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Jennifer Barbara Maria Steffen

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Reviewers:

Prof. Dr. Inna Sokolova, Marine Biology, Institute for Biological Sciences, Faculty of Mathematics and Natural Sciences, University of Rostock

Prof. Dr. Björn Philipp Lehmann, Animal Physiology, Zoological Institute and Museum, University of Greifswald

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Supervisor:

Prof. Dr. Inna Sokolova

Chair of Department of Marine Biology
Faculty of Mathematics and Natural Sciences
University of Rostock
Germany

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Abbreviations

ADP	Adenosine disphosphate
AER	Anisosmotic extracellular osmoregulation
AMP	Adenosine monophosphate
AMPK	AMP-dependent protein kinase
ANT	Adenine nucleotide translocator
AOX	Alternative oxidase
ATP	Adenosine triphosphate
BCL2	B-cell CLL lymphoma 2
BNIP3	BCL2 and adenovirus E1D 19kDa interacting protein 3
CCO	Cytochrome C oxidase
ClpB	Caseinolytic peptidase B
CO_2	Carbon dioxide
CoA	Coenzyme A
CoQ	Coenzyme Q
cytC	Cytochrome C
DEB	Dynamic energy budget theory
DO	Dissolved oxygen
DRP1	Dynamin-related protein 1
e^-	Electron
ETS	Electron transport system
FAA	Free amino acid
FEL	Fractional electron leak
Fis1	Mitochondrial fission protein 1
GC	Gas chromatography
GDP	Guanosine-5'-diphosphate
Glu	Glutamate
GOT	Glutamate oxalacetate transaminase
GPT	Glutamate pyruvate transaminase
GTP	Guanosine-5'-triphosphate
H^+	Proton
H/R	Hypoxia and reoxygenation
HPLC	High performance liquid chromatogrpahy
H_2O_2	Hydrogen peroxide
I/R	Ischemia/reperfusion
IIR	Isosmotic intracellular regulation
IMM	Inner mitochondrial membrane

IPCC	Intergovernmental Panel on Climate Change
LEAK	Mitochondrial proton leak
MCA	Metabolic control analysis
MDH	Malate dehydrogenase
Mff	Mitochondrial fission factor
Mfn2	Mitofusin 2
Mieap	Mitochondria-eating protein
mm-	Methylmalonyl-
MMP	Mitochondrial membrane potential
MQC	Mitochondrial quality control
MRD	Metabolic rate depression
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial desoxyribonucleic acid
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NIX	BNIP3-like
NMR	Nuclear magnetic resonance
O_2^-	Superoxide
OCLTT	Oxygen capacity limited thermal tolerance
OM	Organic matter
OMA1	Mitochondrial metalloendopeptidase
OMM	Outer mitochondrial membrane
OPA1	Mitochondrial dynamin-like 120 kDa protein optic atrophy 1
OXA	Oxalacetate
OXPHOS	Oxidative phosphorylation, mitochondrial respiratory state
OXPHOS CE	OXPHOS coupling efficiency
PC	Pyruvate carboxylase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PGAM5	Phosphoglycerate mutase 5
PINK1	PTEN induced kinase 1
PK	Pyruvate kinase
PKA	Protein kinase A
PKC	Protein kinase C
PL	Proton leak
PLA	Phospho-L-arginine
pmf	Proton motive force
PS	Phosphorylation subsystem

psu	Practical salinity units
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
RCR	Respiratory control ratio
ROS	Reactive oxygen species
q-RT PCR	Quantitative real-time polymerase chain reaction
SFG	Scope for growth
SFP	Aerobic scope for performance
SMR	Basal maintenance costs, standard metabolic rate
SO	Substrate oxidation
SOD	Superoxide dismutase
TCA cycle	Tricarboxylic acid cycle
UCP	Uncoupling protein
W_{max}	Maximum aerobic energy flux
α-KG	α-ketoglutarate
Δp	Protonmotive force
Δψ	Mitochondrial membrane potential

Summary

Shallow coastal habitats are highly dynamic environments characterized by strong fluctuations of multiple abiotic factors such as oxygen concentration and salinity that can negatively affect the oxygen availability of the coastal organisms. Dynamic characteristics are caused by the combined influence of sea, land and wind making these areas highly susceptible to changes. Prior literature suggests that current progression of anthropogenic influences will be detrimental to the coastal ecosystems. Sessile benthic organisms of those coastal ecosystems are most susceptible to changes due to their low motility making them heavily rely on physiological adaptation. Previous studies on coastal hypoxia mostly focused on passive survival of hypoxia-tolerant species, disregarding molecular mechanisms of functions and integrity of mitochondria, a central physiological organelle dependent on oxygen availability. To bridge this gap, this study aims to elaborate the role of mitochondrial mechanisms in hypoxia tolerance in four marine bivalve species differing in hypoxia tolerance (exceptionally hypoxia-tolerant subtidal *Arctica islandica*, strongly hypoxia-tolerant intertidal *Crassostrea gigas* and *Ostrea edulis*, moderate hypoxia-tolerant *Mytilus edulis*) and to investigate how mitochondrial mechanisms are integrated into cellular metabolism to ensure survival and recovery of hypoxia and reoxygenation stress. All bivalve species were exposed to three different oxygen treatments: short-term hypoxia (24h; <0.01% O_2), short-term hypoxia-reoxygenation stress (24h <0.01% O_2 and subsequent 1.5h 21% O_2), and normoxic control group. In addition to the oxygen treatment, *C. gigas* was acclimated to two different salinities before oxygen exposures to assess effects of multiple stressors on mitochondrial mechanisms. Bioenergetics-related parameters of mitochondria such as OXPHOS and LEAK respiration, ROS efflux and control coefficients were investigated in all treatment groups. Additional analysis of the transcriptional response of mitochondrial quality control and metabolic profiling completed the search for mechanisms of mitochondrial hypoxia tolerance in marine bivalves. Our study finds highly robust OXPHOS and ETS activity and avoidance of mitochondrial collapse supported by anaerobic metabolism in *A. islandica* and *C. gigas* characterizing a hypoxia-tolerant mitochondrial phenotype. However, mitochondrial respiration of *A. islandica* remained more susceptible to reoxygenation, which might be attributed to their more submersed lifestyle compared to intertidal life with strong oxygen fluctuations of *C. gigas*. Interestingly, additional salinity stress modulated intrinsic resilience mechanisms of mitochondria in *C. gigas*. According to our transcriptional data, *C. gigas* had a strong and controlled cellular homeostasis and higher resistance to apoptosis, while *M. edulis* relied on removal of dysfunctional mitochondria as their protective mechanism. Despite different metabolic responses of species, the ability to maintain mitochondrial function during H/R stress allowed efficient energy production and recovery in all studied species. Our findings underline the importance of mitochondrial functions in hypoxia tolerance of marine bivalves and highlight features of a hypoxia-tolerant mitochondrial phenotype that is species-specific and modulated by lifestyle and environmental changes. Further research is needed to unravel the complex interaction of respiratory control, metabolic pathways, and protection mechanisms in hypoxia tolerance of mitochondria in marine bivalves.

Zusammenfassung

Flache Küstengewässer sind hochdynamische Lebensräume, die durch starke Schwankungen zahlreicher abiotischer Faktoren wie Sauerstoffkonzentration und Salzgehalt gekennzeichnet sind, welche sich negativ auf die Sauerstoffverfügbarkeit der Küstenorganismen auswirken kann. Die dynamischen Bedingungen werden durch den kombinierten Einfluss von Meer, Land und Wind verursacht, welche diese Gebiete sehr anfällig für Veränderungen macht. Bisher vorliegende Berichte gehen davon aus, dass die derzeitige Entwicklung der anthropogenen Einflüsse sich nachteilig auf die Küstenökosysteme auswirken wird. Sessile benthische Organismen der Küstenökosysteme sind am anfälligsten für Veränderungen, da sie sich aufgrund ihrer geringen Mobilität physiologisch anpassen müssen. Frühere Studien über Hypoxie in Küstengebieten untersuchten weitgehend das passive Überleben Hypoxie-toleranter Arten und vernachlässigten molekulare Mechanismen zur Aufrechterhaltung der Funktionen und Integrität von Mitochondrien, einem zentralen physiologischen Organell, das von der Sauerstoffverfügbarkeit abhängig ist.

Deshalb zielte diese Studie darauf ab, die Rolle mitochondrialer Mechanismen bei der Hypoxie-Toleranz in vier marinen Muschelarten herauszuarbeiten, die sich in ihrer Hypoxie-Toleranz unterscheiden (die außergewöhnlich Hypoxie-tolerante subtidale *Arctica islandica*, die stark Hypoxie-tolerante intertidale *Crassostrea gigas* und *Ostrea edulis* sowie die moderate Hypoxie-tolerante *Mytilus edulis*) und zu untersuchen, wie mitochondriale Mechanismen in den zellulären Stoffwechsel integriert werden, um das Überleben und die Regenerierung bei Hypoxie- und Reoxygenierungsstress zu gewährleisten. Alle vier Muschellarten wurden drei verschiedenen Sauerstoffbedingungen ausgesetzt: kurzzeitige Hypoxie (24 Stunden; <0,01 % O_2), kurzzeitigem Hypoxie- Reoxygenierungsstress (24 Stunden <0,01 % O_2 und anschließend 1,5 Stunden 21 % O_2) und einer normoxischen Kontrollgruppe. Darüber hinaus wurde *C. gigas* vor dem Sauerstoffexperiment an zwei verschiedene Salzgehalte akklimatisiert, um mögliche Auswirkungen mehrerer Stressoren auf mitochondriale Mechanismen zu analysieren. Die bioenergetischen Parameter der Mitochondrien wie OXPHOS- und LEAK-Respiration, ROS-Efflux und Kontrollkoeffizienten wurden in allen Experimentgruppen untersucht. Die zusätzliche Analyse der transkriptionellen Reaktion der mitochondrialen Qualitätskontrolle und die Erstellung von Stoffwechselprofilen vervollständigten die Suche nach Mechanismen der mitochondrialen Hypoxie-Toleranz von marinen Muscheln. Unsere Studie zeigt eine sehr robuste OXPHOS- und ETS-Aktivität und die Vermeidung eines mitochondrialen Kollapses, unterstützt durch einen anaeroben Stoffwechsel in *A. islandica* und *C. gigas*, was einen Hypoxie-toleranten mitochondrialen Phänotyp charakterisiert. Die mitochondriale Respiration von *A. islandica* blieb jedoch anfälliger für Reoxygenierung, das auf ihre eher submerse Lebensweise im Vergleich zum Leben im Gezeitenbereich mit starken Sauerstoffschwankungen von *C. gigas* zurückzuführen sein könnte. Interessanterweise modulierte zusätzlicher Salinitätsstress die intrinsischen Resilienzmechanismen der Mitochondrien in *C. gigas*. Laut unseren Transkriptionsdaten verfügt *C. gigas* über eine starke und kontrollierte zelluläre Homöostase und eine höhere Resistenz gegen Apoptose, während

M. edulis auf die Beseitigung dysfunktionaler Mitochondrien als Schutzmechanismus angewiesen ist. Trotz der unterschiedlichen Stoffwechselreaktionen der Arten ermöglichte die Fähigkeit zur Aufrechterhaltung der Mitochondrienfunktion während des H/R-Stresses bei allen untersuchten Arten eine effiziente Energieproduktion und Erholung. Unsere Ergebnisse unterstreichen die Bedeutung mitochondrialer Funktionen für die Hypoxie-Toleranz mariner Muscheln und heben Merkmale eines Hypoxie-toleranten mitochondrialen Phänotyps hervor, der artspezifisch ist und durch Lebensweise und Umweltveränderungen moduliert wird. Weitere Forschungsarbeit ist erforderlich, um das komplexe Zusammenspiel von Atmungskontrolle, Stoffwechselwegen und Schutzmechanismen bei der Hypoxie-Toleranz von Mitochondrien in Meeresmuscheln zu entschlüsseln.

Part I: Summary of the Doctoral Study

1 Summary of the Doctoral Study

1.1 Role of bioenergetics in adaptations of marine bivalves to environmental stressors

1.1.1 Global change in the shallow ocean as a threat to integrity of coastal benthic ecosystems

The world's oceans experience a variety of natural changes, but human activities threaten to exacerbate these patterns, especially in shallow coastal waters. Coastal areas include near-shore terrestrial areas, estuaries, and coastal waters. Due to combined influence of land and sea, coastal areas are highly dynamic ecosystems. Complex interaction of riverine and terrestrial input and a strong link of deep- and surface-water in shallow coastal systems can result in dynamic shifts of environmental conditions on timescales ranging from daily to seasonal and multi-year periods (Gunderson et al., 2016). Furthermore, coastal areas are among the most productive regions in the world with major economic value, and therefore highly exploited zones susceptible to anthropogenic environmental stressors (Borgwardt et al., 2019).

Anthropogenic climate change is defined as "global-scale changes resulting from the impact of human activity on the major processes that regulate the functioning of the Biosphere" (Duarte, 2014; Steffen, 2006). As outlined by the Intergovernmental Panel on Climate Change (IPCC), carbon emissions due to fossil fuels burning and deforestation cause warming of atmosphere and ocean (IPCC, 2023). Resulting rise in the atmospheric carbon dioxide (CO_2) levels lead to acidification of ocean waters due to the CO_2 uptake and chemical reactions within the ocean carbon cycle (Caldeira and Wickett, 2003). Cooley et al., 2023 predict the pH of ocean waters to decline by 0.08 to 0.37 pH units by 2081-2100 compared to 1995-2014. Rising global temperatures of atmosphere and surface ocean waters (predicted to increase by 0.86 to 2.89°C within the next decade) decrease oxygen solubility of seawater, increase organisms' oxygen consumption, increase stratification and impair ocean circulation resulting in strong deoxygenation of global oceans (Breitburg et al., 2018; Cooley et al., 2023). Sufficient oxygen supply is a key limiting factor for survival in marine environments as oxygen solubility in seawater is 40-times lower than in air. Moreover, seawater oxygen concentration is subject to complex dynamics of oxygen consumption and influx resulting in strong fluctuations of dissolved oxygen (DO) (Gunderson et al., 2016). Warming, acidification, and deoxygenation are known as "deadly trio" that have caused mass extinction events in the past and thus rising major concerns about our future oceans (Bijma et al., 2013). By the end of the 21st century, oceans are predicted to be warmer, more acidic, with less ice coverage, higher sea levels and lower oxygen content (Duarte, 2014). Over past 60 years, the world's oceans lost approximately 2% of their oxygen store leading to a four-fold increase of hypoxic zones (Laffoley and Baxter, 2019). Specifically, a global decrease of 4.1 to 11.2% of oxygen is predicted in 2081-2100 relative to 1995-2014 depending on the climate change scenario (Cooley et al., 2023). Other factors that contribute to and amplify global changes in ocean waters include alteration of ocean mixing, nutrient

cycling, ocean productivity, and reduction of ice cover causing freshwater input into deep sea (Keeling et al., 2010). These multiple stressors are further exacerbated by pollution, eutrophication and overfishing (Hoegh-Guldberg and Bruno, 2010).

1.1.1.1 Coastal hypoxia

Oxygen deficiency occurs in aquatic environments when oxygen depletion due to respiration exceeds oxygen influx via diffusion, mixing, air-sea flux and photosynthesis. Hypoxia is commonly defined as the concentration of DO below 2 ml L^{-1} seawater (=7.4% O_2 at 25°C and salinity 20) and anoxia begins at DO < 0.01 ml L^{-1} seawater (= ~0.03% O_2, at 25°C and salinity 20) (Diaz and Rosenberg, 1995). During the night, organisms' respiration consumes oxygen decreasing oxygen concentration, while photosynthesis restores normoxic levels during daytime. Additionally, oxygen fluctuations can be caused by intertidal and seasonal rhythms (Diaz and Rosenberg, 1995; 2008). Changes in local wind mixing, cloud cover, precipitation, and evaporation exacerbate deoxygenation of shallow coastal waters by global warming (Altieri and Gedan, 2015; Capet et al., 2013; Carstensen et al., 2014; Rabalais et al., 2014; Tyler et al., 2009) (**Fig.1**).

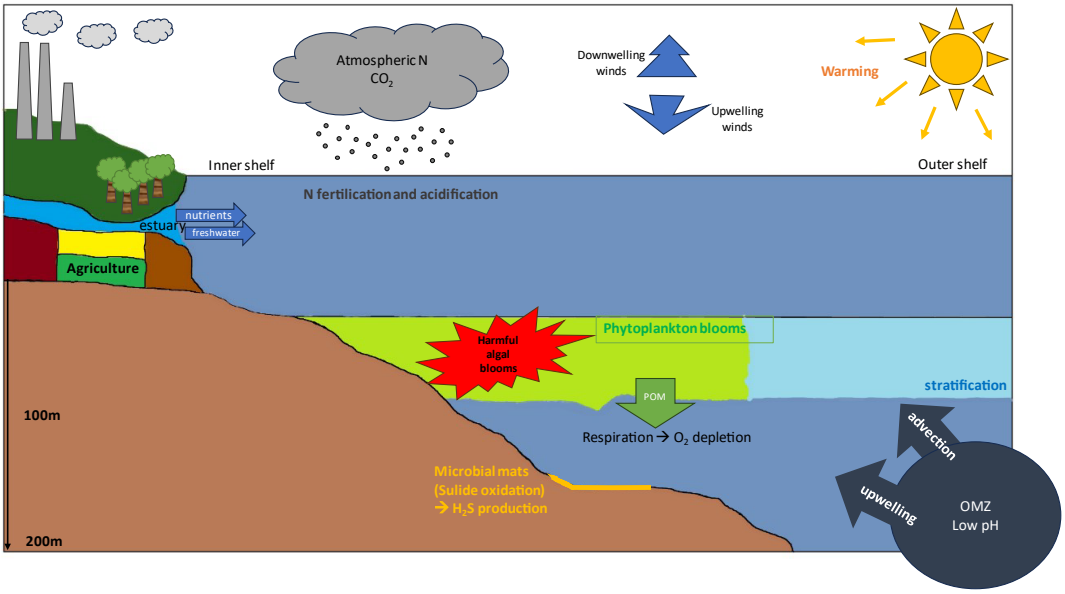

Figure 1: Mechanisms influencing hypoxia formation in benthic habitats along continental shelves (modified from Levin et al., 2009).

Intensive anthropogenic eutrophication including farming, fertilization, deforestation and wastewater discharge combined with local climate change, makes coastal regions prone to increasing hypoxia (Breitburg et al., 2018; Breitburg et al., 2019; Rabalais et al., 2009). Driven by rising eutrophication and pollution, alteration in nutrient fluxes negatively influence phytoplankton communities and thus primary production, which in turn affects coastal ecosystems (Jickells, 1998; Wallace et al., 2014). Increasing nutrient availability stimulates phytoplankton growth resulting in organic matter (OM) production exceeding metabolic capacity of metazoans. Consequently, OM settles to either the pyrocline (the layer, where density gradient is highest within water column) or sediments, where decomposition by heterotrophic bacteria increases. The OM-stimulated microbial respiration surpasses resupply of oxygen from the surface due to inhibited diffusion across density differences

in the water body resulting in intensified oxygen depletion of shallow coastal waters (Levin et al., 2009; Wallace et al., 2014) (**Fig.2**).

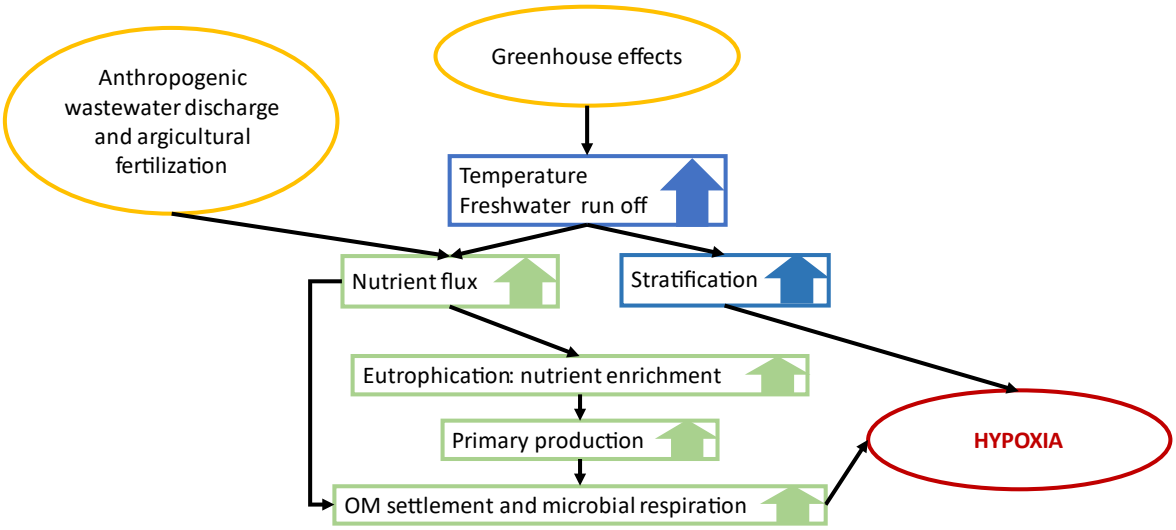

Figure 2: Development of hypoxia in shallow coastal waters.

Effects of global warming contributing to development of hypoxia in coastal waters. Causes of climate change (yellow) result in changes in water column properties (blue) and influences nutrient flux, organic matter (OM) and living organisms (green) leading to hypoxia (red) (modified from Justić et al., 2003; Wu, 2002).

In the recent decades, the rate of oxygen decline in coastal regions exceeded those in the open ocean due to the strong interaction of warming and eutrophication (Gilbert et al., 2010). However, development of hypoxia is highly dependent on individual coastal water bodies due to strong regional variation in temperature effects on precipitation, wind, riverine input, and nutrient pollution (Altieri and Gedan, 2015; Rabalais et al., 2014). Oxygen depletion is more likely in water bodies with strong stratification or isolation from oxygenated waters.

1.1.1.2 Seawater salinity

Increased precipitation, evaporation, freshwater runoff, and seasonal alterations in tidal cycle affect salinity in shallow coastal waters resulting in fluctuating salinity levels (McLusky and Elliott, 2004).On large scale, global sea surface salinity is influenced by changes of surface freshwater fluxes, while advection related to altered circulation is important on a smaller scale (Sathyanarayanan et al., 2021). Regional variation of seawater salinity is mainly shaped by a balance between evaporation and precipitation (Durack and Wijffels, 2010). The hydrological cycle defines the continuous circulation of water in the Earth-Atmosphere System involving evaporation, transpiration, condensation, precipitation, and runoff with oceans as main contributors (noaa.gov). Prior studies unanimously expect changes to the global hydrological cycle due to the anthropogenic climate change as warming of atmosphere increases its ability to store and transport water vapor (Held and Soden, 2006; Meehl et al., 2007; Solomon, 2007; Trenberth et al., 2007). These changes influence the hydrological cycle resulting in altered frequencies of precipitation and evaporation and increased freshwater influx that enhance salinity changes (Durack et al., 2012). Thus, observed

changes in salinity over past decades are connected to warming of surface ocean and an amplification of the hydrological cycle (Durack and Wijffels, 2010). Due to feedback mechanisms of atmospheric changes on precipitation and evaporation, there is a decreasing trend of seawater salinity at low and high latitudes in precipitation-dominated regions, but an increasing trend in subtropical regions, where evaporation dominates (Du et al., 2019; Durack et al., 2012; Talley, 2002).

1.1.1.3 Baltic Sea as model system to investigate the effects of multiple stressors

The unique characteristics of the Baltic Sea makes it a well suited model system to investigate effects of multiple stressors caused by climate change on shallow coastal ecosystems. Due to its natural, geographical, hydrographical, geological and climatic features the Baltic Sea ecosystem is susceptible to change (Snoeijs-Leijonmalm et al., 2017). Particularly the long water residence time, ranging from 30 to 40 years, results in slow response of hydrodynamics and biogeochemical cycles to perturbation and contributing to vulnerability to eutrophication and chemical contamination of the Baltic Sea ecosystem (Gustafsson et al., 2012). Therefore, this semi-enclosed water body is exceptionally endangered by deoxygenation. Hypoxic zones in the Baltic Sea have increased about 10-fold during the last decades, resulting in today's largest "man-made" hypoxic area of approximately 70 000 km2 (Carstensen et al., 2014; Carstensen and Conley, 2019; Conley et al., 2011). In addition, the limited water exchange with the open ocean through the narrow Kattegat together with heavy influence by freshwater runoff of surrounding landmass via >200 rivers, precipitation, and evaporation creates a strong salinity gradient, ranging from 1-2 to 20 (practical salinity units (psu)) (HELCOM, 2023). Coastal regions of the Baltic Sea are further affected by local gradients of temperature and ice cover resulting in a variety of different habitats. Future predictions foresee a warming of 2-4°C, increased precipitation by 30% and consequently increase in land and freshwater run-off through extensive drainage areas along the Baltic Sea by end of 2100, resulting in stronger contamination potential, decreased salinity and further spread of anoxic waters (Andersson et al., 2015).

1.1.2 Marine bivalves as ecosystem engineers in the coastal marine ecosystems – ecological importance and vulnerability to global change

Ecosystem engineers are species that significantly modify habitats by their activities such as reef building, bioturbation and bioirrigation (Kristensen et al., 2012; Norkko and Shumway, 2011). Marine bivalve species are important ecosystem engineers that transport particulate and dissolved material and oxygen into the sediment by bioturbation and bioirrigation (Norkko and Shumway, 2011) and create complex three-dimensional habitats through formation of biogenic reefs (Walles et al., 2015a), thereby increasing biodiversity (Peterson et al., 2003; van der Zee et al., 2012). Bivalves also facilitate bentho-pelagic coupling due to their intense filter feeding (van Leeuwen et al., 2010). Biogenic reefs created by bivalves alter water flow patterns, mitigate waves and stabilize soft-sediment (Borsje et al., 2011; Walles et al., 2015b). Further, accumulation of shells act as sink for calcium carbonate altering chemical conditions in surrounding seawater and buffering against low pH (Green et al., 2009; Waldbusser et al., 2013). These processes have major implications for the

physical and chemical characteristics of soft-sediment habitats (Meysman et al., 2006) and therefore are essential for healthy soft-sediment ecosystems (Dame et al., 2002; Jones et al., 1994; Norkko and Shumway, 2011). Population structure and abundance of bivalves strongly depend on biotic and abiotic factors (Castelli et al., 2004). Alterations in performance or abundance of ecosystem engineer species due to environmental change thus can have cascading effects on ecosystem functioning and health (Norkko et al., 2006). Therefore, understanding how ecosystem engineers, such as marine bivalves, respond to multiple stressors is crucial, as their performance significantly impacts ecosystem functioning and future biodiversity patterns (Meysman et al., 2006).

1.1.3 Role of energy metabolism in response to multiple stressors and stress tolerance

1.1.3.1 Organismal energy budget and effects of stress on energy fluxes in the organism

Living organisms are thermodynamically open systems that are dependent on an external energy source and continuous energy flow (Sokolova et al., 2012). Bioenergetics encompasses all processes of living organisms that can convert external energy sources into chemical, mechanical and transport work of the organism (Skulachev et al., 2013). The dynamic energy budget theory (DEB) hypothesizes that assimilated energy is allocated to vital processes such as basal maintenance costs, reproduction, growth, storage and activity (Kooijman, 2010; Sokolova, 2021) (**Fig. 3**). Among these processes the basal maintenance costs has the highest priority, which comprise cellular and organismal maintenance to drive vital processes (Guderley and Pörtner, 2010; Kooijman, 2010). Overall, energy availability is fundamentally limited for every organism in nature. Limits of energy availability, energy assimilation, metabolic conversion and oxygen delivery inevitably result in trade-offs between basal maintenance costs and energy demand of other fitness-related functions and can limit stress tolerance, e.g. during decreased energy supply (Guderley and Pörtner, 2010; Sokolova et al., 2012; Sokolova, 2021). To ensure survival and fitness, the organism must maintain energy homeostasis, where energy supply and demand are balanced (Sokolova et al., 2012; Sokolova, 2013; Sokolova, 2021). Environmental stress disturbs energy homeostasis by increasing basal maintenance costs due to elevated damage repair, detoxification, and stress protection mechanisms combined with decreasing energy supply caused by stress-induced lower food uptake and impaired metabolism (Sokolova et al., 2012). Ability to maintain energy homeostasis during environmental stress plays a key role in survival and tolerance strategies of marine ectotherms and consequently in population survival (Sokolova et al., 2012; Sokolova, 2013; Sokolova, 2021).

