Mitochondrial responses towards intermittent heat shocks in the Eastern Oyster, *Crassostrea virginica*

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Summary statement:

Intermittent heat shocks modulate mitochondrial and reactive oxygen species metabolism in oysters acclimated to two different temperatures. Oysters acclimated to higher temperatures are struggling to adjust to these thermal challenges.

Abstract

Frequent heat waves caused by climate change can cause 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 Eastern oyster *Crassostrea virginica* which play a key role in setting ectotherms' thermal tolerance. 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°C 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 with an increase 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 ten 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 oyster's capacity to weather intermittent heat shock.

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; Buisson et al., 2008b). 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 the increase in the occurrence of extreme temperature fluctuations, a species' capacity to react towards 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 the aerobic metabolism in eukaryotic cells via oxidative phosphorylation (OXPHOS), are quite sensitive to environmental temperatures, which especially holds true for aquatic ectotherm species (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, possess roles in cell signaling, 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, lipidic 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 organism's death due to 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 towards thermal stress. However, little is known about their response to intermittent heat shocks. While we could 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 (due to thermal stress) and potential increase in ROS production (and thus cellular damages). Moreover, many oyster species, whose optimal temperatures lie between 15°C 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 the Eastern oyster *C. virginica* acclimated to two different temperatures. Specifically, oysters acclimated to either 15 or 22.5°C were challenged with two 7.5°C heat shocks for 12h (reaching 22.5 and 30°C, respectively) at ten-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 these temperatures to represent normal and moderately high temperatures encountered by the oysters in their natural environment (Miramichi Bay, NB, Canada) during summer (15 and 22.5°C, respectively) 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 the mitochondrial responses to the intermittent heat shocks. Specifically, we predicted that in oysters acclimated to 15°C, the first heat shock would cause modulations of mitochondrial and ROS metabolism which would be resolved when these oysters would be exposed to the second heat shock. By contrast, in oysters acclimated to 22.5°C the first heat shock would cause mitochondrial dysfunctions that would be exacerbated when the second heat shock would be 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 shell length ± S.E.M.: 86.14 ± 1.02 mm) were collected from floating trays at the Daigle Aquaculture Inc. farm located in the Miramichi Bay (46°72'N, 64°89'W, NB, Canada) the 18th of 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 2h of collection and transferred in 50 L recirculated aquaria with artificial sea water (26‰) set at either 15°C 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 8mL 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 as well as 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 isolations (1st experiment, control oysters: 1st-C, N = 6). Then, the aquarium temperatures were increased by 7.5°C, reaching 22.5 and 30°C after 2h. The following day, 12h after the start of the temperature increase, 6 oysters were collected again for each temperature (22.5 and 30°C) for mitochondrial isolations (1st experiment, heat-shocked oysters: 1st-HS, N = 6), and the aquarium temperatures were allowed to return to the initial acclimation temperatures (15 and 22.5°C) which were reached after 6h. After 10 days, this procedure was repeated i.e., collection of 6 oysters acclimated to either 15 or 22.5°C (2nd experiment, control oysters: 2nd-C, N = 6) and after the temperatures were raised by 7.5°C (2nd experiment, heat-shocked oysters: 2nd-HS, N = 6). Again, after the heat shock, the aquarium temperatures were cooled down to the initial acclimation temperatures for 10 days before collection of 6 oysters acclimated to either 15 or 22.5°C (3rd experiment, control oysters: 3rd-C, N = 6). An outline of the experimental design is represented in Fig. S1. No changes in gonadal tissues as well as 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 while 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 of the gills were removed, minced and homogenized in an isolation buffer consisting of 100 mM KCl, 400 mM sucrose, 6 mM EGTA, 3 mM EDTA, 70 mM 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 1,250 *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

