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RESEARCH ARTICLE

Mitochondrial responses towards intermittent heat shocks in the eastern oyster, *Crassostrea virginica*

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ABSTRACT

Frequent heat waves caused by climate change can give rise to physiological stress in many animals, particularly in sessile ectotherms such as bivalves. Most studies characterizing thermal stress in bivalves focus on evaluating the responses to a single stress event. This does not accurately reflect the reality faced by bivalves, which are often subject to intermittent heat waves. Here, we investigated the effect of intermittent heat stress on mitochondrial functions of the eastern oyster, *Crassostrea virginica*, which play a key role in setting the thermal tolerance of ectotherms. Specifically, we measured changes in mitochondrial oxygen consumption and H_2O_2 emission rates before, during and after intermittent 7.5°C heat shocks in oysters acclimated to 15 and 22.5°C. Our results showed that oxygen consumption was impaired following the first heat shock at both acclimation temperatures. After the second heat shock, results for oysters acclimated to 15°C indicated a return to normal. However, oysters acclimated to 22.5°C struggled more with the compounding effects of intermittent heat shocks as denoted by an increased contribution of FAD-linked substrates to mitochondrial respiration as well as high levels of H_2O_2 emission rates. However, both acclimated populations showed signs of potential recovery 10 days after the second heat shock, reflecting a surprising resilience to heat waves by *C. virginica*. Thus, this study highlights the important role of acclimation in the oyster's capacity to weather intermittent heat shock.

KEY WORDS: Climate change, Mitochondria, Reactive oxygen species, Aquatic ectotherms, Thermal sensitivity, Metabolism

INTRODUCTION

Climate warming is not only characterized by increased average temperatures all over the world but also by the intermittent occurrence of extreme weather events such as heat waves and droughts (Vasseur et al., 2014). This clearly represents a major challenge for animals that depend on ambient temperature to regulate their body heat. Some species of aquatic ectotherms can somewhat diminish the effects of increasing temperature through behavioral adaptations. For example, certain species of fish can partially mitigate the effects of climate change by migrating into cooler waters (Buisson et al., 2008a,b). However, many aquatic ectotherms such as some bivalve mollusks are sessile and cannot rely on migration to dampen the effects of high temperatures. Consequently, these sessile organisms must entirely rely on altering biochemical and metabolic processes to maintain homeostasis in an era of climate change (Hochachka and Somero, 2002; Pörtner and Farrell, 2008).

With an increase in the occurrence of extreme temperature fluctuations, a species' capacity to react to heat shock becomes a determining factor in its survival. The susceptibility of aquatic ectotherms to climate change (and, consequently, their response to heat shock) has been suggested to be intimately linked to mitochondria (Blier et al., 2014; Chung and Schulte, 2020). These organelles, responsible for aerobic metabolism in eukaryotic cells via oxidative phosphorylation (OXPHOS), are quite sensitive to environmental temperatures (Blier et al., 2014; Chung and Schulte, 2020; Sappal et al., 2014). In bivalves, for example, studies have shown that mitochondrial activity varies significantly across the temperature spectrum (Abele et al., 2002; Hraoui et al., 2020). Moreover, elevated temperatures have been shown to lead to an increase in the production of reactive oxygen species (ROS) in bivalves (Abele et al., 2002; Heise et al., 2003). These molecules, derived from molecular oxygen, have roles in cell signaling and proliferation, and are involved in other functions such as immune response (Franchina et al., 2018; Thannickal and Fanburg, 2000). However, when production and accumulation in cells and tissues overwhelm the ability to detoxify these ROS, oxidative stress (characterized by oxidative damages to proteins, lipid membranes and DNA) occurs, leading to the loss of certain cellular functions and eventually to cell death (Redza-Dutordoir and Averill-Bates, 2016). On a larger scale, this may cause an organism's death as a result of its inability to control the increased ROS proliferation via cellular defense mechanisms. As mitochondria are known to be one of the main producers of ROS in the cell (Starkov, 2010), understanding the interplay between increased temperatures and mitochondrial function is key in characterizing an organism's response to thermal stress.

Many studies have focused on ectotherms' metabolic response to thermal stress. However, little is known about their response to intermittent heat shocks. Although we might expect a sessile organism to weather a single extreme heat shock to a certain extent, the reality is that they are occurring in nature at a greater frequency than ever before (Vasseur et al., 2014). The eastern oyster, *Crassostrea virginica* (Gmelin 1791), is such an organism that is facing the challenges and impacts brought forth by global warming. Increases in temperature have been suggested to induce an increase in mortality rates in *C. virginica* populations (Kimmel and Newell, 2007). This increase may be due to a combination of increased metabolic demand (as a result of thermal

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stress) and a potential increase in ROS production (and thus cellular damage). Moreover, many oyster species, whose optimal temperatures lie between 15 and 30°C, are becoming frequently exposed to temperatures over 35°C, causing adverse effects on pumping, feeding and other critical homeostatic functions (Wang et al., 2015).

In this study, we evaluated the responses of mitochondrial and ROS metabolism to intermittent heat shocks in *C. virginica* acclimated to two different temperatures: 15 or 22.5°C. Specifically, oysters acclimated to either temperature were challenged with two 7.5°C heat shocks for 12 h (reaching 22.5 and 30°C, respectively) at 10 day intervals and mitochondrial oxygen consumption as well as H_2O_2 emission rates were measured in isolated mitochondria of these oysters just before, during and 10 days after the heat shocks. We chose 15 and 22.5°C to represent normal and moderately high temperatures, respectively, encountered by the oysters in their natural environment (Miramichi Bay, NB, Canada) during summer, and 30°C as a stressful temperature when oysters are acclimated to 22.5°C to simulate a heat wave reaching a temperature close to their upper thermal limit. We hypothesized that the acclimation temperature would affect mitochondrial responses to the intermittent heat shocks. Specifically, we predicted that in oysters acclimated to 15°C, the first heat shock would cause modulation of mitochondrial and ROS metabolism, which would be resolved when these oysters were exposed to the second heat shock. By contrast, in oysters acclimated to 22.5°C, the first heat shock would cause mitochondrial dysfunction that would be exacerbated when the second heat shock was initiated because the simulated heat shocks represent a thermal stress close to the upper thermal limit of the animals.