The concept of energy-limited stress tolerance focuses on environmental stress effects on bioenergetics to link cellular stress response to fitness-related performance of organisms and thus to implications on population performance and ecosystem functioning (Sokolova, 2021). Focusing on effects of environmental factors on whole organisms is common and allows to translate responses to population and ecosystem levels. However, underlying mechanisms remain unknown, which limits the ability to extrapolate results to other organisms or multiple stressors. In contrast, results of molecular investigations cannot be directly interpreted in terms of ecological consequences of multiple stressors. Thus, bioenergetics allow a link between physiological effects and ecological

consequences of environmental stress (Sokolova, 2013; Sokolova, 2021). The concept of energy limited stress tolerance integrates the DEB model and the oxygen capacity limited thermal tolerance concept (OCLTT) (Kooijman, 2010; Pörtner, 2010; Pörtner et al., 2017). According to the OCLTT, the ability to maintain aerobic scope is important for survival and differs between stress levels (Pörtner, 2010). Organismal performance during environmental stress can be divided into the four ranges: optimum, pejus, pessimum and lethal range. The so-called tolerance window varies between species, life stages and body size (Sokolova et al., 2012). Additionally, adaptation, acclimation, and acclimatization resulting in cellular adjustments can shift position and width of each range (Hochachka and Somero, 2002). The optimum range is defined by positive energy balance, where ATP production is sufficient to cover maintenance costs and other fitness related costs such as reproduction, growth, activity, development, and storage. Upon stress, the pejus range begins, where a positive energy balance remains, but basal maintenance costs increase due to elevated repair and protection processes limiting energy allocation to both, energy storage and less essential functions. Extreme, sublethal stress conditions, so-called pessimum range, result in complete energy allocation to basal maintenance for short-term survival, while all other essential functions are halted. Onset of anaerobiosis support energy supply to compensate for insufficient aerobic metabolism. Survival of the pessimum range is time limited due to the lack of reproduction and growth. Upon lethal stress, energy homeostasis is completely disrupted resulting in negative energy balance and activation of protective mechanisms that allow only short-term survival (Pörtner, 2010; Pörtner et al., 2017).

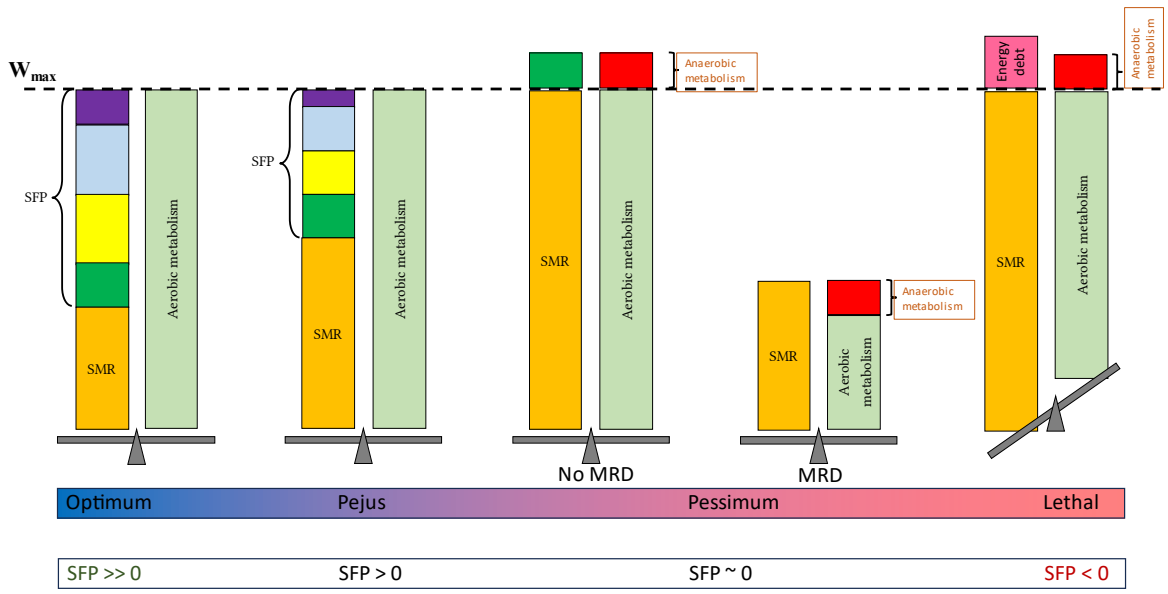

Figure 3: Concept of energy limited stress tolerance.

Level of environmental stress is shown along the X axis, while aerobic scope for energy demand (left bar at individual stress level) and energy supply (right bar at individual stress level) are depicted along the Y axis. SMR – basal maintenance costs as standard metabolic rate, SFP – aerobic scope for performance that is the energy used for fitness-related functions such as locomotion/activity (green), growth/biomass production (yellow), maturity/reproduction (blue), deposition/energy reserves (purple); MRD – metabolic rate depression, W_{max} – maximum aerobic energy flux (modified from Sokolova et al., 2012; Sokolova, 2013; Sokolova, 2021).

Metabolic strategies of stress tolerance differ between the organisms experiencing the pejus or pessimum range of an environmental factor. Transition to pejus range involves compensatory processes by acceleration of metabolism and ATP turnover to cover increasing costs of cellular maintenance and repair, while transition to pessimum range relies on conservation of energy characterized by decreased activity and suppression of metabolic rate (Sokolova et al., 2012). Facultative anaerobes such as marine bivalves are able to enter the state of metabolic rate depression (MRD), where ATP producing and energy demanding processes (such as ion pumping and macromolecule synthesis) are suppressed in a coordinated way (Bayne, 2017; Hochachka et al., 1996). Additionally, these organisms rely on alternative pathways for substrate-level phosphorylation, mitigation of toxic waste accumulation and low intracellular pH (Bayne, 2017). Assessment of these physiological responses creates a framework to relate physiological effects to fitness and survival and further associate them to population and ecosystem consequences.

1.1.3.2 Mitochondria as a central hub of metabolic regulation and stress signalling

Mitochondria are the powerhouse of the cell, producing >90% of total ATP and thus playing a key role in bioenergetics. However, recent research revealed their important function as metabolic and signalling hubs of the cell, where bioenergetics, biosynthesis, stress sensing and signalling are tightly linked. Mitochondrial ATP production is based on the mitochondrial respiration, which includes the electron transport system (ETS) using oxygen as the final electron acceptor, linked to ATP synthesis in the process called oxidative phosphorylation (OXPHOS) (**Fig. 4**). Briefly, respiratory complexes across the inner mitochondrial membrane (IMM), namely NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), ubiquinol-cytochrome C reductase (Complex III) and cytochrome C oxidase (CCO, Complex IV), transfer electrons from metabolic substrates to the final electron acceptor oxygen. This electron transfer is used to pump protons through the IMM from the mitochondrial matrix to the intermembrane space to establish a proton gradient, creating the protonmotive force Δp, which drives ATP synthesis via the F_1,F_O-ATP synthase (Campbell et al., 2014). A wide range of substrates can be used as electron donors to ubiquinone. Ubiquinone transfers electrons between the Complexes I, II and III. Furthermore, electrons can be accepted directly via ubiquinone from different substrates via dehydrogenases not associated to respiratory complexes. This Q-junction facilitates metabolic flexibility of mitochondrial respiration (Banerjee et al., 2022).

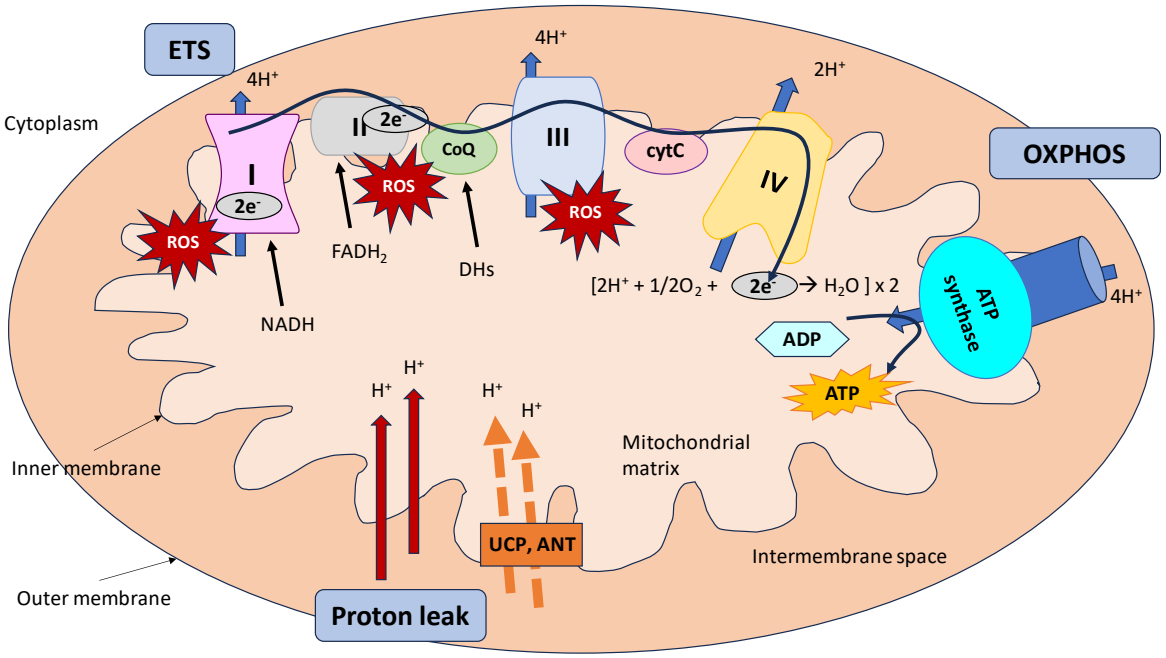

Figure 4: Schematics of key mitochondrial functions.

Electrons (e^-), derived from NADH (Complex I), succinate (FADH2 (Complex II)) and other reduced intermediates (alternative dehydrogenases, DHs), are transported (black arrows) through the electron transport system (ETS), which comprises Complexes I to IV. Reactive oxygen species (ROS) generation sites are Complex I, II and III (red stars). Solid blue arrows show direction of proton (H^+) pumping via ETS complexes. The proton back flow through F_1,F_O-ATPase (ATP synthase) is used for ATP production via OXPHOS. Basal proton leak are indicated by solid red arrows, while inducible proton leak via UCP, uncoupling protein, and ANT, adenosine transporter, are shown as solid broken orange arrows, modified from Sokolova, 2023. CoQ – Coenzyme Q, cytC – Cytochrome C; Complex I – NADH-dehydrogenase, Complex II – succinate dehydrogenase, Complex III – ubiquinol-cytovhrome C reductase, Complex IV – cytochrome C oxidase.

Besides being used for ATP synthesis, Δp is continuously dissipated by the so-called proton leak. Proton leak encompasses both constitutive and inducible processes, where protons return to the mitochondrial matrix disconnected from ATP synthesis (Jastroch et al., 2010). Mitochondrial leak respiration (LEAK) plays an important role in prevention of hyperpolarization and maintenance of mitochondrial membrane potential (MMP or Δψ). The proton leak is considered costly as it depends on constant energy input as part of mitochondrial maintenance (Brand et al., 1994; Brand, 1997; Miwa and Brand, 2003). Moreover, electrons can slip from upstream ETS complexes and get transferred to oxygen before reaching Complex IV resulting in production of superoxide radicals (O_2^-) (Jastroch et al., 2010; Munro and Treberg, 2017). Production of the reactive oxygen species (ROS) such as superoxide by mitochondria was first discovered by Jensen, 1966, and Complexes I, II and III were later identified as main sites of mitochondrial ROS generation (Quinlan et al., 2012). ROS encompasses a range of oxygen free radicals including superoxide anion radical, hydroxyl radical and nonradical oxidants like hydrogen peroxide (H_2O_2) and singlet oxygen (Zorov et al., 2014). Under normal condition, ROS generation and removal are tightly linked and remain controlled maintaining redox homeostasis. Thus, mitochondria protect their function by conversion of O_2^- to H_2O_2 via superoxide dismutase (SOD), which can be detoxified by the glutathione system and thioredoxin (Jastroch et al., 2010). Low physiological levels of ROS act as important cell signals that regulate various cellular processes, including metabolism, transcription, proliferation and apoptosis

(Sies and Jones, 2020; Sies et al., 2022). Stressors like hypoxia or osmotic stress can disrupt this balance and lead to oxidative stress activating protection mechanisms or in the case of severe stress leading to cellular damage (Paradis et al., 2016). When ROS concentrations deviate from the organism- and cell type-specific optimal level, cellular metabolism is disrupted. Low levels result in the loss of important signalling agents, while high levels cause uncontrollable ROS generation that overwhelms detoxification and scavenging processes. (Zorov et al., 2014). Thus, ROS can be seen as a pleiotropic physiological agent, where different ROS have different properties, mechanisms, and roles in organisms' physiology (Sies and Jones, 2020).

The tight link of bioenergetics and stress defence in mitochondria is also evident in regulation of mitochondrial respiration, cellular stress response, and immune response by mitochondrial activation of signalling proteins, and other signalling molecules such as mtDNA and cardiolipin (Lartigue and Faustin, 2013; Monlun et al., 2017). Mitochondria are also involved in generation of precursors for macromolecules and maintenance of redox homeostasis and metabolic waste management (Spinelli and Haigis, 2018). Nucleotides released from mitochondria act as alarming signal to restore cellular energy and metabolic homeostasis, while mitochondrial tricarbocylic acid cycle (TCA cycle) intermediates are strong regulators of cellular signalling cascades via acetylation, succinylation, and phosphorylation of proteins (Bohovych and Khalimonchuk, 2016; Naquet et al., 2016). Stressed mitochondria accumulate unassembled and misfolded proteins that induce unfolded protein response, mitophagic pathways and release an apoptotic regulator, cytochrome C (cytC) (Bohovych and Khalimonchuk, 2016). In addition, mitochondrial protein import machinery is key sensor for mitochondrial fitness and quality due to its tight association to energetic state and protein homeostasis (Harbauer et al., 2014). Rapid modulation of protein import efficiency allows mitochondria to adjust their proteome and actively control mitochondrial activity under physiological stress conditions (Harbauer et al., 2014). The ability of mitochondria to compartmentalize NAD^+ and redox equivalents and to re-purpose toxic cellular waste and metabolic by-products further contributes to cellular survival during stressful conditions (Spinelli and Haigis, 2018; Titov et al., 2016; Yang et al., 2007).

Mitochondria, therefore, represent highly sensitive targets of stress as well as the coordination centre for adaptive cellular response (Sokolova, 2018). The exposure to environmental stress such as oxygen deficiency, salinity and temperature impairs mitochondrial balance shifting from adaptive response to cell death due to energy deficiency and cellular damage (Bohovych and Khalimonchuk, 2016; Vakifahmetoglu-Norberg et al., 2017). However, coevolution and crosstalk of a network of mitochondrial functions together with the ability to establish intra- and extra-cellular communications produce a systematic sensor for mitochondrial fitness and regulation (Becker et al., 2012; Harbauer et al., 2014; Shen et al., 2022). The enormous variety of mitochondrial networks allows enhancement of mitochondrial functions upon stress.

1.1.3.3 Mitochondrial quality control

Mitochondrial integrity and function is regulated and maintained by the mitochondrial quality control (MQC) mechanisms (Berlett and Stadtman, 1997; Zorov et al., 2014). The MQC involves a complex network of proteins regulating mitochondrial dynamics, including mitochondrial proliferation, fission, fusion, and degradation (**Table 1**). The MQC mechanisms are well studied in model organisms like hypoxia-sensitive terrestrial rodents or *Drosophila*, while MQC mechanisms of hypoxia tolerant organisms, especially those from marine habitats, remain poorly understood (Eisner et al., 2018; Li and Liu, 2018).

Under normal conditions, mitochondrial dynamics involve a delicate balance between mitochondrial fusion and fission. To improve mitochondrial efficiency, mitochondria fuse to long interconnected networks sharing metabolites, mtDNA and membrane complexes such as respiratory enzyme complexes (Westermann, 2010; Westermann, 2012). Mitochondrial dynamics is regulated via dynamin-related proteins such as GTPases mitofusin 1, 2 and optic atrophy 1 (OPA1) (Chen et al., 2003; Song et al., 2009). Under stressful conditions, MMP decreases leading to depolarization, which activates the metalloendopeptidase OMA1. OMA1 degrades OPA1 and mediates mitochondrial fission interacting with dynamin-related protein 1 (DRP1) and its adaptor proteins mitochondrial fission factor (Mff) and fission protein 1 (Fis1) (Baker et al., 2014; Losón et al., 2013; Otera et al., 2010; Xiao et al., 2014). Mitochondrial fission allows the removal of damaged mitochondria to avoid dysfunctional mitochondria interfering with mitochondrial function and facilitates apoptosis under extreme cellular stress (Youle and van der Bliek, 2012).

Moreover, multiple ATP dependent and independent proteases support protein quality control including proteolysis, chaperoning, and biosynthesis. The evolutionary highly conserved Lon protease is important in degradation of damaged and oxidized proteins of the mitochondrial matrix. It responds to many stressors such as hypoxia and oxidative stress (Pinti et al., 2015). Its proteolytic activity is involved in downregulation of energy metabolism, control of mitochondrial architecture, ribosome assembly, mtDNA metabolism, and stress response (Pinti et al., 2015). In addition, MQC involves repair mechanisms prohibiting premature cell death and removal of proteins using remodelling and repair processes (Doyle and Wickner, 2008; Ozawa et al., 1999). During extreme stress, proteins aggregate losing functionality. Caseinolytic peptidase B (ClpB) is responsible for disaggregating insoluble protein aggregates, and reactivation of disaggregated proteins (Doyle and Wickner, 2008). Additionally, important roles of m-AAA protease paraplegin and metallopeptidase ATP 23 in assembly and biogenesis of Complex I and F_1,F_O-ATP synthase show a tight control of mitochondrial respiration by MQC (Atorino et al., 2003; Osman et al., 2007). Furthermore, mtDNA maintenance and replication as well as translation mechanisms are involved in MQC (Mai et al., 2017; Spelbrink et al., 2001; Wang et al., 2021).

In case of dysfunctional mitochondria, fusion to mitochondrial network is avoided using mitochondria-specific autophagy - called mitophagy. Mitophagy encompasses many separate pathways, where PINK-Parkin pathway and the mitochondria-eating protein (MIEAP) are the best known. During hypoxia phosphatase and tensin homolog (PTEN) induced kinase 1 (PINK1)

accumulates at dysfunctional mitochondria and recruits Parkin to tag damaged mitochondria for degradation, all under tight regulation by phosphoglycerate mutase 5 (PGAM5) (Chen et al., 2016; Eiyama and Okamoto, 2015). Additional alternative mitophagic pathways are mediated by MIEAP (Kitamura et al., 2011; Nakamura and Arakawa, 2017). Mitochondrial ROS modifies outer mitochondrial membrane (OMM) macromolecules such as lipids and proteins tagging unhealthy mitochondria, which can be recognized by MIEAP followed by elimination or repair of those mitochondria (Kitamura et al., 2011).

Table 1: Summary of important proteins and associated genes involved in mitochondrial quality control.

Mitochondrial quality control function	Name	Protein name	Gene name	Description	References
Fusion	Mitofusin-2	Mfn2	*mfn*2	Mitochondrial membrane GTPase located in the OMM participates in fusion of OMM.	(Chen et al., 2003; Song et al., 2009)
	Mitochondrial dynamin-like 120 kDa protein, optic atrophy 1	OPA1	*opa*1	The GTPase located in IMM regulates mitochondrial fusion and cristae structure of IMM. It cooperates with mitofusin 1 and 2. Its function in cristae fusion contributes directly to respiratory control.	(Song et al., 2009)
Fission	Dynamin-1-like protein	DRP1	*dnm1l*	This GTPase regulates mitochondrial fission, which also facilitates mitophagy.	(Losón et al., 2013; Otera et al., 2010)
	Mitochondrial fission factor	Mff	*mff*	The binding of the OMM anchored protein with DRP1 initiates mitochondrial fission.	(Losón et al., 2013; Otera et al., 2010)
	Mitochondrial fission protein 1	Fis1	*fis*1	The protein integrated in OMM acts as adaptor protein of DRP1 during mitochondrial fission.	(Losón et al., 2013)
	Mitochondrial translation elongation factor EF-Ts	TSFM	*tsfm*	The guanine nucleotide exchange factor is part of the elongation cycle of mitochondrial translation.	(Mai et al., 2017; Wang et al., 2021)

Mitochondrial quality control function	Name	Protein name	Gene name	Description	References
	Mitochondrial metalloendopeptidase OMA1	OMA1	*oma*1	The Zn^{2+}-dependent metalloendopeptidase located in IMM is responsible for stress-dependent OPA1 cleavage (proteolytic activity).	(Baker et al., 2014; Xiao et al., 2014)
MQC and proteolysis	Mitochondrial Lon protease	LonP	*lonp*1	The ATP-dependent protease degrades unfolded or misfolded proteins based on its proteolytic activity, chaperone activity, mtDNA regulation and specifically regulates essential rate-limiting and metabolic processes.	(Kuo et al., 2015; Pinti et al., 2015; Sepuri et al., 2017; Venkatesh et al., 2012)
	Hypoxia upregulated protein 1	HYOU1	*hyou*1	Mitochondrial repair support	(Ozawa et al., 1999)
	Mitochondrial caseinolytic peptidase B	ClpB	*clpB*	The mitochondrial AAA ATPase chaperone of the mitochondrial intermembrane space is involved in protein unfolding, disassembly, disaggregation playing an important role in protein remodelling.	(Doyle and Wickner, 2008)
	Paraplegin		*spg*7	The nuclear-encoded metalloprotease located to the IMM functions proteolytic and chaperon-like contributing to biogenesis of respiratory complex I.	(Atorino et al., 2003)
	Mitochondrial inner membrane protease ATP23	ATP23	*atp*23	This protease associated with IMM/intermembrane space has a dual function in processing and assembly of	(Osman et al., 2007)

Mitochondrial quality control function	Name	Protein name	Gene name	Description	References
				specific subunits of mitochondrial F_1,F_O-ATPase and is thus directly associated to respiratory control.	
Mitophagy	Phosphatase and tensin homolog (PTEN) induced kinase 1	PINK1	*pink*1	The serine/threonine-protein kinase initiates binding of parkin protein to depolarized mitochondria inducing mitophagy.	(Chen et al., 2016)
	E3 ubiquitin-protein ligase parkin	Parkin	*prkn*	The E3 ubiquitin ligase is involved in ubiquitination and mitophagy.	(Chen et al., 2016)
	Mitochondrial serine/threonine protein phosphatase phosphoglycerate mutase 5	PGAM5	*pgam*5	The phosphatase regulates the PINK1-parkin pathway via reversible phosphorylation.	(Chen et al., 2016)
	Mitochondria-eating protein	MIEAP	*mieap*	The interaction of MIEAP with its associated proteins BCL2 and adenovirus E1D 19kDa interacting protein 3 (BNIP3) and BNIP3-like NIX induces a pore, through which lysosomal proteins continue to the intramitochondrial space initiating elimination of unhealthy mitochondria .	(Kitamura et al., 2011; Nakamura and Arakawa, 2017)
mtDNA control	Mitochondrial twinkle mtDNA helicase	Twinkle	*twnk*	The adenine nucleotide dependent DNA helicase is involved in mtDNA maintenance and replication.	(Spelbrink et al., 2001)

1.1.4 Deoxygenation and salinity fluctuations as metabolic stressors for benthic organisms

1.1.4.1 Impacts of hypoxia on bioenergetics and redox balance and adaptations to oxygen deficiency

Deviation of environmental factors from species- and population-specific optimum entails serious consequences for organisms resulting in physiological disturbances and lower fitness (Hoffmann and Parsons, 1991). Responses of benthic organisms to hypoxia depend on duration, predictability, and intensity of oxygen deficiency (Diaz and Rosenberg, 1995; Levin et al., 2009). However, benthic organisms are most vulnerable to coastal hypoxia as they dwell in near bottom areas, where hypoxia strikes first, and their sessile life style requires physiological adaptations to survive in such harsh conditions (Grieshaber et al., 1994). Additionally, organismal responses to hypoxic events depend on the species-specific thresholds of oxygen concentrations, below which physiological functions and metabolic rate cannot be maintained (Richards, 2011). Consequently, organisms show reduced locomotion, reproduction, and growth leading inevitably to possible mortality and ecosystem consequences (Diaz and Rosenberg, 1995; Eriksson and Baden, 1997; Fischer et al., 1992; Schurmann and Steffensen, 1994). Physiologically, initial response to hypoxia includes maintenance of oxygen delivery, followed by conservation of energy expenditure and reduction of energy turn over. Ultimately, supplementation of ATP production with anaerobic metabolism might be required (Boutilier et al., 1988; Boutilier, 2001; Dunn and Hochachka, 1986; Hochachka, 1997). At low oxygen concentrations, hypoxia-sensitive species struggle to maintain energy balance. Low oxygen concentration requires anaerobic ATP production via glycolysis or other substrate level phosphorylation. However, anaerobic metabolism yields around 15 times less ATP than aerobic metabolism (Hochachka and Somero, 2002) and thus, can lead to a mismatch of ATP demand and supply. Coincidently, anaerobic metabolism causes substrate loss and leads to the accumulation of toxic metabolic waste. Especially, hypoxia-sensitive species suffer from an inability to sustain essential ATP dependent processes and impaired energy homeostasis leading to mortality during hypoxic events (Boutilier, 2001; Richards, 2009; St-Pierre et al., 2000). In hypoxia-tolerant species, oxygen supply to the tissues is maintained as long as possible, while MRD occurs when oxygen levels become too low to support aerobic metabolism (Bayne, 2017). In the long term, survival of hypoxia crucially depends on the ability to reduce metabolic demand to avoid a mismatch of energy supply as well as the availability of metabolizable substrate and efficient pathways for O_2 independent ATP generation (Richards, 2009). In turn, sudden reoxygenation can be also dangerous as oxygen surplus causes ROS burst damaging macromolecules (Andrienko et al., 2017).

Organisms such as intertidal and estuarine regions have evolved a wide range of physiological and biochemical tolerance mechanisms against hypoxia. Especially benthic organisms rely on these adaptive traits as their low motility does not allow migration to more favourable habitats (Grieshaber et al., 1994). Thus, physiological and behavioural adaptations are important for these organisms to maintain aerobic metabolism, reduce energy demand and preserve energy homeostasis in hypoxic areas (Levin et al., 2009; Seibel, 2011; Sokolova, 2018).

During short-term exposure to hypoxia, organisms protect themselves by behavioural adaptations depending on their locomotion ability, where mobile species rely on migration and sessile species on mechanisms such as siphon extension or migration to the sediment surface (Diaz and Rosenberg, 1995; Grieshaber et al., 1994). However, survival of longer periods of hypoxia requires morphological and physiological adaptations of ventilatory and circulatory systems to maintain oxygen supply to tissues. During early stages of hypoxia exposure, organisms often increase the ventilation rate to facilitate oxygen uptake (Grieshaber et al., 1994). Enhancement of oxygen uptake by gills is further supported by enhanced blood flow and gill perfusion due to increased cardiac output that involved elevated heart rate and/or stroke volume. However, circulatory responses to hypoxia vary across species (Grieshaber et al., 1994). Under hypoxia, many hypoxia-tolerant organisms such as marine vertebrates and invertebrates as well as high-altitude vertebrates increase surface area and perfusion rate of respiratory organs, elevate hematocrit, increase oxygen affinity of their respiratory pigments, alter capillary density and mitochondrial arrangement near capillaries to improve oxygen transport to mitochondria (Bavis et al., 2007; Devaux et al., 2019; Grieshaber et al., 1994; Richards, 2009; 2011; Storz et al., 2010). Oxygen deficiency below the critical partial pressure of oxygen, where aerobic metabolism cannot be maintained, leads to a decrease in the ventilation rates as species reduce oxygen consumption, while anaerobic metabolism commences to compensate for loss of aerobic ATP production (Grieshaber et al., 1994; Pörtner and Grieshaber, 1993).

