© 2022. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2022) 225, jeb244795. doi:10.1242/jeb.244795

RESEARCH ARTICLE

Metabolic remodeling caused by heat hardening in the Mediterranean mussel *Mytilus galloprovincialis*

Ioannis Georgoulis[1], Christian Bock[2], Gisela Lannig[2], Hans-O. Pörtner[2], Konstantinos Feidantsis[1], Ioannis A. Giantsis[3], Inna M. Sokolova[4] and Basile Michaelidis[1,]*

ABSTRACT

Organisms can modify and increase their thermal tolerance faster and more efficiently after a brief exposure to sublethal thermal stress. This response is called 'heat hardening' as it leads to the generation of phenotypes with increased heat tolerance. The aim of this study was to investigate the impact of heat hardening on the metabolomic profile of *Mytilus galloprovincialis* in order to identify the associated adjustments of biochemical pathways that might benefit the mussels' thermal tolerance. Thus, mussels were exposed sequentially to two different phases (heat hardening and acclimation phases). To gain further insight into the possible mechanisms underlying the metabolic response of the heat-hardened *M. galloprovincialis*, metabolomics analysis was complemented by the estimation of mRNA expression of phosphoenolpyruvate carboxykinase (PEPCK), pyruvate kinase (PK) and alternative oxidase (AOX) implicated in the metabolic pathways of gluconeogenesis, glycolysis and redox homeostasis, respectively. Heat-hardened mussels showed evidence of higher activity of the tricarboxylic acid (TCA) cycle and diversification of upregulated metabolic pathways, possibly as a mechanism to increase ATP production and extend survival under heat stress. Moreover, formate and taurine accumulation provide an antioxidant and cytoprotective role in mussels during hypoxia and thermal stress. Overall, the metabolic responses in non-heat-hardened and heat-hardened mussels underline the upper thermal limits of *M. galloprovincialis*, set at 26°C, and are in accordance with the OCLTT concept. The ability of heat-hardened mussels to undergo a rapid gain and slow loss of heat tolerance may be an advantageous strategy for coping with intermittent and often extreme temperatures.

KEY WORDS: Gene expression, Heat hardening, ^{1}H-NMR spectroscopy, Metabolomics, Metabolic patterns, Mussel

INTRODUCTION

The increase of seawater temperature due to global climate change represents a major threat to global biodiversity by influencing marine organisms' molecular, biochemical and physiological organization (Pörtner, 2002; 2012; 2021; Somero, 2010; 2012; Pörtner et al., 2014). Recently observed marine heat waves have demonstrated the high vulnerability of marine organisms and ecosystems (from geographical species shifts and widespread changes in species composition to harmful algal blooms, mass strandings of mammals and mass mortalities of particular species) to such extreme climate events (Galli et al., 2017; Ummenhofer and Meehl, 2017; Frölicher and Laufkötter, 2018; Androulidakis and Krestenitis, 2022). According to the OCLTT (oxygen- and capacity-limited thermal tolerance hypothesis), once temperature increases beyond an organism's optimum limits, it leads to a mismatch between oxygen supply and demand in the tissues. This mismatch can cause a progressive decline in performance, a compensatory transition from aerobic metabolism to partial anaerobiosis, and energy deficiency (Pörtner and Knust, 2007; Pörtner, 2010; Pörtner et al., 2017). These metabolic shifts might lead to energy reallocation from biomass production (including growth and reproduction) and activity to basal maintenance, which allows the temporary survival of the organism under stressful conditions but negatively affects its fitness (Pörtner et al., 2007; Pörtner, 2012). Extreme (lethal) environmental stress eventually results in an insufficient energy supply from both aerobic and anaerobic pathways, and thus even basal metabolic demand cannot be satisfied, leading to mortality (Pörtner, 2002; 2012; Sokolova et al., 2012; Sokolova, 2013).

Repeated stress exposure could create 'stress memory', allowing an organism to respond faster and more efficiently after re-exposure to similar stress conditions. This can result in phenotypic and stimulus-dependent plasticity of response traits (Ding et al., 2012; Hilker et al., 2016). Hence, such plasticity leads to the adjustment of physiological or developmental phenotype to the environmental stress (Agrawal, 2001; West-Eberhard, 2003). Regarding thermal stress, organisms can increase their thermal tolerance during acclimation to different thermal regimes after a brief exposure to sublethal temperatures (Bilyk et al., 2012). This response (occurring immediately after the initial heat stress) is defined as 'heat hardening' and is a rapid beneficial response that improves heat tolerance, leading to the generation of more resistant phenotypes (Precht et al., 1973; Bowler, 2005). Heat hardening provides a quicker (in comparison to acclimation, which may take days) return to homeostasis, enhanced cell protection and increased survival by engaging cellular responses at transcriptional and post-transcriptional levels, such as elevated antioxidant defense, more efficient electron transfer system (ETS) activity and increased heat shock response (HSR) (Bowler, 2005; Malmendal et al., 2006; Aleng et al., 2015; Georgoulis et al., 2021).

Previous studies on the Mediterranean mussel *Mytilus galloprovincialis* indicated that warming beyond 24°C initiated oxidative stress in conjunction with energy misbalance accompanied by metabolic depression and a shift from aerobic

[1]Laboratory of Animal Physiology, Department of Zoology, School of Biology, Aristotle University of Thessaloniki, GR-54124 Thessaloniki, Greece. [2]Alfred Wegener Institute, Helmholtz-Centre for Polar and Marine Research, Integrative Ecophysiology, Postfach 120161, D-27515 Bremerhaven, Germany. [3]Department of Animal Science, Faculty of Agricultural Sciences, University of Western Macedonia, GR-53100 Florina, Greece. [4]Department of Marine Biology, Institute of Biological Sciences, University of Rostock, D-18055 Rostock, Germany.

*Author for correspondence (michaeli@bio.auth.gr)

I.G., 0000-0002-9012-8061; C.B., 0000-0003-0052-3090; I.M.S., 0000-0002-2068-4302; B.M., 0000-0002-2312-6883

Received 18 July 2022; Accepted 18 November 2022

towards anaerobic metabolism (Feidantsis et al., 2020). In non-heat-hardened mussels, acclimation at temperatures beyond 26°C triggers activation of heat shock proteins (HSPs) and antioxidant enzymes, and eventually leads to increased mortality (Anestis et al., 2007, 2010; Feidantsis et al., 2020, 2021a,b; Georgoulis et al., 2021). In contrast, heat-hardened *M. galloprovincialis* activate antioxidant defense when exposed to temperatures beyond 24°C, enhancing cellular protection and thus increasing survival (Georgoulis et al., 2021). Given the central role of energy homeostasis in stress tolerance (Pörtner, 2002, 2012; Sokolova et al., 2012; Sokolova, 2013), it is likely that differences in the cellular stress response induced by heat hardening are associated with shifts in the cellular energy metabolism of the mussels to support the energy demand of the stress response and minimize the stress-induced energy deficiency. However, the metabolic changes caused by heat hardening and the underlying mechanisms are not yet fully elucidated and require further investigation.

The aim of our present study was to investigate the impact of heat hardening on the metabolomic profile of *M. galloprovincialis* in order to identify adjustments of biochemical pathways that may benefit mussels' thermal tolerance, as previous studies showed that this species is thermally stressed when exposed to 26–27°C, resulting in gradually increased mortality (Anestis et al., 2007; Feidantsis et al., 2020). Although the most extreme heat stress for intertidal species usually occurs during emersion, it should be pointed out that ambient sea surface water temperature recorded in Northern Greece during July–August varies between 27 and 28.5°C (Feidantsis et al., 2018). Thus, our choice of examining heat stress under immersion is ecologically realistic.

Metabolomics is a powerful approach that can provide information about the metabolic responses of an organism to environmental stressors by rapid, unbiased and simultaneous measurements of multiple low molecular weight metabolites (Viant, 2007, 2008). We complemented the metabolomics analysis by measuring the mRNA expression of pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK), which are implicated in the shift from aerobic to anaerobic metabolic pathways in bivalves (Larade and Storey, 2002). Furthermore, we explored the possible involvement of alternative oxidase (AOX) in the mussels' hardening response by measuring transcript levels of AOX under different temperature exposure scenarios. Transcriptional upregulation of AOX is a common response to stress conditions that promote the excess production of reactive oxygen species (ROS) in marine bivalves, including high or low temperatures (Abele et al., 2007; Jacobs and Ballard, 2022). As redox homeostasis plays an important role in heat hardening (Georgoulis et al., 2021), investigation of the transcriptional regulation of AOX upregulation could provide valuable insight into the role of this key enzyme linking metabolic and redox regulation in animal mitochondria (Jacobs and Ballard, 2022) in the heat-hardening response of *M. galloprovincialis*.

MATERIALS AND METHODS

Animals

As described in Georgoulis et al. (2021), *Mytilus galloprovincialis* Lamarck 1819 individuals (mean±s.d.: total mass 25.82±4.62 g, shell length 6.42±0.47 cm, shell width 3.2±0.15 cm) were collected from mussel aquaculture located in Thermaikos Gulf, Greece, in late April 2021, when the ambient seawater temperature was approximately 18°C. Mussels were transferred to the Laboratory of Animal Physiology, Department of Zoology, School of Biology of the Aristotle University of Thessaloniki, and kept in tanks with recirculating aerated natural seawater at 18±0.5°C for 1 week. Salinity was kept at 34±2.85‰ and pH at 8.12±0.05. As explained in detail below (see 'Experimental procedures', 'Acclimation phase'), individuals maintained at 18°C throughout the experimental procedure were used as controls for all experimental conditions. Mussels were fed daily with 0.5% dry weight cultured microalgae *Tisochrysislutea* (CCAP 927/14) per gram total mussel mass.

Experimental procedures

Mussels were exposed sequentially to two different phases (heat-hardening and acclimation phases) as described previously by Georgoulis et al. (2021).

Heat-hardening phase

A group of mussels (referred to below as the heat-hardened group, group H) were exposed to the heat-hardening phase. The experimental design for heat hardening was based on Hutchison's 'repeated–critical thermal maximum' (CTM) method, with minor modifications (Hutchison, 1961). Briefly, ∼300 randomly selected mussels, conditioned as stated above in three aquaria each containing 100 l aerated seawater at 18±0.5°C for 1 week, were exposed to increasing seawater temperature at a rate of 1.5°C min^{-1} and then kept at 27°C for 2.5 h. It has been shown that mussels sense heat stress at 22°C, and cellular stress responses are triggered at 24°C and peak at 30°C (Feidantsis et al., 2020). Therefore, 27°C was chosen as a potent temperature in which physiological responses are induced. Thereafter, water temperature decreased to 18°C (at 1.5°C min^{-1}) and the mussels were left to recover at 18°C for 24 h. This heat-stress bout (including the thermal shock and recovery phase) was repeated 4 times.

Acclimation phase

After the heat-hardening treatments, both non-heat-hardened (referred to below as the control group, group C, for group H) and heat-hardened mussels (group H) were transferred to four 500 l tanks (50–60 individuals from each group per tank, with group C and group H mussels placed in separate baskets within each tank) with recirculating aerated natural seawater at 18°C and left to recover for 4 days. Thereafter, water temperature of the three heat-exposure tanks was increased (1°C h^{-1}) to 24, 26 and 28°C. Mussels in the remaining tank were maintained at 18°C (as this was approximately the ambient seawater temperature at the time of mussel collection) and used as controls for both heat-hardened and non-heat-hardened mussels to evaluate possible temporal effects on the parameters of interest. All exposure conditions were run in triplicate.