MATERIALS AND METHODS

Animal maintenance and experimental design

Adult *C. virginica* (mean±s.e.m. shell length: 86.14±1.02 mm) were collected from floating trays at the Daigle Aquaculture Inc. farm located in Miramichi Bay, NB, Canada (46°72′N, 64°89′W) on 18 July 2018. In this bay, most of the oysters had released their gametes at the end of June (Maxime Daigle, Daigle Aquaculture Inc., personal communication), ensuring that the collected oysters were in a post-reproductive status during the experiments. Animals were transported to the Université de Moncton within 2 h of collection and transferred to 50 l recirculated aquaria with artificial sea water (26‰) set at either 15 or 22.5°C. Fifty oysters were split in two different aquaria (25 oysters per aquarium) for each acclimation temperature. Oysters were fed twice a week with 8 ml Instant Algae Shellfish Diet 1800® (Reed Mariculture Inc., Campbell, CA, USA) per aquarium. Oysters were acclimated to the two different temperatures for 1 month and no mortality and no release of gametes (indicative of reproduction) were detected during the acclimation period. After the preliminary acclimation, 6 oysters were collected for each temperature (15 and 22.5°C) for mitochondrial isolation (first experiment, control oysters: 1st-C, *N*=6). Then, the aquarium temperature was increased by 7.5°C, reaching 22.5 and 30°C after 2 h. The following day, 12 h after the start of the temperature increase, 6 oysters were collected again for each temperature (22.5 and 30°C) for mitochondrial isolation (first experiment, heat-shocked oysters: 1st-HS, *N*=6), and the aquarium temperature was allowed to return to the initial acclimation temperature (15 and 22.5°C), which was reached after 6 h. After 10 days, this procedure was repeated, i.e. collection of 6 oysters acclimated to either 15 or 22.5°C (second experiment, control oysters: 2nd-C, *N*=6) and after the temperature was raised by 7.5°C (second experiment, heat-shocked oysters: 2nd-HS, *N*=6). Again, after the heat shock, the aquarium temperature was cooled down to the initial acclimation temperature for 10 days before collection of 6 oysters acclimated to either 15 or 22.5°C (third experiment, control oysters: 3rd-C, *N*=6). An outline of the experimental design is presented in Fig. S1. No changes in gonadal tissues and no release of gametes were noticed during the entirety of the experiments. At the end of the experimental timeline, oysters acclimated to 15°C had a 22% mortality rate whereas oysters acclimated to 22.5°C had a 34% mortality rate.

Isolation of mitochondria

After collection from the aquarium, the oysters were quickly dissected on ice. Approximately 0.5 g gills was removed, minced and homogenized in an isolation buffer consisting of 100 mmol l^{-1} KCl, 400 mmol l^{-1} sucrose, 6 mmol l^{-1} EGTA, 3 mmol l^{-1} EDTA, 70 mmol l^{-1} Hepes, 0.5% (w/v) BSA and 10 mg ml^{-1} aprotinin, pH 7.6 (Moyes et al., 1985; Munro et al., 2013). The homogenate was centrifuged at 1250 ***g*** for 10 min at 4°C to remove cellular debris, and this procedure was repeated with the resulting supernatant. The final supernatant was centrifuged at 10,500 ***g*** for 15 min at 4°C. The pellet containing mitochondria was then gently resuspended in isolation buffer. Total mitochondrial protein content was determined using the bicinchoninic acid method with BSA as standard (Smith et al., 1985), taking into account that the isolation medium contained 0.5% (w/v) BSA.

Mitochondrial oxygen consumption rate

Mitochondrial oxygen consumption in freshly isolated mitochondria was measured using the Oxygraph O2K (Oroboros Instruments, Innsbruck, Austria). Mitochondria were placed in respirometry chambers set at the different temperatures (15, 22.5 and 30°C) with respiration medium consisting of 550 mmol l^{-1} sucrose, 150 mmol l^{-1} KCl, 70 mmol l^{-1} Hepes, 10 mmol l^{-1} KH_2PO_4 and 0.2% BSA, pH 7.4. A substrate-uncoupler-inhibitor titration (SUIT) protocol was performed using: pyruvate, malate and glutamate (10, 0.5 and 10 mmol l^{-1}, respectively) to measure the leak (non-phosphorylating) state for complex I (CI-LEAK); +ADP (5 mmol l^{-1}) to monitor the phosphorylating state for complex I (CI-OXPHOS); +cytochrome *c* (15 µmol l^{-1}, CIc-OXPHOS) to determine the intactness of the outer mitochondrial membrane (Kuznetsov et al., 2008; Simard et al., 2018); +succinate (10 mmol l^{-1}) to assess maximum phosphorylating state with convergent electrons from complex I and complex II (CI+CII-OXPHOS); +glycerol-3-phosphate (G3P), which allows the transport of electrons to the electron transport system (ETS) via the mitochondrial glycerol-3-phosphate dehydrogenase (CI+CII+mG3PDH-OXPHOS); +rotenone (1 µmol l^{-1})+antimycin A (2.5 µmol l^{-1}) to inhibit complexes I and III, and measure residual oxygen consumption, which was used to correct all the mitochondrial respiration rates. Finally, ascorbate (2 mmol l^{-1})+TMPD (0.5 mmol l^{-1}) were added to evaluate the maximum capacity of complex IV, which was corrected for auto-oxidation of TMPD after inhibition of complex IV by sodium azide (20 mmol l^{-1}). All measurements are presented as pmol O_2 s^{-1} mg^{-1} of protein.

Mitochondrial ratios

The different respiration rates measured were used to calculate several mitochondrial ratios. The OXPHOS coupling ratio was calculated as 1−(CI-LEAK/CI-OXPHOS) and was used as a proxy for mitochondrial quality and mitochondrial coupling

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(Gnaiger, 2009). A large increase in oxygen consumption following injection of ADP results in a coupling ratio close to 1, which indicates a highly coupled system as electrons transported by complex I are highly coupled to oxidative phosphorylation, while an unchanged oxygen consumption rate (close to 0) indicates that oxidative phosphorylation does not exert flux control over the electrons transported from complex I (Gnaiger, 2014). The cytochrome *c* effect was calculated as CIc-OXPHOS/CI-OXPHOS to determine the structural integrity of the outer mitochondrial membrane: an increase of oxygen consumption after injection of cytochrome *c* indicates disruption of the outer mitochondrial membrane (Kuznetsov et al., 2008; Simard et al., 2018). The respiration rate measured following injection of the different substrates was used to evaluate the contribution of each substrate to mitochondrial oxygen consumption during OXPHOS (Cormier et al., 2019, 2021; Jørgensen et al., 2021; Simard et al., 2020a,b): succinate contribution=(CI+CII-OXPHOS−CIc-OXPHOS)/CIc-OXPHOS; and G3P contribution=(CI+CII+mG3PDH-OXPHOS−CI+CII-OXPHOS)/CI+CII-OXPHOS.