Mitochondrial oxygen consumption in freshly prepared mitochondrial isolations was measured using the Oxygraph O2K (Oroboros Instruments, Innsbruck, Austria). Mitochondria were placed in the respirometry chambers set at the different temperatures (15, 22.5 and 30°C) with respiration medium consisting of 550 mM sucrose, 150 mM KCl, 70 mM HEPES, 10 mM KH_2PO_4, 0.2% BSA, pH 7.4. A substrate-uncoupler-inhibitor titration (SUIT) protocol was performed using: pyruvate, malate and glutamate (10 mM, 0.5 mM and 10 mM, respectively) to measure the leak (non-phosphorylating) state for complex I (CI-LEAK); +ADP (5 mM) to monitor the phosphorylating state for complex I (CI-OXPHOS); +cytochrome c (15 µM, CIc-OXPHOS) to determine the intactness of the outer mitochondrial membrane (Kuznetsov et al., 2008; Simard et al., 2018); +succinate (10 mM) to assess maximum phosphorylating state with convergent electrons from complex I and complex II (CI+CII-OXPHOS); +glycerol-3-phosphate that allows the transport of electrons to the ETS via the mitochondrial glycerol-3-phosphate dehydrogenase (CI +CII+G3PDH-OXPHOS); +rotenone (1 µM) + antimycin A (2.5 µM) to inhibit complexes I and III, and measure residual oxygen consumption which was used to correct all the mitochondrial respiration rates. Finally, ascorbate (2 mM) + TMPD (0.5 mM) 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 mM). All measurements are presented as pmol O_2 s^{-1} mg^{-1} of proteins.

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 (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 rates measured with injections of the different substrates were used to evaluate the contribution of each substrate to mitochondrial oxygen consumption during OXPHOS (Cormier et al., 2019; Cormier et al., 2021; Jørgensen et al., 2021; Simard et al., 2020a; Simard et al., 2020b): 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 rates

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). The hydrogen peroxide emission rate was 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 µM Amplex® red, 2 U mL^{-1} horseradish peroxidase and 50 U mL^{-1} superoxide dismutase and three different conditions were measured by adding different substrates and inhibitors. Specifically, we evaluated maximal H_2O_2 emission rates in presence of pyruvate, malate, glutamate, ADP, succinate and G3P (maximal), as well as with the addition of rotenone (maximal+rotenone) and rotenone + antimycin A (maximal+rotenone+antimycin). 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 (R Core Team, 2019). For comparison of oxygen consumption rates, calculated mitochondrial ratios and hydrogen peroxide emission rates, data within acclimation temperature (15 and 22.5°C) were fitted to a mixed model with the treatment (which includes 1^{st}-C; 1^{st}-HS; 2^{nd}-C; 2^{nd}-HS; 3^{rd}-C) as a fixed factor and the aquarium (two different aquarium 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-function (estimated marginal means) 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 the 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 these treatments significantly induced an increase of all mitochondrial respiration rates compared to the control oysters (Fig. 1A-E). Specifically, 1^{st}-HS and 2^{nd}-HS oysters displayed higher CI-LEAK, CI-OXPHOS, CI+CII-OXPHOS, CI+CII+G3PDH-OXPHOS and complex IV activity compared to 1^{st}-C, 2^{nd}-C and 3^{rd}-C (Fig. 1A-E). Moreover, a significant decrease was observed in 2^{nd}-C compared to 1^{st}-C and 3^{rd}-C oysters for all respiration rates (Fig. 1A-E).

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 the 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 (Fig. 1A). CI-OXPHOS, CI+CII-OXPHOS and CI+CII+G3PDH-OXPHOS in 1st-C, 1st-HS and 2nd-HS oysters were all significantly higher than 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 cytochrome c effect were influenced by the 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 to 1st-C and 3rd-C oysters (Fig. 2A). Moreover, 2nd-C oysters had significantly lower coupling ratio compared to 3rd-C oysters (Fig. 2A). Succinate contribution was also significantly higher in 2nd-C oysters compared to 1st-C and 3rd-C oysters, as well as in 1st-HS compared to 3rd-C oysters (Fig. 2C). After addition of G3P, only 2nd-C oysters displayed significantly higher contribution for this substrate compared to 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 the treatment according to the ANOVA (Table 1). The coupling ratio was significantly decreased in 2^{nd}-C oysters compared to 1^{st}-C and 3^{rd}-C oysters, as well as in 2^{nd}-HS compared to 1^{st}-C, 1^{st}-HS and 3^{rd}-C oysters (Fig. 2A). Moreover, the G3P contribution was significantly higher in 2^{nd}-C and 2^{nd}-HS oysters compared to 3^{rd}-C oysters (Fig. 2D).

