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# Combined effects of temperature and emersion-immersion cycles on metabolism and bioenergetics of the Pacific oyster *Crassostrea (Magallana) gigas*

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ABSTRACT

Life on tidal coasts presents physiological major challenges for sessile species. Fluctuations in oxygen and temperature can affect bioenergetics and modulate metabolism and redox balance, but their combined effects are not well understood. We investigated the effects of intermittent hypoxia (12h/12h) in combination with different temperature regimes (normal (15 °C), elevated (30 °C) and fluctuating (15 °C water/30 °C air)) on the Pacific oyster *Crassostrea (Magallana) gigas*. Fluctuating temperature led to energetic costly metabolic rearrangements and accumulation of proteins in oyster tissues. Elevated temperature led to high (60%) mortality and oxidative damage in survivors. Normal temperature had no major negative effects but caused metabolic shifts. Our study shows high plasticity of oyster metabolism in response to oxygen and temperature fluctuations and indicates that metabolic adjustments to oxygen deficiency are strongly modulated by the ambient temperature. Co-exposure to constant elevated temperature and intermittent hypoxia demonstrates the limits of this adaptive metabolic plasticity.

## 1. Introduction

Living in coastal regions presents major physiological challenges for sessile benthic species, which must contend with fluctuating environmental conditions that can disrupt their metabolic and redox homeostasis (Willmer et al., 2005; Abele et al., 2012; Haider et al., 2020). One significant source of variability is the natural tidal cycle, which exposes intertidal organisms to intermittent submersion and emersion coinciding with the changes in oxygen and food availability, temperature and desiccation stress (Vernberg and Vernberg, 1972). Lacking the ability for escape, intertidal sessile organisms like bivalves, limpets and barnacles evolved protective behaviors that involve shell closures to isolate the body from the stressful environment. While shell closure is effective in preventing desiccation, it also limits oxygen supply, leading to hypoxia within the shell (Vernberg and Vernberg, 1972; Platvoet and Pinkster, 1995; Willmer et al., 2005). Consequently, intertidal animals have developed strategies to counteract oxygen deprivation, such as metabolic rate suppression, which can reduce aerobic rates by up to 99% (Hochachka et al., 1993; Hochachka and Lutz, 2001; Storey and Storey, 2004; Abele et al., 2012; Janas et al., 2017; Haider et al., 2020; Steffen et al., 2021). Upon re-submersion, the organism experiences reoxygenation, which can induce a burst in reactive oxygen species (ROS) production due to the rapid reintroduction of oxygen (Kalogeris et al., 2014). While intertidal organisms express high levels of antioxidants, compensating for this ROS burst remains challenging (Hermes-Lima et al., 1998; Sokolova et al., 2011; Abele et al., 2012; Bayne, 2017b).

Temperature is an important stressor that varies on the seasonal, daily and tidal scales in the intertidal zone and can directly affect the physiology and metabolism of ectothermic organisms (Vernberg and Vernberg, 1972; Sommer, 2005; Lushchak, 2011). During summer low tides, body temperature of sessile intertidal organisms rapidly increases due to contact with the warm air and substrate and insolation (Sokolova

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et al., 2000; Ricklefs and Vanselow, 2012; Bayne, 2017d; Sokolova, 2019; Janetzki et al., 2021). As a result, organisms in the intertidal zone experience diurnal temperature fluctuations that occasionally exceed their critical temperatures and can affect energy metabolism, ROS generation and ultimately, fitness (Newell and Bayne, 1973; Sommer et al., 1997; Lushchak, 2011; Strand et al., 2011; Ricklefs and Vanselow, 2012; Bayne, 2017d). Under fluctuating temperature conditions, physiological plasticity plays an important role in an organism's adaptation as described in the "beneficial acclimation hypothesis" (Leroi et al., 1994; Kingsolver and Huey, 1998; Wilson and Franklin, 2002; Seebacher et al., 2015). However, physiological plasticity is limited when stronger or unpredictable fluctuating environments do not provide clear signals for directional adaptation (Kingsolver and Huey, 1998; Kawecki, 2000; Wilson and Franklin, 2002). Earlier studies on the effects of stable vs. fluctuating temperature regimes on adaptive physiological responses and thermal tolerance of different ectotherms species yielded controversial results (Ashmore and Janzen, 2003; Dong and Dong, 2006; Pernet et al., 2007; Byrne et al., 2009; van Dooremalen et al., 2011; Paaijmans et al., 2013; Seebacher et al., 2015; Kingsolver et al., 2020; Slein et al., 2023). This variability in physiological and metabolic responses to temperature might reflect evolutionary adaptations of different species to certain thermal regimes, but may also be modulated by other abiotic factors like oxygen availability. While the individual effects of hypoxia (oxygen deficiency) and temperature have been well studied in intertidal organisms, their combined effects remains poorly understood and require further investigation (Pörtner, 2005; Collins et al., 2023).

The aim of this study was to investigate the combined impacts of intermittent hypoxia and elevated or fluctuating temperature on the metabolism and redox balance of the Pacific oyster, *Crassostrea* (*Magallana*) *gigas* (Thunberg, 1793), an invasive species commonly found in European intertidal waters. The oyster was introduced worldwide for aquaculture purposes but frequently escaped, establishing dense intertidal reefs (Reise et al., 2005; Ruesink et al., 2005; Reid and Valdés, 2011). Invasive populations of *C. gigas* in the German Wadden Sea inhabit major parts of the intertidal zone, where they emerge twice daily for several hours during a semi-diurnal tide (Reise et al., 2017). As a robust and stress-tolerant organism (Zhang et al., 2012), *C. gigas* is a suitable model to investigate the interaction between hypoxia and temperature stress. Here, we hypothesized that intermittent hypoxia will disrupt the redox balance and metabolic profiles of the oysters, with this disruption being intensified by elevated temperature. To test our hypotheses, we exposed Pacific oysters to ten cycles of intermittent hypoxia, with 12 h of hypoxia (air exposure) followed by 12 h of recovery (submersion) per cycle. This regime was chosen to simulate the maximum emersion experienced by oysters in the upper intertidal habitats (Bundesamt für Schiffahrt und Hydrographie, 2023). Extended (12 h) recovery period was chosen to detect the persistent effects of periodic emersion. Additionally, we subjected the oysters to elevated temperatures (30 °C) considered critical for *C. gigas* (Le Gall and Raillard, 1988; Bougrier et al., 1995) and plausible for summer low tide conditions of the German Wadden Sea (Ricklefs and Vanselow, 2012). The temperature was either kept constant at 15 °C or 30 °C throughout the emersion-immersion cycle or fluctuated between the emersion (30 °C) and immersion (15 °C) conditions as would be expected during summer tides. Our results provide insights into the role of metabolic plasticity in responses to intermittent hypoxia and thermal stress in oysters and shows mitigating effects of fluctuating temperatures on the hypoxia-induced metabolic and redox disturbances in this stress-tolerant intertidal species.

## 2. Material and methods

### 2.1. Oyster collection and experimental exposures

Oyster were collected from a mixed intertidal oyster-mussel reef in the intertidal zone of Königshafen (Oddewatt, List, Sylt, Germany) in June 2020. Oysters were cleaned of epibionts and placed in habituation tanks at 15 °C and salinity 28 (practical salinity units) (Pro-REEF Sea Salt; Tropic Marin). These conditions were similar to the habitat water conditions at the time of collection. Starting on day two, salinity was increased to 33 at a rate of ~1 unit per day. After one week, oysters were transferred into a recirculated maintenance system (700l) with biological filtration, aeration and protein skimmer and kept at 15 °C and salinity 33 for 4–9 weeks until experimental exposures. Oysters were continuously fed with a commercial algal mixture (*Nanochloropsis* sp., *Chlorella* sp. *Phaeodactylum* sp., *Cylindrotheca* sp. and *Nitzschia* sp., provided by Aquacopa or Sustainable Aquatics), supplemented with 30–50% (v:v) of freshly cultured *Rhodomonas* sp. using automatic feeding systems (Reefdoser EVO 4, Aqua Medic). The final concentration of algae in the maintenance tanks was kept at approximately 200 μg/l (chlorophyll a equivalents).