Hydrogen peroxide emission rate

Mitochondria are both producers and consumers of ROS and the measurement of hydrogen peroxide emission rates was used to evaluate the absolute amount of hydrogen peroxide emitted from mitochondria, i.e. taking into account mitochondrial H_2O_2 detoxification processes (Munro and Treberg, 2017). Hydrogen peroxide emission rates were determined at room temperature (24°C) using Amplex® red reagent (Invitrogen, Waltham, MA, USA) and a Varioskan™ microplate reader (ThermoScientific™, Mississauga, ON, Canada) with excitation/emission set at 560/587 nm. Briefly, isolated mitochondria were incubated with 10 µmol l^{-1} Amplex® red, 2 U ml^{-1} horseradish peroxidase and 50 U ml^{-1} superoxide dismutase, and H_2O_2 emission under three different conditions was measured by adding different substrates and inhibitors: (1) in the presence of pyruvate, malate, glutamate, ADP, succinate and G3P (maximal), (2) with the addition of rotenone (maximal+rotenone) and (3) with rotenone+antimycin A (maximal+rotenone+antimycin). The results are presented as pmol H_2O_2 min^{-1} mg^{-1} protein.

Statistical analysis

Statistical analyses were performed in R version 3.6.2 (http://www.R-project.org/). For comparison of oxygen consumption rates, calculated mitochondrial ratios and hydrogen peroxide emission rates, data within an acclimation temperature (15 and 22.5°C) were fitted to a mixed model with treatment (1st-C, 1st-HS, 2nd-C, 2nd-HS, 3rd-C) as a fixed factor and aquarium (two different aquaria per acclimation temperature; Fig. S1) as a random factor. A type II ANOVA was then performed, followed by a Tukey's *post hoc* test using the emmeans (estimated marginal means) function to estimate specific differences between treatment in each acclimation group as appropriate. Normality was verified with visualization of the residuals and homogeneity of variances was verified using Levene's test, and data were transformed when required.

RESULTS

Mitochondrial oxygen consumption

Oysters acclimated to 15°C

All the different respiration rates measured in oysters acclimated to 15°C showed the same patterns, with strong effects of treatment according to the ANOVA (Table 1, Fig. 1). Oysters undergoing the heat shock treatments had similar respiration rates but, significantly, these treatments induced an increase of all mitochondrial respiration rates compared with the control oysters (Fig. 1). Specifically, 1st-HS and 2nd-HS oysters displayed higher CI-LEAK, CI-OXPHOS, CI+CII-OXPHOS, CI+CII+G3PDH-OXPHOS and Complex IV activity compared with 1st-C, 2nd-C and 3rd-C oysters (Fig. 1). Moreover, a significant decrease was observed in 2nd-C compared with 1st-C and 3rd-C oysters for all respiration rates (Fig. 1).

Oysters acclimated to 22.5°C

As with oysters acclimated to 15°C, the different respiration rates measured in oysters acclimated to 22.5°C were influenced by treatment according to the ANOVA (Table 1). For CI-LEAK, 1st-C oysters had significantly higher respiration rates than 2nd-C and 3rd-C oysters (Fig. 1A). Moreover, 1st-HS oysters also displayed higher CI-LEAK than 2nd-C and 3rd-C oysters, and 2nd-HS oysters had the highest CI-LEAK, which was significantly different from 1st-HS, 2nd-C and 3rd-C values (Fig. 1A). CI-OXPHOS,

Table 1. Results of separate general linear mixed models, modeling the effects of treatment for each acclimated group of oysters (*Crassostrea virginica*), with aquarium modeled as random effects

	Acclimation to 15°C			Acclimation to 22.5°C		
		Random effect s.d.			Random effect s.d.	
	Fixed effects χ^2	Aquarium	Residual	Fixed effects χ^2	Aquarium	Residual
Mitochondrial respiration rate						
CI-LEAK	295.92***	0.183	0.190	79.84***	13.677×10^{-5}	2.841
CI-OXPHOS	237.69***	0.071	0.200	57.54***	12.226×10^{-4}	22.013
CI+CII-OXPHOS	117.50***	5.812	16.784	77.49***	15.265×10^{-4}	26.733
CI+CII+mG3PDH-OXPHOS	117.02***	6.683	18.777	92.56***	2.732×10^{-6}	0.213
Complex IV	105.45***	0.099	0.160	63.21***	33.40×10^{-4}	49.124
Mitochondrial ratio						
OXPHOS coupling ratio	26.81***	5.917×10^{-3}	18.575×10^{-3}	37.03***	1.445×10^{-6}	34.54×10^{-3}
Cytochrome *c* effect	2.07	6.456×10^{-7}	24.062×10^{-3}	5.10	11.92×10^{-3}	91.15×10^{-3}
Succinate contribution	22.06***	13.780×10^{-3}	59.690×10^{-3}	5.03	8.50×10^{-6}	0.353
G3P contribution	20.36***	4.330×10^{-6}	0.299	27.57***	6.59×10^{-6}	0.264
Hydrogen peroxide emission rate						
Maximal	17.19**	1.064×10^{-6}	0.152	35.33***	0.649	1.243
Maximal+rotenone	8.84	3.972×10^{-6}	0.289	46.00***	0.906	1.265
Maximal+rotenone+antimycin	5.11	3.959×10^{-6}	0.262	20.16***	3.07×10^{-6}	0.227

Chi-square values are reported (treatment d.f.=4), and *P*-values based on a Type II ANOVA, with asterisks indicating significance (**P<0.01, ***P<0.001). For the random effect, standard deviation is given for aquarium and residual. Maximal hydrogen peroxide emission rate was obtained with pyruvate+malate+glutamate+ADP+succinate+G3P.