1.1.4.2 Mitochondrial responses to oxygen fluctuations

Mitochondria are key targets of hypoxic events as they play a central role in ATP production, redox balance and cellular signalling as the main energy producer but also main oxygen consumer (Sokolova et al., 2019). As mitochondria are important metabolic hubs, linking bioenergetics and biosynthesis, maintenance of mitochondrial integrity and functions has high priority and increases organisms' fitness, especially during stress events (Brown et al., 2020; Rodell et al., 2013; Spinelli and Haigis, 2018). Furthermore, long-term survival depends on the ability to rapidly restore aerobic metabolism and cellular homeostasis during post-hypoxic recovery and requires physiological mechanisms to maintain mitochondrial integrity during hypoxia. Characteristically, hypoxia reduces ETS and thus ATP production resulting in ATP deficiency interfering with cellular functions (Hochachka and Lutz, 2001).

During hypoxia, suppression of ETS and CCO activity by passive or active regulation are commonly seen in terrestrial and marine hypoxia-sensitive species (Hickey et al., 2012; Hüttemann et al., 2012; Ivanina et al., 2016; Semenza, 2007). Simultaneously, fast consumption of ATP results in failure of oxygen and ATP dependent processes. Consequently, mitochondrial membrane depolarization and lost intracellular ion balance cause Ca^{2+} overload and ROS production leading to apoptosis and necrosis (Chen et al., 2007; Piper et al., 2003; Solaini et al., 2010). Hypoxia-sensitive bivalves were shown to suffer a similar mitochondrial response involving oxidative damage, collapse of Δp and loss of OXPHOS capacity (Ivanina and Sokolova, 2016; Ivanina et al., 2016). Briefly, protons accumulate in mitochondrial matrix due to failing ETS proton pumping. This proton imbalance causes mitochondrial membranes to depolarize and F_1,F_O-ATP synthase to work reversely becoming

a major ATP consumer (St-Pierre et al., 2000). Due to the missing ATP, vital ATP dependent ion transporters cannot counterbalance ion gradients and a necessary switch to anaerobic metabolism leads to acidosis. Both factors contribute to higher concentration of extracellular Ca^{2+} triggering a Ca^{2+} overload and mitochondrial Ca^{2+} uptake. If energy reserves become exhausted and Ca^{2+} overload persists, mitochondrial swelling results in cell death (Galli and Richards, 2014). In contrast, hypoxia-tolerant species were shown to allow Δp to stabilize at lower values under anoxia to prevent wastage of anaerobically derived ATP (St-Pierre et al., 2000).

The hypometabolism of hypoxia-tolerant species under oxygen deficient conditions is reflected in mitochondrial metabolism, where mitochondria regulate their respiratory capacity to conserve energy, but also as protection against ROS bursts (Galli and Richards, 2014). Typically, activity of Complex I is downregulated, while activity of Complex II is increased or maintained resulting in high mitochondrial activity with reduced ROS production at Complex I. Thus, hypoxia-tolerant organism like freshwater turtles, fish and intertidal bivalves are able to preserve mitochondrial respiration, OXPHOS capacity and Δp and to mitigate oxidative damage during hypoxia-reoxygenation (H/R) stress (Galli and Richards, 2014; Ivanina et al., 2016; Kurochkin et al., 2009; Sokolova, 2018; Sokolova et al., 2019; Sussarellu et al., 2013). Additionally, reversible protein phosphorylation of ETS complexes, upregulated mitochondrial antioxidants and MQC contribute to the maintenance of mitochondrial integrity (Falfushynska et al., 2020b; Ivanina and Sokolova, 2016; Lushchak et al., 2001; Sokolov et al., 2019). Earlier studies on hypoxia-tolerant intertidal bivalves revealed an upregulation of mitochondrial capacity for substrate oxidation (SO) during H/R stress and the ability to recover a normal structure of metabolic control over mitochondrial respiration shortly after recovery (Ivanina et al., 2012; Ivanina et al., 2016; Sokolov et al., 2019). Maintenance of high SO capacity and stable metabolic control over respiration during H/R seems to be a feature of a hypoxia-tolerant mitochondrial phenotype.

Mitochondria of facultative anaerobes including many hypoxia-tolerant mollusks are capable of anaerobic ATP production (Tielens et al., 2002). The redirection of glycolytic flux towards aerobic or anaerobic mitochondrial metabolism occurs at a metabolic branchpoint where two enzymes, pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK), compete for the common substrate, phosphoenolpyruvate (PEP) (**Fig. 5**). In early hypoxia, main anaerobic energy sources are phospho-l-arginine (PLA) and coupling of aspartate transamination to glycogen fermentation resulting in alanine. Under these conditions, PK remains active although pyruvate is then metabolized to alanine or aspartate resulting in fumarate and succinate accumulation. Furthermore, glycogen fermentation and formation of lactate and opines dominate due to anaerobic glycolysis in cytoplasm. During prolonged hypoxia, PEP is converted to ocalacetate by PEPCK and directed (via malate) to anaerobic mitochondria resulting in succinate, acetate, and propionate accumulation (Bayne, 2017). Mitochondrial anaerobic generation of succinate, acetate and propionate yield higher amount of ATP per unit glucose than cytosolic pathways producing lactate or opines, and minimizes metabolic acidosis by producing less metabolic protons or (in the case of propionate) volatile end products that can be easily excreted (Bayne, 2017; Sokolova et al., 2000). Prolonged survival under hypoxic

conditions that rely on anaerobic ATP production is also supported by high tissue stores of anaerobic fuels such as glycogen and amino acids in hypoxia-tolerant marine bivalves (Bayne, 2017).

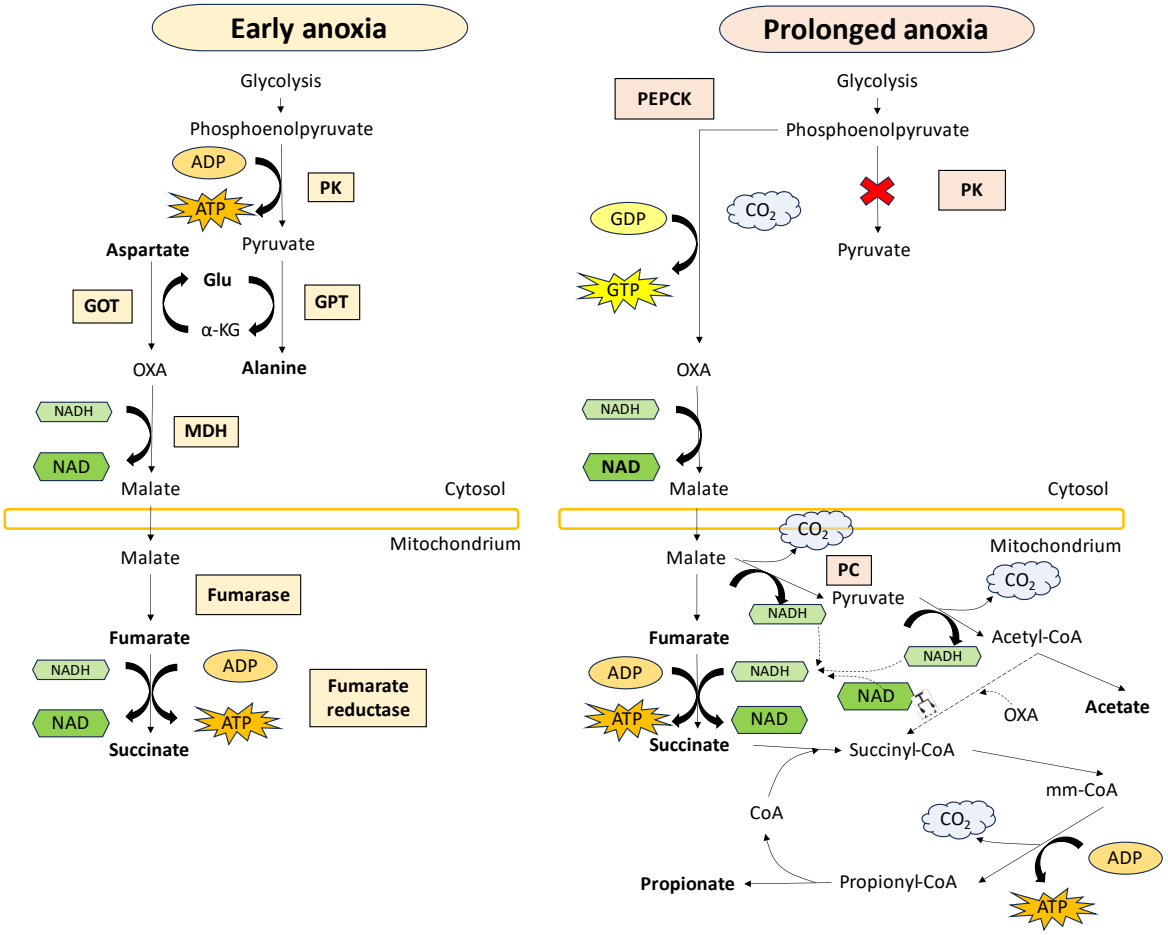

Figure 5: Schematic of anaerobic metabolism in marine bivalves according to Bayne, 2017.

CoA – Coenzyme A; GOT – Glutamate oxalacetate transaminase; GPT – Glutamate pyruvate transaminase; MDH – Malate dehydrogenase; mm – Methylmalonyl; Glu – Glutamate; α-KG – α-ketoglutarate; OXA – Oxalacetate; PC – Pyruvate carboxylase; PEPCK-Phosphoenolpyruvate carboxykinase; PK – Pyruvate kinase (modified after Bayne, 2017)

Reoxygenation restores aerobic ATP synthesis, but comes with the cost of a burst of ROS damaging cellular macromolecules like proteins, lipids and DNA, causing cell death and tissue injury (Cadenas and Davies, 2000; Chouchani et al., 2016; Ivanina et al., 2016; Paradis et al., 2016). However, ETS and Δp remain collapsed in hypoxia sensitive species, thus OXPHOS suppression cannot be reversed (Galli et al., 2013; Hickey et al., 2012; Ivanina et al., 2016; Kurochkin et al., 2009). Furthermore, sudden reoxygenation can be more damaging than hypoxic events as uncontrolled ROS generation causes oxidative injury including the mitochondrial membrane permeability transition, cytC release and apoptosis potentially culminating in organ failure (Kalogeris et al., 2014; Korge et al., 2008). Therefore, hypoxia tolerance is often characterized by high baseline levels of antioxidant defences and the ability to upregulate antioxidant systems during hypoxia as preparation for reoxygenation (Bickler and Buck, 2007; Hermes-Lima et al., 2015). Furthermore, alternative oxidase (AOX)

removes free oxygen without ATP turnover preventing generation of oxygen radicals damaging cellular functions (Bayne, 2017).

Additional costs incurred during post-hypoxic recovery include the allocation of energy to restore cellular and organismal homeostasis, which is disrupted by metabolic arrest, ATP deficiency, and the accumulation of anaerobic end products. These additional energy costs associated with restoring homeostasis manifest as oxygen debt, typically measured by increased organismal oxygen consumption during recovery (Kurochkin et al., 2009; Sokolova, 2021; Vismann and Hagerman, 2008). The reincorporation of anaerobic end products into cellular functions, such as converting succinate and alanine to malate and aspartate via gluconeogenesis, plays a crucial role in restoring energy metabolism. During recovery, the high energy demand can be met through increased utilization of opines, such as strombine in bivalves (Bayne, 2017).

1.1.4.3 Physiological and metabolic responses to salinity fluctuations in marine organisms

Salinity influences the osmotic pressure, relative proportions of solutes, saturation of dissolved gases, density, and viscosity of aqueous media including seawater and extracellular and intracellular fluids (Kinne, 1964) thereby affecting cellular processes and integrity of organelles (Berger and Kharazova, 1997; Prosser, 1991) To maintain osmotic and ion homeostasis during salinity change, organisms evolved osmoregulatory mechanisms such as early gene and protein networks, post-translational modification (PTM), interaction, and compartmentation of osmotic stress response proteins to adjust ion and water fluxes across membranes (Kültz et al., 2007). Based on their osmoregulatory strategy, marine organisms can be classified as osmoregulators or osmoconformers.

Osmoregulators maintain body fluids at levels different to surrounding environment using isosmotic intracellular regulation (IIR) and anisosmotic extracellular osmoregulation (AER). IIR includes increase or decrease of osmolyte concentration (K^+, free amino acids (FAA)) allowing cell volume regulation and modification of membrane-bound transporters (Kirschner, 1991; Péqueux, 1995). In contrast, AER involves maintenance of solute concentration of extracellular fluids. Lower salinity compared to optimal conditions results in hyper-regulation to maintain higher osmolarity of body fluids, while high salinity compared to optimal conditions can either cause isosmotic regulation (body fluids at same osmotic level as surrounding environment) or hypo-regulation (maintenance of lower body osmolarity). In either case, osmoregulation in response to salinity changes requires control of ion fluxes (e.g. Na^+ and Cl^- - ions), control of membrane permeability and active ion pumping (Rivera-Ingraham and Lignot, 2017). However, these processes are energy costly as maintenance of ion gradients is associated with high ATP consumption (Sokolova et al., 2012).

Osmoconformers adjust intracellular osmolarity to match the ambient osmolarity and prevent excessive changes in cell volume (Evans, 2009; Shumway, 1977; Yancey, 2005). Shifts in cellular osmolarity and cell volume caused by salinity stress can result in impaired enzyme activity, potential damage to organelles and membranes and are associated with effects on physiological performance (Berger and Kharazova, 1997). Sensing of osmotic changes involves Ca^{2+}-gradients, transient receptor potential of ion channels, cell volume sensors (Fiol and Kültz, 2007), while

osmoconforming processes are hormonally controlled (Lignot and Charmantier, 2015). In mollusks, the immediate response to a salinity shift involves closing of shell to isolate the mantle cavity and reduce changes in cell volume, later followed by shell opening and IIR (Berger and Kharazova, 1997; Hawkins and Bayne, 1992). IIR includes adjustments of intercellular levels of inorganic (e.g. Na^+, Cl^-) and organic (e.g. FAA) osmolytes. Altered activity of de novo amino acid synthesis and protein breakdown (Meng et al., 2013; Zhao et al., 2012), uptake and efflux of FAAs and changed transport and retention of inorganic ions, particularly sodium, play key roles in IIR (Podbielski et al., 2022). Reliance on IIR is less energetically costly, as upregulation of active transport mechanisms is only transient or altogether avoided. However, frequent salinity alterations may result in cycles of protein breakdown and FAA efflux resulting in the loss of energy and biomass (Haider et al., 2018).

Failure to maintain osmolarity causes osmotic stress leading to changes of plasma hormones, energy metabolism and electrolyte homeostasis, enhanced ROS generation, and oxidative damage (Choi and An, 2008). Increasing salinity stress facilitates oxidative stress damage in marine organisms by increasing lipid peroxidation, protein carbonyls and H_2O_2 levels, while initiating antioxidant defence mechanisms (An and Choi, 2010; Lee et al., 2017; Paital and Chainy, 2010; 2012). Antioxidant enzymes play important roles in reducing oxidative stress under salinity stress (An and Choi, 2010; Choi et al., 2008). Consequently, cellular stress response induced by salinity stress may increase energy demand to effectively regulate cellular antioxidant protection (Sokolova et al., 2012). As marine bivalves rely on IIR, trade-offs between protection mechanisms against salinity changes and hypoxia tolerance likely occur in hypoxia-tolerant phenotypes, specifically in multiple stressor environments such as the coastal benthos.

Effect of oxidative damage and organic osmolytes on protein and membrane stability directly influences enzymatic rates and cellular metabolism (Storey, 1997). Possible ion disbalance and failing redox balance can have severe effects on metabolism causing electron chain dysfunction, reduced coupling and high ROS efflux impairing mitochondrial efficiency and causing oxidative stress (Bal et al., 2022; Rivera-Ingraham et al., 2016a; Rivera-Ingraham et al., 2016b; Sokolov and Sokolova, 2019). Gill mitochondria of mud crab showed elevated activities of ETS enzymes with increasing salinity, while activity of ATP synthase decreased with higher salinity (Paital and Chainy, 2012). These findings suggest that high salinity caused a hypoxia-like state in mitochondria of mud crab gill accompanied with increased H_2O_2 formation and oxidative stress (Paital and Chainy, 2012). In contrast, mitochondria of intertidal bivalves seem to be highly tolerant to shifts in osmolarity (Ballantyne and Storey, 1983; 1984; Ballantyne and Moon, 1985; Ballantyne and Moyes, 1987b; Ballantyne and Moyes, 1987a) involving the strong ability to adjust their salinity optima without negatively affecting mitochondrial capacity (Ballantyne and Moyes, 1987a; Haider et al., 2018). In oyster mitochondria, mitochondrial ATP synthesis, ETS flux and coupling was higher at low osmolarity (450 mOsm) compared to high osmolarity (900 mOsm), although animals were acclimated to high salinity of 30 (~1000 mOsm) (Sokolov and Sokolova, 2019). However, physiological concentrations of taurine, a common osmolyte in marine bivalves, enhanced mitochondrial ATP synthesis and ETS capacity, increased mitochondrial coupling and stimulated

forward electron transfer in Complex I, predominantly under high osmolarity stress (Sokolov and Sokolova, 2019). Consequently, taurine appears to support mitochondrial bioenergetics during hyperosmotic stress in oysters. The compatibility of taurine with metabolic functions explains the broad distribution as osmolyte in marine osmoconformers (Sokolov and Sokolova, 2019). Thus, adaptation of mitochondrial machinery to osmoconformity may be reflected in osmotically robust mitochondria of intertidal bivalves (Sokolova, 2018). However, salinity stress alters glycolysis in *Crassostrea gigas* modulated by AMP-dependent protein kinase (AMPK) effect on the mitochondria (Chen et al., 2022; Fuhrmann et al., 2018). As salinity stress seem to affect mitochondrial bioenergetics and redox balance, it might interfere with mitochondrial tolerance to multiple stressors such as combined salinity and hypoxia.

1.2 Experimental approaches to assess the mitochondrial role in hypoxia tolerance

1.2.1 Gaps in the literature and aims of the studies included in this dissertation

Physiological mechanisms of hypoxia tolerance were extensively studied in hypoxia-tolerant invertebrates, fish and reptiles (Bickler and Buck, 2007; Grieshaber et al., 1994; Hochachka and Lutz, 2001). However, these studies focused predominantly on passive survival of hypoxia-tolerant species (Grieshaber et al., 1994; Hochachka and Lutz, 2001), while molecular mechanisms for maintenance of integrity and recovery of energy homeostasis remain unknown. Specifically, preservation and regulation of mitochondria during hypoxia to sustain energy metabolism and prevent cellular damage during reoxygenation remain to be resolved. The regulation and control of mitochondrial functions play important roles in H/R tolerance of marine bivalves (Ivanina et al., 2016). Thus, mitochondrial reorganization involving upregulation of ETS activity, antioxidants and suppression of ATP-demanding processes contributed to high resilience to hypoxia in an intertidal hypoxia-tolerant clam *Mercenaria mercenaria* compared with hypoxia-sensitive subtidal scallop *Argopecten irradians* (Ivanina et al., 2016). In addition, mitochondria of hypoxia-tolerant species showed evidence of specific intrinsic mechanisms including regulation of OXPHOS and catabolic pathways via reversible phosphorylation important in hypoxia tolerance (Sokolov et al., 2021). However, it remains to be established, which mitochondrial processes are most sensitive to H/R stress and which molecular mechanisms preserve mitochondrial functions of differently tolerant bivalve species during H/R stress. Moreover, it is yet to be determined, if regulatory mechanisms in mitochondria are connected to specific hypoxia-tolerant mitochondrial phenotypes.

Combination of environmental stressors affect mitochondrial tolerance mechanisms differently than a single stressor resulting in trade-offs between different physiological functions. Effects of multiple stressors depend on the intensity and timing of individual abiotic and biotic stressors and on the organisms' responses to the combined stressors (Gunderson et al., 2016). However, exposure to combined stressors can either exacerbate negative effects of individual stressors or activate cross-tolerance mechanisms. For instance, decreasing salinity combined with increasing hypoxia has been shown to negatively affect the physiological performance of *Perna viridis*, compromising immune functions and resulting in severe consequences such as reduced disease resistance, growth and survival (Wang et al., 2011; Wang et al., 2012). Similarly, mitochondrial resistance to H/R stress in *Crassostrea virginica* appeared to be impaired by exposure to cadmium and acute temperature stress, potentially reducing their ability to survive fluctuating oxygen conditions in coastal and estuarine habitats (Ivanina et al., 2012). Despite evidence suggesting that transcriptional frontloading of genes involved in stress indication, immune response, and protein synthesis is important in facilitating cross-tolerance between stressors and enhancing organism fitness (Collins et al., 2021), it remains unclear whether the extent of phenotypic plasticity can buffer the effects of severe anthropogenic changes in coastal habitats. As coastal habitats face increasing frequency and magnitude of anthropogenic eutrophication, freshwater runoff, and other climate-change related factors in the near

future, the question remains: how and to what extent is the mitochondrial resilience of hypoxia-tolerant marine bivalves affected by exposure to multiple stressors?

Mitochondria have a network of quality control mechanisms including mitochondrial dynamics, mitophagy and protease activity. Alteration in abundance and phosphorylation state of proteins related to mitochondrial maintenance during H/R stress indicated their important role in hypoxia tolerance of *C. gigas* (Sokolov et al., 2019). Species-specific reversible phosphorylation of respiratory complexes by protein kinase A (PKA) and protein kinase C (PKC) during H/R stress were observed in *A. islandica*, *M. edulis* and *C. gigas* indicating post-translational regulation of mitochondrial functions during hypoxia (Falfushynska et al., 2020b). In addition, species-specific differences in inflammatory and apoptotic response at the transcriptional level in *M. edulis* and C. gigas revealed importance of cell survival pathways in hypoxia tolerance (Falfushynska et al., 2020a). The molecular mechanisms of MQC in marine bivalves remain to be fully understood, along with whether the degree of hypoxia tolerance can be associated with different regulatory mechanisms of MQC. Furthermore, due to the crucial role of mitochondria as central metabolic hubs that interconnect various cellular pathways, including energy pathways and stress signalling, it is plausible that mitochondrial resilience correlates with specific regulatory patterns in cellular metabolism. This correlation is especially relevant during exposure to multiple stressors, which can affect the emission of cellular signals (such as ROS) and the rates and efficiency of ATP production.

The main objective of this study was to assess mitochondrial mechanisms involved in response to H/R stress of four species of marine bivalves differing in their hypoxia tolerance (i.e. an exceptionally hypoxia-tolerant *A. islandica*, strongly hypoxia-tolerant *C. gigas* and *O. edulis* and moderately hypoxia-tolerant *M. edulis*), and to determine how the mitochondrial mechanisms are integrated into cellular metabolism to ensure recovery and survival. Functional and molecular studies of isolated mitochondria aimed to elaborate whether the mechanisms regulating the mitochondrial respiration during H/R transitions are shared by different hypoxia-tolerant species and contribute to a hypoxia-tolerant mitochondrial phenotype. To shed light on the mitochondrial role in hypoxia tolerance of marine bivalves, we identified the sensitivity of mitochondrial processes (including ETS activity, proton leak and ATP synthesis) and their underlying molecular mechanisms to hypoxia using top-down metabolic control analysis (MCA) and assessed the integration of mitochondrial changes into the cellular metabolic network using non-targeted metabolomics (Ainscow and Brand, 1999a; 1999b; Patti et al., 2012). Furthermore, we investigated, whether combined exposure to salinity and hypoxia can interfere with mitochondrial performance and metabolism of marine bivalves, and which intrinsic mitochondrial mechanisms might be involved in the responses to these combined stressors. To understand the role of the MQC in hypoxia tolerance, transcriptional responses of key genes involved in the mitochondrial dynamics, mitophagy and mitochondrial protein degradation were assessed in bivalves with different degree of hypoxia tolerance.

The present study aimed to test the following hypotheses:

1. The mitochondrial bioenergetics of hypoxia-tolerant bivalve species is reorganized in response to hypoxia to preserve mitochondrial OXPHOS capacity and prevent severe ROS efflux, facilitating rapid restoration of energy homeostasis during reoxygenation.
2. Salinity stress impairs mitochondrial resilience to hypoxia in hypoxia-tolerant bivalve species.
3. The regulation of mitochondrial quality control mechanisms to compensate for oxidative damage increases with decreasing hypoxia tolerance among bivalve species.
4. The modulation of metabolic pathways in response to H/R stress increases with decreasing hypoxia-tolerance in bivalve species.

1.2.2 Experimental design and approaches to hypothesis testing

1.2.2.1 Model organisms

The marine environment encompasses a wide range of diverse habitats characterized by varying lengths, magnitudes, and frequencies of hypoxia and salinity stress. Sessile marine organisms must adapt to these conditions, and their adaptive and tolerance mechanisms are influenced by their lifestyle and specific habitat characteristics. To assess the role of mitochondria in hypoxia and salinity tolerance, it is crucial to compare marine bivalves that exhibit differing degrees of hypoxia tolerance. This approach allows for a comprehensive understanding of the variety of adaptive mechanisms involved. Model organisms are selected for their well-studied adaptive traits, with the assumption that insights gained from these models can be extrapolated to other less-studied species. This study uses four bivalve species with varying degrees of hypoxia tolerance to investigate the mitochondrial basis of hypoxia tolerance and provide insights into these adaptive mechanisms.

The least hypoxia-tolerant of the four studied species, the blue mussel *Mytilus edulis* (Linnaeus, 1758), is native to the North Atlantic Ocean and adjacent seas. The Baltic Sea population of the blue mussel is a hybrid form of *M. edulis* and Mytilus trossulus. However, the population of this study has a 100% mitochondrial and 70% nuclear genetic background of *M. edulis* (Stuckas et al., 2017) and will be thus referred hereafter as *M. edulis*. Hypoxia can compromise growth, respiration rate, and energy acquisition of the blue mussel (Sanders et al., 2014). Activation of inflammatory and apoptotic response in *M. edulis* show strong dependence on cellular stress response mechanisms in response to hypoxia compared to Crassostrea (Magallana) gigas (Thunberg, 1793) (Falfushynska et al., 2020a). Moreover, different regulation of intermediate metabolite homeostasis compared to *C. gigas* might explain weaker hypoxia tolerance of the mussels (Haider et al., 2020). However, hypoxia tolerance varies with populations, especially when comparing intertidal and subtidal populations of the blue mussel (Altieri, 2006). The blue mussels used in the present study are permanently submersed due to the lack of tides in the Baltic Sea. In addition, adaptation to the distinctive salinity conditions of the Baltic Sea might be reflected in the mitochondrial resilience to osmotic stress in *M. edulis* (Noor et al., 2021).

The European flat oyster *Ostrea edulis* (Linnaeus, 1758) appears to be moderately stress-tolerant. Due to overfishing, invasion of *C. gigas* and frequent viral and bacterial diseases, it became mostly extinct in the North Sea (Beck et al., 2011; Da Silva et al., 2005). However, there are projects to re-introduce the European oyster into the Wadden Sea. Remaining populations of *O. edulis* can be found along the Western coast of Europe from Morocco up North to Norway. As estuarine and intertidal species, oysters are commonly seen as hypoxia-tolerant (Stevens and Gobler, 2018). The salinity optima are similar in the European flat oyster *O. edulis* and the Pacific oyster *C. gigas* (Laing et al., 2005; Reise, 1998). Although low salinity negatively affects scope for growth (SFG) in the European flat oyster, especially when combined with thermal stress, adaptation to changing salinity does not cause severe problems for this species (Çelik et al., 2015; Hutchinson and Hawkins, 1992). However, oyster species differ in their ability to overcome chemical and temperature stress (Lemasson et al., 2018; Perić et al., 2020), with *O. edulis* being more susceptible to climate drivers than *C. gigas* (Stechele et al., 2022).