For each experimental treatment group, 19–20 randomly selected oysters (average length 51 mm) were placed into three 5 l tanks (6–7 oysters per tank, salinity 33). Water in the tanks was constantly aerated, and the tanks were kept under the dim light conditions throughout the exposures. For each experimental group, the oysters were exposed to ten cycles of emersion-immersion (12 h: 12 h) under different temperature regimes including either normal (15 °C in water and air), elevated (30 °C in water and air) or fluctuating (15 °C in water and 30 °C in air) temperature. During emersion, the oysters were placed for 12 h in dry plastic containers lined with plastic spacers to allow drainage. Air temperature was controlled by placing the containers into a water bath (15 °C) or temperature-controlled incubator (30 °C). Humidity was not controlled during emersion but was kept high (>80%) by closing the plastic container with a lid. Oysters maintained at 15 °C under normoxia and constant immersion were used as controls. During exposures, oysters were fed every other day with the algal mix (0.3–0.4 ml/tank; Premium Reef Blend, Sustainable Aquatics). Ammonium and nitrite concentrations were monitored and water exchanged if the threshold of 10 mg/l for either compound was exceeded.

Tissue samples were collected from all experimental oysters at the end of the last (10th) immersion cycle. This time point corresponded to 12 h of recovery after hypoxia ensuring that all oysters were sampled under the normoxic conditions. Normoxic controls were collected after 3, 5 and 10 days of exposure. Pilot analyses showed that there was no significant difference in the studied endpoints in the normoxic controls over time ($p > 0.05$; data not shown). Therefore, all control samples were treated as a single experimental group. Gill and digestive gland were dissected on ice, immediately frozen in liquid nitrogen and stored at −80 °C until further analyses.

### 2.2. Determination of energy reserves and electron transport system (ETS) activity

Gill or digestive gland tissues (15.6 ± 3.1 mg per sample) were placed into 2 ml tubes containing zirconium beads (Ø 1 mm and 2 mm, ~420 mg and ~230 mg, respectively) and ice-cold homogenization buffer (0.1 M Tris-HCl, pH 8.5, 1.5 g/l polyvinylpyrrolidone, 153 μM $MgSO_4$ and 0.2% Triton X-100 (w/v) in the tissue to buffer ratio of 1:25 (w/v). Tissues were homogenized using FastPrep-24 homogenizer (MP Biomedicals) with five 60 s cycles at 6.5 m/s and 5 min cooling on ice between the cycles. The homogenate was centrifuged for 10 min at 4 °C and 3000×*g*. A 75 μl aliquot of the supernatant was collected and stored at −80 °C for ETS measurement. The remaining homogenate was lysed using four freeze-thaw cycles (5 min each) alternating between liquid nitrogen and water bath (50–55 °C). The samples were centrifuged for 10 min at 4 °C and 3000×*g* and stored at −80 °C for carbohydrate and protein analyses.

ETS was measured in an assay buffer containing 91 mM Tris-HCl, pH 8.5, 0.21% Triton X-100 (w/v), 0.34 mM NADH and 51 μM NADPH as described elsewhere (King and Packard, 1975; De Coen and Janssen,

1997). To account for the background activity of non-mitochondrial oxidoreductases, blanks were set with addition of mitochondrial ETS inhibitors (1 M KCN and 0.4 mM rotenone) instead of NADH and NADPH. The reaction was started with 2.7 mM P-iodonitrotetrazolium (INT) and monitored at 490 nm (SpectraMax iD3, Molecular Devices). Using the extinction coefficient of the produced INT formazan $\epsilon = 15900\ M^{-1}\ cm^{-1}$, the ETS activity was calculated as the background-corrected oxygen consumption rate assuming the molar ratio 2:1 of formazan to oxygen.

Tissue levels of carbohydrates were measured using phenol-sulfuric acid colorimetric method at 492 nm with glucose as a standard (Masuko et al., 2005). Proteins were measured using the Bradford assay at 595 nm (Bradford, 1976) using bovine serum albumin as a standard.

### 2.3. Oxidative stress markers

Gills or digestive gland tissues (45.5 ± 8.0 mg per sample) were homogenized in phosphate-buffered saline (PBS, 50 mM, pH 7.4) using 1:10 (w/v) tissue to buffer ratio. The homogenate was split into two aliquots and the proteins were precipitated by adding two volumes of 50% trichloroacetic acid (TCA). After 10–15 min of incubation on ice, the samples were centrifuged for 10 min at 4 °C and 5000×*g*. The resulting supernatants were combined. Pellets and supernatant were stored at −80 °C.

Lipid peroxidation products were determined in the supernatant as the concentration of the thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). Thiobarbituric acid (21 mM in 0.1 M HCl) was added 1:1 (v/v) to the supernatant and incubated at 90–100 °C for 20 min. Samples were then cooled on ice and absorbance measured at 532 nm. TBARS level was calculated using $\epsilon = 156000\ M^{-1}\ cm^{-1}$.

Protein carbonyl content was measured in the precipitated protein pellets using 2,4-dinitrophenylhydrazine (DNPH, 10 mM in 2 M HCl) as described before (Levine et al., 1990). The second pellet from each sample was treated with HCl and used as a negative control. The DNPH- or HCl-treated samples were centrifuged for 10 min at 4 °C and 10000×*g*, washed twice with 1 ml 5% TCA and centrifuged for 10 min at 4 °C and 10000×*g*. The pellets were dissolved in 1.5 ml of 8 M urea, centrifuged for 10 min at 4 °C and 10000×*g* and absorbance measured in the supernatant at 370 nm. The sample absorbance was corrected for the absorbance of the negative controls, and the protein carbonyls concentration calculated using $\epsilon = 22000\ M^{-1}\ cm^{-1}$.

Total antioxidant capacity (TAOC) was measured in the homogenates of the gill or digestive gland tissues. Tissues (29.8 ± 6.0 mg) were homogenized 1:15 (w/v) in 50 mM PBS, pH 7.8, containing 0.1 mM phenylmethylsulfonyl fluoride (Re et al., 1999). Samples were centrifuged for 10 min at 4 °C and 5000×*g* and the supernatant stored at −80 °C. For TAOC measurements, samples were mixed with freshly prepared $ABTS^{\cdot+}$ working solution in PBS (the final concentrations: 104 μM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 37 μM K2S2O8) and incubated for 6 min on a shaker at room temperature in the dark. The absorbance was measured at 734 nm and TAOC was calibrated with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as a standard and expressed in μmole Trolox-equivalents per g wet mass.

### 2.4. Metabolite profiles

Metabolite content was measured in the gill as described elsewhere (Haider et al., 2020). Metabolites were extracted from 25.7 ± 4.1 mg of tissue in 1 ml 80 % ethanol with 1 μg/ml 2-(N-morpholino)ethanesulfonic acid (MES) as an internal standard using FastPrep-24 homogenizer (MP Biomedicals). The homogenate was centrifuged for 10 min at 4 °C and 13000×*g* to remove the debris. The supernatants were freeze-dried under vacuum (Alpha 1–4 LSCplus and RVC 2–25 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH) and stored at −80 °C. The samples were then dissolved in ROTISOLV® LC-MS-grade water (Carl Roth), filtered (0.2 μm, Omnifix®-F, Braun, Germany) and analysed with the high-performance liquid chromatograph-mass spectrometer LCMS-8050 (Shimadzu). The sample separation was conducted as described earlier (Haider et al., 2020). The metabolites were identified and quantified with the built-in LC-MS/MS package for primary metabolites (Vers. 2, Shimadzu, P/N 225–24,862-92) and the LabSolutions software package (Shimadzu) (Selim et al., 2018). Each compound was calibrated using the respective standard substances (Merck) and loading-corrected relative to the internal standard (MES) peak. The peaks of citrate and isocitrate could not be distinguished, and the respective concentrations are combined and reported as (iso)citrate.

### 2.5. Statistics

Effects of different exposure regimes on the measured biomarkers were tested using one-way ANOVA. Any outliers with values outside the 1.5x interquartile range were excluded from further analyses. Data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). Data showing lack of homogeneity of variances or not normal distribution were analysed using Welch-ANOVA or Kruskal-Wallis test, respectively. Bonferroni, Games-Howell or Dunn-Bonferroni were used as post-hoc tests for ANOVA, Welch-ANOVA or Kruskal-Wallis analysis, respectively. Data was analysed using IBM SPSS Statistics 27.