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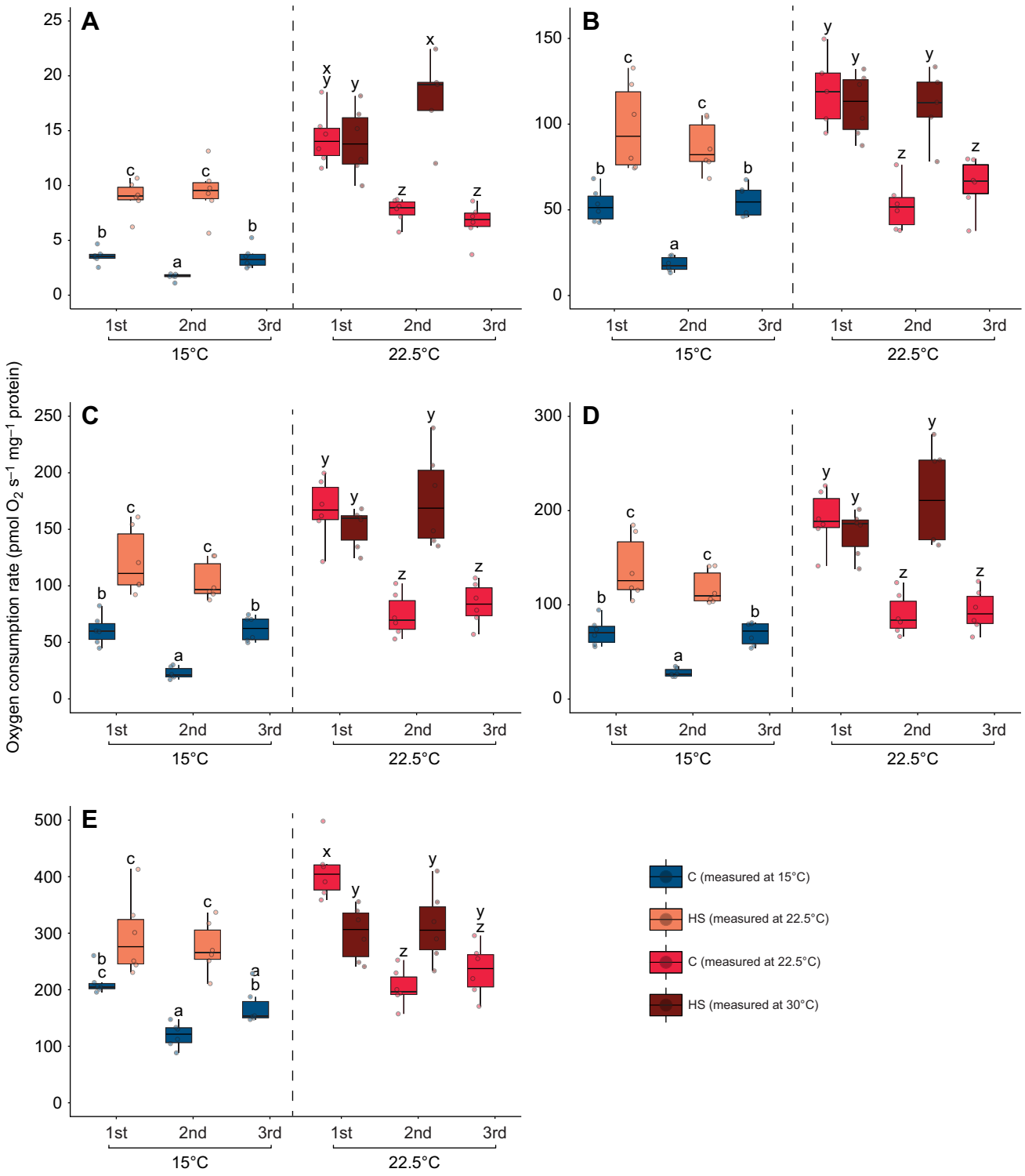

Fig. 1. Mitochondrial oxygen consumption rate measured in isolated gill mitochondria from *Crassostrea virginica* acclimated to either 15 or 22.5°C and exposed to heat shock treatment. Oxygen consumption rates were measured in mitochondria isolated from oysters before (control) and 12 h after the start of a first 7.5°C heat shock (1st-C and 1st-HS, respectively), 10 days after the first heat shock and 12 h after the start of a second heat shock (2nd-C and 2nd-HS, respectively), and 10 days after the second heat shock (3rd-C). For each treatment, measurements were made at the respective temperature experienced by the oysters (1st-C, 2nd-C and 3rd-C measured at 15°C and 1st-HS and 2nd-HS measured at 22.5°C for oysters acclimated to 15°C; 1st-C, 2nd-C and 3rd-C measured at 22.5°C and 1st-HS and 2nd-HS measured at 30°C for oysters acclimated to 22.5°C) in the presence of different substrates and inhibitors. (A) Pyruvate+malate+glutamate (CI-LEAK); (B) +ADP (CI-OXPHOS); (C) +succinate (CI+CII-OXPHOS); (D) +G3P (CI+CII+mG3PDH-OXPHOS); after inhibition of Complexes I and III by rotenone and antimycin A, respectively (for residual oxygen consumption); and (E) TMPD+ascorbate (Complex IV). Box plot values consist of the median (center line) and interquartile range (IQR, upper and lower edges of box), and the whiskers correspond to maximum and minimum values ≤1.5×IQR (Tukey-style) for each condition (N=6). Different letters denote significant differences (P≤0.05) between treatments for each acclimation group (a–c for oysters acclimated to 15°C and x–z for oysters acclimated to 22.5°C; one-way ANOVA followed by Tukey's *post hoc* test).

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CI+CII-OXPHOS and CI+CII+mG3PDH-OXPHOS in 1st-C, 1st-HS and 2nd-HS oysters were all significantly higher than in 2nd-C and 3rd-C oysters (Fig. 1B–D). For Complex IV activity, 1st-C oysters displayed significantly higher respiration rate than 1st-HS, 2nd-C, 2nd-HS and 3rd-C oysters (Fig. 1E), and 1st-HS and 2nd-HS oysters also had significantly higher complex IV activity than 2nd-C oysters (Fig. 1E).