The Pacific oyster *C. gigas* is one of the most successful invaders among bivalves in intertidal ecosystems worldwide, including European coastal waters (Sigwart et al., 2021; Troost, 2010). Its ecological success in both native and invasive ranges is likely attributed to its wide tolerance of various environmental stressors making it a well suited model for studying mechanisms of mitochondrial tolerance to hypoxia and salinity. Previous studies have identified a variety of cellular mechanisms contributing to its high tolerance (David et al., 2005; Le Moullac et al., 2007; Zhang et al., 2016). *C. gigas* exhibits cellular resistance to apoptosis and reduced inflammation, which results in less severe mitochondrial and cellular damage during H/R stress compared to *M. edulis* (Falfushynska et al., 2020a). Furthermore, the Pacific oyster undergoes rearrangements in mitochondrial function in response to H/R stress to maintain energy homeostasis (Sussarellu et al., 2013). Additionally, its tolerance to H/R stress may be further supported by shifts in the mitochondrial proteome that regulate the efficiency of the ETS and modulate electron flow to reduce potential ROS bursts (Falfushynska et al., 2020b; Sokolov et al., 2019). Its wide salinity tolerance range of 12 to 42 psu reflects its strong euryhaline adaptation (Wiltshire, 2007) and is associated with robust osmotic resilience in mitochondrial and cellular functions (Meng et al., 2013; Sokolov and Sokolova, 2019).

Extreme longevity (Wanamaker et al., 2008) and exceptional hypoxia tolerance (Theede et al., 1969; Theede, 1973) characterize the ocean quahog *Arctica islandica* (Linnaeus, 1767), native to the North Atlantic and Baltic Sea. In contrast to other studied species, *A. islandica* experiences unpredictable hypoxic events (Conley et al., 2011; Gustafsson et al., 2012) and spontaneously self-induces hypoxia by digging into sediment where it enters MRD and transitions to anaerobic metabolism (Oeschger and Storey, 1993; Taylor, 1976). The ocean quahog's longevity is linked to a wide range of cellular mechanisms that reduce oxidative stress during aging, thereby increasing resilience to hypoxic events. In addition to the strong influence of extrinsic factors on life expectancy, various physiological and biochemical traits also contribute to its longevity (Strahl et al., 2007; Strahl and Abele, 2010). These putatively adaptive traits (including membrane composition resistant to

oxidation, low mitochondrial ROS production associated with ETS modulation, and high antioxidant capacities) prevent oxidative stress simultaneously delaying ageing and making ocean quahogs hypoxia tolerant (Abele et al., 2008; Blier et al., 2017; Munro and Blier, 2012; Munro et al., 2013; Munro and Blier, 2015; Munro and Treberg, 2017). High capacities for damage repair and removal, along with low tissue oxygenation in both burrowed and non-burrowed states, support the ocean quahog's resilience to hypoxia and oxidative stress (Basova et al., 2012; Begum et al., 2009). *A. islandica* is also tolerant to salinity changes, although combination of low salinity with low temperature can be stressful to this species (Begum et al., 2009; Begum et al., 2010; Hiebenthal et al., 2012).

Overall, the differences in habitat and lifestyle conditions among the four bivalve species, along with previous findings regarding their tolerance mechanisms to hypoxia and salinity stress, make them a well-suited model system for assessing the mitochondrial basis of hypoxia tolerance in marine mollusks.

1.2.2.2 Experimental design and methodology

To address research aims of the present study, bivalves were exposed to short-term hypoxia (24 hours <0.01% O_2) or hypoxia-reoxygenation stress (24 hours <0.01% O_2 and subsequent 1.5 hours 21% O_2). The control group was maintained under normoxic conditions (**Fig. 6**) (**publications 1, 2, 3, manuscript 4**). Oxygen regimes were chosen to reflect tidal and diurnal cycles of oxygen fluctuations and were based on previous findings of the strongest physiological and mitochondrial response within first hours of reoxygenation (Falfushynska et al., 2020a; Haider et al., 2020; Kurochkin et al., 2009). To assess the potential mechanisms of mitochondrial tolerance to hypoxia, we measured bioenergetics-related parameters OXPHOS and LEAK respiration, ROS efflux of isolated mitochondria in *A. islandica* and *C. gigas* (**publication 1 and 2**) and control coefficients and oxygen flux capacity of isolated mitochondria in *A. islandica* (**publication 1**), assessed transcriptional response of MQC genes in *C. gigas* and *M. edulis* (**publication 3**) and conducted metabolic profiling of the gill and the heart tissues in *C. gigas*, *A. islandica* and *O. edulis* (**manuscript 4**). To assess the combined effects of salinity and H/R stress on mitochondrial functions, *C. gigas* individuals were acclimated to two different salinities (high salinity = 33 and low salinity = 15) for three months prior to H/R exposures (**publication 2**). Salinity conditions were used to cover the current salinity experienced by *C. gigas* in its invasive range in the North Sea while addressing the potential of this species to further expand into the brackish seawater of the Baltic Sea (Ewers-Saucedo et al., 2020; Schmidt et al., 2008; Wiltshire, 2007).

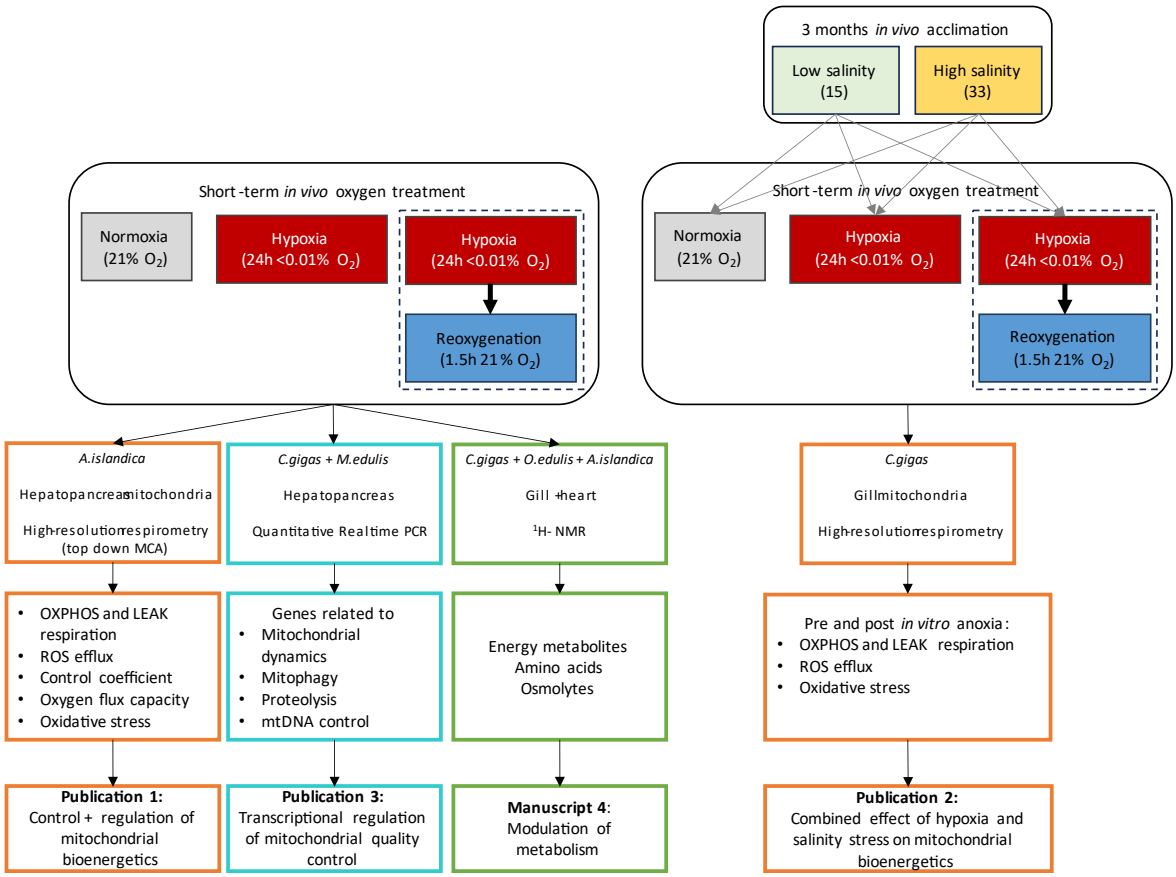

Figure 6: Experimental design and approach to investigate mechanisms of mitochondrial tolerance to short-term hypoxia and reoxygenation stress and effects of salinity acclimation.

MCA – metabolic control analysis, MQC – mitochondrial quality control, NMR – nuclear magnetic resonance spectroscopy; Orange contour – mitochondrial study based on high-resolution respirometry, blue contour – transcriptomic study, green contour – metabolic profiling study. Details of measurement and results are given in chapters 2.1., 2.2., 2.3., and 3.1.

We used high resolution respirometry and fluorometry to assess mitochondrial oxygen consumption and ROS production in different respiratory states (OXPHOS and LEAK) in isolated mitochondria from the bivalves exposed to different oxygen and salinity regimes described in detail in **chapters 2.1. and 2.2.** (**publications 1 and 2**). Additional acute *in vitro* hypoxia during mitochondrial measurements allowed to assess possible intrinsic mechanisms of mitochondrial respiration contributing to hypoxia tolerance (**publication 2**).

Common SUIT protocols primarily focus on LEAK and OXPHOS state functions, often neglecting the metabolic control of individual steps within the respiratory chain. To address this knowledge gap, the effects of H/R stress on the capacity of key mitochondrial subsystems, as well as their regulation and control over mitochondrial respiration, ATP synthesis, and ROS efflux in the hepatopancreas of *A. islandica*, were assessed using top-down MCA (described in detail in **chapter 2.1.**; **publication 1**). The top-down MCA is a simplification of metabolic complexity of mitochondria focusing on the three functionally important and interconnected subsystems of the ETS. The MCA enables the determination of stress effects on the capacities of functional mitochondrial subsystems, including substrate oxidation (SO), proton leak (PL), and phosphorylation (PS). Furthermore, it quantifies the degree of control each subsystem exerts over oxidative phosphorylation and ROS

production under normoxia, intermittent hypoxia and during recovery. All three subsystems are linked by a common intermediate: the proton motive force (pmf or Δp), which influences mitochondrial activities via feedback mechanisms. SO encompasses substrate and nucleotide transporters and the ETS, which generates Δp. The phosphorylation system dissipates this Δp to drive ATP synthesis, while the PL consumes it to maintain MMP within a physiological range (**Fig. 7**) (Ainscow and Brand, 1999a; 1999b). Top down MCA uses an experimental perturbation of Δp coupled with the kinetic response measurement of mitochondrial subsystems to investigate regulatory control of each subsystem over mitochondrial respiration and to identify processes most sensitive to H/R stress (Brand and Kesseler, 1995; Brand, 1998).

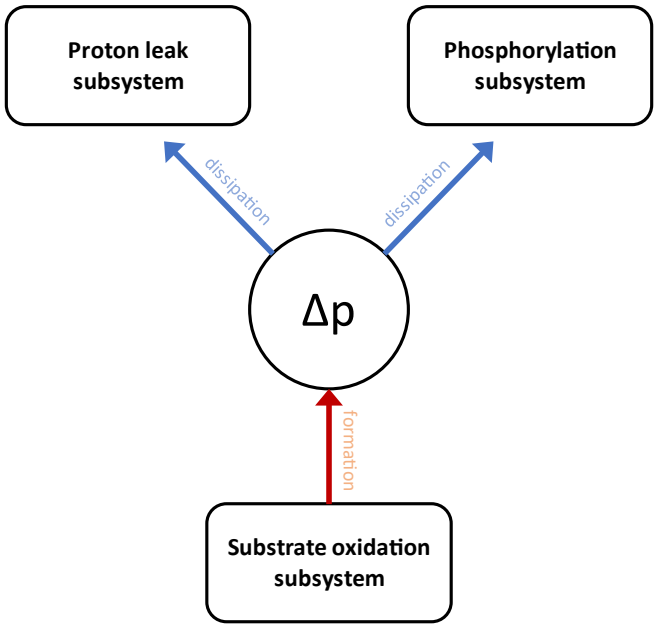

Figure 7: Schematic representation of the concept of top-down metabolic control analysis (MCA).

Substrate oxidation including TCA cycle, electron transport system (ETS), metabolite transport generates protonmotive force (Δp) by pumping protons from mitochondrial matrix to intermembrane space. Two subsystems dissipate Δp: phosphorylation subsystem drives ATP synthesis using this proton gradient, while proton leak subsystem dissipates Δp via cation cycles not linked to ATP synthesis.

Potential protection mechanisms against oxidative stress by MQC and their transcriptomic regulation were assessed by mRNA expression of important markers of MQC during H/R stress using quantitative real-time -PCR (q-RT PCR) in *C. gigas* and *M. edulis* (described in detail in **chapter 2.3.**, **publication 3**). q-RT PCR is a golden standard to investigate gene expression of gene sets in moderate sample number (Gibson et al., 1996; Wang et al., 1989) and is widely used in biomarker analysis and monitoring (VanGuilder et al., 2008).

Metabolism of organisms encompasses an immense network of metabolic pathways, where the mitochondrial ETS plays an important role intertwined in many important energetic pathways. ^{1}H-nuclear magnetic resonance (NMR) spectroscopy is a well-known analytical method first reported by Jungnickel and Forbes, 1963. Over the last decades, importance of NMR has been growing as most suitable for quantitative measurement of complex multi-compound samples (Bharti and Roy, 2012). One advantage of this analytical method over methods like high performance liquid

chromatography (HPLC) and gas chromatography (GC) is its reliance on the structural complexity of molecules for differentiation, rather than depending on the physical properties of molecules (Shaykhutdinov et al., 2009). ^{1}H-NMR allows an overview into changes of a wide range of metabolites associated to different pathways of stress response, energy metabolism and macromolecule synthesis and breakdown feeding energy metabolism and supporting detoxification processes. This study assessed metabolic changes in gill tissue of *C. gigas*, *O. edulis*, *A. islandica* and heart issue of the two former oyster species in response to hypoxia to determine if recovery of energy homeostasis is limited by mitochondrial capacity, cellular metabolism, or combination of both (described in detail in **chapter 3.1.**, **manuscript 4**).

1.3 Role of metabolic plasticity in adaptation and tolerance to hypoxia and salinity stress

Our findings demonstrate the important role of mitochondrial plasticity in hypoxia tolerance of marine mollusks. An intertidal bivalve *C. gigas* showed remarkably robust mitochondrial respiration and redox balance under H/R conditions (**publications 2 and 3**), while mitochondria of the mostly subtidal living bivalves *A. islandica* and *M. edulis* from the Baltic Sea revealed higher susceptibility to sudden reoxygenation (**publications 1 and 3**).

Our studies revealed commonalities in the functional mitochondrial response to hypoxia in all studied marine bivalves, while reoxygenation led to different responses in intertidal and subtidal species, possibly reflecting adaptations to the highly fluctuating oxygen regime in the intertidal habitats (**publication 2**) as compared with the more stable oxygen conditions in subtidal habitats with only occasional self-induced hypoxia as found in *A. islandica* (**publication 1**). The underlying study of **publication 2** found that salinity stress could interfere with intrinsic mitochondrial mechanisms resulting in altered mitochondrial tolerance to H/R stress in marine bivalves (**publication 2**).

In addition to robustness of the respiratory system, MQC plays an important role in hypoxia tolerance at cellular level. Our study revealed higher dependence of *M. edulis* (collected from a permanently submerged Baltic Sea population) on MQC than *C. gigas* from an intertidal population. This suggests higher demand of repair and protection against H/R stress in the less hypoxia-tolerant mussels not experiencing fluctuating oxygen levels in their subtidal habitat (**publication 3**). Furthermore, the analysis of metabolic pathways showed significant differences between two oyster species and the ocean quahog in energy metabolic intermediates and osmolytes in the gills and heart tissues, which may reflect differences in the lifestyle, ecology, and evolution of these species (**manuscript 4**).

1.3.1 How is mitochondrial bioenergetics regulated during H/R stress in hypoxia-tolerant species?

Mitochondrial activity can be divided into different activity states. OXPHOS respiration rate defines mitochondrial oxygen consumption to produce ATP, while LEAK respiration encompasses oxygen consumption due to ion cycling not associated to ATP synthesis. The succinate-driven LEAK respiration was always lower than OXPHOS respiration under ormoxic conditions in both *A. islandica* (~2 and 5 nmol min^{-1} mg^{-1}) and *C. gigas* (~3.5-5 and 20 nmol min^{-1} mg^{-1}) (**publication 1 and 2**). It is worth noticing that data of quahog mitochondria derived from hepatopancreas tissue, while *C. gigas* data are based on gill mitochondria. However, the overall pattern remained unchanged. Our results are in line with previous studies on hard clams and scallops, which report similar low mitochondrial OXPHOS respiration of gill tissue using succinate as a substrate (*M. mercenaria* ~2-5.5 nmol min^{-1} mg^{-1} and *A. irradians* 4.5 nmol min^{-1} mg^{-1}) (Ivanina et al., 2016). Higher respiration rates were measured in the bivalve mitochondria simultaneously oxidizing succinate and Complex I substrates (i.e. pyruvate and malate) (Ouillon et al., 2021; Sussarellu et al., 2013).

Hypoxia mildly increased OXPHOS respiration in mitochondria of *A. islandica*, while *C. gigas*' mitochondria maintained stable OXPHOS respiration rate (**publication 1 and 2**). Maintenance of constant or mildly elevated OXPHOS capacity supports sufficient ATP synthesis during hypoxia. Various other hypoxia-tolerant species such as intertidal bivalves *M. mercenaria* and *C. virginica*, the epaulette shark as well as previous studies on *C. gigas* revealed robust OXPHOS capacities during oxygen deficiency (Hickey et al., 2012; Ivanina et al., 2012; Ivanina et al., 2016; Sokolov et al., 2019). In contrast, hypoxia-sensitive species were found to suffer from mitochondrial depolarization, suppressed OXPHOS and ETS and consequently permanent loss of ATP synthesis capacity resulting in total mitochondrial collapse (Ascensão et al., 2006; Du et al., 2019; Hickey et al., 2012; Ivanina et al., 2016). Avoidance of mitochondrial collapse including mitochondrial depolarization, collapsed ETS and OXPHOS contribute to hypoxia tolerance and support rapid recovery during reoxygenation (Galli et al., 2013; Ivanina et al., 2016; Sussarellu et al., 2013; Zhang et al., 2013). Maintenance of the mitochondrial functions during hypoxia and rapid restoration of energy homeostasis during post-hypoxic recovery in *C. gigas* might be facilitated by their ability of succinate-driven respiration. Elevated rate of succinate driven mitochondrial respiration was attributed to hypoxia tolerance in *Mya arenaria* and *C. gigas* (Adzigbli et al., 2022; Ouillon et al., 2021). In the present study, gill and heart tissue of the intertidal Pacific oyster *C. gigas* and a related European oyster *O. edulis* showed accumulation of succinate during hypoxia followed by fast depletion during reoxygenation (**manuscript 4**). Oxidation of accumulated succinate might facilitate mitochondrial respiration and activity of TCA cycle to rapidly recover energy homeostasis in *C. gigas*. Observation of upregulated fumarate levels during post-hypoxic recovery in gill tissue of the two oyster species in the present study agree with an active forward TCA cycle metabolizing succinate (**manuscript 4**). In contrast, *A. islandica* seems to rely less on succinate-driven respiration during recovery, as succinate was not depleted during reoxygenation (**manuscript 4**). Anaerobic capacities are high in *A. islandica*, allowing survival of prolonged periods of hypoxia due to MRD (Oeschger, 1990; Oeschger and Storey, 1993; Strahl et al., 2011; Taylor, 1976). However, 1.5 hours recovery seems not sufficient to recover from MRD and reincorporate anaerobic end products to restore energy homeostasis, possibly explaining the susceptibility of mitochondria of *A. islandica* to sudden reoxygenation observed in this study (described in **chapter 1.3.2** and **publication 1**).

Mitochondrial proton leak defines the rate of respiration required to counterbalance depolarization caused by ion cycles not linked to ATP synthesis (Brand et al., 1994). A mild elevation of LEAK during reoxygenation is an important control mechanism to regulate ROS production in hypoxia-tolerant species (Brand, 1997; Miwa and Brand, 2003), while high LEAK rates result in energy wastage, impaired OXPHOS capacity and low coupling (Sokolova, 2023). Surprisingly, both studied species, *A. islandica* and *C. gigas*, did not change mitochondrial LEAK respiration rates during H/R stress (**publication 1 and 2**). In contrast, *M. arenaria* appears to have elevated LEAK and lowered OXPHOS capacity resulting in decreased coupling efficiency under fluctuating oxygen regime (Ouillon et al., 2021). Maintenance of relatively stable or slightly elevated mitochondrial LEAK respiration can be interpreted as a safety valve preventing oxidative damage during stress conditions in bivalves.

The coupling efficiency (respiratory control ratio (RCR) and OXPHOS coupling efficiency (CE)) of mitochondria of *C. gigas* and *A. islandica* remained unchanged or were mildly elevated during H/R stress, respectively. High and stable RCR and OXPHOS CE can be attributed to adaptive traits for survival of hypoxic periods in intertidal zone or anoxic sediments. In line with previous studies on marine bivalves, mildly elevated or unchanged mitochondrial coupling facilitates efficient ATP synthesis during oxygen fluctuations and prevents major ETS dysfunction (Cadenas, 2018).

1.3.2 Metabolic control over respiratory fluxes during H/R stress in hypoxia-tolerant species

Control by different mitochondrial subsystems over oxidative phosphorylation plays an important role in regulation of mitochondrial respiration and ATP synthesis in response to environmental stress. MCA is an experimental approach that allows determining, which mitochondrial processes are most sensitive to specific environmental factors. Typically, SO (that encompasses activity of ETS and substrate transporters) exerts the highest degree of control over mitochondrial OXPHOS respiration. This strong control by SO appears to be conserved across ectotherms and is found in hypoxia-tolerant and hypoxia-sensitive bivalve species, as well as in insects and plants (Chamberlin, 2004a; 2004b; Ivanina et al., 2012; Ivanina et al., 2016; Kesseler et al., 1992; Kurochkin et al., 2011). The findings of the present study are consistent with this paradigm and find the dominant control by the SO subsystem over the mitochondrial respiration and OXPHOS rate in *A. islandica* (**publication 1**). Conservation of constant OXPHOS capacity requires continuous function of the ETS system, as it is responsible for the formation of the protonmotive force crucial for ATP synthesis. Consequently, ETS is predominantly responsible for regulating oxidative phosphorylation resulting in highest control of the SO subsystem. Small contribution of the PS and PL subsystems are commonly seen (Ivanina et al., 2012; Ivanina et al., 2016; Kurochkin et al., 2011). The small contribution of PL to the control over OXPHOS may be attributed to the low proton conductance at low Δp of actively phosphorylating mitochondria (Ivanina et al., 2012).

During hypoxia, the SO subsystem, with the ETS activity serving as the primary determinant of SO flux, emerges as a potential focal point for oxygen-related alterations. Specifically, the Complex IV of the mitochondrial ETS, CCO, relies on oxygen as its primary substrate. Thus, a robust ETS system is essential for maintenance of ATP synthesis during oxygen fluctuations (Sokolova et al., 2019). Mitochondria of *A. islandica* maintained the high degree of control by the SO subsystem over OXPHOS during H/R stress (**publication 1**). Stable control distribution over OXPHOS during H/R stress is common to hypoxia-tolerant species, while hypoxia-sensitive species shift control to higher PL and PS contribution in response to environmental stressors (Ciapaite et al., 2009; Ivanina et al., 2012; Ivanina et al., 2016; Kurochkin et al., 2011). Other marine hypoxia-tolerant species, including bivalves, reptiles and fish, maintain or even increase their ETS activity upon H/R stress (Du et al., 2016; Galli et al., 2013; Gerber et al., 2019; Ouillon et al., 2021). Similarly, *C. gigas* appears to have robust ETS functions during hypoxia and reoxygenation in previous studies (Sokolov et al., 2019; Sussarellu et al., 2013). Furthermore, the respiration rates of CCO, the key target of oxygen deficiency, remained unchanged during hypoxia in *C. gigas* (Sokolov et al., 2019). Sokolov et al.,

2019 proposed that modulation of subunits of respiratory complexes might contribute to ETS maintenance in hypoxia-tolerant molluscan mitochondria. Additionally, low control of the PS subsystem over OXPHOS during H/R stress in mitochondria of *A. islandica* resembles the control situation in other hypoxia-tolerant bivalve species in response to H/R stress (Ivanina et al., 2016; Kurochkin et al., 2011). High control by the SO subsystem combined with low control of the PS subsystem in mitochondria of the ocean quahog might reflect an important role of the system generating Δp in setting pace of mitochondrial oxygen flux compared to systems dissipating Δp. Due to the strong oxygen dependence of ETS and the SO subsystem, robustness of these processes are essential for ATP synthesis maintenance and ROS mitigation during H/R stress. This situation implies that stress induced damage to the SO subsystem have a higher impact on OXPHOS and consequently regulation of SO is most likely target for adaptation to achieve hypoxia stress tolerance in marine bivalves.

Unlike other hypoxia-tolerant species, the SO subsystem in mitochondria of *A. islandica* also exerted a high degree of control over the LEAK respiration under the normal (unstressed) conditions (**publication 1**). In a hypoxia-tolerant bivalve species *C. virginica* and in terrestrial insects, control over LEAK respiration is equally shared between subsystems SO and PL (Chamberlin, 2004a; 2004b; Ivanina et al., 2012; Kurochkin et al., 2011). In contrast, mammalian mitochondria show highest degree of control by PL over LEAK respiration (Dufour et al., 1996; Lombardi et al., 2000). For ectotherms unusual distribution of the control over the mitochondrial LEAK respiration found in *A. islandica* might be related to their organization of ETS. Typically, Complexes I and III play key roles in respiratory control in most marine bivalves. In contrast, the mitochondrial respiration flux is controlled by Complexes III and IV in *A. islandica* (Rodriguez, 2021; Rodríguez et al., 2020). The two major ROS-producing Complexes I and II have little control over the quahog mitochondrial respiration. This might be an effective mechanism to control ROS efflux and cope with oxidative stress that contributes to the exceptional longevity of *A. islandica* (Rodriguez, 2021; Rodríguez et al., 2020). Interestingly, hypoxic conditions resulted in reorganization of control over LEAK respiration in mitochondria of *A. islandica*, so that the control became equally distributed between subsystems PL and SO. In contrast to response of the ocean quahog, larvae of the insects and Eastern oysters under stressed conditions such as cadmium exposure increased control of SO over LEAK respiration (Chamberlin, 2004a; 2004b; Kurochkin et al., 2011). As control distribution over LEAK respiration was quickly restored to unstressed conditions in mitochondria of *A. islandica* during post-hypoxic recovery, both subsystems seem to play important roles in control over LEAK respiration and plastic response of mitochondria to environmental stress.