Metabolite data was processed using MetaboAnalyst 5.0 (Pang et al., 2021). Pathway analyses was conducted using auto scaling, *Drosophila melanogaster* as a reference library, global test as the enrichment method and relative-betweeness centrality for topology analysis. (iso)citrate was treated as citrate. Exposure groups were compared to the normoxic control with pathways considered as significantly affected if the pathway impact was >0 and the false discovery rate (FDR) was <0.05. Partial least squares-discriminant analysis (PLS-DA) was conducted to reduce the dimensionality of the data set using autoscaling. Metabolites that contributed most to the separation along 1st and 2nd component were identified based on the loadings (cut-off ±0.2) and were used for further analyses.

## 3. Results

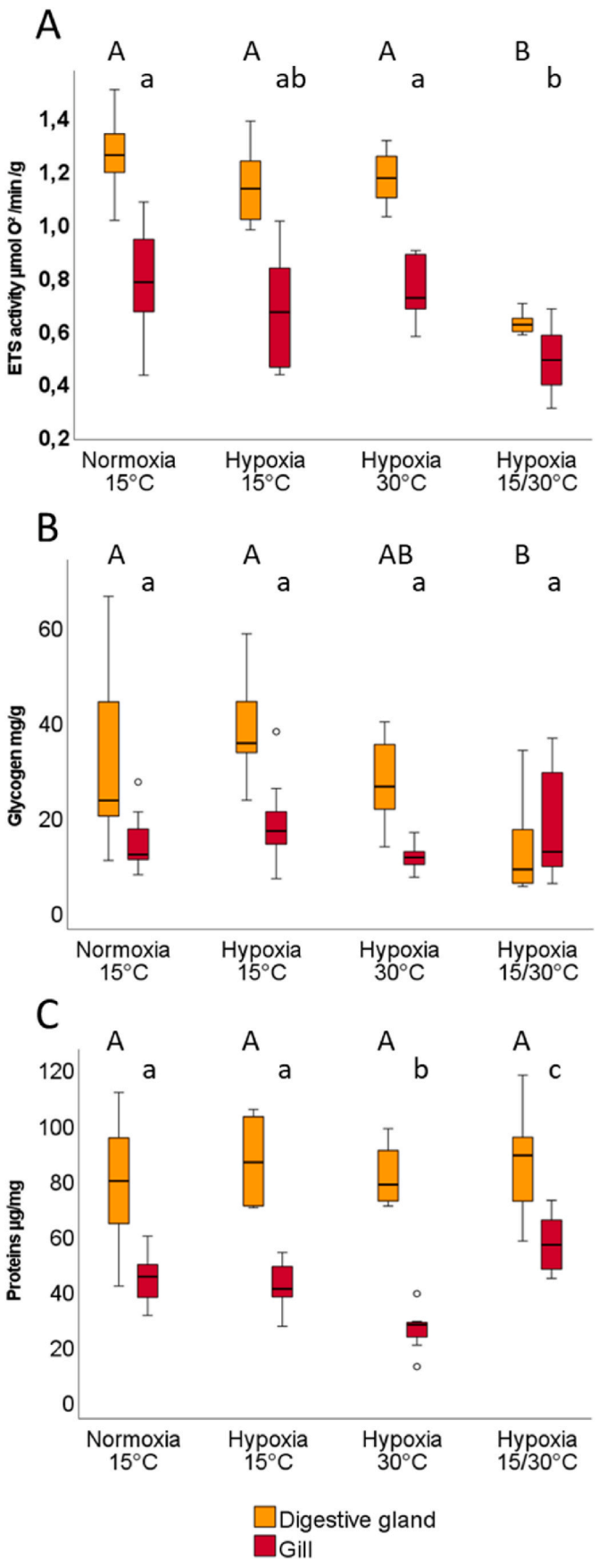
### 3.1. Mortality

No mortality was observed in the control oysters or those exposed to intermittent hypoxia (emersion-immersion cycles) at 15 °C (N = 20). In the group exposed to intermittent hypoxia combined with the fluctuating temperature, one oyster died at exposure day 8 (mortality rate of 5%, N = 19), whereas intermittent hypoxia combined with the constantly elevated temperature (30 °C) led to the mortality rate of 60% which was distributed over the whole course of the exposure (N = 20; 1.7 casualties per day).

### 3.2. Cellular bioenergetic parameters

The oyster gills showed lower ETS activity than the digestive gland (Fig. 1A). The experimental treatment showed significant effects on the ETS activity in oyster tissues (Table 1). This, oysters exposed to intermittent hypoxia combined with fluctuating temperature exhibited a notable decrease in ETS activity, with reductions of 37% and 52% in the gill and digestive gland, respectively, compared to the normoxic control (Fig. 1A). No change of ETS activity was observed in other hypoxia-exposed groups relative to the normoxic controls ($p > 0.05$).

The gills of oysters had generally lower glycogen content compared to the digestive gland (Fig. 1B). Glycogen content in the gills did not change in response to the intermittent hypoxia regardless of the temperature regime (Table 1). However, in the digestive gland, glycogen depletion was observed in oysters exposed to intermittent hypoxia combined with fluctuating temperature relative to the controls and




*(caption on next column)*

**Fig. 1.** Bioenergetic markers: electron transport system (ETS, A), carbohydrate content in glycogen equivalents (B) and protein content (C) are shown for digestive gland (yellow, upper case letters) and gills (red, lower case letters) and scaled to tissue fresh mass; columns that do not share the same letter are significantly different ($p < 0.05$); box plots show median, interquartile range (IQR) as well as maximum and minimum; outliers (>1.5x IQR) are indicated by circles.

hypoxia-exposed oysters kept at 15 °C (Fig. 1B).

The digestive gland had higher levels of proteins compared to the gill in all exposures (Fig. 1C). Intermittent hypoxia, irrespective of the temperature, showed no significant effect on the protein content of the digestive gland in oysters ($p > 0.05$, Table 1). However, in the gills, intermittent hypoxia combined with 30 °C caused protein depletion, and combined with fluctuating temperature - protein accumulation (Table 1, Fig. 1C).

### 3.3. Oxidative stress markers

Lipid peroxidation (LPO) levels were higher in the oyster gills compared to the digestive gland in all experimental groups (Fig. 2A). Moderate elevation of LPO levels was observed in the gills of the oysters exposed to hypoxia at 30 °C, but this increase was only significant when compared to the hypoxic group kept at fluctuating temperature (Fig. 2A). In the digestive gland, LPO levels were lower in oysters exposed to intermittent hypoxia at 15 °C compared to the normoxic controls (Table 1, Fig. 2A).

Protein carbonyl content was generally lower in the gills than in the digestive gland of oysters (Fig. 2B). The experimental treatment showed no evidence of effect on protein carbonyl levels in the gills and the digestive gland (Table 1).

Oysters exposed to intermittent hypoxia at 30 °C exhibited an increase in methionine sulfoxide concentration in the gills compared to those of the normoxic controls (Table 4, Fig. 2C). Hypoxia at constant or fluctuating temperature did not cause significant changes in methionine sulfoxide levels in oyster gills (Fig. 2C).

The total antioxidant capacity (TAOC) was lower in the gill compared to the digestive gland (Fig. 4D). There was no evidence for the effect of the experimental treatment on the TAOC in the gill or the digestive gland (Table 1).

### 3.4. Metabolite profiles in the gill tissue

PLS-DA analysis revealed that two first principal components accounted for 24.7% and 17.2% of the variance in the data, respectively (Fig. 3). All hypoxia-exposed groups were clearly separated from the control group in the scatter plot of the two principal components (Fig. 3). Samples from oysters exposed to intermittent hypoxia at fluctuating temperatures were strongly separated from the controls (along the 1st component axis) as well as from two other hypoxia-exposed groups (along the 2nd component axis) (Fig. 3). Samples from oysters exposed to intermittent hypoxia at constant temperatures (15 °C or 30 °C) showed overlapping positions, suggesting similarity in their metabolite profiles.

Based on the loadings of the PLS-DA analysis, 21 metabolites were identified as contributing to the separation of the model (loadings >0.2 or < −0.2; Table 2). Alanine, citrulline, tryptophan, tyrosine, glutamate, isoleucine, leucine, phenylalanine, succinate, and valine were associated with the 1st component (the fluctuating temperature axis), while aconitate, aspartate, glutamine, lysine, AMP, asparagine, (iso)citrate, malate, methionine sulfoxide, and threonine were associated with the 2nd component (hypoxia and constant temperature axis). Taurine contributed to the separation along both components.