Mitochondrial ratios

Oysters acclimated to 15°C

All mitochondrial ratios except the cytochrome *c* effect were influenced by treatment in oysters acclimated to 15°C according to the ANOVA (Table 1). The coupling ratio was decreased in 2nd-HS oysters, but only significantly compared with 1st-C and 3rd-C oysters (Fig. 2A). Moreover, 2nd-C oysters had a significantly lower coupling ratio compared with 3rd-C oysters (Fig. 2A). Succinate contribution was also significantly higher in 2nd-C oysters compared with 1st-C and 3rd-C oysters, as well as in 1st-HS compared with 3rd-C oysters (Fig. 2C). After addition of G3P, only 2nd-C oysters displayed a significantly higher contribution for this substrate compared with 2nd-HS and 3rd-C oysters (Fig. 2D).

Oysters acclimated to 22.5°C

For oysters acclimated to 22.5°C, only the coupling ratio and the G3P contribution were influenced by treatment according to the ANOVA (Table 1). The coupling ratio was significantly decreased in 2nd-C oysters compared with 1st-C and 3rd-C oysters, as well as in 2nd-HS oysters compared with 1st-C, 1st-HS and 3rd-C oysters (Fig. 2A). Moreover, the G3P contribution was significantly higher in 2nd-C and 2nd-HS oysters compared with 3rd-C oysters (Fig. 2D).

Hydrogen peroxide emission rate

Oysters acclimated to 15°C

Among the three conditions tested in oysters acclimated to 15°C, only maximal H_2O_2 emission was influenced by treatment according to the ANOVA (Table 1). Specifically, maximal H_2O_2

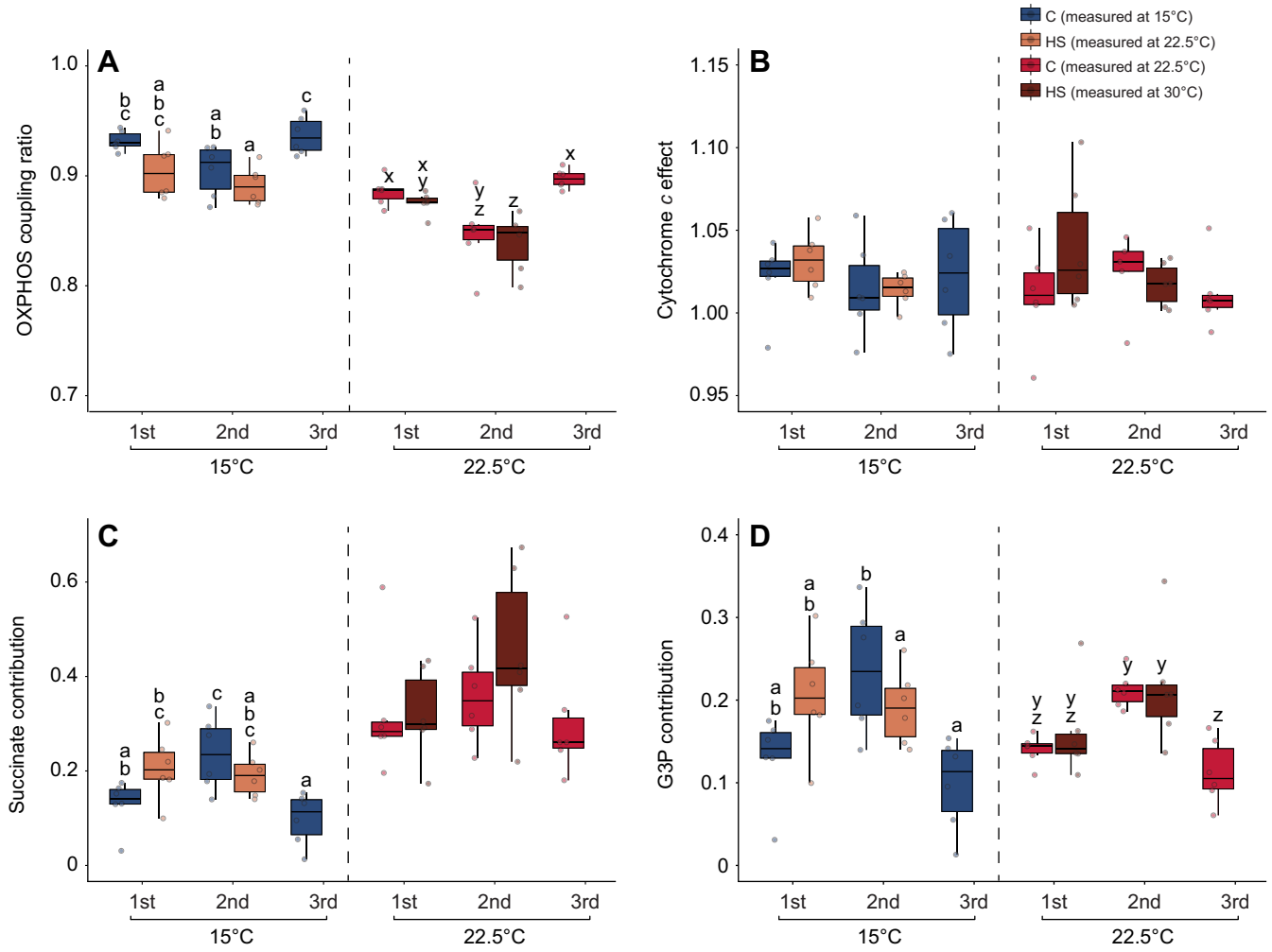

Fig. 2. Calculated mitochondrial ratios and substrate contributions measured in isolated gill mitochondria from *C. virginica* acclimated to either 15 or 22.5°C and exposed to heat shock treatment. Mitochondrial oxygen consumption rates used in calculations were measured as described in Fig. 1. (A) OXPHOS coupling ratio (1−CI-LEAK/CI-OXPHOS). (B) Cytochrome *c* effect (CIc-OXPHOS/CI-OXPHOS). (C) Succinate contribution [(CI+CII-OXPHOS−CIc-OXPHOS)/CIc-OXPHOS]. (D) G3P contribution [(CI+CII+mG3PDH-OXPHOS−CI+CII-OXPHOS)/CI+CII-OXPHOS]. Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values ≤1.5×IQR (Tukey-style) for each condition (N=6). Different letters denote significant differences (P≤0.05) between treatments for each acclimation group (a–c for oysters acclimated to 15°C and x–z for oysters acclimated to 22.5°C; one-way ANOVA followed by Tukey's *post hoc* test).

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emission rate was slightly but significantly decreased in 3rd-C compared with 1st-C, 1st-HS and 2nd-C oysters (Fig. 3A).