Hydrogen peroxide emission rates

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 the treatment according to the ANOVA (Table 1). Specifically, maximal H_2O_2 emission rate was slightly but significantly decreased in 3^{rd}-C compared to 1^{st}-C, 1^{st}-HS and 2^{nd}-C oysters (Fig. 3A).

Oysters acclimated to 22.5°C

When oysters acclimated to 22.5°C were tested for H_2O_2 emission, the 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 2^{nd}-C oysters compared to 1^{st}-C, 1^{st}-HS and 3^{rd}-C oysters, as well as in 2^{nd}-HS compared to 3^{rd}-C oysters (Fig. 3A-B). For maximal+rotenone+antimycin, significantly higher H_2O_2 emission rates were observed in 1^{st}-C and 2^{nd}-C compared to 3^{rd}-C oysters (Fig. 3C).

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 rates and H_2O_2 emission rates of oysters before and during a first heat shock (1^{st}-C and 1^{st}-HS oysters, respectively), 10 days after the first heat shock and during a second heat shock (2^{nd}-C and 2^{nd}-HS oysters, respectively), and another 10 days after the second heat shock (3^{rd}-C oysters; Fig. S1). Our results showed that in oysters acclimated to 15°C, 1^{st}-HS had increased respiration rates compared to 1^{st}-C oysters, but that, across the board, respiration rates for 2^{nd}-C were lower than for 1^{st}-C. This trend (2^{nd}-C lower than 1^{st}-C) was prominent in oysters acclimated to both temperatures. Moreover, a slight decrease of the OXPHOS coupling ratio was observed in 2^{nd}-HS oysters, and contributions of FAD-linked substrates (succinate and G3P) were slightly increased in 2^{nd}-C oysters. OXPHOS reorganization due to thermal stress (shown here by the increased contribution of FAD-linked substrates in 2^{nd}-C) has been suggested to be associated with impairment of metabolic function in bivalves (Hraoui et al., 2020). Recently, it has been shown that 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 throttling 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 2^{nd}-C. 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 1^{st}-HS of oysters acclimated at 22.5°C). One could argue that this may have introduced a bias into the results, but since 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 as well as the potential 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 damages associated with elevated levels of ROS), oyster mitochondria may deliberately throttle oxygen production. 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, the 3rd-C demonstrated a trend towards lower H_2O_2 emission rates in comparison to 1st-C and 2nd-C. This effect was significant in the condition without any mitochondrial complex inhibitors (maximal, Fig. 3A). The other two conditions exhibited similar trends, but results were not significantly different from 1st-C and 2nd-C. This data suggests 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 changes 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 temperatures), this might be indicative of mitochondrial defects caused by the 1st heat shock. The respiration rates were then drastically decreased in 2nd-C (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 damages 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 both oyster groups at 1st-C, H_2O_2 emission rate for 2nd-C was significantly elevated in comparison with 1st-C only for oysters acclimated at 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 already 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 on this end 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*. showed that a long-term exposure of the two bivalve species *C. virginica* and *Mercenaria mercenaria* to moderate warming (going from 22°C to 27°C) did not induce any persistent oxidative stress signals (Matoo et al., 2013).

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 1st-C. This may have represented (as mentioned before) a throttling of oxygen consumption to control ROS levels, but it may also have reflected damages caused to mitochondrial metabolic functions. However, as OXPHOS coupling ratio as well as 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 functions, in spite of the heat shock waves. That being said, it is important to contextualize our results in terms of timing of the heat shocks. Oysters were exposed to heat waves by segments of 12h. 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 up to multiple days (and not just 12h). Many molecular changes in mitochondria might not be noticeable with only 12h 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 12h 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 have 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). On the other hand, the second experimental group was exposed to a temperature of 30°C. This temperature is near the upper thermal tolerance of the studied oysters. As such, in contrast to the group exposed to 22.5°C, these oysters should have been exposed to a more stressful acute temperature shock and thus, may have sustained 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 in 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 singular stress experiments may not tell the whole story. As extreme weather patterns become more frequent due to climate change, studies such as this one may help 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 confronted to 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. Since 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 mitochondrial health of bivalves.