Pathway enrichment analysis revealed alterations in 13, 10, and 5 out of 20 analysed metabolic pathways (with pathway impact >0) in the gills of oysters exposed to intermittent hypoxia under fluctuating

**Table 1**
Analysis of the effects of the experimental treatments on the bioenergetic and oxidative stress markers in the gills and the digestive gland tissues of *C. gigas*. The treatment effects were tested by ANOVA (a), Kruskal-Wallis test (k) or Welch ANOVA (w). Significant effects are highlighted in bold.

| Trait | Gill | DG |
|---|---|---|
| ETS activity | $F(3,39) = 5.700$ **p = 0.002**[a] | $F(3,35) = 50.384$ **p < 0.001**[a] |
| Glycogen | $F(3,17.2) = 2.757$ p = 0.074[a] | $\chi^2 = 15.619$ **p = 0.001**[k] |
| Proteins | $F(3,38) = 18.082$ **p < 0.001**[a] | $F(3,39) = 0.486$ p = 0.694[a] |
| Lipid peroxidation products | $F(3,13.5) = 6.819$ **p = 0.005**[a] | $F(3,10.5) = 3.687$ **p = 0.048**[w] |
| Protein carbonyls | $F(3,10.4) = 1.746$ p = 0.218[a] | $Chi2 = 2.695$ p = 0.441[k] |
| Total antioxidant capacity | $F(2,25) = 0.337$ p = 0.717[a] | $F(2,6.6) = 0.729$ p = 0.518[w] |

temperature conditions, at 15 °C, and at 30 °C, respectively (Table 3, Supplementary Table). Metabolic pathways such as alanine, aspartate, and glutamate metabolism, arginine biosynthesis, tryptophan metabolism, and tyrosine metabolism were affected in all exposure groups (Table 3, Fig. 4 A, B, C). Glycine, serine, and threonine metabolism were altered by intermittent hypoxia at 15 °C, while the TCA cycle changed in response to intermittent hypoxia exposure at 30 °C. In the gills of oysters exposed to intermittent hypoxia under the fluctuating temperature regime, alterations in phenylalanine, tyrosine, and tryptophan biosynthesis, arginine and proline metabolism, and butanoate metabolism were detected (Fig. 4 C).

### 3.5. TCA and urea cycle metabolites

The combined exposure of oysters to intermittent hypoxia and heat at 30 °C resulted in an upregulation of aconitate and (iso)citrate, and a decrease in succinate levels in the gills (Table 4). Additionally, carnitine concentrations were elevated in the gills of oysters exposed to intermittent hypoxia at 30 °C (Table 4). However, there were no significant changes in the concentrations of TCA intermediates and carnitine in other experimental treatment groups compared to the control, except for a decrease in succinate levels observed in all experimental treatments (Table 4). Citrulline content in the gills significantly increased (by 12.8-fold) under the fluctuating temperature regime compared to control (Table 4).

### 3.6. Levels of free amino acids

All exposures led to a significant decrease in tryptophan and tyrosine (Table 4). Moreover, exposure of oysters to hypoxia at 15 °C resulted in a significant increase in the concentrations of taurine, glutamine, glycine, asparagine, and lysine content in the gill tissues (Table 4). Combined exposure to intermittent hypoxia and 30 °C led to an increase in the content of aspartate and asparagine, but a depletion of alanine and proline, in the oyster gills (Table 4). Furthermore, intermittent hypoxia at fluctuating temperature resulted in a significant decrease in the gill content of taurine, alanine, phenylalanine and proline (Table 4).

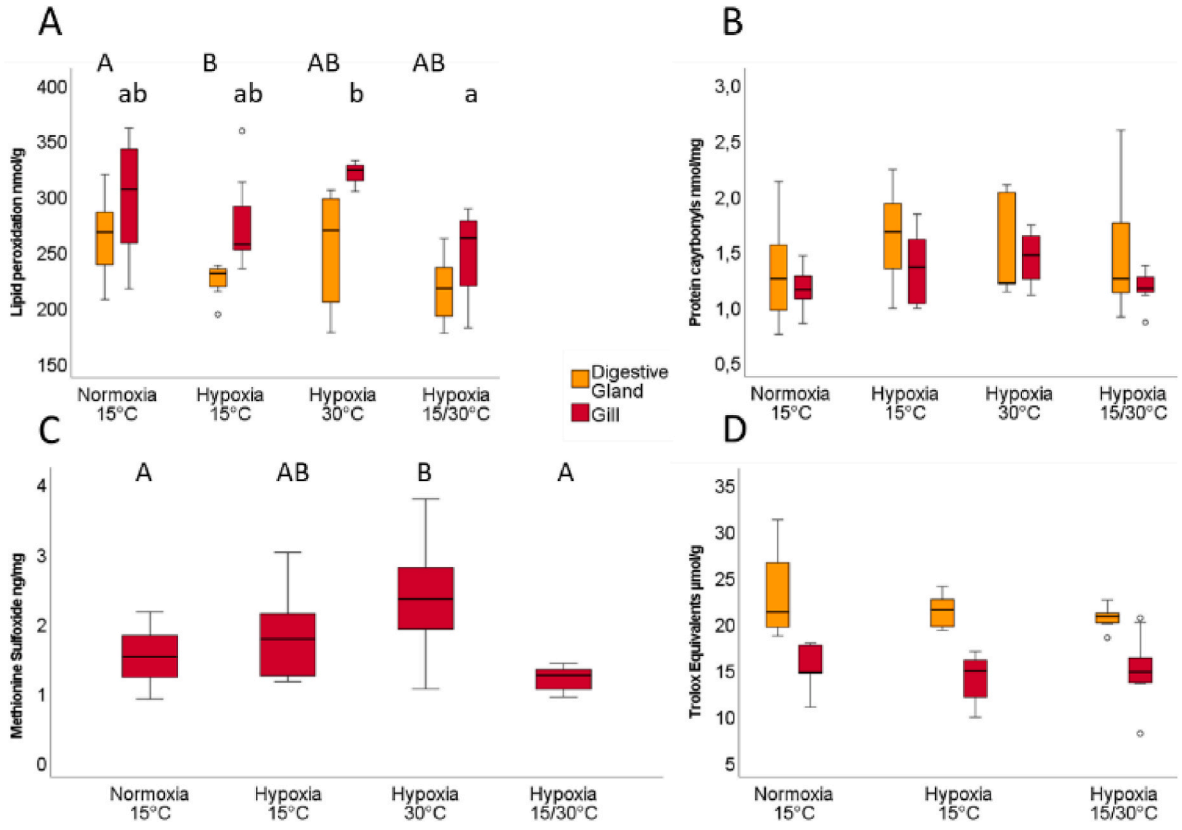



**Fig. 2.** Oxidative stress markers: lipid peroxidation in thiobarbituric acid-reactive substances equivalents (A), protein carbonyls (B), methionine sulfoxide (C) and total antioxidant capacity in trolox equivalents (D) are shown for digestive gland (yellow, upper case letters) and gills (red, lower case letters) and scaled to tissue fresh mass; columns that do not share the same letter are significantly different ($p < 0.05$); box plots show median, interquartile range (IQR) as well as maximum and minimum; outliers (>1.5x IQR) are indicated by circles.

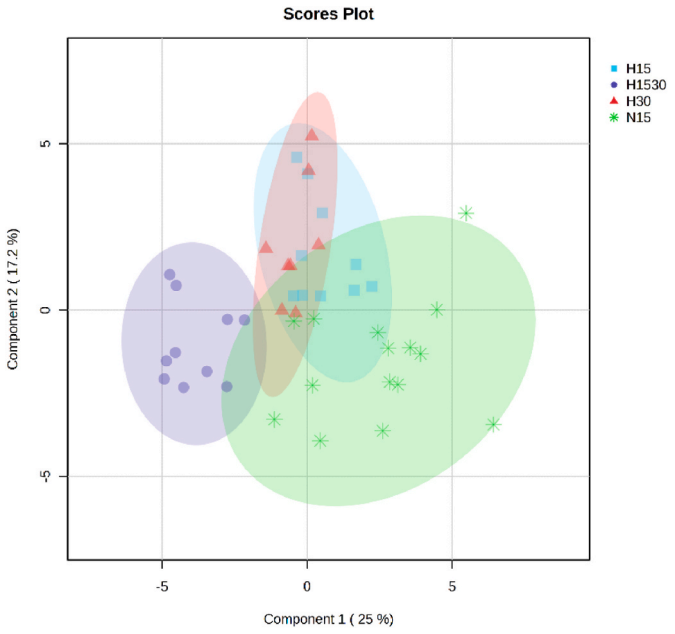



**Fig. 3.** PLS-DA scores plot: results for the first two components based on LC-MS data of the gill samples. Hypoxia exposure combined with fluctuating temperature (H1530) is separated along the second component from hypoxia exposure with constant normal (H15) or elevated (H30) temperature. Normoxic control (N15) and the hypoxia exposures are separated along the first component.