Oysters acclimated to 22.5°C

When oysters acclimated to 22.5°C were tested for H_2O_2 emission, treatment influenced all three conditions measured according to the ANOVA (Table 1). Significantly higher H_2O_2 emission rates for maximal and maximal+rotenone were detected in 2nd-C oysters compared to 1st-C, 1st-HS and 3rd-C oysters, as well as in 2nd-HS compared with 3rd-C oysters (Fig. 3A,B). For maximal+rotenone+antimycin, significantly higher H_2O_2 emission rates were observed in 1st-C and 2nd-C compared with 3rd-C oysters (Fig. 3C).

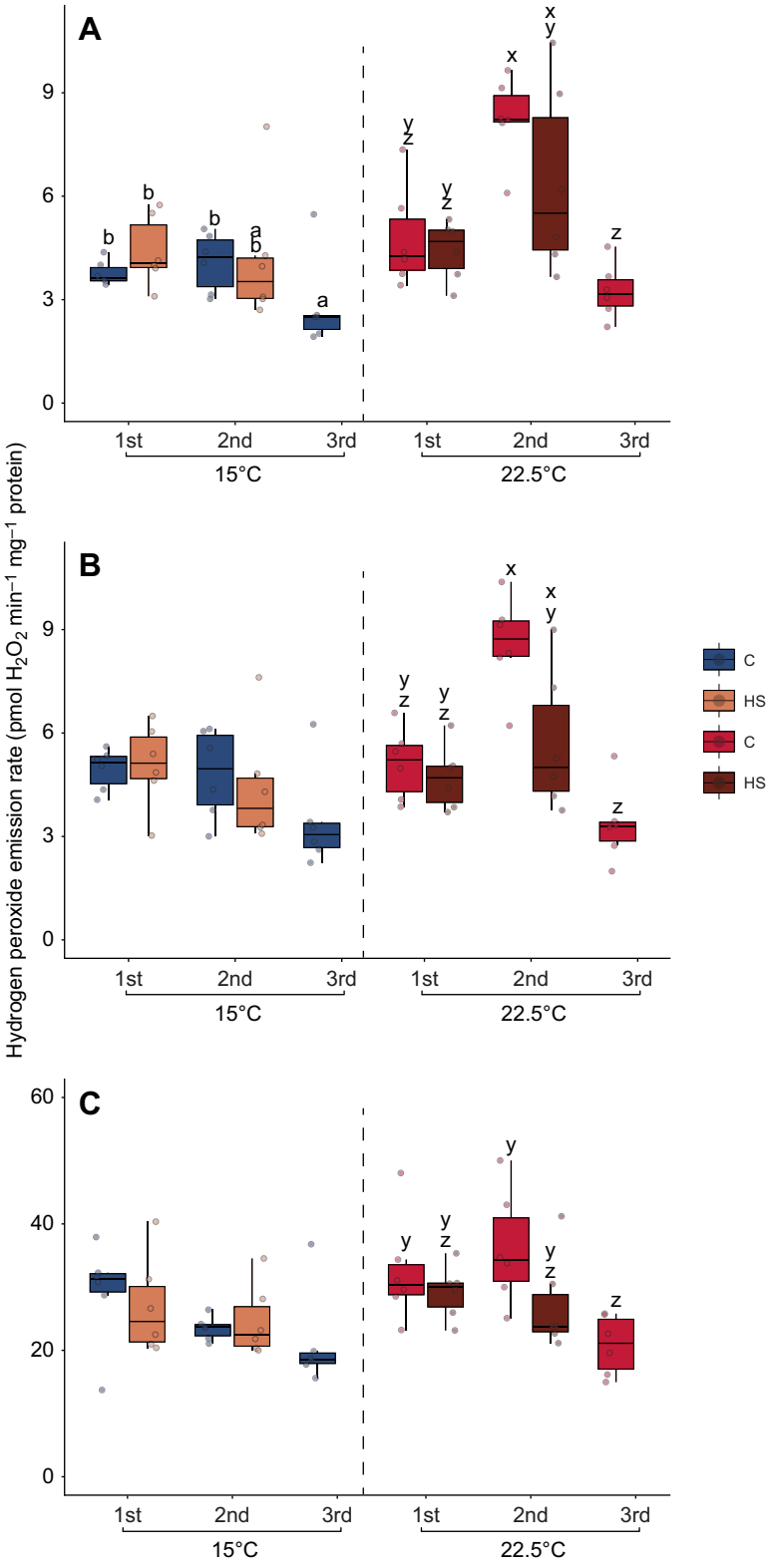

Fig. 3. Hydrogen peroxide emission rates measured in isolated gill mitochondria from *C. virginica* acclimated to either 15 or 22.5°C and exposed to heat shock treatment. Hydrogen peroxide emission rates were measured at room temperature (24°C) for mitochondria isolated from oysters before and 12 h after the start of a first 7.5°C heat shock (1st-C and 1st-HS, respectively), 10 days after the first heat shock and 12 h after the start of a second heat shock (2nd-C and 2nd-HS, respectively), and 10 days after the second heat shock (3rd-C). Measurements were made in the presence of different substrates and inhibitors. (A) Maximal: pyruvate+malate+glutamate+ADP+succinate+G3P. (B) Maximal+rotenone. (C) Maximal+rotenone+antimycin A. Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values ≤1.5×IQR (Tukey-style) for each condition (N=6). Different letters denote significant differences (P≤0.05) between treatments for each acclimation group (a and b for oysters acclimated to 15°C and x–z for oysters acclimated to 22.5°C; one-way ANOVA followed by Tukey's *post hoc* test).