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Competing interests

No competing interests declared.

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Data availability

Data are available at Mendeley Data, doi: 10.17632/9p79wm268c.1.

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Figures

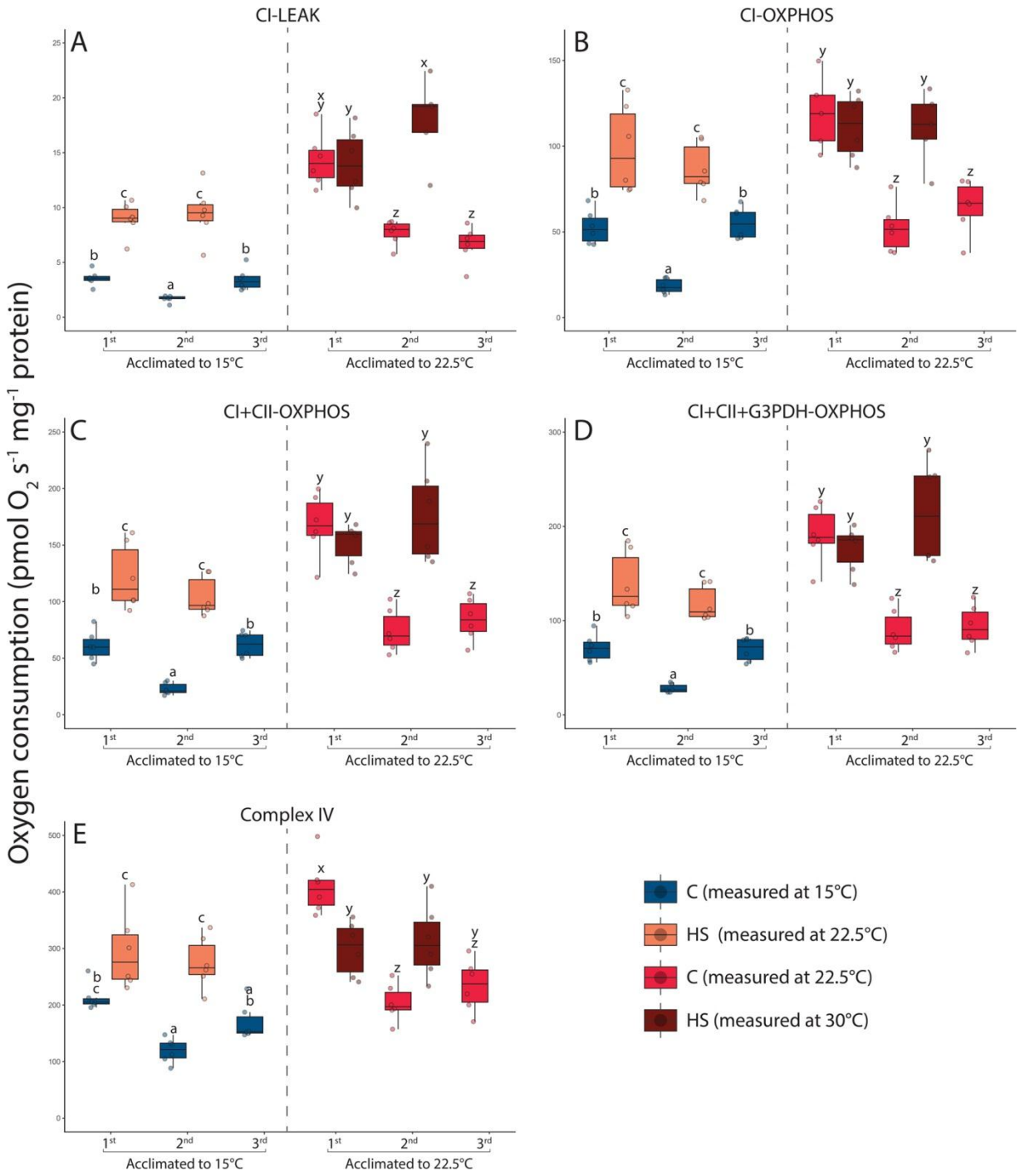

Figure 1. Mitochondrial oxygen consumption rates measured in isolated mitochondria from *Crassostrea virginica*'s gills acclimated to either 15 or 22.5°C and exposed to different heat shock treatments. Mitochondrial oxygen consumption rates were measured before and during a first 7.5°C heat shock of 12h (1^{st}-C and 1^{st}-HS, respectively), 10 days after the first heat shock and during a second heat shock (2^{nd}-C and 2^{nd}-HS, respectively), and 10 days after the second heat shock (3^{rd}-C). For each treatment, mitochondrial oxygen consumption was measured at the respective temperature experienced by the oysters: 1^{st}-C, 2^{nd}-C and 3^{rd}-C measured at 15°C and 1^{st}-HS and 2^{nd}-HS measured at 22.5°C for oysters acclimated to 15°C; 1^{st}-C, 2^{nd}-C and 3^{rd}-C measured at 22.5°C and 1^{st}-HS and 2^{nd}-HS measured at 30°C for oysters acclimated to 22.5°C. Mitochondrial oxygen consumption was measured in presence of (A) pyruvate+malate+glutamate (CI-LEAK), (B) +ADP (CI-OXPHOS), (C) +succinate (CI+CII-OXPHOS), (D) +G3P (CI+CII+G3PDH-OXPHOS), as well as after inhibition of complexes I and III by rotenone and antimycin A, respectively, and addition of (E) TMPD + ascorbate (Complex IV). Box plot values consist of the median (center line), the inter-quartile range (IQR, upper and lower edges of box), and the whiskers corresponds to maximum and minimum values no further than 1.5*IQR (Tukey-style) for each condition (N=6). Letters denote significant differences ($P \leq 0.05$) between treatments for each acclimated group with 'a', 'b' and 'c' representing differences between treatments for oysters acclimated to 15°C and 'x', 'y' and 'z' representing differences between treatments for oysters acclimated to 22.5°C (one-way ANOVA followed by Tukey's post hoc test).