## 4. Discussion

### 4.1. Temperature regime modulates bioenergetic effects of intermittent hypoxia

Oysters can tolerate short hypoxic events without adverse effects on their energy metabolism (Ivanina and Sokolova, 2016; Bayne, 2017b; Haider et al., 2020). Hypoxic survival in oysters and other hypoxia-tolerant facultative anaerobes is commonly associated with metabolic rate suppression (Hochachka, 1986, 1988), which is enhanced in hypometabolic states caused by low temperatures (Gorr, 2017). In our present study, exposure of *C. gigas* to intermittent hypoxia at a low temperature (15 °C) had little impact on tissue bioenergetic parameters. Specifically, there was no change in the electron transport system (ETS) activity, which serves as an index of cellular energy demand, and glycogen was preserved in both gill and digestive gland tissues. In addition, the level of AMP in the gill tissue remained stable, indicating that the energy status of the cell was not severely compromised (Hardie, 2003). A similar pattern was observed in oysters exposed to intermittent hypoxia at a constant elevated temperature (30 °C), where ETS activity, glycogen reserves, and AMP levels remained stable.

The lack of negative shifts in tissue energy status at 15 °C may be attributed to the combination of cold-induced hypometabolism and the metabolic rate suppression during hypoxic periods, which are typical for intertidal bivalves like oysters (Storey and Storey, 1990; Storey and Churchill, 1995; Samain, 2011; Bayne, 2017d). However, at a constant elevated temperature (30 °C), higher energy expenditure is expected during both hypoxic and recovery phases, which could potentially lead to energy deficiency, particularly since feeding is restricted to the recovery periods. This deficiency may be supplied by the breakdown of glycogen, which serves as the primary energy fuel for anaerobic metabolism during emersion (de Zwaan and Wijsman, 1976; Bayne, 2017c). Despite the energetically challenging conditions of heat combined with intermittent hypoxia, the lack of glycogen loss indicates a low rate of glycogen breakdown during emersion in hypoxia-adapted organisms like oysters (Gäde et al., 1975; Ivanina et al., 2016; Bayne, 2017b; Janas et al., 2017) and/or a rapid glycogen resynthesis during the recovery

**Table 2**
PLS-DA loadings of selected metabolites (cutoff ±0.2). Loadings exceeding the cutoff are presented bold.

| | C1 | C2 |
|---|---|---|
| Aconitate | 0.0387 | **0.2986** |
| Alanine | **0.2649** | 0.0957 |
| Aspartate | 0.0937 | **0.3305** |
| Citrulline | **−0.2613** | −0.0460 |
| Glutamine | −0.0914 | **0.2415** |
| Lysine | −0.0948 | **0.2394** |
| Taurine | **0.2161** | **0.2550** |
| Tryptophan | **0.3039** | −0.0667 |
| Tyrosine | **0.2987** | 0.01500 |
| AMP | 0.1770 | **0.2011** |
| Asparagine | −0.0356 | **0.3414** |
| (Iso)citrate | 0.0843 | **0.3362** |
| Glutamate | **0.2670** | 0.1765 |
| Isoleucine | **0.3248** | 0.0041 |
| Leucine | **0.3179** | 0.0048 |
| Malate | −0.0098 | **0.2232** |
| Methionine sulfoxide | 0.0373 | **0.2807** |
| Phenylalanine | **0.3196** | 0.0577 |
| Succinate | **0.2466** | −0.1634 |
| Threonine | 0.0483 | **0.2757** |
| Valine | **0.3260** | −0.0197 |

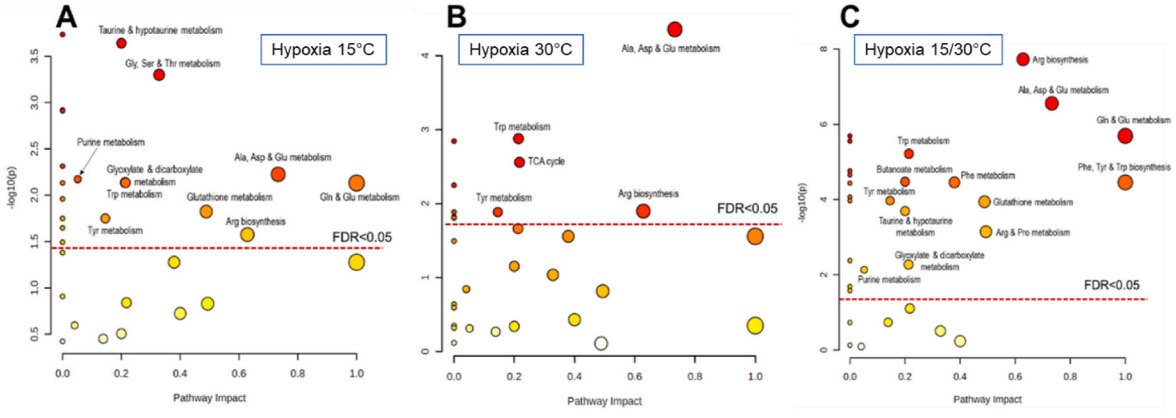



**Fig. 4.** Pathway enrichment: analyses based on based on LC-MS data of gill samples. Hypoxia exposure at constant normal (15 °C, A), elevated (30 °C, B) or fluctuating (15/30 °C, C) temperature are compared to normoxic control at 15 °C. Y axis: log10 (p), X axis: pathway impact. Horizontal red dashed line indicates the false discovery rate (FDR) defined as the threshold p value (0.05) after adjustment for multiple comparisons. Symbol size corresponds to the pathway impact, colour shading (yellow to red) reflects the relative significance (with red showing the highest significance).

**Table 3**
Summary of the significantly enriched pathways in different experimental treatment groups relative to the normoxic controls. "+" indicates significant differences for the respective treatment relative to the control (FDR<0.05). "-" indicates that the certain pathway was not significantly altered in the respective treatment group relative to the control (FDR>0.05). Only pathways with the impact >0 were considered.

| | Hypoxia 15 °C | Hypoxia 30 °C | Hypoxia 15/ 30 °C |
|---|---|---|---|
| Alanine, aspartate and glutamate metabolism | + | + | + |
| Arginine biosynthesis | + | + | + |
| Tryptophan metabolism | + | + | + |
| Tyrosine metabolism | + | + | + |
| D-Glutamine and D-glutamate metabolism | + | – | + |
| Glutathione metabolism | + | – | + |
| Glyoxylate and dicarboxylate metabolism | + | – | + |
| Purine metabolism | + | – | + |
| Taurine and hypotaurine metabolism | + | – | + |
| Phenylalanine metabolism | – | – | + |
| Phenylalanine, tyrosine and tryptophan biosynthesis | – | – | + |
| Arginine and proline metabolism | – | – | + |
| Butanoate metabolism | – | – | + |
| Glycine, serine and threonine metabolism | + | – | – |
| Citrate cycle (TCA cycle) | – | + | – |
| Total # of altered pathways | 10 | 5 | 13 |

phase (Ouillon et al., 2022). It is worth noting that due to the high mortality (~60%) in co-exposures to constant elevated temperature and hypoxia, the less tolerant individuals (and those that presumably show the largest disruptions of metabolic and energetic bias) were likely those that died first leading to the survivor bias to the more stress-tolerant individuals in our data set. Overall, our findings suggest that oysters are capable of maintaining their energy homeostasis when exposed to intermittent hypoxia at a constant temperature of 15 °C, and a certain fraction of the population is also capable of doing so when co-exposed to intermittent hypoxia at a constant temperature of 30 °C.