Tissue sampling and water quality monitoring

Individuals (n=8 at each time point) from heat-hardened and non-heat-hardened groups were collected from each tank at 12 h (day 0) and 1, 3, 5, 10 and 15 days after the target temperature (24, 26 or 28°C) was reached. At 24°C, both heat-hardened and non-heat-hardened mussels exhibited no mortality, and at 26°C, mortality of non-heat-hardened heat-hardened mussels reached 50% and 40%, respectively, by day 15. However, because mortality of non-heat-hardened mussels was 100% by day 15 at 28°C (in contrast to heat-hardened mussels, which exhibited 45% mortality on day 15) (Georgoulis et al., 2021), samplings on day 15 were not carried out. Thereafter, mussels were dissected, and the mantle was removed, immediately frozen in liquid nitrogen and stored at −80°C for later analysis. Physicochemical water parameters were measured daily including salinity (g l^{-1}), O_2 (mg l^{-1}) and pH (using a Consort C535, Multiparameter Analyser, Consort bvba, Turnhout,

Journal of Experimental Biology

Belgium), and concentrations of NH_3 (µg l^{-1}), NO_2^- (µg l^{-1}) and NO_3^- (µg l^{-1}) using commercial kits (Tetra Werke, Melle, Germany) (Table 1) (Georgoulis et al., 2021).

Tissue extraction

Mantle tissue was extracted using a methanol–chloroform extraction protocol after Rebelein et al. (2018). As previously reported (Feidantsis et al., 2020), mantle exhibits higher aerobic capacity and a more intense physiological stress response compared with posterior adductor muscle (PAM) and thus was chosen for this study. The frozen tissue (about 100 mg) was homogenized in 400 µl ice-cold methanol and 125 µl ice-cold Milli-Q water. Centrifugation (20 s, 2500 ***g***; Kubota Corporation 3520) followed and then 400 µl ice-cold chloroform and 400 µl ice-cold Milli-Q water were added, and samples were vortexed for 15 s and incubated on ice for 10 min. Methanol–chloroform was added to the samples and after centrifugation (10 min, 3000 ***g***, 4°C; Kubota Corporation 3520) three phases resulted: the upper methanol layer with the polar metabolites, the lower chloroform layer with the lipids and a thin layer of proteins in the middle. The upper layer was transferred to a new tube and was dried in a vacuum centrifuge at room temperature overnight. The lower layer was dried in a fume hood at room temperature. The resulting water-soluble fractions of the samples containing the metabolites of cytosol were sent to the AWI in Bremerhaven, where they were dried using a rotational vacuum concentrator (RVC 2-18 HCl, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at room temperature for metabolomic studies.

Metabolomic profiling based on ^{1}H-NMR spectroscopy

Untargeted metabolite profiling based on NMR spectroscopy was performed similar to Götze et al. (2020). The NMR spectroscopy measurements were all performed on a vertical 9.4 T wide bore magnet operating at 400.13 MHz with Avance III HD electronics (Bruker GmbH, Bremen, Germany). For this, the dried tissue extracts were dissolved in deuterated water (D_2O) containing 0.05% trimethylsilyl propionate (TSP, Sigma-Aldrich, St Louis, MO, USA) as standard with a 1:1 ratio and transferred into 1.7 mm NMR tubes. The samples were all placed in a 1.7 mm diameter triple-tuned (^{1}H–^{13}C–^{15}N) probe and measured using a classical Call–Purcell–Meiboom–Gill (Bruker protocol cpmgpr1d) sequence with water suppression at room temperature using the following measurement parameters: acquisition time (AQ) 4.01 s, sweep width (SW) 8802 Hz (22 ppm), delay (D1) 4 s, dummy scan (DS) 4, number of scans (ns) 256.

Metabolite profiles and concentrations were determined using Chenomx NMR suite 8.1 (Chenomx Inc., Edmonton, AB, Canada). For this purpose, the ^{1}H-NMR spectra were phase and baseline corrected and set to the chemical shift of the TSP standard (0.0 ppm). The TSP signal served as line width and concentration standard (2.3 mmol l^{-1}). Metabolites were identified using their corresponding NMR signals from the Chenomx database and profiled to the NMR spectrum of each individual spectrum for quantification. For alanopine and strombine, additional ^{1}H-NMR measurements were performed on the pure substance (Akos GmbH, Stuttgart, Germany) and the respective spectra were included in the Chenomx database for identification and quantification. ATP and ADP signals were combined and are presented as cumulative concentrations, as a unidirectional assignment from the ^{1}H-NMR spectra was not possible.

Gene expression

RNA was extracted from 50 mg of mantle tissue using NucleoZOL reagent (Macherey-Nagel, Düren, Germany) following the manufacturer's guidelines. Concentration of the total RNA was estimated in a Q5000 spectrophotometer (Quawell Technology Inc., San Jose, CA, USA) and approximately 50 ng RNA was utilized for cDNA synthesis using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan). Quantitative real-time PCR (qPCR) for PEPCK, PK and AOX cDNA was carried out in an Eco 48 (PCRmax, Cole-Parmer North America, Vernon Hills, IL, USA) qPCR cycler, in 10 µl volume reactions using a KAPA SYBR FAST qPCR Master Mix (2X) kit (KAPA Biosystems, Wilmington, MA, USA) and primers based on conserved sequences from *Mytilus*, *Crassostrea* and *Ruditapes* (Table 2). Confirmation of the amplified product lengths was performed by 1.5% agarose gel electrophoresis. The qPCR regime was as follows: 95°C for 2 min, followed by 40 cycles of 95°C, 60°C and 72°C for 5, 10 and 20 s, respectively. Relative quantification was carried out using the actin gene for reference, as described in our previous study (Georgoulis et al., 2021).

Statistics

The treatment effects and parameters, i.e. hardening, acclimation temperature and time, were considered as independent variables and statistically compared by applying a general linear model (GLM) ANOVA and a one-way ANOVA that were performed to detect significant differences at 5% (P<0.05) probability level (SPSS Scientific Inc. software, version 21). Values are presented as means±s.d.

Differences in metabolite profiles and concentrations were analyzed using MetaboAnalyst 5.0 (Chong et al., 2019). Metabolite concentrations were transformed and scaled to achieve normalization. Normalized concentrations were tested for differences using univariate (ANOVA) and multi-variate statistics [principal component analysis (PCA) and partial least square with discriminant analysis (PLS-DA)] within MetaboAnalyst. The models were tested for overfitting using cross-validation and permutation analysis and SAM (significant analysis of metabolomics based on F-statistics; the false discovery rate was set to a delta of 0.2). Heatmaps and dendrograms were generated to better visualize shifts and changes in metabolite groups.

RESULTS

No time-dependent changes were found in control (18°C) mussels in any of the measured parameters (metabolites, gene expression) in

Table 1. Mean values of seawater parameters

	Temperature (°C)	Salinity (g l^{-1})	pH	O_2 (mg l^{-1})	NH_3 (mg l^{-1})	NO_2^- (mg l^{-1})	NO_3^- (µg l^{-1})
18°C	18±0.1	33.2±0.03	8.04±0.02	7.9±0.06	0.2±0.01	0.2±0.01	<12.5
24°C	24±0.3	33.4±0.03	8.03±0.02	7.8±0.07	0.2±0.01	0.3±0.02	14±0.6
26°C	26±0.4	33.3±0.01	8.02±0.01	7.8±0.05	0.2±0.02	0.3±0.01	14±0.3
28°C	28±0.3	33.2±0.04	8.02±0.01	7.8±0.07	0.3±0.02	0.2±0.02	13±0.5

Seawater parameters as measured in all experimental conditions. Values are presented as means±s.d. (n=10).

Table 2. Primers used for amplification of the target genes

Primer name	Primer sequence (5′–3′)	Amplified product length (bp)
AOXf	GGAGAAAGAACGGAGAAGAAATG	299
AOXr	CTTTCGTTTTCTGCTTCCTCTA	
PKf	GACATGRTTTTYGCSTCCTTCA	231
PKr	TCATCATCTTCTGKGCVAGGAA	
PEPCKf	AGATCATGCAYGACCCAATGG	128
PEPCKr	CGGAACCAGTTCACATGGAA	

mantle tissue of *M. galloprovincialis*. The thermal response to time-dependent exposure to elevated temperature (24, 26 and 28°C) varied significantly between non-heat-hardened (group C) and heat-hardened (group H) mussels.

Metabolites

In total, 46 (plus two unassigned) metabolites were assigned in mantle tissue of *M. galloprovincialis* independent of groups. The levels of aspartate (a substrate for anaerobic fermentation) and those of the metabolic intermediates and end products of this fermentation (alanine, fumarate and succinate) were increased when group C mussels were exposed to either of the three elevated temperatures compared with 18°C (Fig. 1). Compared with the group C mussels, the group H mussels exhibited a quicker and higher increase in aspartate concentration, throughout the 15 days of exposure regardless of temperature (Figs 1A and 2). Alanine levels peaked in the first (1–5) days of exposure in both group C and group H mussels (Fig. 1B) and decreased after 10–15 days of exposure in the group C mussels at 24 and 28°C (Figs 1B and 2). However, in the group H mussels, alanine continued to increase throughout the exposure, reaching significantly higher levels compared with the non-hardened mussels (specifically on day 15 at 24°C and the day 10 at 28°C). Regarding fumarate and succinate, exposure to elevated temperature led to their strong accumulation in group C mussels throughout the exposure (except for a decrease back to the baseline after 15 days at 26°C) (Figs 1C,D and 2). In contrast, succinate and fumarate concentrations were lower in the group H mussels relative to their group C counterparts, and were generally similar to or lower than the baseline levels found in mussels kept at the control temperature of 18°C (Fig. 1C,D).

Elevated temperatures (24, 26 and 28°C) differentially modulated the concentrations of alanopine, lactate, strombine and taurine in the group H and group C mussels (Fig. 3). Specifically, at 24°C, alanopine concentration showed an initial decrease below baseline levels (obtained at 18°C) and returned to the baseline after 15 days of exposure (Fig. 3A). However, in group H mussels exposed to 24°C, alanopine levels increased after 10–15 days of exposure (Figs 3A and 2). Similarly, in the group H mussels at 26 and 28°C, alanopine concentration peaked after 1–5 days of exposure, reaching considerably higher levels compared with those of their group C counterparts. Alanopine levels subsequently decreased in both group C and group H, reaching levels similar to or lower than the baseline after 10–15 days (Fig. 3A).