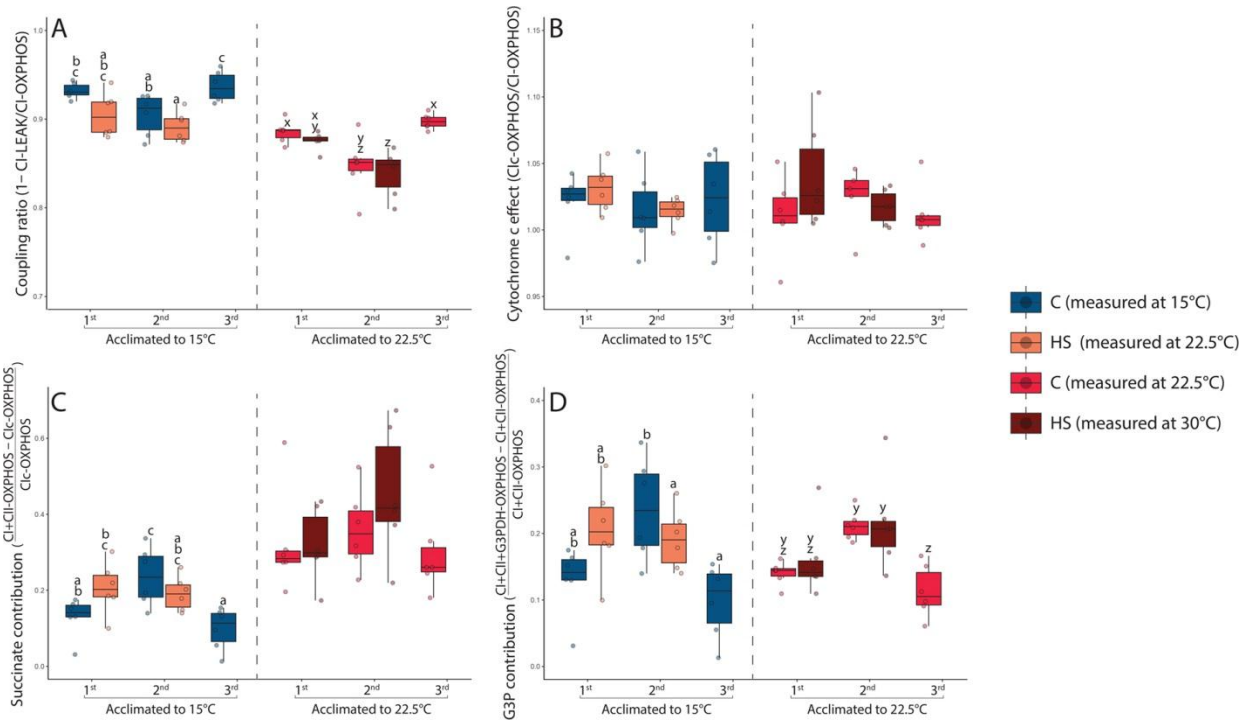

Figure 2. Calculated mitochondrial ratios and substrate contributions measured in isolated mitochondria from *Crassostrea virginica*'s gills acclimated to either 15 or 22.5°C and exposed to different heat shock treatments. Mitochondrial oxygen consumption rates were measured before and during a first 7.5°C heat shock of 12h (1^{st}-C and 1^{st}-HS, respectively), 10 days after the first heat shock and during a second heat shock (2^{nd}-C and 2^{nd}-HS, respectively), and 10 days after the second heat shock (3^{rd}-C). For each treatment, mitochondrial oxygen consumption rates used in calculations were measured at the respective temperature experienced by the oysters: 1^{st}-C, 2^{nd}-C and 3^{rd}-C measured at 15°C and 1^{st}-HS and 2^{nd}-HS measured at 22.5°C for oysters acclimated to 15°C; 1^{st}-C, 2^{nd}-C and 3^{rd}-C measured at 22.5°C and 1^{st}-HS and 2^{nd}-HS measured at 30°C for oysters acclimated to 22.5°C. (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+G3PDH-OXPHOS – CI+CII-OXPHOS)/ CI+CII-OXPHOS). Box plot values consist of the median (center line), the inter-quartile range (IQR, upper and lower edges of box), and the whiskers corresponds to maximum and minimum values no further than 1.5*IQR (Tukey-style) for each condition (N=6). Letters denote significant differences ($P \leq 0.05$) between treatments

for each acclimated group with ‘a’, ‘b’ and ‘c’ representing differences between treatments for oysters acclimated to 15°C and ‘x’, ‘y’ and ‘z’ representing differences between treatments for oysters acclimated to 22.5°C (one-way ANOVA followed by Tukey’s post hoc test).