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DISCUSSION

In this study, we sought to characterize the effects of intermittent 7.5°C heat shocks on mitochondrial and ROS metabolism of gills from *C. virginica* acclimated to either 15 or 22.5°C. For this purpose, we measured mitochondrial oxygen consumption rate and H_2O_2 emission rate of oysters before and 12 h after the start of a first heat shock (1st-C and 1st-HS oysters, respectively), 10 days after the first heat shock and 12 h after the start of a second heat shock (2nd-C and 2nd-HS oysters, respectively), and another 10 days after the second heat shock (3rd-C oysters; Fig. S1). Our results showed that in oysters acclimated to 15°C, 1st-HS oysters had increased respiration rates compared with 1st-C oysters, but that, across the board, respiration rates for 2nd-C oysters were lower than for 1st-C oysters. This trend (2nd-C lower than 1st-C) was prominent in oysters acclimated to both temperatures. Moreover, a slight decrease of the OXPHOS coupling ratio was observed in 2nd-HS oysters, and contributions of FAD-linked substrates (succinate and G3P) were slightly increased in 2nd-C oysters. OXPHOS reorganization due to thermal stress (shown here by the increased contribution of FAD-linked substrates in 2nd-C oysters) has been suggested to be associated with impairment of metabolic function in bivalves (Hraoui et al., 2020). Recently, it has been shown that the contribution of FAD-linked substrates increases with temperature and that this increase is correlated with whole-organism thermal tolerance in *Drosophila* (Jørgensen et al., 2021). However, while we may presume that lower oxygen consumption may be indicative of metabolic damage (likely due to increased ROS production) (Venditti et al., 2013), things may not be so straightforward. The lower oxygen consumption may also consist of a deliberate suppression by the mitochondria to better regulate ROS production. It has been shown that elevated temperatures induce a significant increase in ROS production in bivalves (Abele et al., 2002; Heise et al., 2003). As such, lowering oxygen consumption may represent a means by which ROS production is limited. Consequently, the necessity to avoid deleterious effects induced by ROS coupled with a potentially impaired OXPHOS may explain the results found in 2nd-C oysters. This hypothesis was supported by our results for H_2O_2 emission rates.

H_2O_2 emission rates were measured at 24°C for all cases, whereas mitochondrial respiratory functions were measured at acclimation and treatment temperatures (e.g. at 30°C for the 1st-HS of oysters acclimated at 22.5°C). One could argue that this may have introduced bias into the results, but as this was a comparative study focused on the effect of intermittent heat shocks in groups acclimated to two different temperatures, it should not have affected the comparisons that were made or the conclusions that were drawn from the results. Our results for H_2O_2 emission showed no significant effect between 1st-C and 2nd-C in oysters acclimated to 15°C despite decreased mitochondrial respiration rates. To mitigate the increase in ROS production (and therefore, the potential oxidative damage associated with elevated levels of ROS), oyster mitochondria may deliberately reduce oxygen consumption. The 2nd-HS seemed to elicit the same type of reaction from oyster mitochondria acclimated at 15°C regarding H_2O_2 emission. These results suggested that oyster mitochondria were most likely able to recover from the first heat shock and successfully weather the second shock. Interestingly, 3rd-C oysters demonstrated a trend towards lower H_2O_2 emission rates in comparison to 1st-C and 2nd-C oysters. This effect was significant in the condition without any mitochondrial complex inhibitors (maximal, Fig. 3A). The other two conditions exhibited similar trends, but the results were not significantly different from 1st-C and 2nd-C oysters. These data suggest that, after weathering two heat shocks, cellular defense mechanisms may have been established and may regulate ROS production more effectively. These mechanisms may have included (but are not limited to) induction of heat shock proteins as well as activation of antioxidant genes, as these processes have been identified in other bivalve species (Truebano et al., 2010).

In oysters acclimated to 22.5°C, the first heat shock did not induce any significant change in respiration rates except for a decrease of Complex IV maximal oxygen consumption. Considering the effects that the thermodynamics should have had on respiration rates (increased oxygen consumption with increasing temperature), this might be indicative of mitochondrial defects caused by the first heat shock. Respiration rates were then drastically decreased in 2nd-C oysters (likely as a consequence of the first heat shock) and increased in 2nd-HS oysters. This coincided with the lowest OXPHOS coupling ratio as well as the highest H_2O_2 emission rate, further suggesting mitochondrial defects in 2nd-C and 2nd-HS oysters. Overall, our results were consistent with our hypothesis that predicted damage after 1st-HS exacerbated by 2nd-HS in 22.5°C acclimated oysters. These results were also further supported by the change in H_2O_2 emission rates.

While ROS levels were similar for the two oyster groups at 1st-C, H_2O_2 emission rate for 2nd-C oysters was significantly elevated in comparison with 1st-C oysters only for those acclimated to 22.5°C. This elevated H_2O_2 emission aligned with lower respiratory rates. At first glance, these elevated ROS levels may have represented a failure to cope with the adverse effects of the 1st-HS (as suggested by our results on respiratory rates). However, this also could be due to a more effective establishment of ROS scavenging mechanisms at higher acclimation temperatures. Acclimation temperature has been shown to affect the thermal tolerance of bivalves (Galbraith et al., 2012). As elevated ROS levels directly contribute to cellular dysfunction (and therefore thermal tolerance), our results were congruent with previous findings. Our results were also in line with previous studies in terms of ROS production across the 20 days of the experiment. For example, Matoo et al. (2013) showed that long-term exposure of the two bivalve species *C. virginica* and *Mercenaria mercenaria* to moderate warming (from 22°C to 27°C) did not induce any persistent oxidative stress signals.

One key result from our data stemmed from the 3rd-C for 22.5°C acclimated oysters. For the 15°C acclimation, oyster respiration levels were able to return to pre-HS levels at 3rd-C. Contrarily, respiration levels at 3rd-C for oysters acclimated to 22.5°C seemed to be significantly lowered in comparison with those at 1st-C. This may have represented (as mentioned before) a reduction of oxygen consumption to control ROS levels, but it may also have reflected damage caused to mitochondrial metabolic functions. However, as OXPHOS coupling ratio and H_2O_2 emission rates were similar to initial values despite these decreased respiration rates, this suggests that with more time, these oysters might have been able to restore their mitochondrial function, in spite of the heat shock waves. That being said, it is important to contextualize our results in terms of the timing of the heat shocks. Oysters were exposed to heat waves in periods of 12 h. This was a relatively short (but not acute) exposure time, although intertidal oysters might experience more drastic temperature changes. Indeed, many heat waves can last multiple days (and not just 12 h). Many molecular changes in mitochondria might not be noticeable with only 12 h of exposure, and it may take a longer exposure time to fully understand how mitochondria change/react when faced with thermal stress, although changes in RNA levels and expression of certain genes can also be discernible in few hours in bivalves (Lang et al., 2009; Li et al., 2013; Zhang et al., 2020).