Lactate levels were found to be higher in the group H mussels compared with the group C ones at all experimental temperatures. Specifically, while lactate levels of group C mussels showed an increase until day 5 of exposure at 24°C, and thereafter a decrease until day 15, group H individuals exhibited a steady increase until day 15 of exposure (Fig. 3B). Similarly, at 26 and 28°C, group H

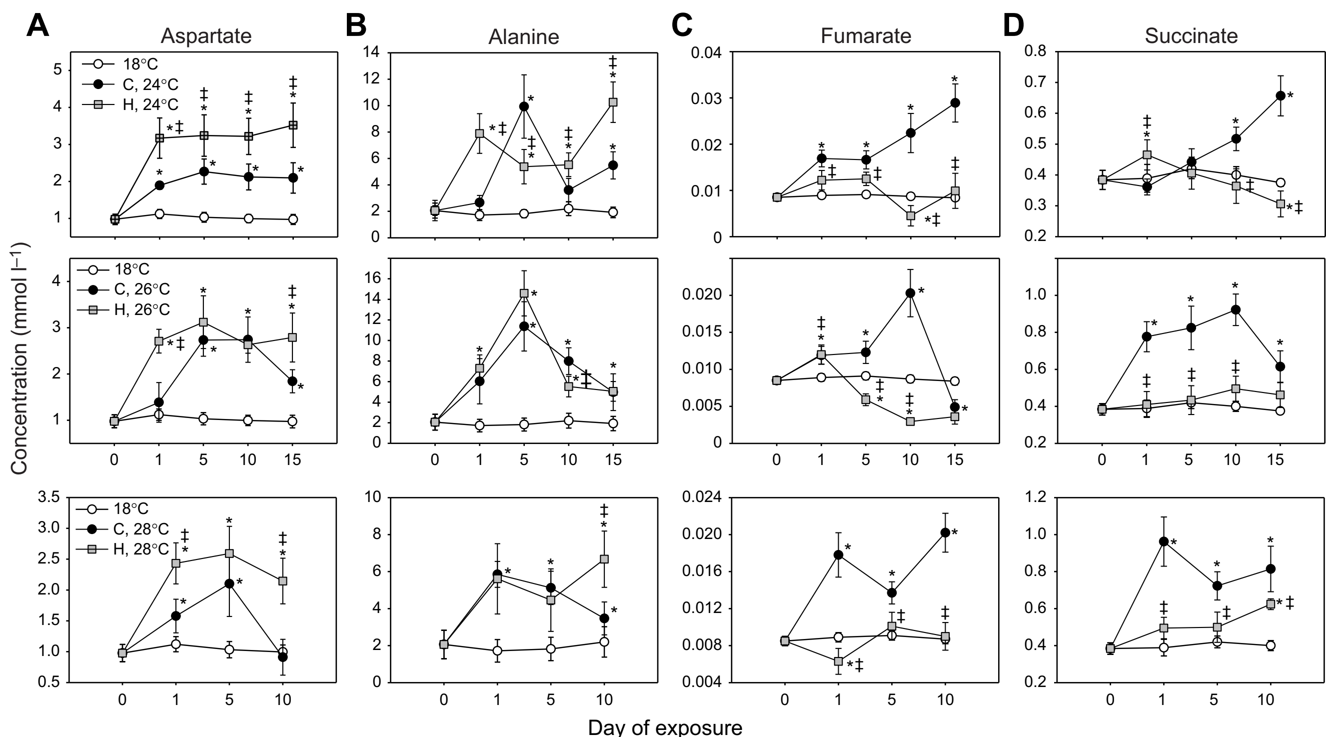

Fig. 1. Changes in aspartate, alanine, fumarate and succinate levels in mantel from heat-hardened and non-heat-hardened *Mytilus galloprovincialis* exposed to different temperatures. (A) Aspartate, (B) alanine, (C) fumarate and (D) succinate levels in the mantle of heat-hardened (group H) and non-heat-hardened (group C) mussels when exposed to 24°C (top row), 26°C (middle row) and 28°C (bottom row) compared with the control at 18°C, sampled at 12 h (day 0) and 1, 5, 10 and 15 days, as indicated. Values are means±s.d., *n*=8 preparations from different animals. *P<0.05 compared with 18°C-acclimated (control) mussels, ‡P<0.05 compared with non-heat-hardened (group C) mussels.

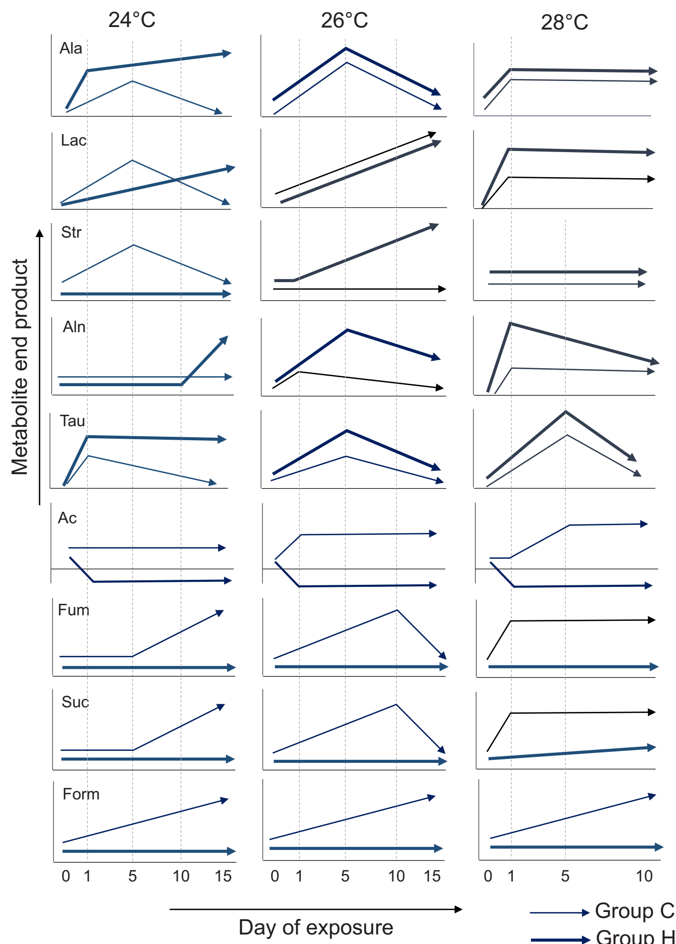

Fig. 2. Trends of metabolite accumulation in non-heat-hardened and heat-hardened mussels exposed to different temperatures.

mussels showed higher lactate levels compared with group C individuals (Figs 3B and 2). In contrast to the above, strombine concentration in both group C and group H mussels exposed to 24 and 28°C remained similar to that of 18°C. However, at 26°C, both groups displayed a slight increase after day 5 of exposure, with group H mussels exhibiting higher levels compared with group C mussels (Figs 3C and 2). At all elevated temperatures, taurine concentration increased on day 1 of exposure, with a peak until day 5 and then decreased until day 15 in group C and group H mussels. Nevertheless, group H mussels showed slightly higher levels compared with group C mussels (Figs 3D and 2).

Acetate and formate concentrations were higher in the group C mussels than in their group H counterparts in all tested elevated temperatures (24, 26 and 28°C) (Fig. 4). Interestingly, acetate and formate levels in the group H mussels were below the 18°C levels throughout the exposure, whereas in the group C mussels, acetate and formate levels were elevated above the 18°C baseline (except for a transient decline of acetate during the first 5 days at 24°C) (Figs 4 and 2).

The summed concentration of high-energy phosphates (ATP+ADP) decreased in both group C and group H when mussels were exposed at all elevated temperatures. However, a stronger decrease in group C compared with group H was observed throughout the experimental exposure (Fig. 5A). Inosine concentrations were elevated in group H mussels exposed to the elevated temperatures compared those in group C, which remained similar to those in the 18°C-acclimated mussels (Fig. 5B). Hypoxanthine levels in the mantle tissue of group C *M. galloprovincialis* were significantly affected by exposure to elevated temperatures. However, only on day 5 of exposure was the most significant change observed – levels peaked at 24°C and decreased at 26 and 28°C. In contrast, hypoxanthine levels in the group H mussels exposed to all elevated temperatures decreased over time, with levels lower than in those exposed to 18°C (Fig. 5C).

Gene expression

Heat-hardened mussels exposed to 24 and 26°C exhibited an increased expression of all three studied genes (Fig. 6). The mRNA levels of *PEPCK* peaked early (12 h) during the exposure and declined afterwards (Fig. 6B), whereas *PK* transcript levels remained elevated until day 3 (24°C) and day 5 (26°C) (Fig. 6A). In contrast, in the group C mussels exposed to 24 and 26°C, *PK* transcript levels remained low and mostly below the 18°C baseline, and a transient modest increase in *PEPCK* transcript levels was exhibited at 24°C (days 3–5) and 26°C (day 1) (Fig. 6A,B). Regarding exposure at 28°C, the transcript levels of both *PK* and *PEPCK* were similar to or below the 18°C baseline in the group C mussels (Fig. 6A,B). However, *PK* transcript levels were initially (12–24 h) elevated in the group H mussels, returning to baseline later during the exposure (Fig. 6A). Moreover, *PEPCK* mRNA levels showed an irregular pattern with modest increases after days 1 and 10 in group H (Fig. 6B).

Transcript levels of *AOX* were noticeably elevated above the respective 18°C baseline in the group H mussels at all studied elevated temperatures (except for day 10 at 28°C), whereas in their group C counterparts, no induction (or even suppression) of the *AOX* transcripts was observed (Fig. 6C).

Multivariate analyses of metabolite profiles

The heatmaps in Fig. 7 (top) show the differences in the metabolome shifts between group C and group H mussels as well as between the different exposure temperatures. At 24°C, three groups of metabolites, showing correlated patterns of change, were identified based on the dendrograms. The first group included metabolites which increased toward the end of exposure in the group C mussels (acetate, formate, fumarate, succinate and hypoxanthine). The second group included metabolites more abundant during the mid- to late-exposure period in the hardened mussels (adenylates, taurine, aspartate and inosine) and the third group included those peaking early in the exposure in the group C mussels and later in the group H ones (alanine, alanopine, strombine and lactate) (Fig. 7A). At 26°C, acetate, formate, fumarate, succinate and hypoxanthine showed correlated patterns with higher enrichment in group C, similar to what was found at 24°C (Fig. 7B). Other studied metabolites peaked during early (adenylates, taurine, aspartate, inosine, alanine and alanopine) or late (lactate and strombine) exposure at 26°C in the group H mussels and remained generally low in the group C ones (Fig. 7B). At 28°C, the comparison between differently treated mussels revealed a group of metabolites (mainly inosine, adenylates, lactate, alanine, aspartate and strombine) that were relatively more enriched in the group H mussels, and a second group (mainly hypoxanthine, taurine, fumarate, succinate, alanopine, acetate and fumarate) with higher levels in the group C mussels (Fig. 7C).