In contrast to the unchanged energy status during exposure to intermittent hypoxia at constant temperatures, we detected negative energetic shifts in oysters co-exposed to hypoxia and fluctuating temperature. Aerobic capacity (ETS) and glycogen levels decreased significantly in this group. Glycogen depletion in the digestive gland is especially meaningful, as it is a key storage site for this carbohydrate (de Zwaan and Wijsman, 1976; Bayne, 2017c). These findings indicate higher energy demand during hypoxic episodes in the oysters exposed to the combination of hypoxia and fluctuating temperature relative to other hypoxia-exposed groups. The glycogen used up during hypoxia does not appear to be resynthesized during recovery (possibly due to the low temperature in the normoxic phase) leading to a mismatch between the glycogen utilization and synthesis and a decline in the overall glycogen content of the digestive gland. Further, the AMP decrease in the gills might signal misbalance of the cellular energy status (Hardie, 2003). Alternatively, this decrease may be a result of enhanced synthesis rates of nucleic acids, since AMP is a precursor of RNA (Jauker et al., 2015). Upregulated RNA synthesis would align with the observed protein accumulation in the gills of this exposure group (Bolster et al., 2002; Horman et al., 2002; Chan et al., 2004; Rider, 2016). Taken together, these findings show high energy costs of survival in the fluctuating environments for the oysters, and indicate that upregulation of protein synthesis might contribute to this costs as shown in other organisms in extreme fluctuating environments (Blewett et al., 2022).

### 4.2. Effects on oxidative stress and protein levels

The impact of intermittent hypoxia on the protein content of oysters tissues varied depending on the tissue and temperature conditions. Protein content of the digestive gland remaining unchanged across all treatments, indicating a balance between protein synthesis and degradation. However, the gill protein content varied in different hypoxia-temperature treatments. Exposure to intermittent hypoxia combined with a constant elevated temperature (30 °C) resulted in gill protein loss accompanied by elevated levels of methionine sulfoxide (MetO), an oxidized form of the amino acid methionine. Both markers indicate proteome damage. Methionine is particularly susceptible to oxidation by reactive oxygen and nitrogen species (Maisonneuve et al., 2009; Lee and Gladyshev, 2011), and thus might be more sensitive marker of oxidative damage than protein carbonyl levels that did not change in any of our experimental treatments. The protein loss and oxidative damage observed in the gill tissue under the combined hypoxia and heat stress may have negative consequences for the function of this vital organ and, ultimately, the survival and performance of the organism (Friedman et al., 1991, 2005; Goulletquer et al., 1998; Bouchet et al., 2007; Samain, 2011). It is worth noting that the observed effects of hypoxia-heat combination may underestimate the physiological stress experienced by oysters under these conditions, as the high mortality observed in this treatment likely resulted in a survivor bias, with only the most resilient individuals being included in the analyses (Ioannidis, 2005).

Unexpectedly, the increase in protein accumulation in the gill of oysters exposed to intermittent hypoxia and fluctuating temperatures indicate that protein synthesis outstrips the degradation. The mechanisms behind this increase are not fully understood. Dehydration and water loss as a reason for protein accumulation (McCarthy et al., 2013) can be excluded since we did not see a corresponding increase in the concentrations of other biomolecules. Instead, it is more likely that the elevated protein content in the gills is due to the de novo synthesis of protective proteins, such as heat shock proteins (HSPs). HSPs accumulate in response to various stressors, including elevated temperatures and hypoxia, and can account for up to 10% of the total protein mass (Finka and Goloubinoff, 2013; Karademir and Sari-Kaplan, 2018). In oysters, a strong upregulation of HSPs has been reported after exposure to hypoxia or temperature stress, suggesting that HSP accumulation may explain the elevated protein content in the gills (David et al., 2005; Ivanina et al., 2009; Kawabe and Yokoyama, 2012). Interestingly, no protein accumulation was observed in the digestive gland, which may reflect its higher hypoxia tolerance and delayed stress response compared to the gills (Oehler et al., 2000; David et al., 2005; Willmer et al., 2005; Kawabe and Yokoyama, 2012).

There was no evidence for the oxidative damage to the membrane lipids or change in total antioxidant capacity induced by the exposures to intermittent hypoxia regardless of the temperature (constant 30 °C not tested due to the lack of samples). Taken together, our data indicate that oxidative stress is not a major contributor to the physiological stress response of *C. gigas* under the studied exposure conditions, consistent with generally high stress tolerance of this species (Samain, 2011; Zhang et al., 2012; Bayne, 2017a).

### 4.3. Hypoxia-induced shifts in oyster metabolome: amino acid metabolism

Gluconeogenesis was strongly modulated by intermittent hypoxia in oysters as demonstrated by shifts in the Ala, Asp and Glu metabolism. Specifically, certain temperature combinations resulted in increased levels of key amino acids such as Asn, Asp, and Glu, while Gln and Ala showed a decrease. Given that we collected samples after 12 h of normoxic recovery, stimulation of gluconeogenesis might assist in replenishing of glycogen stores used during hypoxia like shown in another hypoxia-tolerant bivalve, the soft-shell clam *Mya arenaria* (Picard et al., 2014; Ouillon et al., 2022). However, glycogen resynthesis was

**Table 4**
Effects of the experimental treatments on the concentrations of metabolites in the gills of *C. gigas*. Tests: A -ANOVA, KW - Kruskal-Wallis test, W – Welch ANOVA. Experimental groups: N - normoxia, H15 - cyclic hypoxia at 15 °C, H30 - cyclic hypoxia at 30 °C, H15/30 - cyclic hypoxia with immersion at 15 °C and emersion at 30 °C. Different letters in "Post hoc" column indicates significant differences between the treatment groups listed in the order: N – H15 – H30 – H15/30. Methionine sulfoxide data are also presented in Fig. 2. "n" specifies number of replicates.