While it is true that both oyster groups were subjected to a temperature increase of 7.5°C, the effect of this increase was expected to differ between the experimental groups. For the first group, the increase led to a 12 h exposure at 22.5°C. This temperature is well within the species' thermal range and, as such, the impact of this increase on the mitochondrial metabolism should have been easier to recover from. Our results led us to surmise that this is indeed the case, as oysters of the 15°C group seemed to show signs of recovery (in regard to oxygen consumption and ROS emission). In contrast, the second experimental group was exposed to a temperature of 30°C, which is near the upper thermal tolerance of the studied oysters. As such, in contrast to the group exposed to 22.5°C, these oysters would have been exposed to a more stressful acute temperature shock and, thus, may have experienced mitochondrial dysfunction. Interestingly, while respiration rates at 3rd-C for the 22.5°C acclimated oysters seemed to support this prediction, our other results (H_2O_2 emission rates and OXPHOS coupling ratios) seemed to point towards a potential recovery.

Overall, our key findings reflected a surprising resilience demonstrated by *C. virginica*. Signs of mitochondrial impairment were found for both acclimation temperatures but, surprisingly, both acclimated populations showed signs of potential recovery, given enough time. Moreover, the contrast in the results obtained for 1st-C, 1st-HS, 2nd-C, 2nd-HS and 3rd-C highlight the importance of studying intermittent thermal stress, as single-stress experiments may not tell the whole story. As extreme weather patterns become more frequent as a result of climate change, studies such as this one may help us to better understand the challenges that sessile organisms such as some bivalve species must face. Lastly, this study highlights the plasticity of oyster's mitochondria when confronted with intermittent heat shocks and uncovers the potential detrimental effects of a combination of increased average temperatures and higher heat wave frequency predicted by climate change. As anthropogenic activity has been directly linked with acidification due to increased uptake of CO_2 (Caldeira and Wickett, 2003), it would be particularly interesting to follow up this study with an experiment that investigates the combined effect of temperature increase and pH acidification on the mitochondrial health of bivalves.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.H., S.B., N.P.; Methodology: G.H., G.M., L.H.B., F.H., N.P.; Validation: S.B., N.P.; Formal analysis: G.H., N.P.; Investigation: G.H., N.P.; Resources: G.M., L.H.B., N.P.; Data curation: N.P.; Writing - original draft: G.H., S.B., N.P.; Writing - review & editing: G.H., S.B., G.M., L.H.B., F.H., N.P.; Visualization: N.P.; Supervision: N.P.; Funding acquisition: N.P.

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Data availability
Data are available from Mendeley Data: https://data.mendeley.com/datasets/9p79wm268c/1

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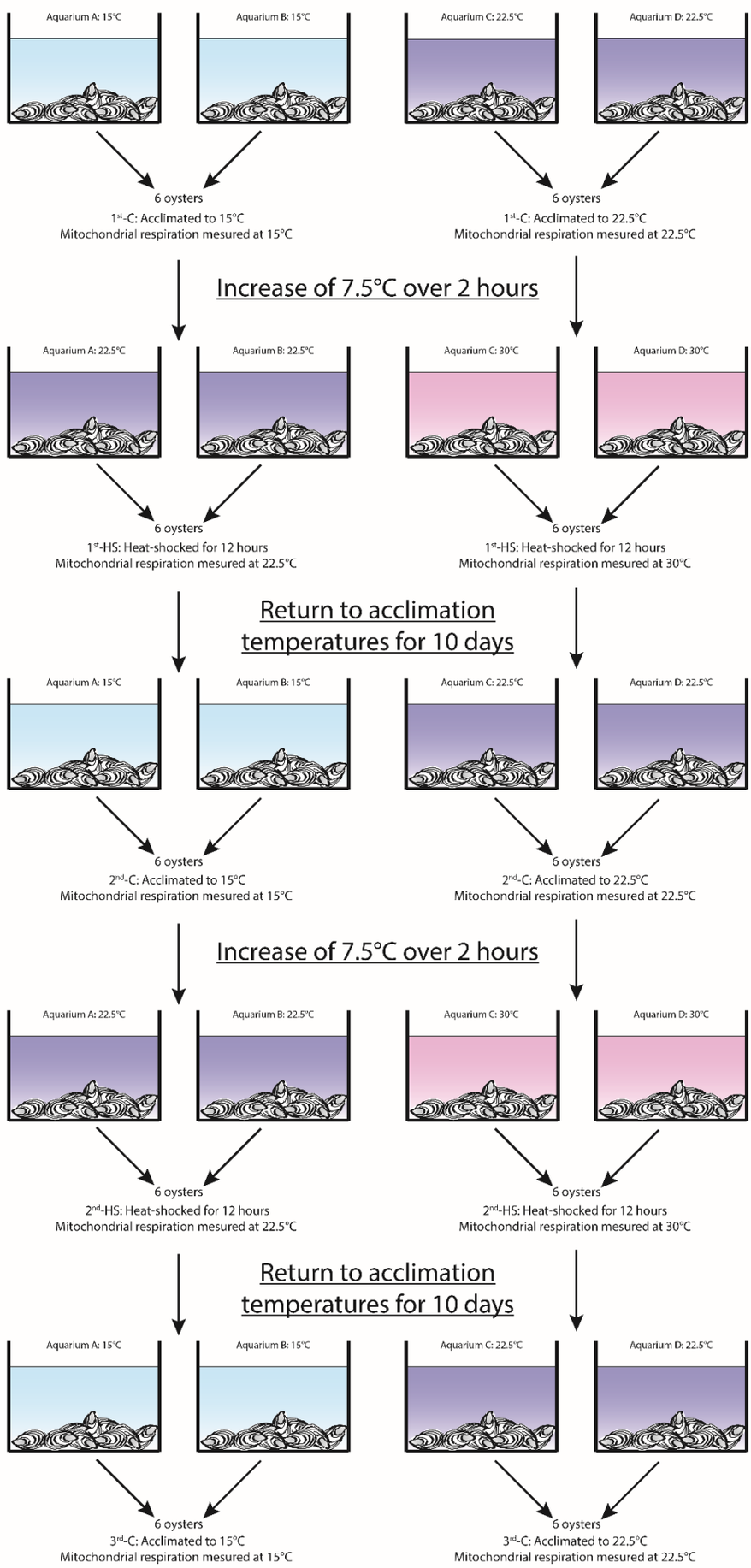

Fig. S1. Outline of experimental design.