1.3.3 Intrinsic mechanisms of mitochondrial tolerance are modulated by salinity stress

In natural habitats, environmental changes encompass multiple stressors. In coastal regions, the combination of human activities and land-based influences significantly exacerbates hypoxia. This is further compounded by salinity fluctuations caused by substantial freshwater influx and varying rates of evaporation and precipitation. Consequently, these factors collectively affect the organisms inhabiting these highly variable and dynamic ecosystems. Acclimation to low and high salinity did

not change mitochondrial performance after *in vivo* exposure of *C. gigas* to different oxygen regimes. However, exposure to different salinity regimes affected *in vitro* post-hypoxic responses of mitochondria indicating modulation of intrinsic mitochondrial functions in the Pacific oyster. In *C. gigas*, acclimation to low salinity resulted in a better performance and a higher intrinsic phenotypic plasticity of mitochondria compared to mitochondria from the high salinity acclimated oysters (**publication 2**). A strong increase of LEAK and OXPHOS respiration during *in vitro* post hypoxic recovery of oyster mitochondria from low salinity resulted in better coupling efficiency. Our findings agree with known broad osmotic tolerance of mitochondria from euryhaline marine osmoconformers including *C. gigas* (Ballantyne and Storey, 1983; Ballantyne and Moyes, 1987b; Haider et al., 2018; Noor et al., 2021; Sokolov and Sokolova, 2019). Beyond their tolerance range, mitochondria of euryhaline species experience ETS dysfunction, high ROS production resulting in oxidative stress (Bal et al., 2022; Paital and Chainy, 2012). However, although high salinity acclimated oyster mitochondria did not show upregulation of oxygen consumption during *in vitro* post-hypoxic recovery, there was no evidence of mitochondrial dysfunction or oxidative stress indicating that the osmotic tolerance range has not been surpassed. In mitochondria of blue mussel, intrinsic regulation via PTM of proteins during H/R stress ensured high respiration and mitigation of oxidative stress during post-hypoxic recovery (Sokolov et al., 2021). Our findings in mitochondria of low salinity acclimated oysters resembled the situation observed in *M. edulis* (Sokolov et al., 2021). Consequently, we hypothesize that intrinsic regulatory mechanisms strongly contribute to the hypoxia-tolerant mitochondrial phenotype in *C. gigas* acclimated to low salinity, while high salinity interfered with these intrinsic functions. The high metabolic flexibility of mitochondria from *C. gigas* previously observed in response to acute *in vitro* anoxia might contribute to resilient mitochondrial mechanisms of this species (Adzigbli et al., 2022). However, higher plasticity of mitochondria of *C. gigas* under low salinity came with cost of higher ROS efflux and fractional electron leak (FEL), where ROS production exceeded mitochondrial ability of oxidative stress mitigation. The question remains, if the increase in ROS efflux is damaging or plays a regulatory role via ROS signalling to activate stress response pathways in oysters under different salinity and oxygen conditions (Forman et al., 2010; Sies and Jones, 2020; Sies et al., 2022; Sokolova, 2018).

1.3.4 Mechanisms to mitigate oxidative stress in hypoxia-tolerant species

Mitigation of oxidative stress involves a large network of processes ranging from direct regulation of ROS efflux during mitochondrial respiration to specific metabolic pathways and MQC mechanisms that detoxify ROS or repair oxidatively damaged macromolecules. ROS plays a pleiotropic role in living organisms by acting as signal molecule, but also causing damage to macromolecules including DNA, proteins and membrane lipids (Paradis et al., 2016; Sies and Jones, 2020; Sies et al., 2022; Zorov et al., 2014). As ROS producers and consumers, mitochondria play key roles in ROS mitigation and signalling (Munro and Treberg, 2017; Treberg et al., 2019).

In our study, both the baseline and H/R stress induced levels of ROS were species-specific among the studied species *A. islandica* and *C. gigas*. Baseline FEL (ratio of consumed O_2 released as H_2O_2) of *C. gigas* mitochondria was lower than in mitochondria of *A. islandica* (**publication 1 and 2**). The

species-specific ROS regulation might be associated to specific adaptation to different hypoxia regimes in subtidal or intertidal environments. Overall, ocean quahogs of **publication 1** showed relatively high ROS efflux compared to other bivalves, fish and terrestrial organisms (Gerber et al., 2021; Hickey et al., 2012; Li Puma et al., 2020; Ouillon et al., 2021). The strong hypoxia tolerance of *A. islandica* is commonly associated to its life-prolonging physiology accompanied by low metabolic activity, low ROS efflux, high antioxidant capacity and slow cell turnover rates as well as with its energy saving mechanisms of self-induced MRD (Abele et al., 2008; Strahl et al., 2007; Strahl et al., 2011). However, the population of this study originated from the Baltic Sea. Previous studies on individuals of *A. islandica* in the Bay of Kiel have shown significant shorter life spans associated with higher metabolic rates and higher cellular damage accumulation over age as well as less reliance on MRD (Philipp et al., 2012). In contrast, higher physiological flexibility and stress hardening of this population lead to stronger stress response (Philipp et al., 2012). The higher baseline ROS levels observed in this study might be related to the population-specific phenotype of the Baltic Sea *A. islandica* that evolved stress tolerance at the expense of higher oxidative stress.

Furthermore, ROS efflux was not severely affected by mitochondrial respiratory state in *A. islandica*, despite its being dependent on MMP, which is typically higher in LEAK than in OXPHOS state (**publication 1**). In contrast, *C. gigas* showed higher ROS efflux during LEAK respiration (**publication 2**), which resembles the common pattern in mammals and aquatic animals, where LEAK state results in higher ROS efflux compared to OXPHOS (Gerber et al., 2021; Liu et al., 2002; Munro et al., 2013; Ouillon et al., 2021). Depending on mitochondrial state, complexes producing ROS vary (Quinlan et al., 2012). The unusually low contribution of two major ROS-generating complexes (Complex I and III) in respiratory control in *A. islandica* compared to other marine bivalves (described in **chapter 1.3.2** and **publication 1**) might partially explain the uniform ROS efflux in LEAK and OXPHOS state and act as a mechanism to limit mitochondrial oxidative damage (Rodriguez, 2021; Rodríguez et al., 2020).

During post-hypoxic recovery, hypoxia-sensitive species suffer from extensive ROS burst damaging macromolecules together with suppression of ETS and OXPHOS activity (Cadenas, 2018; Honda et al., 2005). Resulting collapse of ETS increases FEL and exacerbates ROS damage (Zorov et al., 2014). Thus, suppression of ROS efflux and following FEL is considered beneficial in response to H/R stress and contributes to hypoxia tolerance (Sokolova et al., 2019). Overall, the link between ROS production, antioxidant capacity and hypoxia tolerance is still poorly understood (Sokolova et al., 2019). In the present study, effect of H/R stress on ROS efflux and FEL varied across the two studied species *A. islandica* and *C. gigas*. In *C. gigas*, ROS efflux and FEL remained unchanged during H/R stress independent of respiratory state of mitochondria (**publication 2**). *A. islandica* mildly suppressed ROS efflux and FEL during hypoxia in OXPHOS state, whereas the ROS efflux (but not FEL) was enhanced during reoxygenation (**publication 1**). In LEAK state, ROS efflux and FEL were upregulated during hypoxia, recovering back to normoxic baseline levels during reoxygenation in *A. islandica*. The suppression of FEL and ROS efflux in OXPHOS state is commonly seen as defence mechanism in hypoxia-tolerant species to prevent oxidative damage (Du

et al., 2016; Hickey et al., 2012; Milton and Prentice, 2007; Pamenter et al., 2007; Sokolov et al., 2021). Our study did not show a consistent suppression of FEL in OXPHOS state after H/R stress (which was suppressed in *A. islandica* but unchanged in *C. gigas*), yet no detectable oxidative stress was found in mitochondria of these species. It remains to be investigated, if this variation in ROS regulation relates to their adaptation to different environments (subtidal with unpredictable fluctuations vs intertidal predictability) and life spans.

Our study also provided novel insights into the species-specific differences in intermediary metabolism of bivalves and their potential links with hypoxia tolerance and ROS regulation. The differences in mitochondrial ROS regulation are commonly related to the activity of other protective mechanisms against oxidative stress such as antioxidants and cytoprotective molecules (Abele et al., 2008; Hermes-Lima et al., 1998; Hermes-Lima et al., 2015; Ivanina and Sokolova, 2016; Sokolov et al., 2019; Sokolova et al., 2019; Wei et al., 2022; Zhang et al., 2012). Metabolic profiling of gill tissue of *C. gigas*, *O. edulis* and *A. islandica* showed signs of altered glutamate-glutamine metabolism (**manuscript 4**). Regulation of glutamate and glutamine levels varied between species. Hypoxia increased glutamate levels in gill of *O. edulis* and *A. islandica*. During reoxygenation, glutamate levels increased in gill of *C. gigas*, remained elevated in gill of *O. edulis* and recovered back to baseline levels in gill of *A. islandica*. Solely in gill of *O. edulis*, glutamine levels were increased by hypoxia and reoxygenation. Similarly, glutamate levels were high in gill of *M. edulis* after short and long-term H/R stress, while *C. gigas* did not seem to upregulate glutamate levels in previous studies (Bruhns et al., 2023; Haider et al., 2020). Glutamate has a variety of different functions and effects in metabolism of organisms. In excitatory tissues such as brain, glutamate can cause oxidative stress, called glutamate neurotoxicity (Atlante et al., 2001; Nguyen et al., 2011). However, studies on mammalian model systems (rat and piglet) revealed that oxidative stress induced by herbicide-contaminated food was reduced by glutamate supplementation (Liu et al., 2016; Yin et al., 2015). In mitochondria of *C. gigas*, glutamate caused not only highest OXPHOS respiration, but allowed also mitigation of succinate-driven ROS production under normoxic conditions (Adzigbli et al., 2022). Briefly, introduction of glutamate into the TCA cycle increases reduced NADH pool, which in turn might increase the forward electron flow through Complex I, while simultaneously mitigating reverse electron transfer and thus ROS production (Robb et al., 2018). However, only combination of succinate and glutamate (or other NADH-linked substrates) mitigated increased FEL during post-hypoxic recovery in the previous study (Adzigbli et al., 2022). In the present study, all three studied species showed strong regulation of succinate and glutamate levels in response to H/R stress, which might be an adaptive trait to mitigate oxidative stress. Overall, succinate has been proposed to act as stress fuel in ectotherm mitochondria (Guo et al., 2020). Alternatively, accumulation of glutamate and glutamine might be associated to other mechanisms such as ammonium detoxification during H/R stress. Various anaerobic pathways result in ammonia as highly toxic end product, requiring fast removal (Limón et al., 2021). Fixation of ammonia to alanine and α-ketoglutarate involves glutamate-dehydrogenase and glutamine synthetase. This process is well studied in mammalian models (Hakvoort et al., 2017; Voss et al., 2021), but involvement of it in mitigation of oxidative stress in marine invertebrates is yet to be determined.

MQC can also contribute to mitochondrial resilience to hypoxia and mitigation of oxidative stress in marine bivalves. Comparison of *C. gigas* and *M. edulis* mRNA expression of MQC revealed muted response in oysters, while blue mussels appeared to switch to stress responses during short-term exposure. *M. edulis* shifted to mitochondrial fission, suppressed fusion, increased mitochondrial proteolysis and activated mitophagic processes during H/R stress (**publication 3**). Upregulation of protective mechanisms might facilitate elimination of dysfunctional mitochondria and oxidative damaged proteins and organelles in *M. edulis*. In contrast, the missing response of *C. gigas* might indicate a better ability to maintain cellular homeostasis and higher resistance to apoptosis. As described above, mitochondria of *C. gigas* appear to possess strong regulation and control mechanisms to prevent collapse of respiration and oxidative damage during short-term H/R stress. In *M. edulis*, H/R stress induced upregulation of fission related genes (*dnm*1*l*, *mff*, *fis*1) and genes associated to degradation of fusion proteins (*oma*1), while fusion-related genes were downregulated (*mfn*2) or unaffected (*opa*1). The shift in mitochondrial dynamics towards predominantly fission observed in *M. edulis* is a common cellular stress response that facilitates degradation of damaged mitochondria, while preventing re-joining of dysfunctional mitochondrial to mitochondrial network (Ali and McStay, 2018; Eisner et al., 2018; Sokolova et al., 2019). The important role of mitochondrial dynamics in adjustments to interrupted oxygen supply is reflected in ischemia/reperfusion (I/R) induced regulation of transcription and PTM of fusion and fission proteins in mammals (Li and Liu, 2018). However, reoxygenation showed signs of rapid re-balancing of mitochondrial fusion in *M. edulis*. In contrast, transcriptional response of important proteases (*lonp*1 and *spg*7) was unaffected by H/R stress. These proteases are triggered by oxidative stress. However, thresholds of oxidative stress are species-specific and thus lead to wide variation of transcriptional patterns across species. Hypoxic conditions seem to not have reached this threshold of *M. edulis* reflected in the lack of transcriptional regulation. Earlier findings of upregulated apoptosis in *M. edulis* during H/R stress (Falfushynska et al., 2020a), agree with our present findings of upregulated transcriptional response of the genes of the PINK1-parkin pathway (*pink*1, *prkn*, *pgam*5) during reoxygenation. Metabolic profiling of *M. edulis* indicated early onset of severe stress response during H/R exposure by onset of anaerobiosis, downregulation of gluconeogenesis and alterations in protein turnover (Haider et al., 2020). The present study on MQC and earlier study on pro-apoptotic and inflammatory response in *M. edulis* show clear shifts of hypoxic and recovery transcriptomic response compared to normoxic baselines (Haider et al., 2020) (**publication 1**). In contrast, muted transcriptomic response of *C. gigas* corresponds to the lack of apoptotic and pro-inflammatory response previously reported in this oyster species (Falfushynska et al., 2020a). However, indication of strong PTM in *C. gigas* in response to H/R stress might explain the lack of transcriptomic response as well as its strong tolerance and rapid response to sudden oxygen fluctuations common in intertidal environments (Falfushynska et al., 2020b; Sokolov et al., 2019; Sussarellu et al., 2013).

1.3.5 Conclusions

Mitochondrial tolerance encompasses a wide range of mechanisms including regulatory control of mitochondrial respiration on cellular and mitochondrial level, ROS mitigation, antioxidant systems and MQC responses. Characteristics of these mechanisms vary across species. However, the present study together with previous studies suggest that natural exposure to hypoxia resulted in development of hypoxia-tolerant mitochondrial phenotypes, a combination of features commonly found in hypoxia-tolerant marine bivalves.

In *C. gigas* and *A. islandica,* hypoxia-tolerance is associated to highly robust mitochondrial OXPHOS and ETS activity against H/R stress. However, response to reoxygenation shows variations of typical phenotype response between species, which might be attributed to an adaptation to intertidal life with frequently strong oxygen fluctuations (*C. gigas*) or to more submersed life with occasional self-burrowing events (*A. islandica*). The observed hypoxia-tolerant mitochondrial phenotype was associated to stable and mildly increased OXPHOS and LEAK respiration resulting in constant or even elevated coupling efficiency in *A. islandica* and *C. gigas* during H/R stress. Maintenance of mitochondrial respiration and avoidance of mitochondrial collapse during H/R stress seemed to be supported by reliance on anaerobic metabolic pathways (succinate accumulation) in *C. gigas*, *A. islandica* and *O. edulis.* Metabolic profiling revealed involvement of anaerobic end products in rapid recovery of energy homeostasis contributing to hypoxia tolerance in intertidal bivalves *C. gigas* and *O. edulis*, while *A. islandica* appears to not eliminate anaerobic activity and process end products within the studied short time (1.5 hours) of reoxygenation. Although metabolic responses differed between species, the ability of the studied bivalve species to maintain mitochondrial respiration allowed efficient ATP synthesis and preservation and recovery of energy homeostasis during H/R stress.

Especially, ETS is highly susceptible to oxygen deficiency due to its strong oxygen dependence. Thus, robust ETS and dominant control of the SO subsystem over OXPHOS respiration observed in this study, are essential in hypoxia tolerance of *A. islandica* and other hypoxia-tolerant marine bivalves (Ivanina et al., 2016; Kurochkin et al., 2011). Although control distribution over mitochondrial LEAK respiration appeared to be different in *A. islandcia* compared to other hypoxia-tolerant marine species, remarkable control pattern might be associated to plastic response mechanisms that allow their unparalleled longevity and exceptional lifestyle (Rodriguez, 2021). Avoidance of mitochondrial collapse including robust OXPHOS respiration, ETS and control distribution attributed to a hypoxia-tolerant mitochondrial phenotype prevents energy loss. According to the concept of energy limited stress tolerance, environmental stress can limit energy supply and its metabolic conversion resulting in trade-offs between basal maintenance costs and energy demand of fitness related functions. Limitation of energy availability and metabolic conversion can thus limit stress tolerance (Sokolova, 2021). Thus, mitochondria appear to play key roles in environmental stress tolerance of marine bivalves (Sokolova et al., 2012). However, combined exposure of *C. gigas* to hypoxia and salinity stress suggests that salinity modulated features of the hypoxia-tolerant mitochondrial phenotype, specifically intrinsic mechanisms. One

venue for future research is to explore how different combinations of multiple stressors modify intrinsic mechanisms of a hypoxia-tolerant mitochondrial phenotype.

Furthermore, mitochondrial hypoxia tolerance relies on ROS mitigation processes reflected in unchanged or suppressed ROS efflux in *C. gigas* and *A. islandica*, respectively. Additionally, metabolites such as glutamate appear to play important roles in mitigation processes to avoid oxidative stress by regulation of ROS efflux or taking part in detoxification processes. MQC appears to play another key role in hypoxia tolerance of mitochondria in *C. gigas* and *M. edulis*. Responses of the two studied species showed differences in regulation of these mechanisms. *M. edulis* showed strong transcriptomic regulation and adjustment in response to H/R stress suggesting strong requirement of upregulated protection from MQC. In contrast, transcriptomic response of *C. gigas* was not strongly affected by H/R stress. However, previous studies found high involvement of PTM and allosteric regulation in response to H/R stress in mammalian models (Gowthami et al., 2019; Hüttemann et al., 2012; Mathers and Staples, 2019) and in marine bivalve species (Falfushynska et al., 2020b; Sokolov et al., 2019; Sokolov et al., 2021; Sokolova et al., 2019). Therefore, future research is necessary to investigate the involvement of other regulatory mechanisms such as PTM to complete our knowledge of the role of MQC in hypoxia tolerance of marine mitochondria.

Overall, our study emphasizes the importance of mitochondrial functions in hypoxia tolerance of marine bivalve species. Our findings underline features of a hypoxia-tolerant mitochondrial phenotype that are species-specific and modulated by lifestyle and environmental factors. Moreover, hypoxia tolerance of mitochondria is based on a complex network of respiratory control, metabolic pathways, and protection mechanisms such as MQC and antioxidant capacity. It warrants further investigations to better understand the interactions of these important mechanisms in mitochondrial tolerance and responses to environmental change.

1.4 References

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Part II: Publications

2 Publications

2.1 Publication I: Mitochondrial capacity and reactive oxygen species production during hypoxia and reoxygenation in the ocean quahog, *Arctica islandica*

Published in the Journal of Experimental Biology

Contribution letter

The contents of the first publication chapter from this dissertation have been published in 2021 in the Journal of Experimental Biology under the title "Mitochondrial capacity and reactive oxygen species production during hypoxia and reoxygenation in the ocean quahog, *Arctica islandica*".

I predominantly contributed to this publication and was involved in experimental design and planning, performance of incubation experiments, tissue collection, isolation of mitochondria for respiratory rates. Assessment of respiration rates and ROS efflux of isolated mitochondria was done in collaboration with Dr. Fouzia Haider, during which Dr. Fouzia Haider and me shared performance of mitochondrial respirometry assays 2:3. Additionally, I have also performed the complete statistical analysis and prepared all graphical output. I wrote the first draft of the manuscript and contributed to the revisions of the draft in response to the comments of the peer reviewers.

Contribution of the candidate in % of workload:	
Experimental concept and design	95%
Experimental work and data acquisition	75%
Data analysis and interpretation	95%
Preparation of figures and tables	100%
Drafting the manuscript	85%

Signature of consent

Supervisor	Doctoral candidate
_____	_____
Prof. Dr. Inna Sokolova	Jennifer Barbara Maria Steffen

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RESEARCH ARTICLE

Mitochondrial capacity and reactive oxygen species production during hypoxia and reoxygenation in the ocean quahog, *Arctica islandica*

Jennifer B. M. Steffen[1], Fouzia Haider[1], Eugene P. Sokolov[2], Christian Bock[3] and Inna M. Sokolova[1,4,*]

ABSTRACT

Oxygen fluctuations are common in marine waters, and hypoxia–reoxygenation (H–R) stress can negatively affect mitochondrial metabolism. The long-lived ocean quahog, *Arctica islandica*, is known for its hypoxia tolerance associated with metabolic rate depression, yet the mechanisms that sustain mitochondrial function during oxygen fluctuations are not well understood. We used top-down metabolic control analysis (MCA) to determine aerobic capacity and control over oxygen flux in the mitochondria of quahogs exposed to short-term hypoxia (24 h <0.01% O_2) and subsequent reoxygenation (1.5 h 21% O_2) compared with normoxic control animals (21% O_2). We demonstrated that flux capacity of the substrate oxidation and proton leak subsystems were not affected by hypoxia, while the capacity of the phosphorylation subsystem was enhanced during hypoxia associated with a depolarization of the mitochondrial membrane. Reoxygenation decreased the oxygen flux capacity of all three mitochondrial subsystems. Control over oxidative phosphorylation (OXPHOS) respiration was mostly exerted by substrate oxidation regardless of H–R stress, whereas control by the proton leak subsystem of LEAK respiration increased during hypoxia and returned to normoxic levels during reoxygenation. During hypoxia, reactive oxygen species (ROS) efflux was elevated in the LEAK state, whereas it was suppressed in the OXPHOS state. Mitochondrial ROS efflux returned to normoxic control levels during reoxygenation. Thus, mitochondria of *A. islandica* appear robust to hypoxia by maintaining stable substrate oxidation and upregulating phosphorylation capacity, but remain sensitive to reoxygenation. This mitochondrial phenotype might reflect adaptation of *A. islandica* to environments with unpredictable oxygen fluctuations and its behavioural preference for low oxygen levels.

KEY WORDS: Hypoxia tolerance, Mitochondria, Aerobic capacity, Top-down metabolic control analysis, High-resolution respirometry, Oxidative stress marker

INTRODUCTION

Hypoxic zones (characterized by low concentrations of dissolved oxygen, DO, <2 mg O_2 l^{-1}) are on the rise worldwide (Breitburg et al., 2018, 2019). Closed basins such as the Baltic Sea are especially prone to deoxygenation, as shown by a 10-fold increase in hypoxic zones over the last decade caused by nutrient pollution and climate change (Carstensen et al., 2014). Hypoxic events can occur daily for a few hours, or seasonally, extending over weeks to months (Diaz and Rosenberg, 1995, 2008). Oxygen deficiency can impair organisms' performance and survival, leading to cascading effects in marine ecosystems (Diaz and Rosenberg, 1995). Sessile benthic organisms cannot escape hypoxic zones, which makes them prone to hypoxia and reliant on physiological adaptations to withstand the stress (Grieshaber et al., 1994; Vaquer-Sunyer and Duarte, 2008). Many hypoxia-adapted marine organisms such as benthic bivalves can temporarily survive oxygen deficiency by transitioning to anaerobic metabolism and conserving energy by reversible suppression of ATP turnover rates (Hochachka, 1993; Storey, 2002). However, long-term survival, growth and reproduction depends on the organism's ability to restore aerobic metabolism and energy homeostasis once oxygen returns. This ability critically depends on the physiological mechanisms that maintain mitochondrial integrity during hypoxia and allow rapid recovery during reoxygenation.

Mitochondria play a central role in ATP generation, redox balance and cellular signalling and are a key target of hypoxia–reoxygenation (H–R) stress (Sokolova et al., 2019). Generally, oxygen deficiency suppresses the electron transport system (ETS) and impairs oxidative phosphorylation (OXPHOS) in mitochondria. In hypoxia-sensitive species such as terrestrial mammals, hypoxia causes mitochondrial depolarization, oxidative damage and Ca^{2+} overload (Piper et al., 2003; Solaini et al., 2010). Reoxygenation poses additional challenges to the mitochondrial integrity as a result of a burst in production of reactive oxygen species (ROS) (Honda et al., 2005; Paradis et al., 2016). In hypoxia-sensitive organisms, these changes can result in lasting damage to mitochondria, impair recovery (Chouchani et al., 2016; Ivanina et al., 2016; Piper et al., 2003), and cause cell death and tissue injury (Paradis et al., 2016). Studies show that hypoxia-tolerant facultative anaerobes such as intertidal marine bivalves sustain mitochondrial function under oxygen fluctuations and show stable or increased ETS and OXPHOS activity during post-hypoxic recovery (Ivanina et al., 2016; Kurochkin et al., 2009; Sussarellu et al., 2013). Reversible protein phosphorylation of ETS complexes (Falfushynska et al., 2020b; Sokolov et al., 2019), upregulation of mitochondrial antioxidants (Ivanina and Sokolova, 2016; Lushchak et al., 2001) and protein quality control mechanisms (Sokolov et al., 2019; Steffen et al., 2020) might contribute to the maintenance of mitochondrial integrity during H–R stress in hypoxia-tolerant marine bivalves. However, the mechanisms that regulate the key aspects of mitochondrial function (including proton leak, OXPHOS capacity and ROS efflux) during H–R stress are not yet fully understood in these organisms.

[1]Department of Marine Biology, Institute of Biological Sciences, University of Rostock, 18059 Rostock, Germany. [2]Leibniz Institute for Baltic Research, Leibniz Science Campus Phosphorus Research Rostock, Warnemünde, 18119 Rostock, Germany. [3]Integrative Ecophysiology, Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research, 27570 Bremerhaven, Germany. [4]Department of Maritime Systems, Interdisciplinary Faculty, University of Rostock, 18059 Rostock, Germany.

*Author for correspondence (inna.sokolova@uni-rostock.de)

F.H., 0000-0002-5388-6453; C.B., 0000-0003-0052-3090; I.M.S., 0000-0002-2068-4302

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Ocean quahogs, *Arctica islandica* are common benthic bivalves in the North Atlantic and the Baltic Sea inhabiting depths from 4 to 482 m. They are extremely long lived (>400 years in some populations) (Wanamaker et al., 2008) and exceptionally tolerant of hypoxia (50% survival rate reported after 55 days of anoxia) (Theede et al., 1969; Theede, 1973). *Arctica islandica* can experience unpredictable hypoxic episodes such as those reported in shallow depths of the Baltic Sea (Conley et al., 2011; Gustafsson et al., 2012). Furthermore, this species spontaneously undergoes extended periods of hypoxia by digging deep into the sediment where it enters a metabolically depressed state and transitions to anaerobic metabolism (Oeschger and Storey, 1993; Taylor, 1976). These unique physiological and behavioural features make *A. islandica* an excellent model to investigate mitochondrial mechanisms of tolerance to prolonged hypoxia and subsequent reoxygenation. To date, studies of the mitochondrial responses of marine facultative anaerobes to hypoxia have been conducted on intertidal molluscs adapted to frequent and predictable cycles of hypoxia and reoxygenation (Sokolova et al., 2019). These studies showed that hypoxia-tolerant intertidal bivalves such as oysters and hard-shell clams upregulate the mitochondrial capacity for substrate oxidation during H–R stress and recover the normal structure of metabolic control over mitochondrial respiration shortly after reoxygenation (Ivanina et al., 2012, 2016; Sokolov et al., 2019). These findings indicate that maintenance of the high substrate oxidation capacity (that tightly correlates with the maximum OXPHOS rate) and the stability of the metabolic control over mitochondrial respiration during H–R stress might be a feature of hypoxia-tolerant mitochondrial phenotype in intertidal bivalves. However, it remains unclear whether these potentially adaptive mitochondrial traits are also found in an exceptionally tolerant subtidal species with marked preference for hypoxic environments such as the ocean quahog, *A. islandica*.