A PCA analysis was applied to statistically define differences in metabolite responses studied herein. According to the results for different acclimation temperatures (24, 26 and 28°C), it seems that

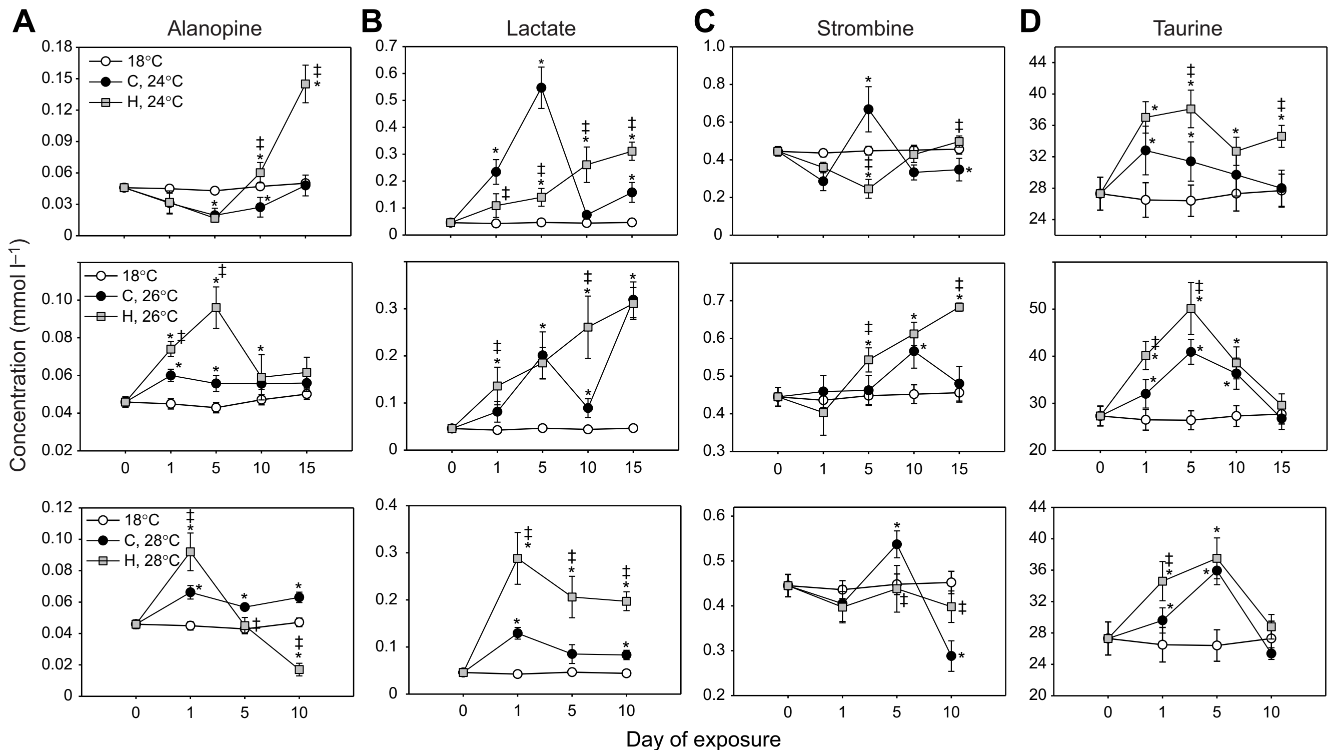

Fig. 3. Changes in alanopine, lactate, strombine and taurine levels in mantle of H- and C-group mussels exposed to different temperatures. (A) Alanopine, (B) lactate, (C) strombine and (D) taurine levels following exposure to 24°C (top row), 26°C (middle row) and 28°C (bottom row) compared with the control at 18°C. Values are means±s.d., n=8 preparations from different animals. *P<0.05 compared with 18°C-acclimated (control) mussels, ‡P<0.05 compared with group C mussels.

in most of the cases, group H individuals were diverted in different clusters from both control and group C individuals (Fig. 7, bottom). Specifically, at 24°C, PC1 explained 32.2% of the variance, while PC2 explained 14.9% (Fig. 7A). At 26°C, PC1 explained 30.1% of the variance, while PC2 explained 14.7% (Fig. 7B). Lastly, at 28°C, PC1 explained 25.8% of the variance, while PC2 explained 13.7%.

Interactions between variables

Table 3 exhibits the overall effect of all variables and their interactions. All variables (hardening, acclimation temperature and time) and their interactions (hardening×acclimation temperature, and hardening×time) were statistically significant.

DISCUSSION

Temperature-dependent metabolic shifts and the effects of heat hardening

According to the OCLTT hypothesis, the range of the optimal thermal window is determined by the organism's ability to sustain oxygen needs for aerobic metabolism so that activation of anaerobic metabolism resulting from respiratory limitations may indicate the limits of the thermal window for sustainable metabolic performance of an organism (Pörtner, 2010, 2021; Pörtner et al., 2017). During the first stage of anaerobiosis in mussels, aspartate and alanine are particularly important, with alanine being an early biomarker of anaerobosis produced in the cytosol from aspartate and potentially protecting the cell from hypoxic damage (Eymann et al., 2020; Chen et al., 2021). Other end products of early anaerobiosis in marine bivalves include opines (such as alanopine and strombine) and lactate (Sato et al., 1993). Opines are typically produced during functional anaerobiosis characterized by high ATP turnover (such as during intensive muscle contraction), whereas lactate production is generally slow and less important in marine mollusks than alternative anaerobic pathways (Sato et al., 1993). At a later stage, the anaerobic metabolism transitions to succinate and acetate production in the mitochondria, which provides higher ATP yield per unit substrate compared with the alanine-, opine- and lactate-generating pathways (Hochachka and Mustafa, 1972; Bayne, 2017). This metabolic flexibility allows facultative anaerobes such as mussels to fine-tune the cellular ATP production depending on the tissue's energy demand and oxygen supply.

Our present study shows that the pattern of metabolic changes in the non-heat-hardened mussels exposed to 24°C is consistent with the activation of cytosolic anaerobiosis (with accumulation of alanine, lactate and strombine) during the first 5 days of exposure, and transition to succinate- and acetate-producing anaerobiosis at a later (15 days) stage. The observed increase in fumarate (a precursor of anaerobic succinate production), succinate and acetate concentrations indicates insufficient oxygen supply to mitochondria and the onset of anaerobic metabolism in these thermally stressed mussels (Zurburg and Zwaan, 1981; Zandee et al., 1985; Haider et al., 2020). This metabolic response is in line with the oxygen consumption in *M. galloprovincialis*, which also follows two phases during exposure to 24°C, initially increasing by day 5 and then decreasing by 10% until day 30 of exposure (Isani et al., 1995). Taken together, our present data and earlier published research (Isani et al., 1995) indicate suppression of aerobic metabolism and increasing reliance on anaerobiosis during prolonged exposure to 24°C in naive (non-hardened) mussels. This hypothesis is supported by our findings of transcriptional changes in non-hardened mussels exposed to 24°C with an upregulation of PEPCK (which channels glycolytic product towards anaerobic succinate production) after 3–5 days and

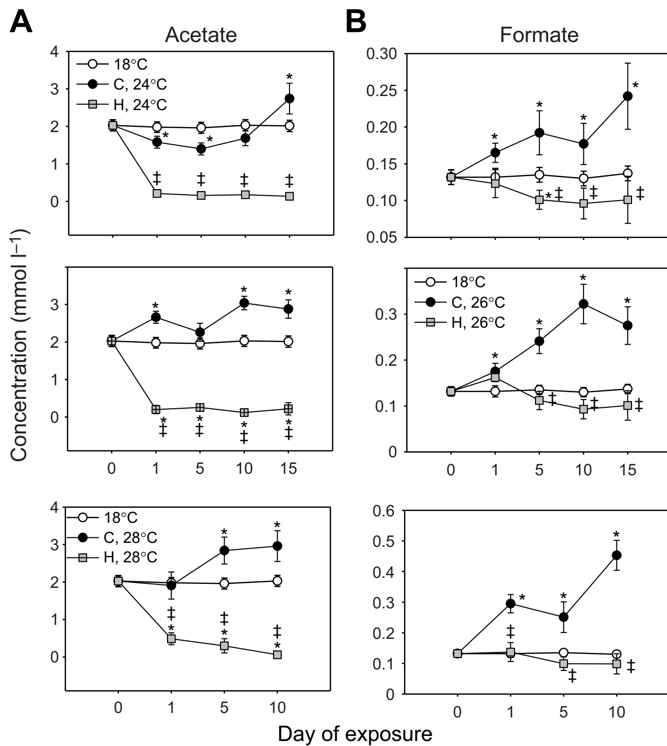

Fig. 4. Changes in acetate and formate levels in the mantle of H- and C-group mussels exposed to different temperatures. (A) Acetate and (B) formate levels following exposure to 24°C (top row), 26°C (middle row) and 28°C (bottom row) compared with the control at 18°C. Values are means±s.d., *n*=8 preparations from different animals. **P*<0.05 compared with 18°C-acclimated (control) mussels, ‡*P*<0.05 compared with group C mussels.

suppression of the mRNA levels of PK (an aerobic marker enzyme) during prolonged exposure (Anestis et al., 2007, 2010). These transcriptional alterations might result in a lower ratio of PK/PEPCK activities and a shift to succinate accumulation in *M. galloprovincialis* during prolonged exposure to 24°C (Anestis et al., 2007, 2010). Similarly, Le Moullac et al. (2007) showed a switch at the PEP branchpoint (on day 10) from the aerobic to the anaerobic pathway of ATP production in the muscle of *Crassostrea gigas*, leading to the induction of PEPCK activity and succinate accumulation during prolonged hypoxia.

In the heat-hardened mussels, the transition to anaerobiosis at 24°C was delayed relative to that in the naive (non-heat-hardened) mussels. Thus, heat-hardened *M. galloprovincialis* showed evidence of cytosolic anaerobiosis (indicated by accumulation of alanine and lactate) only after prolonged (15 days) exposure, and no evidence of fumarate, succinate or acetate accumulation. Both PK and PEPCK were transcriptionally upregulated during the early period (12 h to 3 days), indicating overall upregulation of aerobic and anaerobic metabolic pathways in this group when exposed to 24°C. This metabolic response is in accordance with the findings of Dunphy et al. (2018), who found no increase of succinate in heat-hardened mussel *Perna canaliculus*. They suggested that the delayed upregulation of succinate production in heat-hardened mussels might be due to more robust aerobic pathways and/or the delayed switch to anaerobiosis (Dunphy et al., 2018).

