maintenance or growth of freshwater bivalves. The complex processes that make up the mitochondrial machinery render it difficult to pinpoint specific bottlenecks that may mediate thermal tolerance. Thus, a better understanding of these functions may help future conservation efforts by promoting the development of more adapted and effective predictive models.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S. Breton; Methodology: G.H., S. Bettinazzi, S. Breton; Validation: G.H.; Formal analysis: G.H.; Investigation: G.H., S. Bettinazzi;

Resources: A.D.G., S. Breton; Data curation: G.H.; Writing - original draft: G.H.; Writing - review & editing: G.H., S. Bettinazzi, A.D.G., D.B., S. Breton; Supervision: D.B., S. Breton; Project administration: S. Breton; Funding acquisition: S. Breton.

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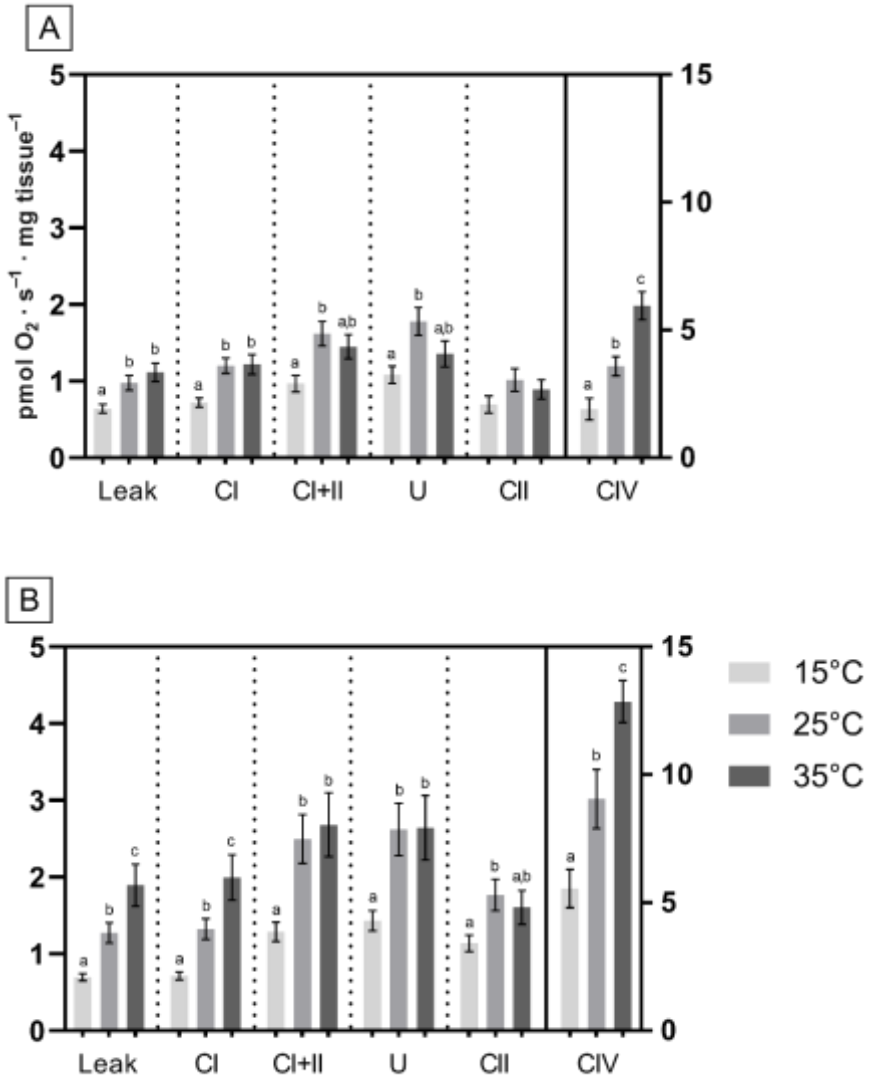

Figure s1. Mitochondrial respiratory capacity measured in permeabilized gills at three different temperatures in *Dreissena bugensis* (**A**) and *Elliptio complanata* (**B**). Data, normalized for mg of tissue, are means ± SEM (n = 9 for *D. bugensis*, n = 12 for *E. complanata*). Respiratory parameters: **Leak**, presence of CI-linked substrates without ADP; **CI**, presence of CI-linked substrates with ADP; **CI+II**, presence of CI/CII-linked substrates with ADP; **U**, presence of CI/CII-linked substrates with ADP and added uncoupler (FCCP); **CII**, presence of CI/CII-linked substrates with ADP and added uncoupler (FCCP) as well as CI-linked inhibitor; **CIV**, presence of CI/CIII-linked inhibitors with Ascorbate/TMPD for sustained activity. Significance was set as $P \leq 0.05$; letters denote differences between temperatures within a parameter with **a** statistically significant from **b** & **c** and **b** statistically different from **a** & **c**. All parameters are associated with the left y axis except for CIV which is associated with the right y axis.