Based on the earlier findings in hypoxia-tolerant intertidal bivalves (Ivanina et al., 2012, 2016; Sokolov et al., 2019), we hypothesized that the hypoxia-tolerant ocean quahogs will upregulate the mitochondrial substrate oxidation during H–R stress and suppress the efflux of ROS, thereby minimizing the oxidative damage to mitochondria during oxygen fluctuations. We also hypothesized that the mitochondria of the quahogs either retain or quickly restore the normal metabolic control over mitochondrial respiration following a hypoxic episode. To test these hypotheses, we used high resolution respirometry coupled with top-down metabolic control analysis (MCA) to determine the effects of H–R exposure on the kinetics of mitochondrial respiration and ROS efflux in the mitochondria of *A. islandica*. The top-down MCA simplifies the metabolic complexity of mitochondria by focusing on three functionally important, interconnected subsystems (Fig. 1): the substrate oxidation subsystem (encompassing the ETS, Krebs cycle and substrate transport) that generates the protonmotive force (Δp); the phosphorylation subsystem (including the mitochondrial F_o,F_1-ATPase, and phosphate and adenylate transporters) that uses Δp for ATP synthesis; and the proton leak subsystem (including all futile proton and cation cycles) that dissipates Δp without ATP synthesis. All three subsystems are linked by a common intermediate, Δp, that influences mitochondrial activities via complex feedback mechanisms. The MCA uses experimental perturbation of Δp coupled with measurement of the kinetic responses of mitochondrial subsystems to this perturbation to investigate the mechanisms of regulatory control over mitochondrial metabolism and identify the processes sensitive to external stressors such as H–R

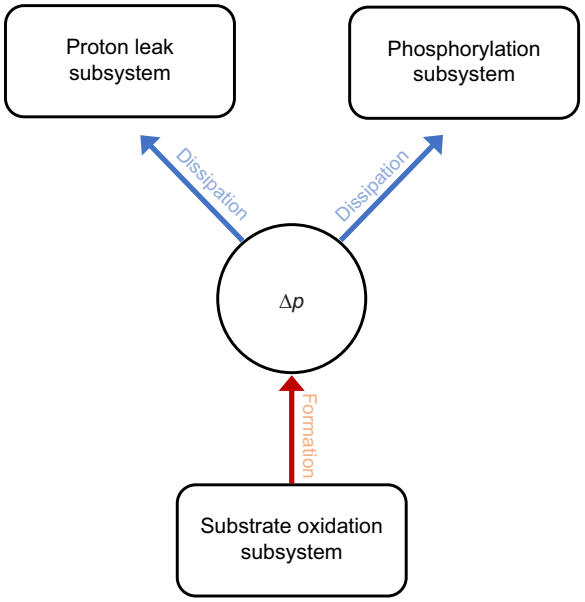

Fig. 1. Mitochondrial subsystems used in top-down metabolic control analysis. Processes of the substrate oxidation subsystem [composed of the TCA cycle, electron transport system (ETS) and metabolite transport] that generates the protonmotive force (Δp) by pumping protons from the mitochondrial matrix into the intermembrane space. Δp is dissipated by two major subsystems: the phosphorylation subsystem uses this proton gradient for ATP synthesis, while the proton leak subsystem dissipates Δp via cation cycles uncoupled from ATP synthesis.

(Brand and Kesseler, 1995; Brand, 1998). Here, we experimentally manipulated Δp in mitochondria from *A. islandica* exposed to normoxia, acute hypoxia (24 h <0.01% O_2) and subsequent reoxygenation (1.5 h 21% O_2) to determine whether H–R stress affects the relationship between Δp, oxygen consumption and ROS efflux in the quahog mitochondria and whether the control over mitochondrial respiration remains stable, indicating resilience to H–R stress. This study provides insights into Δp-dependent kinetics and regulatory mechanisms of mitochondrial functions in an exceptionally hypoxia-tolerant marine bivalve and, combined with the results of earlier studies on intertidal species (Ivanina et al., 2012, 2016; Kurochkin et al., 2009; Sokolov et al., 2019; Sussarellu et al., 2013), sheds light on the common traits of the hypoxia-tolerant mitochondrial phenotype in marine bivalves.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise noted, and were of analytical grade or higher.

Animal maintenance

Individuals of *A. islandica* (Linnaeus 1767) (mean±s.e.m. shell length 44.38±0.76 mm) were collected off the coast of Kühlungsborn, Germany (54°17.145′N, 11°47.143′E) and transported to the University of Rostock (Germany) within 6 h of collection. During transport, quahogs were maintained in aerated cooled water from the site of collection. The quahogs were kept in recirculated temperature-controlled aquarium systems (Kunststoff-Spranger GmbH, Plauen, Germany) with artificial seawater (ASW) (Tropic Marin®, Wartenberg, Germany) at 15±0.5°C and maintained

at a salinity of 15 psu for 2 weeks prior to experiments. These conditions were similar to the quahog's habitat conditions at the time of collection. The quahogs were fed *ad libitum* by continuous addition of a commercial live algal blend (DTs Premium Blend Live Marine Phytoplankton, Coralsands, Mainz Kastel, Germany) according to the manufacturer's instructions (80 ml per 500 l ASW, 3 times a week).

Experimental exposures

Randomly chosen quahog individuals were exposed to 24 h of severe hypoxia (<0.01% O_2) in air-tight 2 l chambers containing eight quahogs at 15±0.5°C and 15 psu. Chambers were bubbled with pure nitrogen with open release valves until an oxygen concentration <0.01% O_2 was achieved (Westfalen AG, Münster, Germany) and then closed with air-tight lids. Oxygen concentration was continuously monitored with an Intellical™ LDO101 Laboratory Luminescent/Optical Dissolved Oxygen Sensor (HACH, Loveland, CO, USA). During exposure, animals were not fed to prevent bacterial growth in the chambers. After hypoxia exposure, a subset of animals was allowed to recover in normoxic ASW (21% O_2) for 1.5 h. Incubation periods of 24 h hypoxia and 1.5 h reoxygenation were chosen based on previous reports demonstrating a strong physiological response within the first few hours of reoxygenation (Falfushynska et al., 2020a,b). The control group was maintained under normoxic conditions in recirculating temperature-controlled aquarium systems (21% O_2). Throughout experiments, no mortality was observed.

Mitochondrial assays

Mitochondria were isolated from hepatopancreas as described elsewhere (Ivanina et al., 2016; Kurochkin et al., 2011). Hepatopancreas was chosen as one of the most metabolically active organs and an energy storage site in marine bivalves. Pilot experiments were conducted to optimize the mitochondrial assay conditions to achieve high respiratory coupling efficiency and mitochondrial integrity revealed by a cytochrome *c* addition test. Based on these pilot studies, the isolation buffer of 760 mOsm and assay buffer of 525 mOsm were used in all subsequent experiments. Briefly, 1.1–1.4 g of hepatopancreas tissue (pooled from 3–4 quahogs) was homogenized in ice-cold isolation buffer [760 mOsm; 30 mmol l^{-1} Hepes, pH 7.5, 100 mmol l^{-1} sucrose, 200 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl, 8 mmol l^{-1} EGTA, 30 mmol l^{-1} taurine, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF), 2 µg ml^{-1} aprotinin and 2 mmol l^{-1} sodium orthovanadate] using a Potter–Elvenhjem homogenizer at 200 rpm. The homogenate was centrifuged at 4°C and 2000 ***g*** for 8 min to remove debris and the supernatant was centrifuged at 4°C and 8500 ***g*** for 8 min to collect mitochondria. The pellet was resuspended in ice-cold assay buffer (525 mOsm) containing 30 mmol l^{-1} Hepes, pH 7.5, 165 mmol l^{-1} sucrose, 50 mmol l^{-1} taurine, 10 mmol l^{-1} NaCl, 130 mmol l^{-1} KCl, 10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 10 mmol l^{-1} KH_2PO_4 and 1% (w/v) fatty acid-free bovine serum albumin (BSA).

The mitochondrial membrane potential (Δψ) and emission of hydrogen peroxide (H_2O_2) were measured in aliquots of the same mitochondrial suspension using two parallel chambers of a 2k Oxygraph (Oroboros, Innsbruck, Austria). Oxygen consumption was monitored in both chambers. Protein concentrations were measured using the Bradford assay (Thermo Fisher Scientific) and corrected for the BSA content of the media. Oxygen consumption rate ($\dot{M}_{O_2}$) and ROS efflux rate were standardized to the mitochondrial protein and expressed in nmol O_2 min^{-1} mg^{-1} protein and nmol H_2O_2 min^{-1} mg^{-1} protein, respectively. Four to six mitochondrial isolates were measured for each of the three subsystems within each oxygen treatment.

We used succinate as a substrate to energize mitochondria, which is a standard approach in top-down MCA as it permits gradual titration of the ETS activity with a competitive inhibitor of succinate dehydrogenase, malonate (Affourtit and Brand, 2006; Brand and Kesseler, 1995; Brand, 1998; Brand and Curtis, 2002; Chamberlin, 2004a,b; Dufour et al., 1996; Kurochkin et al., 2011). Bivalve mitochondria have a strong capacity for succinate oxidation, which might play an important role in post-hypoxic recovery because of the accumulation of succinate under hypoxic conditions in bivalves (Haider et al., 2020; Ivanina et al., 2010; Kurochkin et al., 2009). Based on these considerations, we focused on analysis of the metabolic control over succinate-driven respiration and ROS production in the mitochondria of *A. islandica*.

Determination of $\dot{M}_{O_2}$ and Δψ

The oxygen electrodes were calibrated to 100% and 0% air saturation using air-saturated buffer and saturated dithionite solution, respectively. TPP electrodes were calibrated by a stepwise titration with tetraphenylphosphonium (TPP^+; 0.9–4.6 µmol l^{-1}). To assess the protonmotive force ($\Delta p = \Delta\psi + \Delta pH$), ΔpH was converted into the electrochemical membrane potential (Δψ) by adding the H^+/K^+-ionophore nigericin (123 nmol l^{-1}). Δψ was calculated by the Nernst equation using an estimated mitochondrial matrix volume of 1 µg mg^{-1} and corrected for non-specific binding of TPP^+ as described elsewhere (Ivanina et al., 2016; Kurochkin et al., 2011). The potential contribution of alternative oxidase (AOX) to KCN-insensitive oxygen consumption in *A. islandica* was tested by addition of 20 mmol l^{-1} KCN to inhibit activity of cytochrome *c* oxidase (CCO) followed by addition of 1 mmol l^{-1} of an AOX inhibitor, salicylhydroxamic acid (SHAM). There was no effect of SHAM on $\dot{M}_{O_2}$ in isolated mitochondria of *A. islandica* in any of the experimental groups (data not shown), showing that AOX does not contribute to $\dot{M}_{O_2}$ under the assay conditions of the present study. Therefore, SHAM was omitted in all further assays. Mitochondrial OXPHOS coupling efficiency (OXPHOS CE) was calculated using the $\dot{M}_{O_2}$ of the mitochondria in the LEAK and OXPHOS state as described elsewhere (Ouillon et al., 2021).

Δψ-dependent kinetics of oxygen consumption and top-down MCA

For top-down MCA, kinetic responses of three major mitochondrial subsystems (substrate oxidation, proton leak and phosphorylation) were measured as changes in oxygen consumption during experimentally induced changes in Δψ. Oxygen consumption was measured in the presence of 20 µmol l^{-1} rotenone, 10 mmol l^{-1} succinate and 1.1 mmol l^{-1} ADP. Mitochondrial Δψ was manipulated so as to avoid affecting the activity of the studied subsystem. This was achieved by titration of the mitochondria with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) for the substrate oxidation (SO) subsystem, and with malonate for the proton leak (PL) and phosphorylation (PS) subsystems. Details of the top-down MCA are reported elsewhere (Ivanina et al., 2012, 2016; Kurochkin et al., 2011). Briefly, substrate oxidation was measured in the presence of 5 µmol l^{-1} oligomycin using a stepwise titration with CCCP (0.1–2 µmol l^{-1}) to depolarize the mitochondria via stimulation of the proton leak. Kinetic responses of the proton leak subsystem were measured in the presence of 5 µmol l^{-1} oligomycin and a stepwise addition of malonate to inhibit substrate oxidation. Finally, kinetics of the phosphorylation

subsystem was measured in the presence of a saturating concentration of ADP (1.1 mmol l^{-1}) without oligomycin using titration with malonate (1.4–20 mmol l^{-1}). In the last case, 70 µmol l^{-1} ADP was added during each titration step to ensure that ADP did not become limiting.

ROS efflux

ROS efflux was assessed as described elsewhere (Ouillon et al., 2021). Briefly, assay media contained 5 U ml^{-1} superoxide dismutase (SOD) to convert superoxide into H_2O_2, 10 µmol l^{-1} AmplexRed and 1 U ml^{-1} horseradish peroxidase (HRP) to catalyse the H_2O_2-dependent conversion of AmplexRed to its fluorescent form. Fluorometric sensors were calibrated with the addition of 0.2 µmol l^{-1} H_2O_2 to the assay media. ROS efflux was measured in non-phosphorylating mitochondria in the LEAK state (with 20 µmol l^{-1} rotenone, 10 mmol l^{-1} succinate, 1.1 mmol l^{-1} ADP and 5 µmol l^{-1} oligomycin) and in ADP-stimulated mitochondria in the OXPHOS state (20 µmol l^{-1} rotenone, 10 mmol l^{-1} succinate and 1.1 mmol l^{-1} ADP). The mitochondrial membrane potential was manipulated using malonate titration (1.4–20 mmol l^{-1}) as described above. In a second aliquot of the same sample, $\dot{M}_{O_2}$ and Δψ were measured using identical titration steps, and the data were combined with the H_2O_2 measurements to assess the Δψ dependence of ROS efflux and the rate of electron leak (i.e. the fraction of consumed O_2 converted into H_2O_2).

Oxidative stress markers

Concentrations of malondialdehyde (MDA)–protein conjugates and protein carbonyls (PC) were determined in mitochondria from quahogs maintained under normoxic conditions or exposed to H–R stress using indirect enzyme-linked immunosorbent assays (ELISA) (Ivanina and Sokolova, 2016; Matoo et al., 2013). Mitochondrial suspensions were diluted in 1× phosphate-buffered saline (PBS) to protein concentrations of 0.1 mg ml^{-1} for MDA and 0.01 mg ml^{-1} for PC. Dilutions were sonicated (Sonicator S-4000, Misonix, Farmingdale, NY, USA; amplitude 24, 30 s) to prevent protein aggregation. A MDA standard dilution series was prepared from a 1 mg ml^{-1} MDA–BSA standard (Cell Biolabs, San Diego, CA, USA) in 10 µg ml^{-1} fatty acid- and immunoglobulin-free BSA suspension. PC standards were prepared by oxidizing fatty acid- and immunoglobulin-free BSA with 30% H_2O_2. The concentration of protein carbonyls in the oxidized BSA was assessed spectrophotometrically as described elsewhere (Levine et al., 1990). Oxidized BSA standard was diluted to 10 µg ml^{-1} protein with 1× PBS and used to prepare the standard dilution series. Mitochondrial protein samples and standards were incubated on ELISA plates at 4°C overnight and washed with 1× PBS prior to incubation with antibodies.

For MDA, the plates were blocked with 1 mg ml^{-1} fatty acid- and immunoglobulin-free BSA for 2 h at 37°C, and treated with anti-MDA antibody (1:1000, ab27642, abcam, Cambridge, UK) followed by anti-rabbit antibody conjugated with horseradish peroxidase (1:10,000, 111-035-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). For PC, samples and standards were derivatized by incubation for 45 min with 5 mmol l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in the dark to form dinitrophenylhydrazone–protein carbonyl (DNP). The plates were washed with 1× PBS–ethanol (1:1 v/v) and blocked with 1 mg ml^{-1} fatty acid- and immunoglobulin-free BSA for 2 h at room temperature. The plates were treated with anti-DNP antibody (1:1000, MAB2223, Merck Millipore, Burlington, MA, USA) followed by goat anti-mouse antibody (1:10,000, 115-035-003, Jackson ImmunoResearch Laboratories Inc.). Antibody incubations (1 h each) were conducted at room temperature. Antibodies were detected by addition of horseradish peroxidase substrate 3,3′,5,5′-tetramethybenzidine (TMB/E) Ultra Sensitive (Merck Millipore) and 2 mol l^{-1} sulfuric acid to stop the reaction. Absorbance was measured at 450 nm using a spectrophotometer (SpectraMax iD3, Molecular Devices, LLC, San José, CA, USA).

Data analysis and statistics

Kinetic responses of $\dot{M}_{O_2}$ and ROS efflux rates to changes in Δψ were described using polynomial regressions using the Matlab curve fitting tool (version R2018b, MathWorks Inc., Natick, MA, USA). The kinetic curves were generated for each mitochondrial isolate (Figs S1–S3 and Table S1). Based on the goodness-of-fit, third and second order polynomials were used for oxygen consumption and ROS efflux kinetics, respectively. Because the initial Δψ values (and therefore, Δψ for the corresponding titration steps) differed between mitochondrial isolates (Fig. S1), the individual kinetic curves were used to calculate oxygen or H_2O_2 flux for fixed Δψ values (in 2 mV increments) within the sample-specific Δψ range. The predicted flux values were used to generate the mean kinetic curve for each mitochondrial subsystem, and calculate the flux control coefficients and the oxygen and ROS flux rates at a common Δψ (180 mV for the LEAK and 155 mV for the OXPHOS state) as described earlier (Hafner et al., 1990; Kurochkin et al., 2011).

Normal distribution of data was tested by the Shapiro–Wilk test, and outliers were removed using the 1.5-fold and 3-fold interquartile range of box–whisker plots in IBM® SPSS® Statistics (v.27, IBM Corp., Armonk, NY, USA). In the case of non-normal distribution and/or non-homogeneity of the variances, data were transformed using Box–Cox or Johnson transformation in Minitab (v.19, Minitab LLC., State College, PA, USA). The effects of experimental oxygen regime (treated as a fixed factor with three levels: normoxia, hypoxia and reoxygenation) on the studied mitochondrial traits and oxidative stress markers were tested by one-way ANOVA or Kruskal–Wallis test using SigmaPlot (v.13, Systat Software Inc., San Jose, CA, USA). Significant differences between the pairs of means were determined using Tukey's Honest Significant Differences *post hoc* test. For non-normally distributed data, Dunn's *post hoc* test was used to determine differences between pairs of means.

RESULTS

Mitochondrial $\dot{M}_{O_2}$ and Δψ

Exposure to H–R stress had no significant effect on respiration rates or membrane potential (Δψ) of resting (LEAK) or ADP-stimulated (OXPHOS) quahog mitochondria (P>0.05) (Fig. 2A,B). Exposure to hypoxia and subsequent reoxygenation led to a slight but significant increase in OXPHOS CE in quahog mitochondria (Fig. 2C).

Δψ-dependent kinetics of oxygen consumption and control over respiration flux

The kinetic response of the SO subsystem to changes in Δψ showed an effect of hypoxia on SO kinetics in the range of high (>180 mV) but not low (160–180 mV) Δψ (Fig. 3A). At high Δψ (>180 mV), the oxygen flux of the SO subsystem decreased in the mitochondria from hypoxia-exposed quahogs compared with their normoxic counterparts. After reoxygenation, $\dot{M}_{O_2}$ of the SO subsystem was lower compared with that of normoxic mitochondria across the full experimental Δψ range (Fig. 3A). The effects of H–R stress on the kinetics of the PL subsystem were only apparent in the high Δψ range (>200 mV), where exposure to hypoxia decreased and reoxygenation

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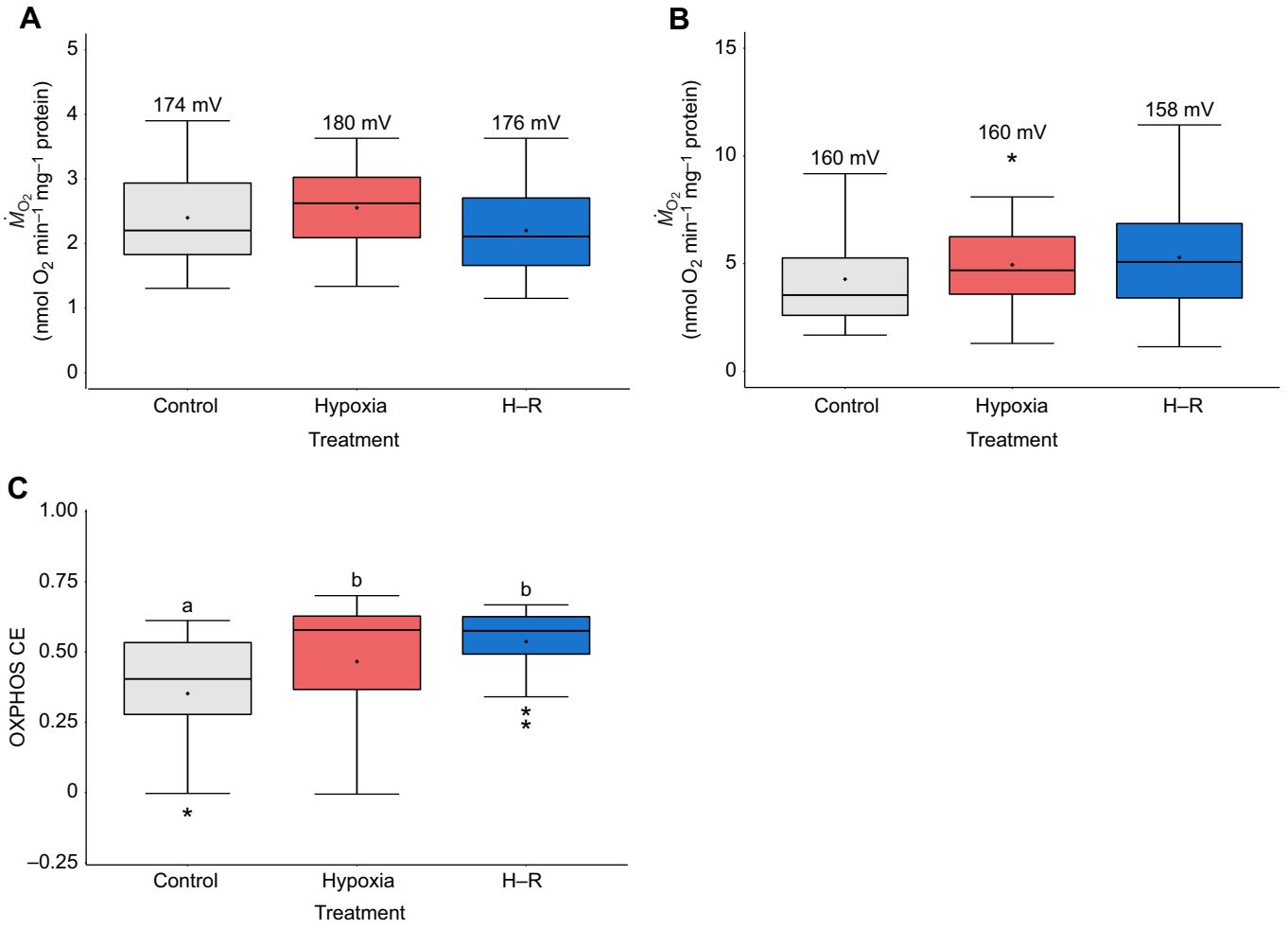

Fig. 2. Effect of short-term hypoxia–reoxygenation (H–R) stress on oxygen consumption rate ($\dot{M}_{O_2}$) and membrane potential (Δψ) of *Arctica islandica* mitochondria in active (OXPHOS) and resting (LEAK) state. (A) $\dot{M}_{O_2}$ of mitochondria in the LEAK state. (B) $\dot{M}_{O_2}$ of ADP-stimulated mitochondria (OXPHOS). (C) OXPHOS coupling efficiency: OXPHOS CE=1−(LEAK/OXPHOS). Succinate was used as a substrate in all measurements. Mean Δψ value for each group in A and B is shown above the respective box plots. Experimental groups: control (21% O_2); short-term (24 h) severe (<0.01% O_2) hypoxia; short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2) (H–R). Means are depicted as points within corresponding box–whisker plots. Outliers are shown as asterisks. Statistical significance (P<0.05) is indicated by different letters above plots. Plots with the same letter or not marked with a letter are not significantly different (P>0.05). N=21–22, 20 and 18–20 in control, hypoxia and H–R, respectively.

elevated the proton leak flux above the normoxic values (Fig. 3B). The strongest response to hypoxia exposure was detected for the PS subsystem kinetics. Hypoxia led to a depolarization of the mitochondrial membrane of phosphorylating mitochondria associated with increased $\dot{M}_{O_2}$ (Fig. 3C). During reoxygenation, the kinetics of the PS subsystem returned to normoxic levels (Fig. 3C). It is worth noting that quahog mitochondria showed considerable individual variability in the kinetic responses of the mitochondrial subsystems (Fig. S1).

Top-down MCA showed that at a physiological Δψ of phosphorylating mitochondria (155 mV), control over OXPHOS respiration (expressed as control coefficient, cc) was mainly exerted by the SO subsystem in all experimental treatments (cc_{SO}>0.95; Fig. 4A). The PS subsystem exerted little control over OXPHOS respiration (cc_{PS}<0.03) under all experimental conditions. PS subsystem control over OXPHOS respiration became negative in mitochondria of hypoxia-exposed quahogs, returning to baseline (normoxic) values during reoxygenation (Fig. 4A). Control over OXPHOS by the PL subsystem was negative in mitochondria of control (cc_{PL}=−0.08) and recovering (H–R; cc_{PL}=−0.30) quahogs, and close to zero (cc_{PL}=0.002) in mitochondria of the hypoxia-exposed quahogs (Fig. 4A).

Control over LEAK respiration was dominated by the SO subsystem (cc_{SO}=0.85), with a minor contribution of the PL subsystem (cc_{PL}=0.15) in mitochondria of control quahogs at a physiologically relevant resting Δψ (180 mV). Hypoxia exposure decreased the SO subsystem control over the LEAK respiration so that control was almost equally shared between the SO and PL subsystems (cc_{SO}=0.58 and cc_{PL}=0.42). In mitochondria of quahogs exposed to H–R, the SO subsystem was mostly responsible for control over the LEAK flux (cc_{SO}=0.97), with a negligible contribution of the PL subsystem (Fig. 4B).

The SO flux capacity of quahog mitochondria remained similar regardless of exposure conditions when assessed at a common Δψ for OXPHOS (155 mV) or LEAK (180 mV) states (Fig. 4C,D). The PS subsystem flux strongly increased in mitochondria of quahogs exposed to hypoxia (by ∼18- and ∼11-fold when compared at 155 and 180 mV, respectively) (Fig. 4C,D). After reoxygenation, the PS flux capacity returned to levels similar to (at 180 mV) or slightly lower than (at 155 mV) those in control quahogs (Fig. 4C,D). At

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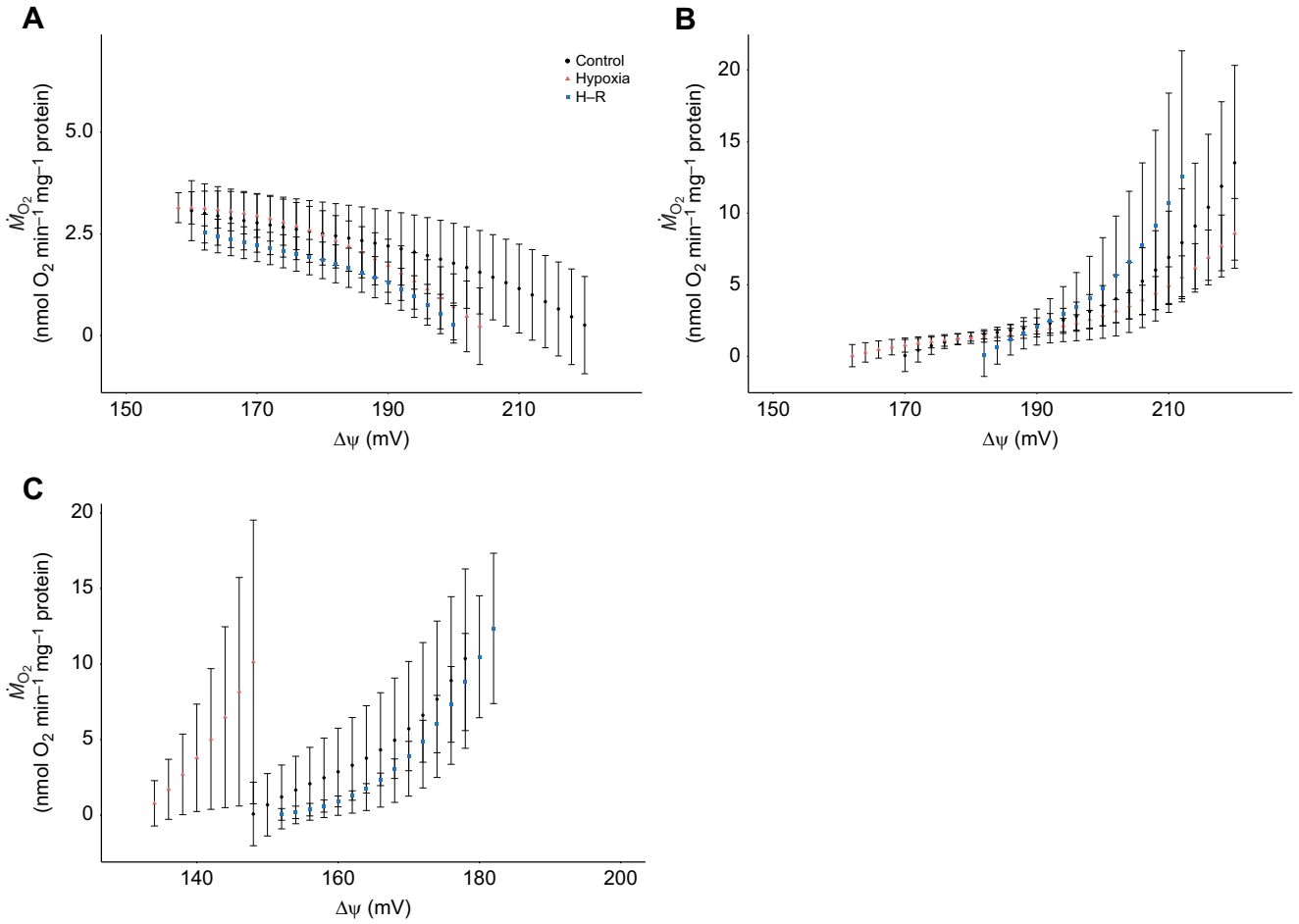

Fig. 3. Kinetic response of the substrate oxidation, proton leak and phosphorylation subsystems to H–R stress. Curves were obtained by averaging the predicted $\dot{M}_{O_2}$ values for a common Δψ (in 2 mV increments based on individual titration curves linking Δψ and $\dot{M}_{O_2}$ for each mitochondrial isolation). (A) Substrate oxidation (SO) subsystem. (B) Proton leak (PL) subsystem. (C) Phosphorylation (PS) subsystem. Experimental groups: control (21% O_2); short-term (24 h) severe (<0.01% O_2) hypoxia; short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2) (H–R). Each data point of the mean curve represents an average of 4–8 mitochondrial isolations. Error bars are s.e.m.