At 26°C, the metabolic pattern showed evidence of an earlier onset of anaerobic metabolism in the naive (non-heat-hardened) mussels compared with those kept at 24°C. Thus, tissue levels of fumarate, succinate and acetate increased after 1 day of exposure at 26°C and continued increasing until day 10. This was accompanied by an earlier increase in PEPCK transcript at day 1 compared with controls at 26°C. After prolonged (15 days) exposure, tissue levels

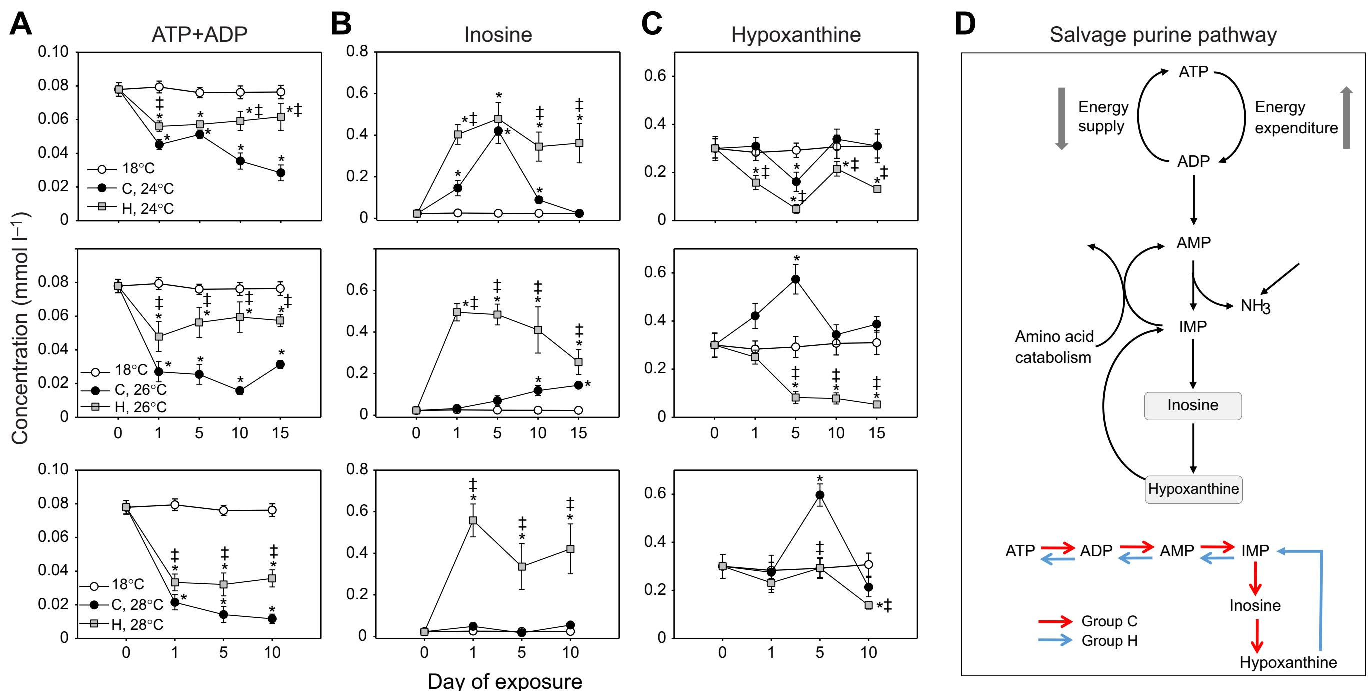

Fig. 5. Changes in ATP+ADP, inosine and hypoxanthine levels in the mantle of H- and C-group mussels exposed to different temperatures. (A) ATP+ADP, (B) inosine and (C) hypoxanthine levels following exposure to 24°C (top row), 26°C (middle row) and 28°C (bottom row) compared with the control at 18°C. Values are means±s.d., *n*=8 preparations from different animals. **P*<0.05 compared with 18°C-acclimated (control) mussels, ‡*P*<0.05 compared with group C mussels. (D) The salvage purine pathway responsible for ATP and hypoxanthine interplay in group H and group C mussels. IMP, inosine monophosphate.

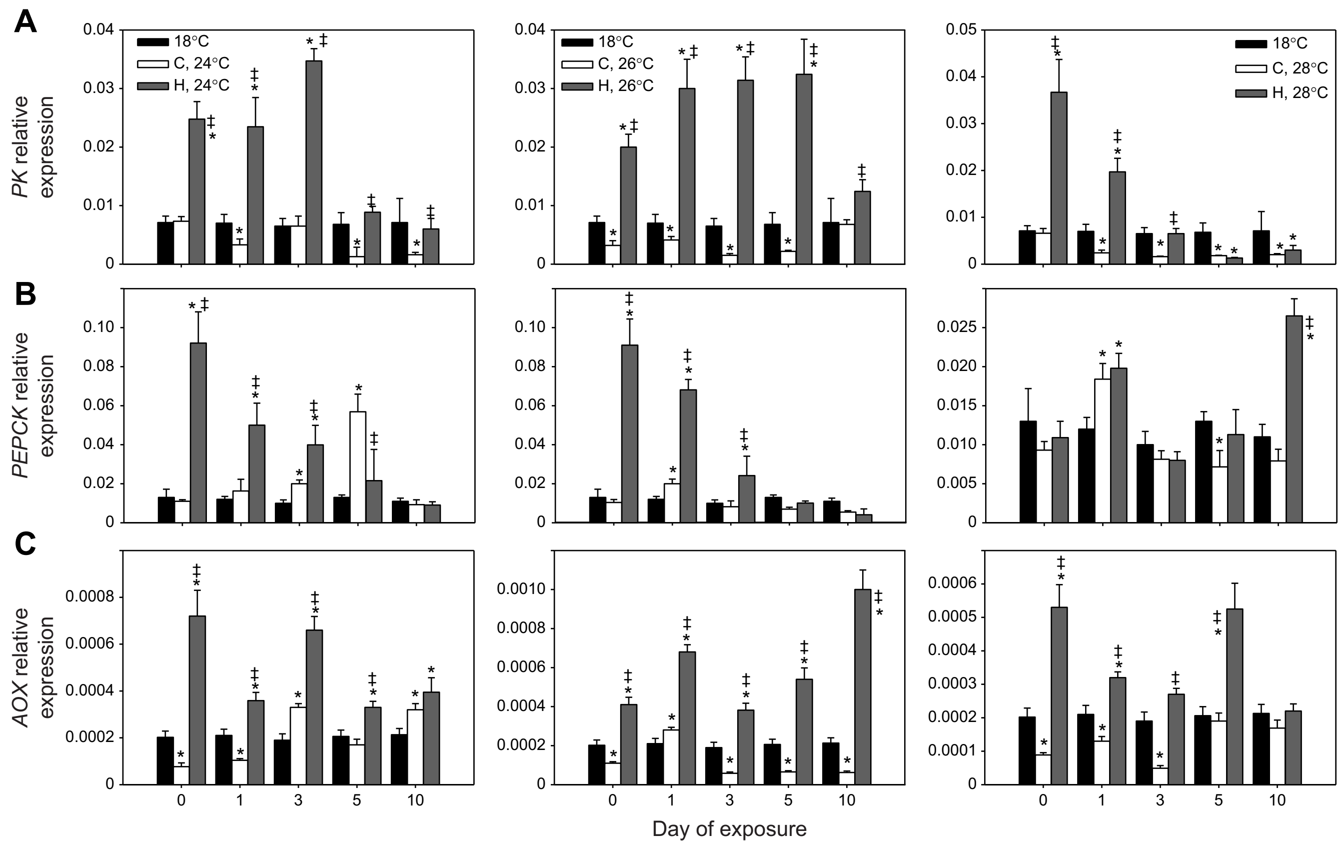

Fig. 6. Changes in *PK*, *PEPCK* and *AOX* mRNA levels in the mantle of H- and C-group mussels exposed to different temperatures. (A) *PK*, (B) *PEPCK* and (C) *AOX* relative gene expression following exposure to 24°C (left), 26°C (middle) and 28°C (right) compared with the control at 18°C, sampled at 12 h (day 0) and 1, 3, 5 and 10 days. Values are means±s.d., *n*=8 preparations from different animals. **P*<0.05 compared with 18°C-acclimated (control) mussels, ‡*P*<0.05 compared with group C mussels.

of fumarate and succinate (but not acetate) declined, possibly indicating mitochondrial damage. Interestingly, the end products of fast glycolytic pathways showed either transient (i.e. opines) or continuous (i.e. lactate) increase throughout the exposure to 26°C in the non-hardened mussels. This indicates activation of opine and lactate dehydrogenases, underlining a more active metabolic rate. The apparently elevated ATP turnover is in line with the initiation of HSP expression, a highly energy-demanding process, which was observed in the mantle of *M. galloprovincialis* at 26°C (Anestis et al., 2007). In the heat-hardened mussels exposed to 26°C, the transition to mitochondrial anaerobiosis was delayed relative to that in naive mussels, similar to the pattern found at 24°C. Thus, this group showed no evidence of deviation of the metabolic flux towards succinate or acetate production during prolonged exposure to 26°C, despite an early transient increase in PEPCK mRNA levels (12 h to 3 days). Elevation of alanine, strombine and lactate levels indicated higher metabolic demand during the early stages of 26°C exposure that was later downregulated and/or compensated for by aerobic metabolism. This notion is supported by the finding of consistently elevated PK mRNA expression in the heat-hardened mussels throughout the exposure to 26°C.

Similar to 26°C, exposure to 28°C led to a sequential activation of cytosolic and later mitochondrial anaerobiosis, which was maintained in the naive mussels throughout the thermal stress exposure, and mitigation of this anaerobiosis activation in the heat-hardened mussels. This shows that 28°C does not appear to result in a metabolic collapse at the cellular level in *M. galloprovincialis*, even though our earlier study revealed severe energy deficiency and a negative scope for growth (SFG) in *M. galloprovincialis* after 30 days at 28°C (Anestis et al., 2010). This discrepancy might be explained by the decreased feeding rate and assimilation efficiency of mussels as that can suppress energy intake and contribute to mortality of around 50% after 30 days at 28°C (Anestis et al., 2007, 2010). Notably, expression of PK mRNA increased during the first days of exposure at 28°C in the heat-hardened mussels, but unlike 24 or 26°C, this increase could not be sustained at 28°C, indicating limits for aerobic metabolic compensation at this extreme temperature.

Interestingly, despite significant accumulation of alanine during the early period of thermal stress exposure, there was no depletion of aspartate in the naive or heat-hardened mussels at any of the studied elevated temperatures. In fact, tissue levels of aspartate increased in the thermally stressed mussels, particularly in those that were heat hardened prior to the elevated temperature exposures. In marine mollusks including mussels and oysters, aspartate is commonly used as an energy fuel during environmental stress, resulting in alanine accumulation (Haider et al., 2020; Hu et al., 2022). Therefore, elevated levels of aspartate stored in the mantle tissue of the heat-hardened mussels might contribute to their improved performance during thermal stress as this amino acid serves as an important substrate for anaerobic ATP production through succinate pathway as well as via gluconeogenesis.

An intriguing and unexpected observation of the present study was the accumulation of formate during thermal stress in the non-hardened mussels. The physiological role of formate and the metabolic pathways that it is involved in are unknown in marine