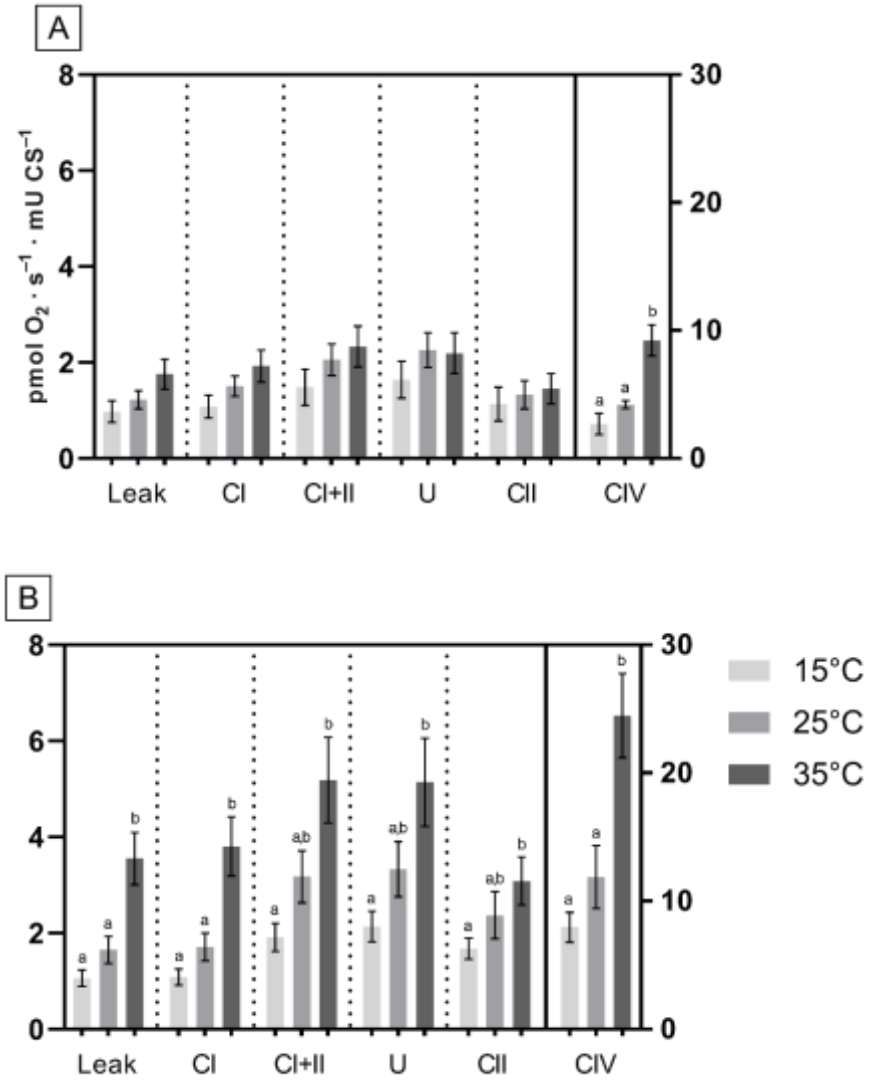

Figure s2. Mitochondrial respiratory capacity measured in permeabilized gills at three different temperatures in *Dreissena bugensis* (**A**) and *Elliptio complanata* (**B**). Data, normalized for mIU of citrate synthase, are means ± SEM (n = 9 for *D. bugensis*, n = 12 for *E. complanata*). Respiratory parameters: **Leak**, presence of CI-linked substrates without ADP; **CI**, presence of CI-linked substrates with ADP; **CI+II**, presence of CI/CII-linked substrates with ADP; **U**, presence of CI/CII-linked substrates with ADP and added uncoupler (FCCP); **CII**, presence of CI/CII-linked substrates with ADP and added uncoupler (FCCP) as well as CI-linked inhibitor; **CIV**, presence of CI/CIII-linked inhibitors with Ascorbate/TMPD for sustained activity. Significance was set as $P \leq 0.05$; letters denote differences between temperatures within a parameter with **a** statistically significant from **b**. All parameters are associated with the left y axis except for CIV which is associated with the right y axis.

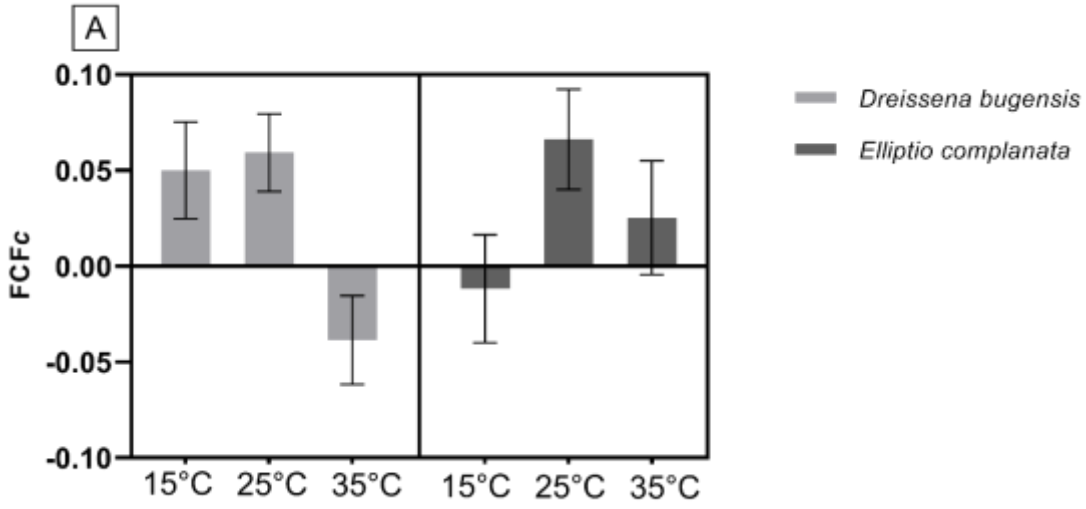

Figure s3. Integrity of the mitochondrial outer membrane (**A**) calculated as the flux control factor for cytochrome *c* (FCFc) ascertained from respiratory values in the presence of complex I substrates; 0 indicates full integrity of the outer mitochondrial membrane and 1 indicates a fully damaged outer mitochondrial membrane ($FCF_c = (CI_c - CI) \cdot CI_c^{-1}$). Data are means ± SEM ($n = 9$ for *D. bugensis*, $n = 12$ for *E. complanata*). Significance was set as $P \leq 0.05$.