LEAK state Δψ (180 mV), the mitochondrial PL flux capacity did not change in response to hypoxia but strongly (by ∼3-fold) decreased during reoxygenation (Fig. 4D).

Δψ-dependent kinetics of ROS efflux and H_2O_2:O_2 ratio

The rate of mitochondrial ROS efflux (measured as H_2O_2 efflux rate) was dependent on Δψ in mitochondria in the LEAK and OXPHOS state (Fig. 5; Figs S2 and S3). In the LEAK state, mitochondrial ROS efflux increased with increasing Δψ in all experimental treatment groups (Fig. 5A). In actively phosphorylating (OXPHOS) mitochondria of recovering quahogs, ROS efflux was minimal in the physiological range of Δψ (150–155 mV) and increased at both higher and lower Δψ (Fig. 5B). In hypoxic quahogs, mitochondrial ROS efflux increased with increasing Δψ (Fig. 5B). The Δψ dependence of ROS efflux of the OXPHOS-state mitochondria from control quahogs showed an intermediate pattern relative to mitochondria from H–R- and hypoxia-exposed quahogs (Fig. 5B). The ratio of H_2O_2 efflux to O_2 consumption (indicative of the electron leak) was similar in quahog mitochondria in the LEAK and OXPHOS states and decreased with increasing Δψ in all experimental conditions (Fig. 5C,D).

When assessed at a common Δψ (180 and 155 mV for the LEAK and OXPHOS states, respectively), mitochondrial ROS efflux and H_2O_2:O_2 ratio were elevated in resting mitochondria and suppressed in phosphorylating mitochondria of quahogs exposed to hypoxia relative to the normoxic baseline (Fig. 6). The ROS efflux and H_2O_2:O_2 ratio returned to normoxic levels within 1.5 h of reoxygenation (Fig. 6).

Despite variations in ROS efflux, levels of oxidative lesions (MDA and protein carbonyls) in the mitochondrial proteins of *A. islandica* remained at baseline during H–R stress (Fig. 7).

DISCUSSION

Distribution of control over mitochondrial respiration in quahogs

Top-down MCA showed that the SO subsystem exerted the highest degree of control over the succinate-driven OXPHOS and LEAK respiration in mitochondria of control (normoxic) *A. islandica*, with only minor contributions of the PL and the PS subsystems. The predominant control of the SO subsystem over the succinate-driven OXPHOS respiration has been found in diverse ectotherm mitochondria including those of bivalves (Ivanina et al., 2016; Kurochkin et al., 2011) and insects (Chamberlin, 2004a,b), as well as in plants (Kesseler et al., 1992; Kesseler and Brand, 1994a, 1994b, 1994c). Specifically, the control coefficients of the substrate oxidation subsystem (cc_{SO}) over the OXPHOS flux were

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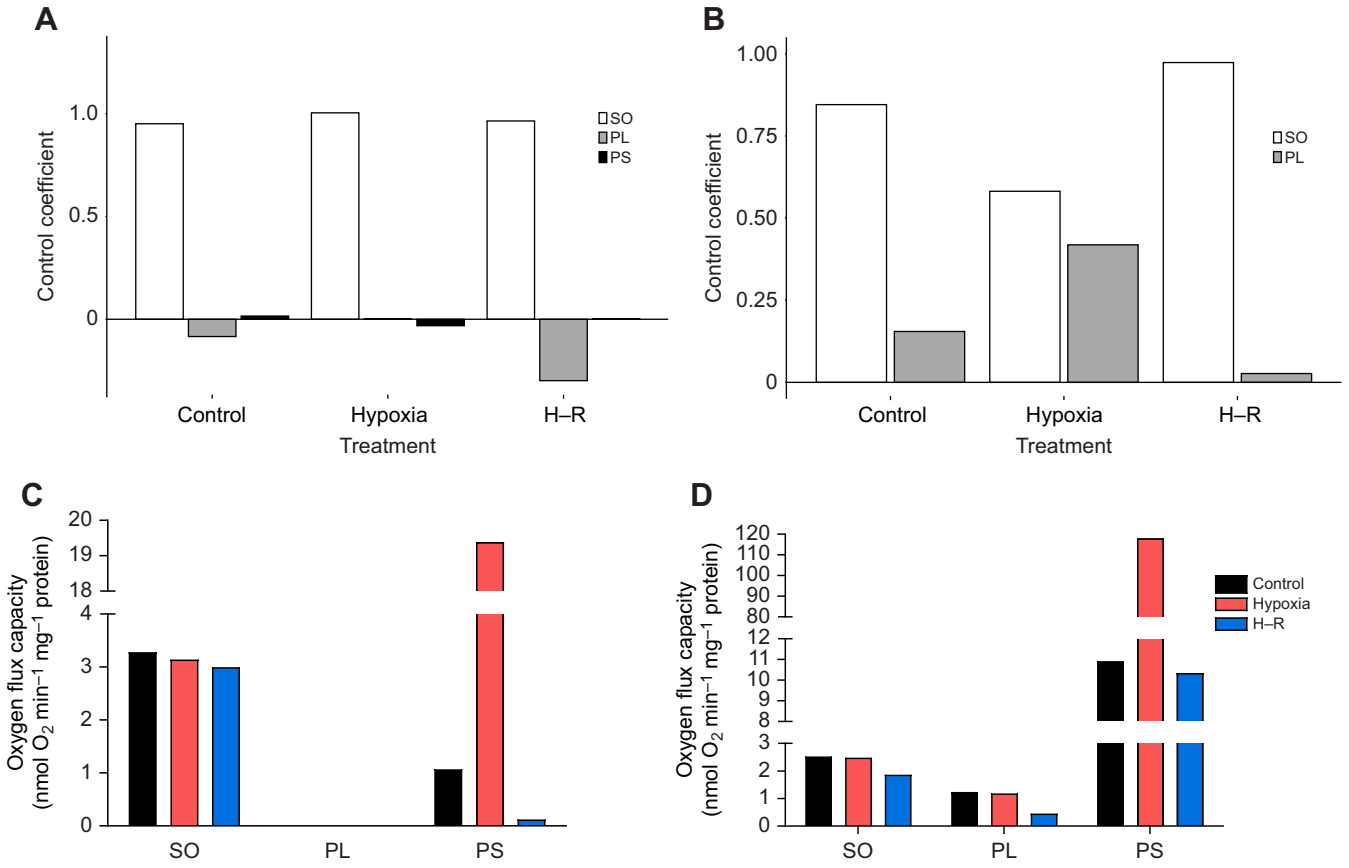

Fig. 4. Control coefficient and oxygen flux capacity of mitochondrial subsystems in OXPHOS and LEAK state in response to H–R stress. (A) Control coefficients over OXPHOS state at 155 mV. (B) Control coefficients over LEAK state at 180 mV. (C) Oxygen flux capacity in the OXPHOS state calculated at a common $\Delta\psi$ of 155 mV. (D) Oxygen flux capacity in the LEAK state calculated at a common $\Delta\psi$ of 180 mV. Experimental groups: control (21% O_2); short-term (24 h) severe (<0.01% O_2) hypoxia; short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2) (H–R). Calculations are based on kinetic curves representing an average of 4–8 mitochondrial isolations per group. The value of the control coefficients shows the degree of control that each subsystem exerts over the oxygen consumption of OXPHOS and LEAK state mitochondria.

~0.75–0.95 in bivalves (including >0.95 in quahogs in the present study) (Ivanina et al., 2012, 2016; Kurochkin et al., 2011), ~0.90–0.95 in insects (Chamberlin, 2004a,b) and ~0.90 in potato tuber mitochondria (Kesseler et al., 1992; Kesseler and Brand, 1994a). The degree of control over the OXPHOS flux was somewhat lower in mammalian mitochondria as shown in liver of rodents (cc_{SO}=0.53–0.68) (Ciapaite et al., 2009; Dufour et al., 1996) and skeletal muscle of pigs (cc_{SO}=0.34–0.45) (Lombardi et al., 2000). Overall, the substantial control of the SO subsystem over OXPHOS respiration appears to be a conserved feature of ectotherm mitochondria, indicating that ETS activity plays an important role in setting pace to the maximum ATP synthesis capacity. This might reflect the dependence of ATP synthesis on the protonmotive force generated by ETS and the important role of the ETS in the regulation of ATP synthesis under the conditions of high ADP:ATP ratio and near-maximum respiration rates.

A high degree of control of the SO subsystem over the LEAK respiratory flux (>80%) in hepatopancreas mitochondria of *A. islandica* energized with succinate sets them apart from mitochondria of other animals studied so far. In *Crassostrea virginica*, control over the LEAK respiratory flux was distributed between the SO (~0.32–0.44) and PL (~0.56–0.68) subsystems (Ivanina et al., 2012; Kurochkin et al., 2011). Similarly, shared control over LEAK respiration of the SO and PL subsystems (0.50–0.55 and 0.45–0.50, respectively) has been reported in succinate-energized insect mitochondria (Chamberlin, 2004a; 2004b). In mammalian (pig and rodent) mitochondria respiring on succinate, the PL subsystem exerted predominant control (>0.7–0.8) over the LEAK respiratory flux (Dufour et al., 1996; Lombardi et al., 2000). The exceptionally high control of the SO subsystem over both the LEAK and the OXPHOS respiration of *A. islandica* might be due to the ETS organization of mitochondria. In *A. islandica* mitochondria, OXPHOS activity is mostly controlled by Complexes III and IV with negligible contributions by Complexes I and II (Rodríguez et al., 2021). In other marine bivalves (*Mya arenaria*, *Spisula solidissima* and *Mercenaria mercenaria*), Complexes I and III play a key role in respiratory control (Rodríguez et al., 2021). This unusual pattern of ETS regulation in *A. islandica* with comparatively low contributions of two major ROS-generating complexes (Complex I and II) might be related to the exceptionally high longevity of this species and act as a mechanism to limit mitochondrial oxidative damage (Rodríguez et al., 2021).

The unusual control features of *A. islandica* mitochondria with a major regulatory role of ETS in the baseline (LEAK) respiration as well as OXPHOS (ATP synthesis) has implications for the mitochondrial responses to H–R stress in this species. Overall, exposure to H–R stress had little effect on the distribution of control over OXPHOS respiration in mitochondria of quahogs. The SO subsystem retained the greatest degree of control (cc_{SO}=0.95–1.00) over the succinate-driven OXPHOS respiration, regardless of H–R

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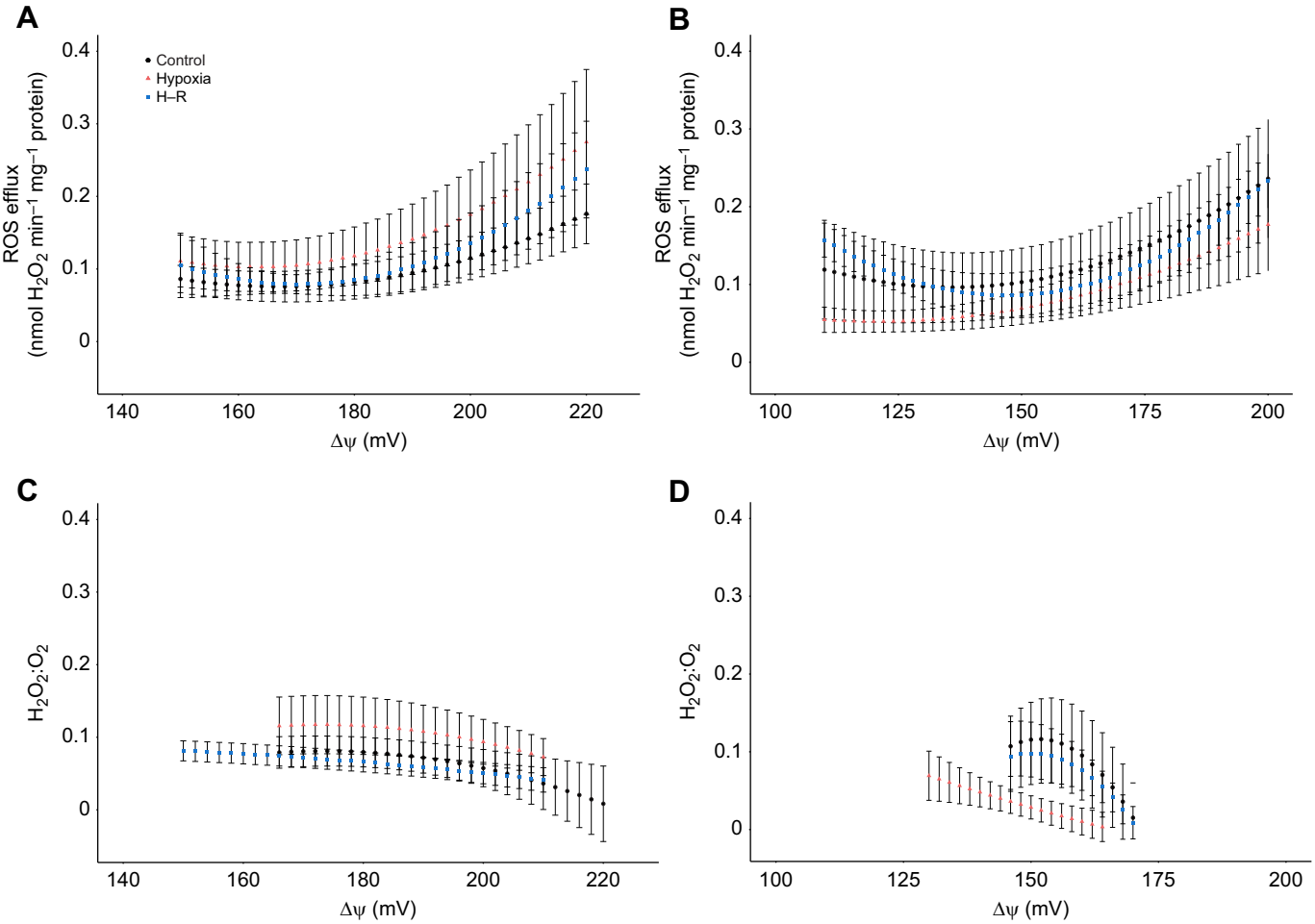

Fig. 5. Kinetic response of reactive oxygen species (ROS) efflux and associated H_2O_2:O_2 ratio in OXPHOS and LEAK state mitochondria during H–R stress. Curves were obtained by averaging the predicted ROS efflux and H_2O_2:O_2 ratios for set Δψ values (in 2 mV increments) based on individual titration curves linking Δψ and ROS efflux or H_2O_2:O_2 ratio for each mitochondrial isolation. ROS efflux was measured as emission of hydrogen peroxide (H_2O_2). (A,B) ROS efflux and (C,D) H_2O_2:O_2 ratio in the (A,C) LEAK state and (B,D) OXPHOS state. Experimental groups: control (21% O_2); short-term (24 h) severe (<0.01% O_2) hypoxia; short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2) (H–R). Each data point of the mean curve reflects an average of 4–6 mitochondrial isolations. Error bars are s.e.m.

exposure. The only notable shift was an increase in the degree of (negative) control of the PL subsystem over OXPHOS respiration in the mitochondria of quahogs during post-hypoxic recovery. Similar to quahogs, the hypoxia-tolerant intertidal bivalves *C. virginica* and *M. mercenaria* preserved a stable distribution of control over OXPHOS respiration during hypoxia and reoxygenation, while in a hypoxia-sensitive species (*Argopecten irradians*), the PL and PS subsystems gained control over OXPHOS under H–R stress (Ivanina et al., 2012, 2016). Unlike OXPHOS, control over the LEAK respiration shifted in mitochondria of quahogs during hypoxia as a result of a decrease in SO subsystem control (from 0.85 to 0.58) and a major gain of control (from 0.15 to 0.42) by the PL subsystem. This mitochondrial response to stress differs from other studied ectotherm and endotherm species, where the contribution of the SO subsystem to control over LEAK respiration increased during exposure to stressors such as Cd (Kurochkin et al., 2011) and low temperature (Brown et al., 2007), or during energy-demanding processes such as metamorphosis (Chamberlin, 2004a,b). The normal control structure over LEAK respiration was rapidly restored in quahog mitochondria during post-hypoxic reoxygenation, indicating that the observed shift in the mitochondrial control distribution under hypoxia represents a plastic response rather than a permanent damage to mitochondria.

Effects of H–R stress on mitochondrial OXPHOS and ETS activity

Mitochondrial OXPHOS of quahogs was resistant to H–R stress as shown by the lack of major effects of H–R on steady-state OXPHOS respiration or Δψ measured in isolated mitochondria. Furthermore, a mild but significant increase of the OXPHOS CE during hypoxia and reoxygenation indicated stronger OXPHOS coupling during oxygen fluctuations that might support efficient ATP synthesis in quahog mitochondria. Similarly, mitochondrial OXPHOS respiration remained stable during H–R stress in hypoxia-tolerant marine bivalves such as the hard shell clam, *M. mercenaria*, and the Pacific oyster, *Crassostrea gigas* (Ivanina et al., 2016; Sokolov et al., 2019), or in the hypoxia-tolerant epaulette shark, *Hemiscyllium ocellatum* (Hickey et al., 2012). In hypoxia-sensitive species of molluscs and fish (Hickey et al., 2012; Ivanina et al., 2016), as well as in terrestrial mammals (Ascensão et al., 2006; Boutilier, 2001; Du et al., 1998), H–R stress results in mitochondrial depolarization accompanied by suppressed OXPHOS respiration and a decrease in ETS activity. This might result in a permanent loss of ATP synthesis capacity and requires involvement of the mitochondrial quality control mechanisms to repair, remove or replace damaged organelles (Anzell et al., 2018; Bohovych et al., 2015; Steffen et al., 2020).

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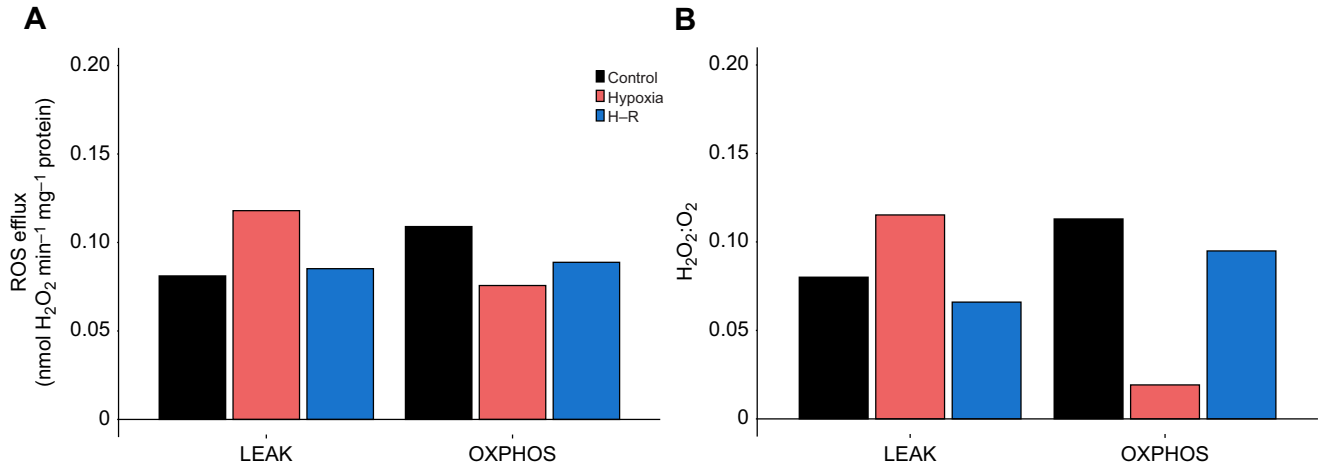

Fig. 6. Effect of short-term H–R stress on ROS efflux and H_2O_2:O_2 ratio in LEAK and OXPHOS state at a common Δψ. (A) ROS efflux at a common Δψ. (B) H_2O_2:O_2 ratio at a common Δψ. The common Δψ was 180 mV for LEAK state and 155 mV for OXPHOS state. ROS efflux was measured as emission of hydrogen peroxide (H_2O_2). Experimental groups: control (21% O_2); short-term (24 h) severe (<0.01% O_2) hypoxia; short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2) (H–R). Calculations are based on kinetic curves representing an average of 4–6 mitochondrial isolations per group.

Avoidance of mitochondrial collapse during H–R stress such as found in quahogs (present study), oysters (Ivanina et al., 2016; Sussarellu et al., 2013), high-altitude locusts (Zhang et al., 2013) and freshwater turtles (Galli et al., 2013) can therefore contribute to the tolerance of these species to fluctuating oxygen conditions.

The SO subsystem that exerts the greatest control over the succinate-driven OXPHOS respiration in ectotherms (Chamberlin, 2004a,b; Ivanina et al., 2012, 2016), including quahogs (this study), is potentially sensitive to oxygen availability because oxygen serves as a substrate of the terminal ETS complex, CCO. Therefore, robust ETS tolerance to oxygen fluctuations is essential for the maintenance of ATP synthesis and mitigation of ROS efflux in hypoxia-tolerant organisms (Sokolova et al., 2019). Furthermore, the ability to rapidly oxidize succinate might be especially important for facultative anaerobes such as marine bivalves that accumulate high concentrations of succinate during hypoxia as an end-product of anaerobic metabolism (Bayne, 2017; Haider et al., 2020; Hochachka and Mommsen, 1983; Hochachka and Mustafa, 1972; Ivanina et al., 2010; Kurochkin et al., 2009). In *A. islandica*, SO flux capacity did not differ in mitochondria of normoxic and hypoxic quahogs, when compared at a common Δψ representative for LEAK state mitochondria (180 mV). Therefore, even though mitochondrial functional properties were changed by hypoxia, as reflected in deviating kinetics, these changes have most likely no relevance to normal conditions at a physiological Δψ. It is worth noting that the ocean quahog maintains low oxygen partial pressure (P_{O_2}) in their mantle cavity even under normoxic conditions (Abele et al., 2010; Strahl et al., 2011). Thus, the hypoxic exposures used in this study might not induce changes of the oxygen-dependent SO subsystem adapted to function at low oxygen levels.

Reoxygenation led to a decrease of SO flux in *A. islandica* mitochondria when assessed at a Δψ of 180 mV, representative of the LEAK state mitochondria. This finding differs from reports in other hypoxia-tolerant marine bivalves where the flux capacity of the SO subsystem remains unchanged or increases during reoxygenation (Ivanina et al., 2012, 2016). The decline of the mitochondrial SO flux during reoxygenation in *A. islandica* is modest (by 26%) as opposed to an almost complete collapse of the SO flux in hypoxia-sensitive bivalves such as scallops (Ivanina et al., 2016). This indicates that the SO subsystem (and by implication ETS) of *A. islandica* is robust to

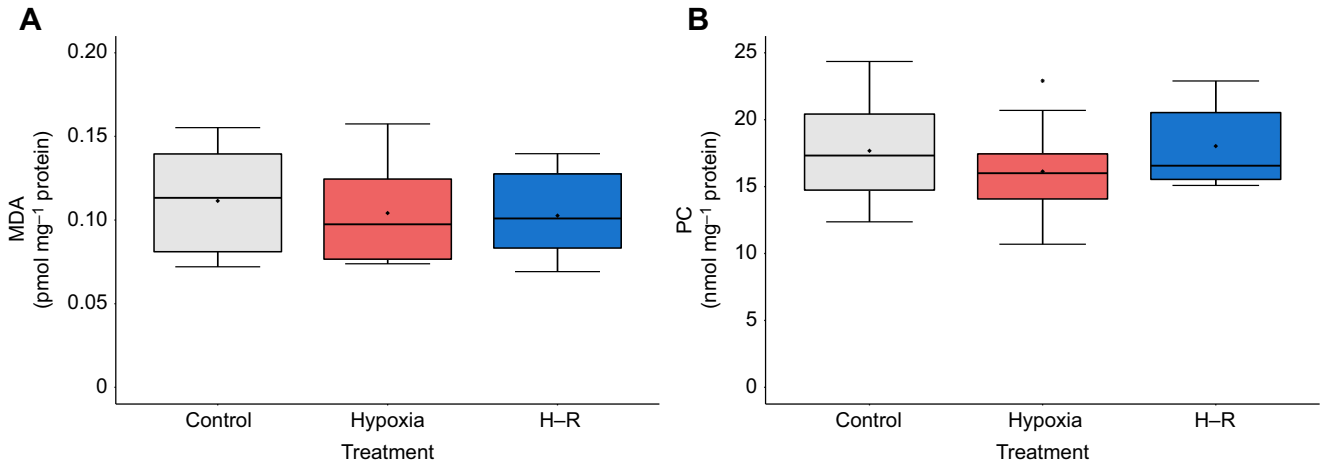

Fig. 7. Oxidative damage in mitochondrial proteins of quahogs exposed to short-term H–R stress. (A) Malondialdehyde (MDA)–protein conjugates. (B) Protein carbonyls (PC). Experimental groups: control (21% O_2); short-term (24 h) severe (<0.01% O_2) hypoxia; short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2) (H–R). Means are depicted as points within corresponding box–whisker plots. Outliers are shown as asterisks. No significant between-group differences in these traits were found (P>0.05). N=12.

oxygen fluctuations. Furthermore, behavioural adaptations that maintain low P_{O_2} levels in the mantle cavity of *A. islandica* (Abele et al., 2010; Strahl et al., 2011) and lead to a periodic migration into low-oxygen environments might further mitigate the potential negative impacts of reoxygenation on the mitochondrial ETS of this species (Oeschger and Storey, 1993; Taylor, 1976).