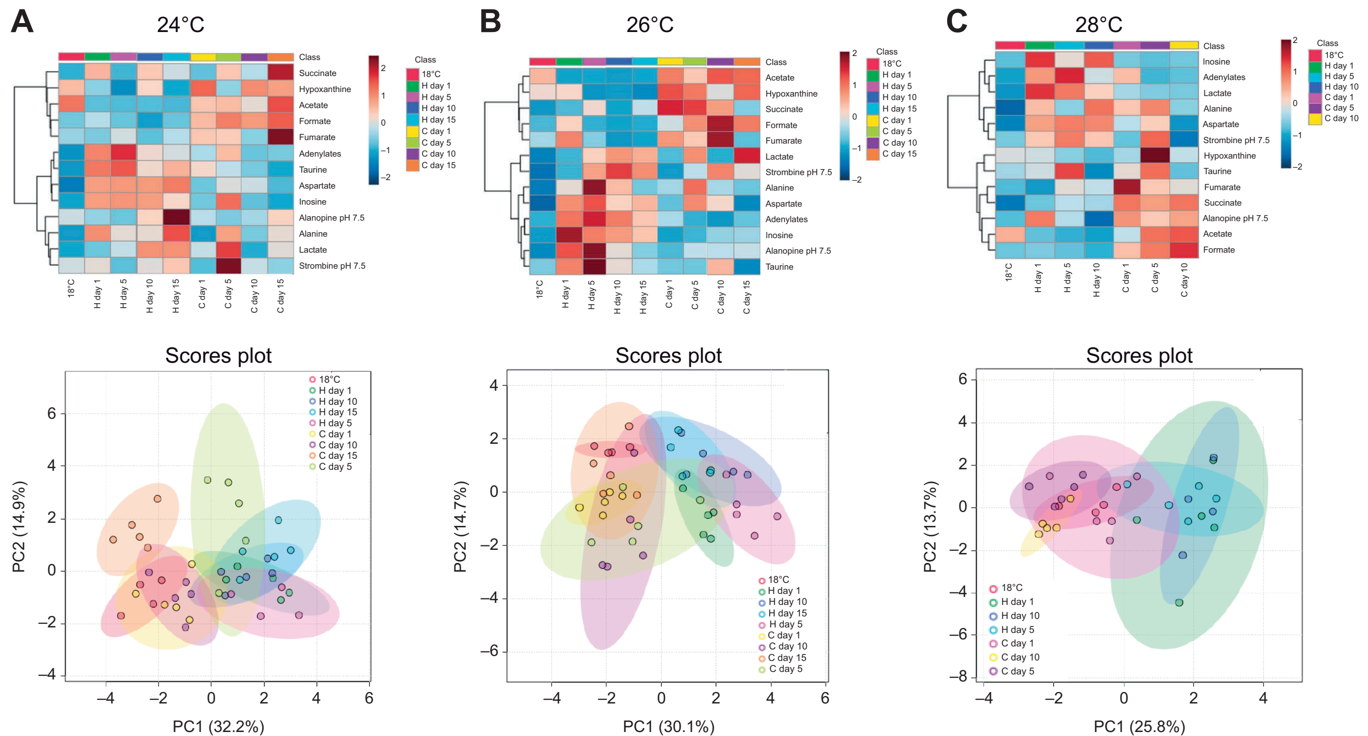

Fig. 7. Summary of the change in metabolites in the mantle of H- and C-group mussels exposed to different temperatures. Top: heatmaps depicting the fold-change of metabolites following exposure to 24°C (A), 26°C (B) and 28°C (C). Bottom: dendograms indicating the correlation between metabolites with each of the first two principal components (PCs) in the multivariate analysis: 18°C day 1; group H at 24, 26 or 28°C on days 1, 5, 10 and 15; group C at 24, 26 or 28°C on days 1, 5, 10 and 15, as indicated.

mollusks and, to the best of our knowledge, no published research reports stress-induced formate accumulation in mollusks. In mammals, formate is a precursor for the *de novo* synthesis of purine and deoxythymidine nucleotides and is therefore indirectly involved in energy metabolism by promoting the synthesis of adenine nucleotides (Oizel et al., 2020). Moreover, formate is involved in the regulation of the host–microbiome interactions in mammals as a byproduct of anaerobic fermentation of bacteria populating the gut microbiome (Pietzke et al., 2020). Formate participates in the synthesis of NADPH and contributes to the reduction of fumarate to succinate, biological reactions that ensure the survival of the microbe in an oxidative milieu (Appanna et al., 2014; Thomas et al., 2016). Tomanek and Zuzow (2010) have shown that metabolic patterns during thermal stress are involved in the putative switch from NADH- to NADPH-producing pathways in mussels. The present metabolomic analysis showed that the pattern of formate and acetate changes during the exposure of mussels at the elevated temperatures coincides well with that of fumarate and succinate. It is not known whether formate accumulation provides an antioxidant role in mussels during hypoxia and thermal stress. We hypothesize that such a mechanism might have been evolutionarily

Table 3. Results of general linear model ANOVA analyses

Variable	d.f.	Type III SS	F	P
Hardening	2	0.146	6.789	0.004*
Acclimation temperature	2	0.174	6.673	0.009*
Time	2	0.456	3.675	0.035*
Hardening×Acclimation temperature	4	0.178	5.782	0.014*
Hardening×Time	4	0.286	4.889	0.022*

*Statistically significant effect.

conserved in facultative anaerobes such as mussels. However, the importance of formate as a substitute for NADH in environments where the tricarboxylic acid (TCA) cycle and oxidative phosphorylation are ineffective needs further investigation.

Interestingly, thermal stress also led to the accumulation of taurine, particularly in the heat-hardened mussels. In the naive (non-hardened) mussels, taurine accumulation was transient at all studied temperatures, returning to baseline after 10–15 days of exposure. In the heat-hardened mussels, higher levels of taurine were accumulated and this increase was sustained throughout the 15 day exposure at 24°C, but transient at 26 and 28°C. Taurine is an important metabolite supporting mitochondrial function and minimizing ROS production in eukaryotic organisms from yeast to mammals (Jong et al., 2021). It stabilizes proteins and phospholipid membranes (Huxtable, 1992; Williams and Somero, 1996) and the mitochondrial electron transport chain, acting like an antioxidant (Jong et al., 2012). In marine mollusks, taurine enhances oxidative phosphorylation in the mitochondria and improves mitochondrial coupling efficiency (Sokolov and Sokolova, 2019). Furthermore, the ability to maintain high levels of taurine was associated with improved hypoxia survival in marine mollusks, whereas depletion of taurine correlated with higher sensitivity to hypoxia (Haider et al., 2020). Taurine also accumulates in response to thermal stress and serves as an endogenous thermoprotectant in mussels (Gleason et al., 2017). Thus, taurine enrichment in the heat-hardened mussels might support proteome and membrane integrity and enhance aerobic metabolism, thereby contributing to the thermotolerant phenotype of this group.

High-energy phosphates and purine metabolism

Thermal stress led to the depletion of high-energy phosphates (ATP and ADP) in the mussels, with a stronger decrease found with

Glycogen
PEP
Strombine
Alanopine
Tauropine
Taurine
Pyruvate
Lactate
Aspartate
α-Ketoglutarate
Glutamate
Alanine
PFL ?
Formate
Oxaloacetate
Acetyl-CoA
ADP
ATP
Malate
Acetate
Fumarate
Formate
?
O_2
H_2O
Respiratory chain-ETC
CO_2
Succinate

Succinate pathway
Towards Krebs cycle and mitochondrial metabolism
Opine DH and end products
Limited oxygen (hypoxia) (?)

Fig. 8. Summary of the metabolic response in mantle tissue of non-heat-hardened and heat-hardened *Mytilus galloprovincialis* exposed to thermal stress. DH, dehydrogenase; PFL, pyruvate formate-lyase.

increasing exposure temperatures. This indicates energy deficiency at the cellular level and is consistent with the notion of the mismatch between energy demand and supply in thermally stressed mussels. In the heat-hardened mussels, the tissue levels of high-energy phosphates were partially protected during the thermal stress, and the depletion was consistently less than in their naive counterparts, particularly at the less extreme temperatures (24 and 26°C). This finding indicates that the metabolic shifts caused by heat hardening ensure the more efficient maintenance of the cellular energy balance in the mussels.

A potential mechanism contributing to the ATP replenishment under the thermally induced hypoxia in the mussels might involve the purine salvage pathway. The depletion of the adenylated pool during thermally induced hypoxemia results in an accumulation of inosine and hypoxanthine (DeWall et al., 1971). Further oxidation of hypoxanthine by xanthine oxidase can cause oxidative damage as a result of the generation of superoxide (Quinlan et al., 1997; Gutteridge and Halliwell, 2000; Rodrigues et al., 2014). Our results showed increased levels of inosine but no accumulation of hypoxanthine in the non-hardened mussels during the first days of exposure to 24°C. At 26 and 28°C, this pattern was reversed with little inosine accumulation and high levels of hypoxanthine, indicating a higher flux towards hypoxanthine production. In contrast, in the heat-hardened mussels, inosine levels increased at all elevated temperatures, while the levels of hypoxanthine were suppressed below the baseline. This might indicate inhibition of the purine breakdown pathway in the heat-hardened mussels, which may be beneficial for heat tolerance and hypoxia survival. Haider et al. (2020) also reported an increase in the levels of all the purine metabolism intermediates, including AMP, adenosine and inosine during prolonged hypoxia in mussels. Adenosine and inosine have protective roles in the cells (Ralevic, and Burnstock, 1998), can provide an alternative source of energy under glucose deficiency, and delay accumulation of $NADH^+$ and H^+, preventing inhibition of glycolysis and the Krebs cycle (Haskó et al., 2004). Furthermore, hypoxic rabbit kidney cells treated with adenosine or inosine exhibited elevated cellular ATP levels (Weinberg and Humes, 1986; Takeo et al., 1988; Haun et al., 1996; Mandel et al., 1998), most likely as a result of ATP generation via the purine salvage pathway (Szokodi, 2005; Frenguelli, 2019). Our findings suggest that a similar pathway operates in the heat-hardened mussels (Fig. 5D) but its activity and contribution to the ATP balance requires further investigation.

AOX as a potential regulator of redox homeostasis during thermal stress and hardening

Exposure to the moderately elevated temperatures (24°C) led to a slight upregulation of AOX expression in the naive (non-heat-hardened) mussels, in line with the putative shift from NADH- to NADPH-producing pathways and reduction of ROS production under hypoxia.

This response was mitigated or reversed during exposure to 26 or 28°C. Notably, heat hardening enhanced transcriptional upregulation in the mussels, and this response was sustained throughout the entire exposure at all temperatures except for the last time point (10 days) at 28°C. AOX is found in most animal phyla including hypoxia-tolerant marine bivalves (McDonald et al., 2009) and is induced by stressors including toxins, hypoxia, nutrient deficiency, temperature, desiccation and pathogen attack (Jacobs and Ballard, 2022). AOX protects anaerobic mitochondria from respiratory poisoning by hydrogen sulfide and prevents mitochondrial membrane hyperpolarization and concomitant ROS production by diverting electrons away from the classical phosphorylation sites at complexes III and IV during metabolic shutdown (Abele et al., 2007). High AOX activity combined with the suppression of the pathways channeling electrons to ubiquinone (Sokolov et al., 2019) might minimize electron leak from highly reduced electron carriers and thus suppress mitochondrial ROS production during hypoxia–reoxygenation stress (Adzigbli et al., 2022). Similar to our results, Sussarellu et al. (2012) reported up-regulation of the AOX mRNA which becomes the main electron transport route under anoxic conditions in *C. gigas*. The beneficial role of AOX in mussels under metabolic depression is also evidenced by transcriptional suppression of AOX in the mussels exposed to 26°C, when they exhibit reactivation of metabolic rate (Anestis et al., 2007). Respirometry using inhibitors showed that AOX accounted for a greatly increased proportion of the electron flow to oxygen in *C. gigas* during post-hypoxic recovery (Sussarellu et al., 2012). This may reflect a mechanism to minimize the burst of ROS production that is associated with re-oxygenation (Storey, 1996). Taken together, these findings point to an important role of AOX in regulating the cellular redox balance during stress-induced metabolic shifts in mollusks, and show that stimulation of AOX expression might contribute to the thermotolerant phenotype caused by heat hardening.