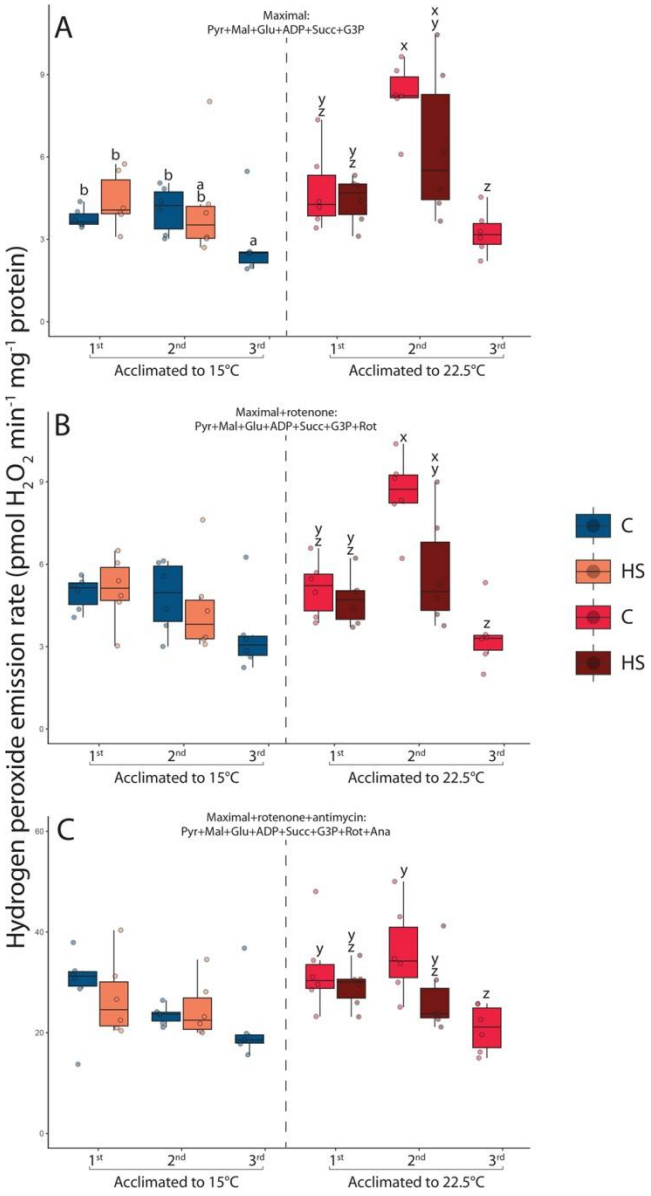

Figure 3. Hydrogen peroxide emission rates measured in isolated mitochondria from *Crassostrea virginica*'s gills acclimated to either 15 or 22.5°C and exposed to different heat shock treatments. Mitochondrial oxygen consumption rates were measured at 24°C before and during a first 7.5°C heat shock of 12h (1^{st}-C and 1^{st}-HS, respectively), 10 days after the first heat shock and during a second heat shock (2^{nd}-C and 2^{nd}-HS, respectively), and 10 days after the second heat shock (3^{rd}-C). Hydrogen peroxide emission measured in presence of (A) Maximal: pyruvate+malate+glutamate+ADP+succinate+glycerol-3-phosphate, (B) Maximal+rotenone: pyruvate+malate+glutamate+ADP+succinate+glycerol-3-phosphate+rotenone, (C) Maximal+

rotenone+antimycin: pyruvate+malate+glutamate+ADP+succinate+glycerol-3-phosphate+ rotenone +antimycin A. Box plot values consist of the median (center line), the inter-quartile range (IQR, upper and lower edges of box), and the whiskers corresponds to maximum and minimum values no further than 1.5*IQR (Tukey-style) for each condition (N=6). Letters denote significant differences ($P \leq 0.05$) between treatments for each acclimated group with ‘a’ and ‘b’ representing differences between treatments for oysters acclimated to 15°C and ‘x’, ‘y’ and ‘z’ representing differences between treatments for oysters acclimated to 22.5°C (one-way ANOVA followed by Tukey’s post hoc test).

Table

Table 1. Results of separate general linear mixed models, modelling effects of the treatment for each acclimated group of oysters (*Crassostrea virginica*); with Aquarium modelled as random effects. Chi-square values are reported, and p values based on a Type II ANOVA, with ** indicating significance at P < 0.01, and * at P < 0.001. For the random effect, values of SD are given for Aquarium and Residual.**

	Oysters acclimated to 15°C			Oysters acclimated to 22.5°C		
	Fixed effects X^2	**Random effect SD**		**Fixed effects X^2**	**Random effect SD**	
	Treatment *Df = 4*	Aquarium	Residual	Treatment *Df = 4*	Aquarium	Residual
Mitochondrial respiration rates						
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+G3PDH-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 ratios						
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 rates						
Maximal : Pyr+Mal+Glu+ADP+Succ+G3P	17.19**	1.064×10^{-6}	0.152	35.33***	0.649	1.243
Maximal+rotenone : Pyr+Mal+Glu+ADP+Succ+G3P+Rot	8.84	3.972×10^{-6}	0.289	46.00***	0.906	1.265
Maximal+rotenone+antimycin : Pyr+Mal+Glu+ADP+ Succ+G3P+Rot+Ana	5.11	3.959×10^{-6}	0.262	20.16***	3.07×10^{-6}	0.227