| Metabolite | Test | Statistic | Average, ng/mg ± standard error | | | | Post hoc |
|---|---|---|---|---|---|---|---|
| | | | N | H15 | H30 | H15/30 | |
| Aconitate | A | $F(3,38) = 4.8$ $p = 0.006$ | 26.41 ± 3.1 (n = 15) | 32.11 ± 2.6 (n = 9) | 47.34 ± 7.7 (n = 8) | 28.11 ± 2.8 (n = 10) | A-AB-B-A |
| Alanine | A | $F(3,37) = 30.9$ $p < 0.001$ | 138.99 ± 10.5 (n = 15) | 157.11 ± 7.1 (n = 10) | 75.55 ± 5.8 (n = 7) | 43.78 ± 7.4 (n = 9) | A-A-B-B |
| AMP | A | $F(3,35) = 5.1$ $p = 0.005$ | 222.37 ± 9.6 (n = 12) | 207.14 ± 14.8 (n = 10) | 214.79 ± 11.9 (n = 8) | 164.78 ± 7.3 (n = 9) | AB-ABC-B-C |
| Arginine | A | $F(3,37) = 0.5$ $p = 0.652$ | 42.53 ± 3.0 (n = 15) | 39.21 ± 3.6 (n = 9) | 44.48 ± 4.8 (n = 8) | 45.27 ± 1.9 (n = 9) | Not tested |
| Asparagine | KW | $Chi2 = 17.5$ $P < 0.001$ | 5.13 ± 0.43 (n = 14) | 7.97 ± 0.86 (n = 10) | 9.90 ± 1.26 (n = 8) | 5.85 ± 0.42 (n = 9) | A-B-B-AB |
| Aspartate | A | $F(3,38) = 14.1$ $p < 0.001$ | 505.13 ± 36.7 (n = 15) | 615.24 ± 25.0 (n = 10) | 744.48 ± 55.9 (n = 8) | 387.97 ± 22.5 (n = 9) | AC-AB-B-C |
| Carnitine | KW | $Chi2 = 19.5$ $P < 0.001$ | 46.44 ± 3.2 (n = 14) | 43.46 ± 2.0 (n = 8) | 85.49 ± 4.2 (n = 8) | 47.43 ± 2.6 (n = 9) | A-A-B-A |
| (Iso)citrate | A | $F(3,37) = 6.1$ $p = 0.002$ | 337.11 ± 48.9 (n = 15) | 504.76 ± 47.3 (n = 9) | 546.33 ± 61.2 (n = 7) | 266.73 ± 40.8 (n = 10) | AC-AB-B-C |
| Citrulline | KW | $Chi2 = 28.3$ $P < 0.001$ | 0.20 ± 0.009 (n = 14) | 0.27 ± 0.019 (n = 8) | 0.29 ± 0.015 (n = 8) | 2.62 ± 0.113 (n = 9) | A-A-A-B |
| Cystine | KW | $Chi2 = 9.7$ $P = 0.021$ | 0.30 ± 0.040 (n = 15) | 0.41 ± 0.067 (n = 10) | 0.28 ± 0.087 (n = 8) | 0.15 ± 0.030 (n = 9) | AB-A-AB-B |
| GABA | A | $F(3,37) = 1.1$ $p = 0.379$ | 18.45 ± 3.9 (n = 14) | 26.54 ± 7.1 (n = 9) | 13.45 ± 4.4 (n = 8) | 16.30 ± 5.0 (n = 10) | Not tested |
| Glutamate | W | $F(3,19.5) = 31.1$ $p < 0.001$ | 266.95 ± 17.7 (n = 15) | 265.90 ± 9.9 (n = 9) | 258.59 ± 15.3 (n = 8) | 151.34 ± 8.5 (n = 10) | A-A-A-B |
| Glutamine | KW | $Chi2 = 11.1$ $P = 0.011$ | 16.82 ± 1.5 (n = 15) | 31.94 ± 4.5 (n = 10) | 20.06 ± 2.2 (n = 8) | 26.42 ± 4.4 (n = 10) | A-B-AB-AB |
| Glycine | A | $F(3,39) = 6.1$ $p = 0.002$ | 102.32 ± 6.9 (n = 15) | 145.87 ± 7.5 (n = 10) | 109.93 ± 8.9 (n = 8) | 119.37 ± 8.1 (n = 10) | A-B-A-AB |
| GSH | A | $F(3,38) = 0.5$ $p = 0.71$ | 59.2 ± 5.3 (n = 15) | 66.32 ± 6.7 (n = 10) | 68.05 ± 7.8 (n = 8) | 65.85 ± 4.8 (n = 9) | Not tested |
| Histidine | A | $F(3,34) = 1.6$ $p = 0.219$ | 5.94 ± 0.36 (n = 14) | 6.59 ± 0.32 (n = 8) | 5.63 ± 0.40 (n = 8) | 5.45 ± 0.32 (n = 8) | Not tested |
| Isoleucine | W | $F(3,20.8) = 15.5$ $p < 0.001$ | 10.03 ± 0.04 (n = 15) | 7.86 ± 0.52 (n = 10) | 7.83 ± 0.54 (n = 8) | 4.53 ± 0.44 (n = 10) | A-A-A-B |
| Lactate | A | $F(3,36) = 1.1$ $p = 0.358$ | 33.2 ± 2.4 (n = 13) | 30.89 ± 2.2 (n = 9) | 33.15 ± 3.2 (n = 8) | 38.39 ± 3.8 (n = 10) | Not tested |
| Leucine | KW | $Chi2 = 18$ $P < 0.001$ | 11.64 ± 0.93 (n = 15) | 8.92 ± 0.45 (n = 10) | 8.54 ± 0.61 (n = 8) | 5.85 ± 0.64 (n = 10) | A-AB-AB-B |
| Lysine | W | $F(3,17.8) = 4.2$ $p = 0.021$ | 16.29 ± 1.5 (n = 15) | 31.33 ± 4.5 (n = 10) | 19.31 ± 2.2 (n = 8) | 26.29 ± 4.4 (n = 10) | A-B-AB-AB |
| Malate | A | $F(3,37) = 5.9$ $p = 0.002$ | 3980 ± 436 (n = 14) | 5726 ± 4716 (n = 10) | 6270 ± 412 (n = 8) | 3700 ± 666 (n = 9) | A-AB-B-A |
| Methionine | KW | $Chi2 = 5$ $P = 0.173$ | 2.14 ± 0.20 (n = 15) | 2.01 ± 0.24 (n = 10) | 2.47 ± 0.16 (n = 8) | 1.84 ± 0.20 (n = 10) | Not tested |
| Methionine Sulfoxide | A | $F(3,38) = 7.6$ $p < 0.001$ | 1.51 ± 0.11 (n = 15) | 1.79 ± 0.19 (n = 10) | 2.35 ± 0.29 (n = 8) | 1.20 ± 0.06 (n = 9) | A-AB-B-A |
| OH-Proline | A | $F(3,36) = 2.5$ $p = 0.078$ | 2.82 ± 0.27 (n = 14) | 3.91 ± 0.53 (n = 9) | 4.03 ± 0.27 (n = 8) | 4.27 ± 0.70 (n = 9) | Not tested |
| Ornithine | A | $F(3,36) = 3.1$ $p = 0.04$ | 6.62 ± 0.44 (n = 14) | 5.25 ± 0.45 (n = 9) | 6.78 ± 0.28 (n = 7) | 5.62 ± 0.33 (n = 10) | Not tested |
| Phenylalanine | W | $F(3,21.1) = 17.3$ $p < 0.001$ | 11.04 ± 0.81 (n = 15) | 9.72 ± 0.51 (n = 10) | 8.97 ± 0.46 (n = 8) | 5.79 ± 0.43 (n = 10) | A-A-A-B |
| Proline | KW | $Chi2 = 25.4$ $P < 0.001$ | 77.75 ± 13.3 (n = 15) | 122.75 ± 9.2 (n = 9) | 17.32 ± 2.4 (n = 7) | 27.03 ± 7.1 (n = 8) | A-A-B-B |
| Serine | A | $F(3,38) = 6.7$ $p = 0.001$ | 13.73 ± 0.9 (n = 15) | 14.97 ± 0.8 (n = 9) | 18.15 ± 1.8 (n = 8) | 21.53 ± 2.0 (n = 14) | A-A-AB-B |
| Succinate | W | $F(3,15.5) = 13.8$ $p < 0.001$ | 61.13 ± 11.69 (n = 14) | 37.07 ± 1.54 (n = 10) | 8.24 ± 1.83 (n = 8) | 2.58 ± 0.33 (n = 9) | A-B-BC-C |
| Taurine | A | $F(3,33) = 57.4$ $p < 0.001$ | 9059 ± 270 (n = 12) | 11796 ± 119 (n = 7) | 8339 ± 410 (n = 8) | 6143 ± 262 (n = 10) | A-B-A-C |
| Threonine | A | $F(3,37) = 3.2$ $p = 0.035$ | 10.38 ± 0.6 (n = 14) | 14.61 ± 1.2 (n = 10) | 13.76 ± 1.9 (n = 7) | 11.09 ± 1.4 (n = 10) | Not tested |
| Tryptophan | W | $F(3,19.2) = 20$ $p < 0.001$ | 1.61 ± 0.139 (n = 15) | 1.03 ± 0.117 (n = 10) | 0.87 ± 0.067 (n = 8) | 0.59 ± 0.038 (n = 10) | A-B-B-C |
| Tyrosine | W | $F(3,21.3) = 9.1$ $p < 0.001$ | 10.17 ± 0.85 (n = 15) | 7.27 ± 0.52 (n = 10) | 6.86 ± 0.43 (n = 8) | 4.83 ± 0.56 (n = 10) | A-B-B-C |
| Valine | W | $F(3,18.7) = 13$ $p < 0.001$ | 10.67 ± 0.74 (n = 15) | 8.29 ± 0.40 (n = 10) | 7.92 ± 0.12 (n = 6) | 5.22 ± 0.52 (n = 10) | A-B-B-C |

insufficient to fully compensate for the breakdown in the digestive gland of the oysters exposed to hypoxia-heat combination. Apart from that, our findings agree with the earlier studies that report enhanced carbohydrate metabolism during hypoxia in human endothelial cells (Oehler et al., 2000), polychaetes (Kamp and Juretschke, 1989) and bivalves (Isani et al., 1989; Greenway and Storey, 1999).