Conclusions

Exposure of *M. galloprovincialis* to elevated temperatures resulted in metabolic disturbances including shifts in multiple essential metabolic pathways, which were largely mitigated in mussels that experienced heat hardening prior to thermal stress exposure (Fig. 8). Overall, the observed metabolic responses of heat-hardened mussels may indicate higher activity of the TCA cycle in order to increase their ATP production as much as possible and prolong their survival until environmental conditions return to optimum. Furthermore, observed low concentrations of fumarate and succinate and the accumulation of end products of alanopine, lactate and strombine indicate increased oxidative capacities via the corresponding anaerobic pathways in order to supplement the energy supply. At severe thermal stress (28°C), the maintenance of lactate levels with a concomitant progressive accumulation of succinate underlies the initiation of metabolic depression after the first 3–5 days of exposure. Overall, the metabolic responses in non-heat-hardened and heat-hardened mussels underline the upper thermal limits of *M. galloprovincialis*, set at 26°C (Anestis et al., 2007), and are in accordance with the OCLTT concept. The ability of heat-hardened mussels to undergo a rapid gain and slow loss of heat tolerance may be an advantageous strategy for coping with intermittent and often extreme temperatures (Moyen et al., 2020).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: B.M.; Methodology: I.G., C.B., G.L., K.F., I.A.G.; Software: C.B., G.L., K.F., I.A.G.; Validation: B.M.; Formal analysis: I.G., K.F., I.A.G.; Investigation: I.G., K.F., I.A.G.; Resources: B.M.; Data curation: I.G., K.F., I.A.G.; Writing - original draft: I.G., K.F., I.A.G., B.M.; Writing - review & editing: I.G., C.B., G.L., H.P., K.F., I.A.G., I.M.S., B.M.; Visualization: I.G., K.F., I.A.G., B.M.; Supervision: B.M.; Project administration: B.M.; Funding acquisition: I.G.

Funding

The implementation of the doctoral thesis was co-financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme 'Human Resources Development, Education and Lifelong Learning' in the context of the Act 'Enhancing Human Resources Research Potential by undertaking a Doctoral Research' Sub-action 2: IKY Scholarship Programme for PhD candidates in the Greek Universities.

References

Abele, E., Philip, E., Gonzalez, P. M. and Puntarulo, S. (2007). Marine invertebrate mitochondria and oxidative stress. *Front. Biosci.* **12**, 933-946. doi:10.2741/2115

Adzigbli, L., Sokolov, E. P., Ponsuksili, S. and Sokolova, I. M. (2022). Tissue- and substrate-dependent mitochondrial responses to acute hypoxia–reoxygenation stress in a marine bivalve (*Crassostrea gigas*). *J. Exp. Biol.* **225**, jeb243304. doi:10.1242/jeb.243304

Agrawal, A. A. (2001). Phenotypic plasticity in the interaction and evolution of species. *Science* **294**, 321-326. doi:10.1126/science.1060701

Aleng, N. A., Sung, Y. Y., Macrae, T. H. and Wahid, M. E. A. (2015). Non- lethal heat shock of the Asian green mussel, *Perna viridis*, promotes Hsp70 synthesis, induces thermotolerance and protects against vibrio infection. *Plos One* **10**, e0135603. doi:10.1371/journal.pone.0135603

Androulidakis, Y. S. and Krestenitis, Y. N. (2022). Sea surface temperature variability and marine heat waves over the Aegean, Ionian, and Cretan Seas from 2008–2021. *J. Mar. Sci. Eng.* **10**, 42. doi:10.3390/jmse10010042

Anestis, A., Lazou, A., Portner, H. O. and Michaelidis, B. (2007). Behavioral and metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *Am. J. Physiol.* **293**, R911-R921. doi:10.1152/ajpregu.00124.2007

Anestis, A., Portner, H., Karagiannis, D., Angelidis, P., Staikou, A. and Michaelidis, B. (2010). Response of *Mytilus galloprovincialis* (L.) to increasing seawater temperature and to marteliosis: metabolic and physiological parameters. *Comp. Biochem. Physiol. A* **156**, 57-66. doi:10.1016/j.cbpa.2009.12.018

Appanna, V. P., Auger, C., Thomas, S. C. and Omri, A. (2014). Fumarate metabolism and ATP production in *Pseudomonas fluorescens* exposed to nitrosative stress. *Antonie Leeuwenhoek* **106**, 431-438. doi:10.1007/s10482-014-0211-7

Bayne, B. L. (2017). *Biology of Oysters*. Academic Press.

Bilyk, K. T., Clive, W. E. and Devries, A. L. (2012). Heat hardening in Antarctic notothenioid fishes. *Pol. Biol.* **35**, 1447-1451. doi:10.1007/s00300-012-1189-0

Bowler, K. (2005). Acclimation, heat shock and hardening. *J. Therm. Biol.* **30**, 125-130. doi:10.1016/j.jtherbio.2004.09.001

Chen, Y., Wang, J., Liao, M., Li, X. and Dong, Y. (2021). Temperature adaptations of the thermophilic snail *Echinolittorina malaccana*: insights from metabolomics analysis. *J. Exp. Biol.* **224**, jeb238659. doi:10.1242/jeb.238659

Chong, J., Wishart, D. S. and Xia, J. (2019). Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr. Protoc. Bioinform.* **68**, e86. doi:10.1002/cpbi.86

Dewall, R. A., Vasko, K. A., Stanley, E. L. and Kezdi, P. (1971). Responses of the ischemic myocardium to allopurinol. *Am. Heart J.* **82**, 362-370. doi:10.1016/0002-8703(71)90302-4

Ding, Y., Fromm, M. and Avramova, Z. (2012). Multiple exposures to drought 'train' transcriptional responses in *Arabidopsis*. *Nat. Commun.* **3**, 740. doi:10.1038/ncomms1732

Dunphy, B. J., Ruggiero, K., Zamora, L. N. and Ragg, N. L. C. (2018). Metabolomic analysis of heat-hardening in adult green-lipped mussel (*Perna canaliculus*): A key role for succinic acid and the GABAergic synapse pathway. *J. Therm. Biol.* **74**, 37-46. doi:10.1016/j.jtherbio.2018.03.006

Eymann, C., Götze, S., Bock, C., Guderley, H., Knoll, A. H., Lannig, G., Sokolova, I. M., Aberhan, M. and Pörtner, H.-O. (2020). Thermal performance of the european flat oyster, *Ostrea edulis* (Linnaeus, 1758)— explaining ecological findings under climate change. *Mar. Biol.* **167**, 1-15. doi:10.1007/s00227-019-3620-3

Feidantsis, K., Pörtner, H. O., Vlachonikola, E., Antonopoulou, E. and Michaelidis, B. (2018). Seasonal changes in metabolism and cellular stress phenomena in the gilthead sea bream (*Sparus aurata*). *Physiol. Biochem. Zool.* **91**, 878-895. doi:10.1086/697170

Feidantsis, K., Giantsis, I. A., Vratsistas, A., Makri, S., Pappa, A. Z., Drosopoulou, E., Anestis, A., Mavridou, E., Exadactylos, A., Vafidis, D. et al. (2020). Correlation between intermediary metabolism, Hsp gene expression, and oxidative stress-related proteins in long-term thermal-stressed

Journal of Experimental Biology

Mytilus galloprovincialis. *Am. J. Physiol.* **319**, R264-R281. doi:10.1152/ajpregu.00066.2020

Feidantsis, K., Michaelidis, B., Raitsos, D. E., Vafidis, D. (2021a). Seasonal cellular stress responses of commercially important invertebrates at different habitats of the North Aegean Sea. *Comp. Biochem. Physiol. A* **250**, 110778. doi:10.1016/j.cbpa.2020.110778

Feidantsis, K., Georgoulis, I., Giantsis, I. A. and Michaelidis, B. (2021b). Treatment with ascorbic acid normalizes the aerobic capacity, antioxidant defense, and cell death pathways in thermally stressed *Mytilus galloprovincialis*. *Comp. Biochem. Physiol. B.* **255**, 110611. doi:10.1016/j.cbpb.2021.110611

Frenguelli, B. G. (2019). The purine salvage pathway and the restoration of CerebralATP: implications for brain slice physiology and brain injury. *Neurochem. Res.* **44**, 661-675. doi:10.1007/s11064-017-2386-6

Frölicher, T. L. and Laufkötter, C. (2018). Emerging risks from marine heat waves. *Nat. Commun.* **9**, 1-4. doi:10.1038/s41467-018-03163-6

Galli, G., Solidoro, C. and Lovato, T. (2017). Marine heat waves hazard 3D maps and the risk for low motility organisms in a warming Mediterranean sea. *Front. Mar. Sci.* **4**, 136. doi:10.3389/fmars.2017.00136

Georgoulis, I., Feidantsis, K., Giantsis, I. A., Kakale, A., Bock, C., Pörtner, H. O., Sokolova, I. M. and Michaelidis, B. (2021). Heat hardening enhances mitochondrial potential for respiration and oxidative defense capacity in the mantle of thermally stressed *Mytilus galloprovincialis*. *Sci. Rep.* **11**, 17098. doi:10.1038/s41598-021-96617-9

Gleason, L. U., Miller, L. P., Winnikoff, J. R., Somero, G. N., Yancey, P. H., Bratz, D. and Dowd, W. W. (2017). Thermal history and gape of individual *Mytilus californianus* correlate with oxidative damage and thermoprotective osmolytes. *J. Exp. Biol.* **220**, 4292-4304. doi:10.1242/jeb.168450

Götze, S., Bock, C., Eymann, C., Lannig, G., Steffen, J. B. M. and Pörtner, H. O. (2020). Single and combined effects of the "Deadly trio" hypoxia, hypercapnia and warming on the cellular metabolism of the great scallop *Pecten maximus*. *Comp. Biochem. Physiol. B.* **243-244**, 110438. doi:10.1016/j.cbpb.2020.110438

Gutteridge, J. M. C. and Halliwell, B. (2000). Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann. N. Y. Acad. Sci.* **899**, 136-147. doi:10.1111/j.1749-6632.2000.tb06182.x

Haider, F., Falfushynska, H. I., Timm, S. and Sokolova, I. M. (2020). Effects of hypoxia and reoxygenation on intermediary metabolite homeostasis of marine bivalves *Mytilus edulis* and *Crassostrea gigas*. *Comp. Biochem. Physiol. A* **242**, 110657. doi:10.1016/j.cbpa.2020.110657

Haskó, G., Sitkovsky, M. V. and Szabó, C. (2004). Immunomodulatory and neuroprotective effects of inosine. *Trends Pharmacol. Sci.* **25**, 152-157. doi:10.1016/j.tips.2004.01.006

Haun, S. E., Segeleon, J. E., Trapp, V. L., Clotz, M. A. and Horrocks, L. A. (1996). Inosine mediates the protective effect of adenosine in rat astrocyte cultures subjected to combined glucose-oxygen deprivation. *J. Neurochem.* **67**, 2051-2059. doi:10.1046/j.1471-4159.1996.67052051.x

Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Bäurle, I., Geiselhardt, S., Hincha, D. K., Kunze, R., Mueller-Roeber, B., Rillig, M. C. et al. (2016). Priming and memory of stress responses in organisms lacking a nervous system. *Biol. Rev.* **91**, 1118-1133. doi:10.1111/brv.12215

Hochachka, P. W. and Mustafa, T. (1972). Invertebrate Facultative Anaerobiosis: a reinterpretation of invertebrate enzyme pathways suggests new approaches to helminth chemotherapy. *Science* **178**, 1056-1060. doi:10.1126/science.178.4065.1056

Hu, Z., Feng, J., Song, H., Zhou, C., Yang, M., Shi, P., Yu, Z., Guo, Y., Li, Y. and Zhang, T. (2022). Metabolic response of *Mercenaria mercenaria* under heat and hypoxia stress by widely targeted metabolomic approach. *Sci. Total Environ.* **809**, 151172. doi:10.1016/j.scitotenv.2021.151172