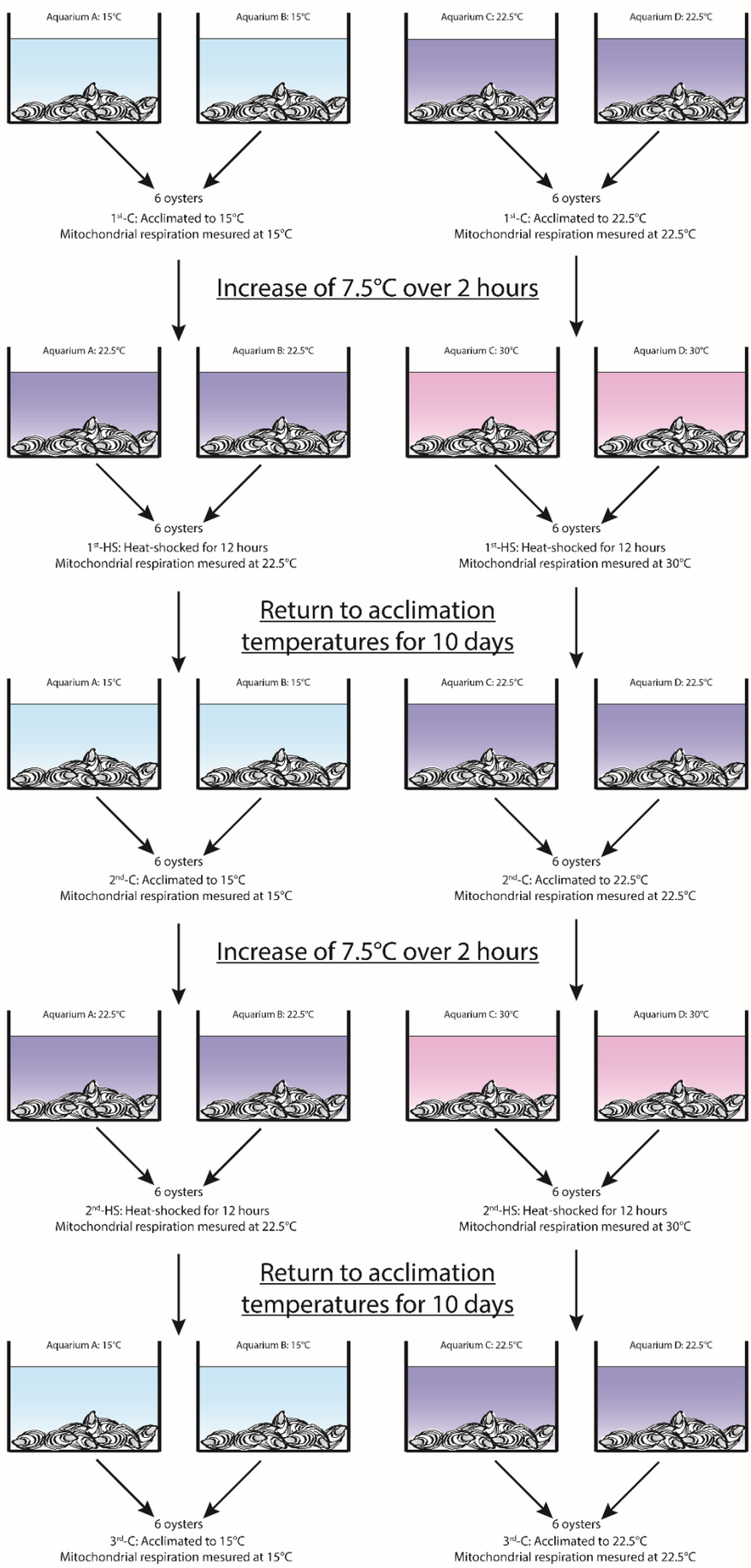

Fig. S1. Outline of experimental design.