Arginine biosynthesis was modulated in all exposure groups demonstrated by shifts of the key amino acids Asp, Gln, Glu and citrulline. A modulation of urea cycle is unlikely as urea contributes only a small fraction of the total nitrogen excretion in oysters (Bayne, 2017b). Therefore, alteration of Arg biosynthesis under hypoxic conditions might reflect other functions of the pathway intermediates such as the role synthesis of phosphagens (phospho-L-arginine) or signaling functions. Interestingly, we detected a massive increase (12.8 times) of citrulline in oysters exposed to hypoxia and fluctuating temperature compared to the normoxic controls. In *C. gigas*, the conversion of Arg to citrulline via nitric oxide synthase forms nitric oxide radicals (NO·) which acts as an important signaling molecule for metabolic regulation, immune response, and inflammation in different animals (Jacklet, 1997; Scholz and Truman, 2000; Hermes-Lima, 2004; Storey, 2004; Lambert et al., 2007; Swamy et al., 2010). Nitric oxide is involved in metabolic suppression and cytoprotection during hypoxia in bivalves (Strahl and Abele, 2020) and vertebrates (Fu et al., 2013; Heidorn et al., 2018), respectively. Thus, the high citrulline accumulation observed in our study might reflect elevated NO production (Johansson and Carlberg, 1995) to support metabolic arrest during the hypoxic phase and provide cellular protection during the recovery. The lack of citrulline accumulation in the oysters exposed to hypoxia under constant temperature regime indicates that unlike the fluctuating temperature and hypoxia combinations that mimic summer tidal conditions, less realistic scenarios of intermittent hypoxia at constant temperature might not provide all the necessary cues to trigger this physiological response in oysters.

Gill tissue levels of essential branched-chain amino acids (leucine, isoleucine and valine; BCAAs) and aromatic amino acids (phenylalanine, tyrosine, tryptophan; AAAs) decreased in all hypoxia exposures in the oysters (Manahan, 1990; Fitzgerald and Szmant, 1997). BCAAs are critical for cellular metabolism, growth, and stress signaling, as well as stimulating protein synthesis and inhibiting proteolysis, which aids protein deposition (De Bandt and Cynober, 2006; Holeček, 2018; Nambara et al., 1998). Aromatic amino acids (AAAs) play a crucial role in protein synthesis (Han et al., 2019; Li et al., 2021, 2023) and serve as

precursors for signaling molecules including hormones and neurotransmitters (Kutchko and Siltberg-Liberles, 2013). Our data do not allow determining whether the observed decline in BCAAs and AAAs are due to their impaired uptake from the food, increased incorporation into the proteins or elevated catabolism for energy during hypoxia (Willmer et al., 2005; Haider et al., 2019). Regardless of the underlying mechanisms, the decrease in the tissue levels of these important amino acids might have negative consequences for protein synthesis, and endocrine and neural function of oysters (Kutchko and Siltberg-Liberles, 2013; Adeva-Andany et al., 2017).

### 4.4. Temperature modulates TCA cycle in combined exposures

PLS-DA analysis showed significant shifts in the TCA cycle metabolite profiles along the 2nd component that could be assigned to temperature effects. Further, pathway enrichment analysis identified the TCA cycle as a pathway significantly altered in the oysters co-exposed to intermittent hypoxia with constant elevated temperature. Here, three of the measured key metabolites of this pathway (aconitate, (iso)citrate and malate) increased in the oyster gills. These findings indicate activation of TCA cycle during temperature stress, possibly to support the elevated energy demand or biosynthetic needs of the cells. Furthermore, TCA cycle metabolites are important for retrograde signaling between the mitochondria and other cell components (Martínez-Reyes and Chandel, 2020) and regulation of the immune response (reviewed in Choi et al., 2021). Accumulation of the TCA metabolites might therefore signal the heat-induced mitochondrial damage (Choi et al., 2021) and stimulate immune response and other cellular protective mechanisms as already reported for other invertebrates (Wang et al., 2008; Matozzo and Marin, 2011; Catalán et al., 2012; Dang et al., 2012; Applebaum et al., 2014; Bayne, 2017d). Further investigations are needed to determine whether TCA-related signaling plays a role in this immune activation. Notably, exposure to fluctuating temperature did not lead to accumulation of TCA cycle intermediates in hypoxia-exposed oysters indicating milder mitochondrial stress in this environmentally relevant exposure scenario.

The only studied TCA metabolite that consistently responded to intermittent hypoxia rather than temperature stress, was succinate, which was consistently depleted in all hypoxia-exposed groups compared to the normoxic baseline. Succinate is a common anaerobic end product that accumulates during hypoxia in marine bivalves (Schiedek, 1997a, 1997b; Storey, 2004; Bayne, 2017b; Haider et al., 2020) and is rapidly depleted during recovery (Ouillon et al., 2021, 2022). This depletion often demonstrates overshoot with succinate levels decreasing below the normoxic baseline (Ouillon et al., 2021, 2022), similar to the pattern found in our present study. This might reflect overactivation of respiration (the so called oxygen debt) commonly observed after hypoxia and interpreted as the metabolic costs of restoring cellular and systemic homeostasis disrupted by hypoxia (Herreid, 1980; Ellington, 1983). This hypothesis is supported by the enhanced mitochondrial ability to oxidize succinate during post-hypoxic recovery in *C. gigas* (Adzigbli et al., 2022) and might be an adaptive mechanism that allows this hypoxia-tolerant species to use accumulated anaerobic end-product (succinate) as a mitochondrial fuel to rapidly restore ATP levels.

### 4.5. Taurine metabolism is modified by temperature and hypoxia

Loadings of taurine were above the threshold for the two first PLS-DA components, indicating combined effects of hypoxia and temperature on this metabolite. Pathway enrichment analysis also identified taurine and hypotaurine metabolism as a significantly modulated pathway in hypoxia exposures combined with normal or fluctuating temperature involving an increase or decrease in taurine content, respectively. In bivalves including oysters, taurine acts as an important compatible osmolyte (Yancey, 2005). Furthermore, taurine enhances mitochondrial performance and mitigates the mitochondrial ROS production in bivalves and other organisms (Hansen et al., 2006; Jong et al., 2012; Bin et al., 2017; Sokolov and Sokolova, 2019). In cross-species comparisons of bivalves, high taurine levels were associated with higher tolerance to hypoxia-reoxygenation stress (Haider et al., 2020). Depletion of taurine might therefore partially explain the lower mitochondrial performance (shown by suppressed ETS activity levels) in oysters exposed to intermittent hypoxia combined with fluctuating temperature observed in our present study. No decrease in ETS activity was found in the other two experimental groups that maintained normal or elevated taurine concentrations. Overall, our present study and earlier published research indicate that *C. gigas* effectively maintain taurine homeostasis with only mild variation under most stress scenarios (Hummel et al., 1996; Kube et al., 2007; Haider et al., 2020). This ability might contribute to exceptionally high stress tolerance of *C. gigas* (Zhang et al., 2012; Bayne, 2017a) and requires further investigation.

## 5. Conclusion and outlook

Our present study demonstrated that intermittent hypoxia combined with fluctuating temperature caused metabolic shifts in *C. gigas*, which was accompanied by depletion of energy reserves. Studies show that 30 °C is the upper thermal limit of *C. gigas* (Le Gall and Raillard, 1988; Bougrier et al., 1995), albeit submerged North Sea oysters can survive this temperature for at least three days without mortality (Bruhns, unpublished data). However, the combination of this high temperature with cyclic hypoxia exceeded the oysters' stress tolerance leading to tissue damage and high mortality. In contrast, exposure to cyclic hypoxia at 15 °C caused metabolic shifts in different pathways in the Pacific oyster but no negative changes in bioenergetics or oxidative stress. This study highlights the importance of investigating combined stressors affecting metabolic modulation caused by different combinations of stress intensities or modes. In general, we showed that *C. gigas* is quite robust against natural stress conditions but gains this robustness from energetically costly adaptations. Further studies are needed to test the putative mechanisms of phenotypic plasticity and their role in stress adaptation in oysters, like the role of HSPs synthesis in the protein accumulation in the gills, the implications of the altered BCAAs and AAAs metabolism in endocrine signaling and proteome maintenance, and the role of NO as a metabolic regulator during fluctuating temperature and oxygen regimes.

## Authors' statement

Conceptualization – IMS, TB
Methodology – TB, ST, IMS
Validation – TB, ST
Formal analysis - TB
Investigation – TB, ST, NF, SE
Resources – IMS, ST
Data Curation – TB, ST
Writing - Original Draft - TB
Writing - Review & Editing – TB, ST, NF, SE, ML, MW, IMS
Visualization – TB, IMS
Supervision – IMS, ML, MW
Project administration – IMS, ML, MW
Funding acquisition – IMS, ML, MW

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2023.106231.

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