Hutchison, V. H. (1961). Critical thermal maxima in salamanders. *Physiol. Biochem. Zool.* **34**, 92-125. doi:10.1086/physzool.34.2.30152688

Huxtable, R. J. (1992). Physiological actions of taurine. *Physiol. Rev.* **72**, 101-163. doi:10.1152/physrev.1992.72.1.101

Isani, G., Cattani, O., Zurzolo, M., Pagnucco, C. and Cortesi, P. (1995). Energy metabolism of the mussel, *Mytilus galloprovincialis*, during long-term anoxia. *Comp. Biochem. Physiol. B* **110**, 103-113. doi:10.1016/0305-0491(94)00132-E

Jacobs, H. T. and Ballard, J. W. O. (2022). What physiological role(s) does the alternative oxidase perform in animals? *Biochim. Biophys. Acta* **1863**, 148556. doi:10.1016/j.bbabio.2022.148556

Jong, C. J., Azuma, J. and Schaffer, S. (2012). Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production. *Amino Acids* **42**, 2223-2232. doi:10.1007/s00726-011-0962-7

Jong, C. J., Sandal, P. and Schaffer, S. W. (2021). The role of taurine in mitochondria health: more than just an antioxidant. *Molecules* **26**, 4913. doi:10.3390/molecules26164913

Larade, K. and Storey, K. B. (2002). A profile of the metabolic responses to anoxia in marine. *Cell Mol. Res. Stress* **3**, 27-46. doi:10.1016/S1568-1254(02)80005-5

Le Moullac, G., Bacca, H., Huvet, A., Moal, J., Pouvreau, S. and Van Wormhoudt, A. (2007). Transcriptional regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase in the adductor muscle of the oyster *Crassostrea gigas* during prolonged hypoxia. *J. Exp. Zool. A* **307A**, 371-382. doi:10.1002/jez.390

Malmendal, A., Overgaard, J., Bundy, J. G., Sørensen, J. G., Nielsen, N. C., Loeschcke, V. and Holmstrup, M. (2006). Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *Am. J. Physiol.* **291**, R205-R212. doi:10.1152/ajpregu.00867.2005

Mandel, J. L., Takano, T., Soltoff, S. P. and Murdaugh, S. (1998). Mechanisms whereby exogenous adenine nucleotides improve rabbit renal proximal function during and after anoxia. *J. Clin. Investig.* **81**, 1255-1264. doi:10.1172/JCI113443

McDonald, A. E., Vanlerberghe, G. C. and Staples, J. F. (2009). Alternative oxidase in animals: unique characteristics and taxonomic distribution. *J. Exp. Biol.* **212**, 2627-2634. doi:10.1242/jeb.032151

Moyen, N. E., Crane, R. L., Somero, G. N. and Denny, M. W. (2020). A single heat-stress bout induces rapid and prolonged heat acclimation in the California mussel, *Mytilus californianus*. *Proc. R. Soc. B.* **287**, 20202561. doi:10.1098/rspb.2020.2561

Oizel, K., Tait-Mulder, J., Fernandez-De-Cossio-Diaz, J., Pietzke, M., Brunton, H., Lilla, S., Dhayade, S., Athineos, D., Rodriguez Blanco, G., Sumpton, D. et al. (2020). Formate induces a metabolic switch in nucleotide and energy metabolism. *Cell Death Dis.* **11**, 310. doi:10.1038/s41419-020-2523-z

Pietzke, M., Meiser, J. and Vazquez, A. (2020). Formate metabolism in health and disease. *Mol. Metab.* **33**, 23-37. doi:10.1016/j.molmet.2019.05.012

Pörtner, H. O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol. A* **132**, 739-761. doi:10.1016/S1095-6433(02)00045-4

Pörtner, H. O. (2010). Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* **213**, 881-893. doi:10.1242/jeb.037523

Pörtner, H. O. (2012). Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. *Mar. Ecol. Prog. Ser.* **470**, 273-290. doi:10.3354/meps10123

Pörtner, H. O. (2021). Climate impacts on organisms, ecosystems and human societies: integrating OCLTT into a wider context. *J. Exp. Biol.* **224**, jeb238360. doi:10.1242/jeb.238360

Pörtner, H. O. and Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science.* **315**, 95-97. doi:10.1126/science.1135471

Pörtner, H. O., Peck, L. and Somero, G. (2007). Thermal limits and adaptation in marine Antarctic ectotherms: an integrative view. *Philos. Trans. R. Soc. Lond. B* **362**, 2233-2258. doi:10.1098/rstb.2006.1947

Pörtner, H. O., Karl, D. M., Boyd, P. W., Cheung, W. W. L., Lluch-Cota, S. E., Nojiri, Y., Schmidt, D. N. and Zavialov, P. O. (2014). Ocean systems. In: *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (ed. C. B. Field, V. R. Barros, D. J. Dokken, K. J. Mach, M. D. Mastrandrea, T. E. Bilir, M. Chatterjee, K. L. Ebi, Y. O. Estrada, R.C. Genova, et al.), pp. 411-484. Cambridge University Press.

Pörtner, H. O., Bock, C. and Mark, F. C. (2017). Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. *J. Exp. Biol.* **220**, 2685-2696. doi:10.1242/jeb.134585

Precht, H., Christophersen, J., Hensel, H. and Larcher, W. (1973). *In Temperature and Life*, pp. 302-348. Springer.

Quinlan, G. J., Lamb, N. J., Tilley, R., Evans, T. W. and Gutteridge, J. M. (1997). Plasma hypoxanthine levels in ARDS: implications for oxidative stress, morbidity, and mortality. *Am. J. Respir. Crit. Care Med.* **155**, 479-484. doi:10.1164/ajrccm.155.2.9032182

Ralevic, V. and Burnstock, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50**, 413-492.

Rebelein, A., Pörtner, H. O. and Bock, C. (2018). Untargeted metabolic profiling reveals distinct patterns of thermal sensitivity in two related notothenioids. *Comp. Biochem. Physiol. A. Mol. Integr.* **217**, 43-54. doi:10.1016/j.cbpa.2017.12.012

Rodrigues, A. F., Roecker, R., Junges, G. M., De Lima, D. D., Da Cruz, J. G. P., Wyse, A. T. S. and Dal Magro, D. D. (2014). Hypoxanthine induces oxidative stress in kidney of rats: protective effect of vitamins E plus C and allopurinol. *Cell Biochem. Funct.* **32**, 387-394. doi:10.1002/cbf.3029

Sato, M., Takeuchi, M., Kanno, N., Nagahisa, E. and Sato, Y. (1993). Distribution of opine dehydrogenases and lactate dehydrogenase activities in marine animals. *Comp. Biochem. Physiol. B* **106**, 955-960. doi:10.1016/0305-0491(93)90057-C

Sokolova, I. M. (2013). Energy-Limited Tolerance to Stress as a Conceptual Framework to Integrate the Effects of Multiple Stressors. *Integr. Comp. Biol.* **53**, 597-608. doi:10.1093/icb/ict028

Sokolov, E. P. and Sokolova, M. I. (2019). Compatible osmolytes modulate mitochondrial function in a marine osmoconformer *Crassostrea gigas* (Thunberg, 1793). *Mitochondrion* **45**, 29-37. doi:10.1016/j.mito.2018.02.002

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Mar. Environ. Res.* **79**, 1-15. doi:10.1016/j.marenvres.2012.04.003

Sokolov, E. P., Markert, S., Hinzke, T., Hirschfeld, C., Becher, D., Ponsuksili, S. and Sokolova, I. M. (2019). Effects of hypoxia reoxygenation stress on mitochondrial proteome and bioenergetics of the hypoxia-tolerant marine

bivalve *Crassostrea gigas*. *J. Proteomics.* **194**, 99-111. doi:10.1016/j.jprot.2018.12.009

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers.' *J. Exp. Biol.* **213**, 912-920. doi:10.1242/jeb.037473

Somero, G. N. (2012). The physiology of global change: linking patterns to mechanisms. *Annu. Rev. Mar. Sci.* **4**, 39-61. doi:10.1146/annurev-marine-120710-100935

Storey, K. B. (1996). Oxidative stress: animal adaptations in nature. *Braz. J. Med. Biol. Res.* **29**, 1715-1733.

Sussarellu, R., Fabioux, C., Camacho, S. M., Le Goïc, N., Lambert, C., Soudant, P. and Moraga, D. (2012). Molecular and cellular response to short-term oxygen variations in the Pacific oyster *Crassostrea gigas*. *J. Exp. Mar. Biol. Ecol.* **412**, 87-95. doi:10.1016/j.jembe.2011.11.007

Szokodi, I. (2005). Purine metabolism in the reperfused porcine myocardium. *Acta Physiol. Scand.* **185**, 3. doi:10.1111/j.1365-201X.2005.01476_1.x

Takeo, S., Tanonaka, K., Miyake, K. and Imago, M. (1988). Adenine nucleotide metabolites are beneficial for recovery of cardiac contractile force after hypoxia. *J. Mol. Cell. Cardiol.* **20**, 187-199. doi:10.1016/S0022-2828(88)80052-X

Thomas, S. C., Alhasawi, A., Auger, C., Omri, A. and Appanna, V. D. (2016). The role of formate in combatting oxidative stress. *Antonie Leeuwenhoek* **109**, 263-271. doi:10.1007/s10482-015-0629-6

Tomanek, L. and Zuzow, M. J. (2010). The proteomic response of the mussel congeners *Mytilus galloprovincialis* and M. trossulus to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. *J. Exp. Biol.* **213**, 3559-3574. doi:10.1242/jeb.041228

Ummenhofer, C. C. and Meehl, G. A. (2017). Extreme weather and climate events with ecological relevance: a review. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **372**, 20160135. doi:10.1098/rstb.2016.0135

Viant, M. R. (2007). Metabolomics of aquatic organisms: the new 'omics' on the block. *Mar. Ecol. Prog. Ser.* **332**, 301-306. doi:10.3354/meps332301

Viant, M. R. (2008). Recent developments in environmental metabolomics. *Mol. Biosyst.* **4**, 980-986. doi:10.1039/b805354e

Weinberg, J. M. and Humes, H. D. (1986). Increases of cell ATP produced by exogenous adenine nucleotides in isolated rabbit kidney tubules. *Am. J. Physiol.* **250**, F720-F733. doi:10.1152/ajpcell.1986.250.5.C720

West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. Oxford University Press.

Williams, E. and Somero, G. (1996). Seasonal-, tidal-cycle- and microhabitat related variation in membrane order of phospholipid vesicles from gills of the intertidal mussel *Mytilus californianus*. *J. Exp. Biol.* **199**, 1587-1596. doi:10.1242/jeb.199.7.1587

Zandee, D., Holwerda, D., Kluytmans, J. and De Zwaan, A. (1985). Metabolic adaptations to environmental anoxia in the intertidal bivalve mollusk *Mytilus edulis* L. *Neth. J. Zool.* **36**, 322-343. doi:10.1163/002829686X00117

Zurburg, W. and Zwaan, A. (1981). The role of amino acids in anaerobiosis and osmoregulation in bivalves. *J. Exp. Zool.* **215**, 315-325. doi:10.1002/jez.1402150309