Effects of H–R stress on the PS subsystem

The mitochondrial PS subsystem (in particular, F_o,F_1-ATPase) has been proposed as an important player in metabolic responses to hypoxia (Solaini et al., 2010). Under hypoxic conditions when $\Delta\psi$ decreases as a result of the low ETS activity, mitochondrial F_o,F_1-ATPase can work in reverse, hydrolysing ATP to maintain $\Delta\psi$ (Solaini et al., 2010; St-Pierre et al., 2000). Suppression of the hydrolytic activity of mitochondrial F_o,F_1-ATPase (e.g. by the mitochondrial ATPase inhibitory protein IF1) has therefore been proposed as a protective mechanism preventing ATP wastage during hypoxia (García-Aguilar and Cuezva, 2018; Lebowitz and Pedersen, 1993). A decrease in F_o,F_1-ATPase activity during hypoxia has been reported in hypoxia-tolerant vertebrates such as the freshwater turtle *Trachemys scripta* (Galli and Richards, 2014) and the killifish *Austrofundulus limnaeus* (Duerr and Podrabsky, 2010). However, our present study in *A. islandica* and earlier published research in bivalves and crustaceans (Ivanina et al., 2012, 2016; Martinez-Cruz et al., 2012) indicates that this mechanism is not universal in hypoxia-tolerant species. In *A. islandica*, exposure to hypoxia resulted in strong stimulation of the PS oxygen flux capacity associated with a decrease in $\Delta\psi$. The observed depolarization of actively phosphorylating mitochondria of *A. islandica* cannot be explained by lower ETS activity or elevated proton leak (which were both preserved in hypoxia-exposed quahogs) and probably reflects elevated proton flux via more active F_o,F_1-ATPase. Similarly, an increase in F_o,F_1-ATPase activity during hypoxia was found in the Pacific white shrimp, *Litopenaeus vannamei* (Martinez-Cruz et al., 2012). In the hypoxia-tolerant *C. virginica*, the PS subsystem capacity remained unchanged under hypoxia (Ivanina et al., 2012), whereas in *M. mercenaria*, the PS flux capacity decreased (Ivanina et al., 2016). During reoxygenation, the PS oxygen flux capacity of *A. islandica* was suppressed (this study), unlike in *M. mercenaria* and *C. virginica*, where the PS subsystem activity was upregulated (Ivanina et al., 2012, 2016). Taken together, these data indicate that the PS subsystem activity can be differentially regulated during hypoxia and reoxygenation in different species without a clear link to a hypoxia-tolerant metabolic phenotype. However, given that the PS subsystem exerts a low degree of control over OXPHOS respiration, this variability in the PS subsystem responses to H–R stress is unlikely to have a major effect on mitochondrial ATP synthesis in *A. islandica* and other marine bivalves.

Effects of H–R stress on mitochondrial proton leak

Mitochondrial LEAK respiration represents the oxygen consumption required to offset depolarizing effects of proton and cation cycles not linked to ATP generation (Brand, 1997; Jastroch et al., 2010). Excessive proton leak might lead to energy wastage and decrease the OXPHOS efficiency, but normal physiological levels of proton leak are essential to prevent hyperpolarization and excessive ROS formation at high $\Delta\psi$ (Brand et al., 2004; Miwa and Brand, 2003). Mild uncoupling as a result of elevated proton leak has been proposed as a protective mechanism against oxidative stress during H–R stress (Cadenas, 2018; Cheng et al., 2017), although alternative mechanisms (such as a decrease in $\Delta\psi$ due to lower ETS activity) have also been reported (Boutilier and St-Pierre, 2002). In *A. islandica*, $\Delta\psi$-dependent kinetics of the PL subsystem changed in response to H–R stress. The strongest changes were observed in mitochondria from quahogs exposed to reoxygenation, while hypoxia had relatively little effect. Thus, at physiologically relevant $\Delta\psi$ (180 mV) typical for non-phosphorylating mitochondria, no difference in the mitochondrial PL flux was observed between quahogs exposed to normoxia or hypoxia. Similarly, hypoxia exposure had no effect on the PL flux rate and proton conductance of mitochondria from hypoxia-tolerant oysters *C. virginica* (Ivanina et al., 2012) and hard shell clams, *M. mercenaria* (Ivanina et al., 2016). The PL flux of *A. islandica* mitochondria decreased during reoxygenation, similar to earlier reports in the hypoxia-tolerant hard shell clam, *M. mercenaria*, whereas in *C. virginica*, the PL flux rate was slightly elevated above normoxic baseline (Ivanina et al., 2012, 2016). In hypoxia-sensitive scallops, *A. irradians*, the PL flux rate decreased in hypoxia and collapsed during reoxygenation, reflecting mitochondrial damage and the loss of ETS capacity (Ivanina et al., 2016). In hypoxia-sensitive terrestrial mammals, H–R stress led to a strong increase in PL flux, reflecting the loss of the inner mitochondrial membrane integrity and opening of the mitochondrial permeability transition pore (Honda et al., 2005). Overall, our present study and earlier published research (Boutilier and St-Pierre, 2002; Cheng et al., 2017; Ivanina et al., 2016) indicate that the changes in the mitochondrial PL flux during H–R stress might have different underlying mechanisms, from physiological modulation of the proton conductance to pathological consequences of the ETS collapse or membrane damage. Understanding the causes and the bioenergetic implications of PL changes must therefore be considered in the context of other mitochondrial traits such as ETS activity, membrane integrity and oxidative damage.

ROS efflux in response to H–R stress

Mitochondria are both ROS producers and ROS consumers (Munro and Treberg, 2017; Treberg et al., 2019), and the balance between ROS generation and detoxification in mitochondria determines the net ROS efflux across the mitochondrial membrane. The ROS escaping from mitochondria play an important role in cell signalling; however, excessive ROS release can result in oxidation of proteins, lipids and DNA, potentially leading to inflammation and cell death (Paradis et al., 2016; Zorov et al., 2014).

In the hepatopancreas mitochondria of *A. islandica*, ROS efflux increased with increasing $\Delta\psi$ regardless of mitochondrial state or exposure condition. The $\Delta\psi$-dependent increase in ROS efflux implies that *A. islandica* mitochondria will have higher ROS efflux rates in the LEAK state than in the OXPHOS state. This notion is supported by earlier studies in *A. islandica* that showed higher mitochondrial ROS efflux in the LEAK state (Munro et al., 2013) as well as reports of elevated ROS efflux in the LEAK (compared with the OXPHOS) state mitochondria of mammals (Liu et al., 2002; Munro et al., 2019; Quinlan et al., 2012), fish (Gerber et al., 2021) and marine bivalves (Ouillon et al., 2021; Philipp et al., 2005). However, when compared at a typical $\Delta\psi$ (155 mV for OXPHOS and 180 mV for LEAK state), the ROS efflux and electron leak rates (measured as H_2O_2 to O_2 ratio) were similar in the LEAK and OXPHOS mitochondria of *A. islandica*. This indicates that despite the measurable $\Delta\psi$ dependence, ROS efflux is not strongly affected by the mitochondrial activity state in quahogs.

Notably, studies in mammalian models showed that mitochondrial production sites differ between mitochondrial respiratory states. In phosphorylating rat mitochondria, the specific superoxide-producing I_F site of Complex I was the dominant H_2O_2 producer, while in non-phosphorylating mitochondria, several active sites of ROS efflux (I_F

and III_{Q0}, and possibly I_Q) were found on Complexes I and III (Quinlan et al., 2012). It remains to be investigated whether this switch of the predominant sites of ROS efflux has implications for the electron leak rates and to what degree it is driven by the change in $\Delta\psi$. In our present study, an inhibitor of Complex I (rotenone) was used to measure succinate-driven respiration and ROS efflux; therefore, ROS generation at Complex I would be negligible in our assays. Thus, the observed ROS efflux in *A. islandica* mitochondria in both the LEAK and OXPHOS states is probably due to Complex III and, possibly, Complex II, which is also a ROS-generating site in some species (Dröse, 2013; Grivennikova et al., 2017). Notably, the fraction of O_2 converted to H_2O_2 in *A. islandica* mitochondria is relatively high (1.9–11.5%) compared with that in other species, such as the hypoxia-tolerant bivalve *M. arenaria* with 5% (Ouillon et al., 2021), *Salmo salar* with 0.5% (Gerber et al., 2021), the hypoxia-tolerant epaulette shark with 2.5% (Hickey et al., 2012) and terrestrial organisms such as mice with 3% (Li Puma et al., 2020). This indicates that low ROS efflux in *A. islandica* is a by-product of slow mitochondrial metabolism rather than exceptionally low electron leak.

Hypoxia exposure caused a notable change in ROS dynamics of *A. islandica* mitochondria, suppressing the ROS efflux and electron leak rate. Suppression of ROS efflux during hypoxia exposure is commonly seen in hypoxia-tolerant species and has been interpreted as a defence mechanism to prevent oxidative damage during H–R stress (Du et al., 2016; Hickey et al., 2012; Milton et al., 2007; Pamenter et al., 2007). Mitochondrial antioxidants also commonly increase during hypoxia in hypoxia-tolerant species (Du et al., 2016; Ivanina and Sokolova, 2016; Ivanina et al., 2016), termed the 'preparation for oxidative stress hypothesis' (Hermes-Lima et al., 1998). It is worth noting that the levels of antioxidants do not increase during hypoxia in *A. islandica* (Strahl et al., 2011), possibly because this species has high baseline levels of antioxidant capacity (Abele et al., 2008; Strahl et al., 2007). Notably, a decreased ROS efflux in hypoxic clams was only observed in the OXPHOS state. As this drop was accompanied by a strong upregulation of the PS flux capacity, a decrease in the ROS efflux during hypoxia might be predominantly determined by the regulation of ETS activity in *A. islandica* mitochondria. Regardless of the underlying mechanisms, suppressed ROS efflux in hypoxia might reflect adaptation of *A. islandica* to its unique lifestyle that involves self-induced hypoxia as a result of burrowing into anoxic sediment and valve closure to maintain low internal P_{O_2} (Abele et al., 2010; Strahl et al., 2011; Taylor and Brand, 1975; Taylor, 1976).

Overall, mitochondria of *A. islandica* show high tolerance to hypoxia by maintaining stable ETS flux, upregulating phosphorylation capacity and suppressing ROS efflux, but remain sensitive to reoxygenation. This might reflect the behavioural adaptations of *A. islandica* that lead to prolonged exposure of quahogs to low oxygen tensions but only infrequent reoxygenation events (Taylor and Brand, 1975; Taylor, 1976). This pattern contrasts with earlier findings in other hypoxia-tolerant bivalves such as intertidal species that show robust mitochondrial function during both hypoxia and reoxygenation, and commonly upregulate the ETS and/or phosphorylation capacity during post-hypoxic recovery (Sokolova et al., 2019). These differences might reflect adaptations to environments with different predictability of oxygen fluctuations (e.g. rare and unpredictable fluctuations in subtidal benthic habitats versus frequent and predictable H–R events in the intertidal), reinforced by the peculiarities of behavioural regulation of oxygen levels in *A. islandica*. It is also worth noting that the studied population of *A. islandica* from the Baltic Sea shows metabolic adaptations to low salinity, indicated by enhanced metabolic rates and lower capability of metabolic rate depression than found in populations from high salinity sites such as the North Sea (Basova et al., 2012; Philipp et al., 2012). The Baltic Sea *A. islandica* populations also demonstrate higher physiological flexibility and stress hardening than North Sea populations (Philipp et al., 2012). Whether these adaptations to low salinity contribute to the unusual mitochondrial responses to H–R in the Baltic Sea *A. islandica* compared with earlier studied hypoxia-tolerant bivalves (Ivanina et al., 2012, 2016; Ouillon et al., 2021; Sokolov et al., 2019) is presently unknown and requires further investigation.

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Competing interests
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Author contributions
Conceptualization: J.B.S., C.M.B., I.M.S.; Methodology: J.B.S., E.S., I.M.S.; Validation: J.B.S., E.S.; Formal analysis: J.B.S., F.H., I.M.S.; Investigation: J.B.S., F.H., E.S.; Resources: I.M.S.; Data curation: J.B.S., F.H.; Writing - original draft: J.B.S., I.M.S.; Writing - review & editing: J.B.S., F.H., E.S., C.M.B., I.M.S.; Visualization: J.B.S.; Supervision: E.S., I.M.S.; Project administration: I.M.S.; Funding acquisition: C.M.B., I.M.S.

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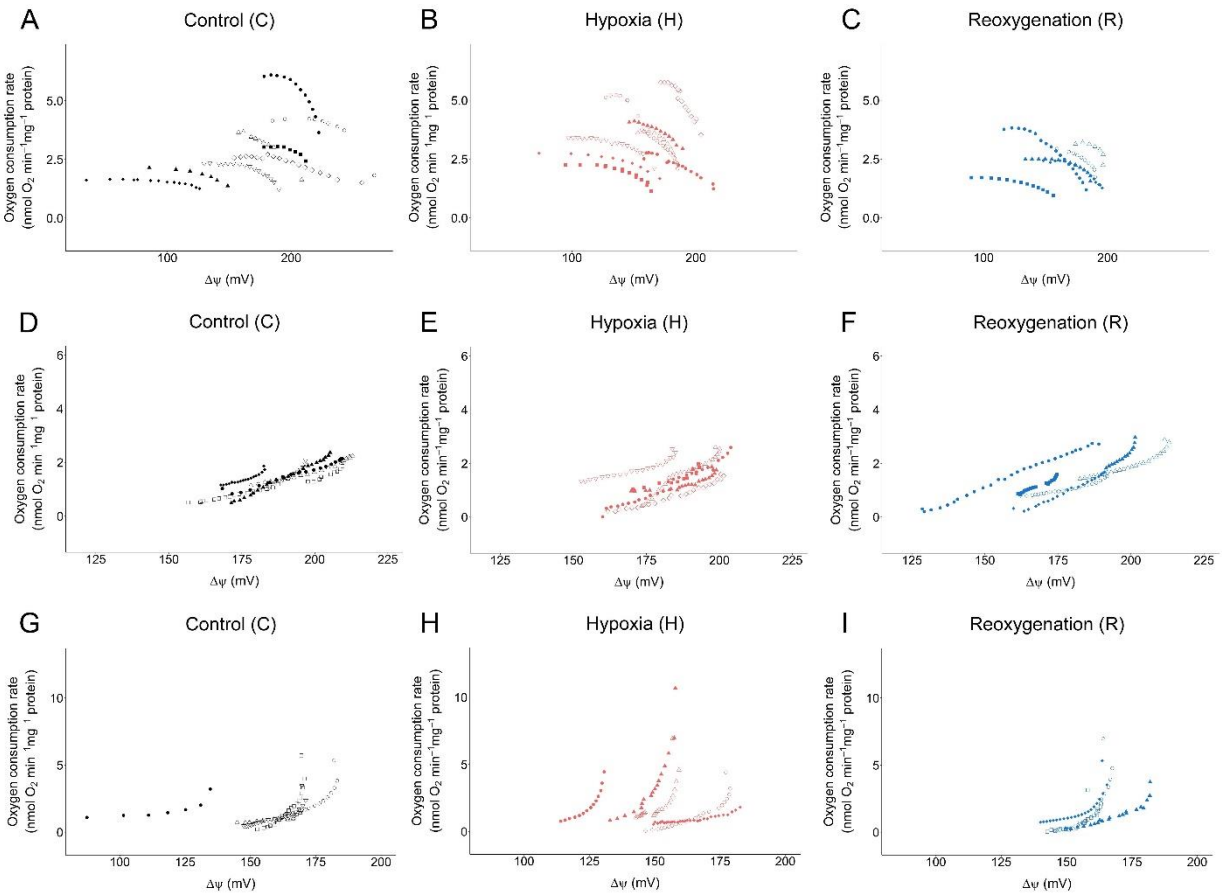

Fig. S1. Individual kinetic response of the mitochondrial subsystems substrate oxidation (SO), proton leak (PL) and phosphorylation system (PS) to H/R stress. Single points depict measured MO_2 at the corresponding measured mitochondrial membrane potential (Δψ). Oxygen consumption and Δψ were calculated per mass of mitochondrial protein. Subsystems (A,B,C) substrate oxidation; (D,E,F) proton leak; (G,H,I) phosphorylation system. Experimental groups: C, control (21% O_2); H, short-term (24 h) severe (<0.01% O_2) hypoxia; R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2).

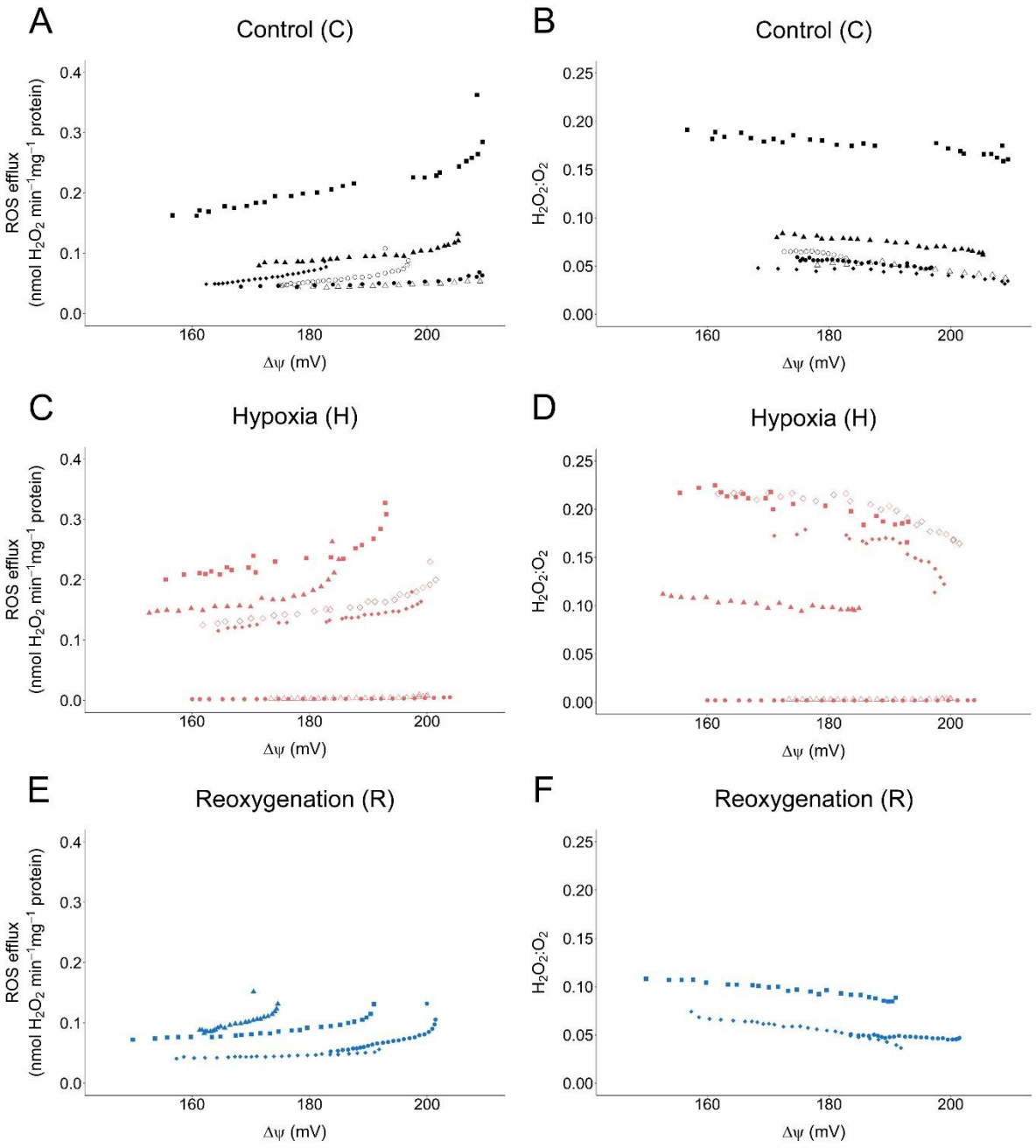

Fig. S2. Individual kinetic response of reactive oxygen species (ROS) efflux and associated H_2O_2:O_2 ratio in resting mitochondria state (LEAK state) during H/R stress. Single points depict measured H_2O_2 emission (ROS) at the corresponding measured mitochondrial membrane potential (Δψ). Oxygen consumption, H_2O_2 emission and Δψ were calculated per mass of mitochondrial protein. (A,C,E) ROS production; (B,D,F) H_2O_2:O_2 ratio; (A,B) control (normoxia); (C,D) hypoxia; (E.F) reoxygenation. Experimental groups: C, control (21% O_2); H, short-term (24 h) severe (<0.01% O_2) hypoxia; R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2).

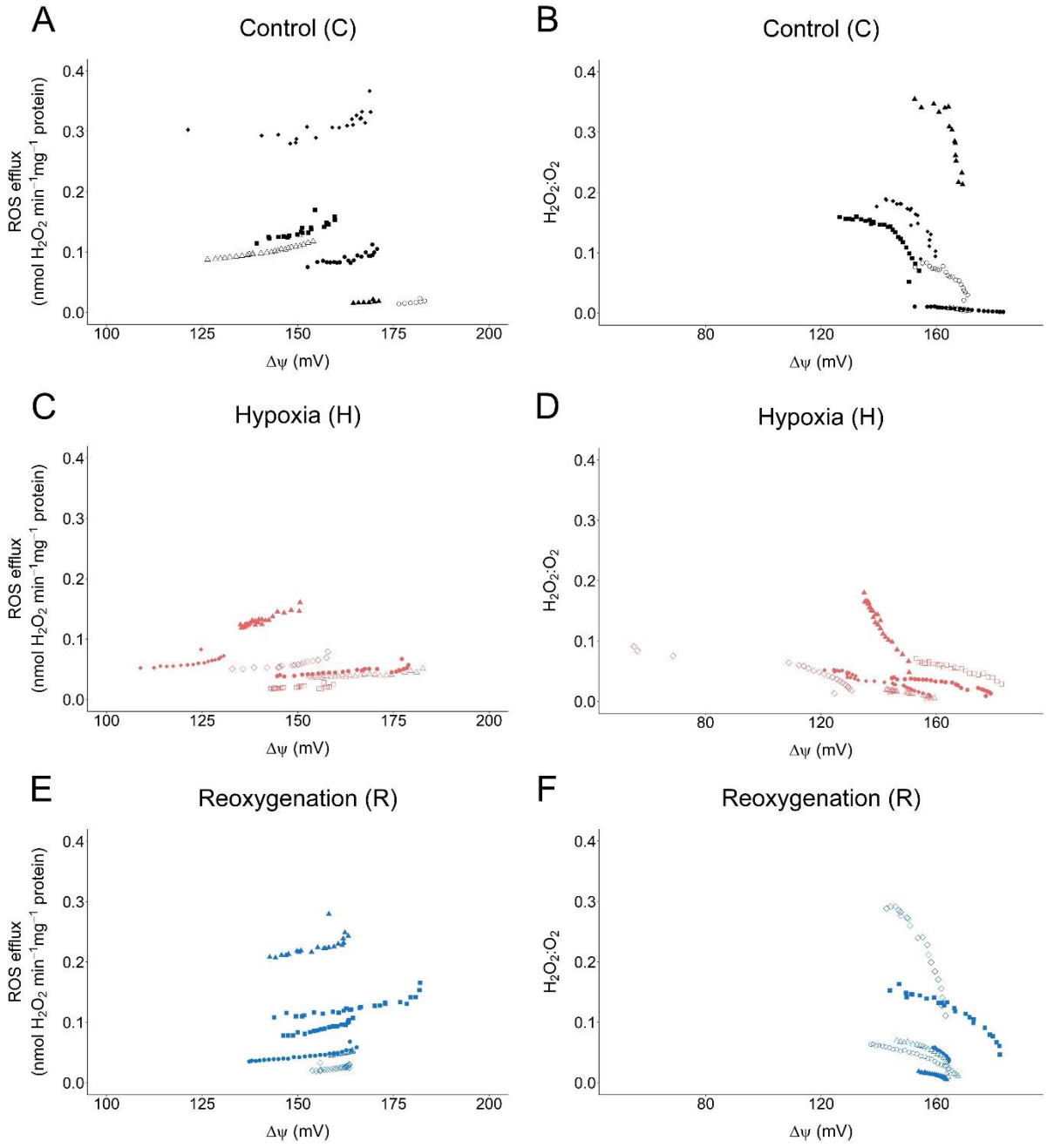

Fig. S3. Individual kinetic response of reactive oxygen species (ROS) efflux and associated H_2O_2:O_2 ratio in active mitochondria (OXPHOS state) during H/R stress. Single points depict measured H_2O_2 emission (ROS) at the corresponding measured mitochondrial membrane potential (Δψ). Oxygen consumption, H_2O_2 emission and Δψ were calculated per mass of mitochondrial protein. (A,C,E) ROS production; (B,D,F) H_2O_2:O_2 ratio; (A,B) control (normoxia); (C,D) hypoxia; (E.F) reoxygenation. Experimental groups: C, control (21% O_2); H, short-term (24 h) severe (<0.01% O_2) hypoxia; R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2)

Table S1. Equations of individual and mean kinetic response of mitochondrial respiration, ROS efflux and H_2O_2:O_2 ratio. a) Mitochondria in LEAK state, b) mitochondria in OXPHOS state and c) uncoupled mitochondria during substrate oxidation in response to H/R stress. ROS measurement and H_2O_2:O_2 ratio could not be measured with uncoupled mitochondria and are thus missing in table S1 c). Equations were fitted by Matlab's curve fitting tool by means of 3rd polynomials and 2nd polynomials, respectively. Empty, gray-shaded cells are missing non-successful mitochondrial measurements.

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a)	Sample ID	O_2 consumption					ROS efflux				H_2O_2:O_2 ratio			
		a	b	c	d	R^2	a	b	c	R^2	a	b	c	R^2
Control (C)	AI1 N PL	-1.521E-05	0.00891	-1.70200	107.50000	0.9783	1.660E-05	-0.00585	0.56040	0.9261	-1.080E-05	0.00377	-0.28090	0.9507
	AI2 N PL	2.307E-05	-0.01287	2.41400	-150.70000	0.996	1.720E-05	-0.00626	0.61280	0.9177	-1.900E-05	0.00686	-0.56760	0.9605
	AI3 N PL	2.308E-05	-0.01297	2.48000	-159.70000	0.9958	3.620E-05	-0.01264	1.18700	0.918	-1.070E-05	0.00350	-0.20270	0.9659
	AI4 N PL	3.418E-04	-0.17710	30.62000	-1764.00000	0.9687	5.370E-05	-0.01708	1.40700	0.9594	-1.492E-04	0.05234	-4.52300	0.9362
	AI5 N PL	1.494E-05	-0.00795	1.42500	-85.18000	0.9759	3.870E-05	-0.01204	1.10800	0.816	-2.850E-06	0.00060	0.16370	0.8398
	AI6 N PL	8.837E-04	-0.50220	95.18000	-6014.00000	0.991	5.280E-05	-0.01801	1.58300	0.7113	-9.440E-06	0.00306	-0.18890	0.9128
	mean	**2.119E-04**	**-0.11740**	**21.74000**	**-1344.00000**	**1**	**3.590E-05**	**-0.01198**	**1.07600**	**1**	**-3.370E-05**	**0.01169**	**-0.93320**	**1**
Hypoxia (H)	AI1 A PL	5.316E-06	-0.00256	0.45190	-28.47000	0.9786	2.140E-06	-0.00072	0.06299	0.9352	3.450E-07	-0.00013	0.01329	0.3938
	AI2 A PL	1.684E-04	-0.09559	18.13000	-1148.00000	0.9801	9.480E-06	-0.00339	0.30550	0.8371	1.740E-06	-0.00063	0.05996	0.6708
	AI3 A PL	9.426E-06	-0.00459	0.76350	-43.19000	0.9895	5.030E-05	-0.01655	1.49300	0.8916	-5.660E-05	0.01940	-1.44500	0.9642
	AI4 A PL	5.710E-05	-0.03016	5.31200	-311.10000	0.9292	3.830E-05	-0.01272	1.17700	0.8859	-1.346E-04	0.04826	-4.14600	0.879
	AI5 A PL	2.104E-04	-0.11310	20.29000	-1212.00000	0.9439	8.450E-05	-0.02737	2.42700	0.8379	-1.850E-05	0.00535	-0.16410	0.8742
	AI6 A PL	6.620E-05	-0.03269	5.39400	-295.80000	0.9651	1.328E-04	-0.04273	3.58200	0.8072	1.100E-05	-0.00420	0.49660	0.9021
	mean	**8.614E-05**	**-0.04645**	**8.39000**	**-506.40000**	**1**	**5.290E-05**	**-0.01725**	**1.50800**	**1**	**-3.280E-05**	**0.01134**	**-0.86420**	**1**
Reoxygenation (R)	AI1 R PL	-6.944E-06	0.00323	-0.45250	19.71000	0.9973								
	AI2 R PL	8.492E-05	-0.04905	9.46300	-608.10000	0.9723								
	AI3 R PL	-4.086E-05	0.02214	-3.94300	231.60000	0.9922	3.640E-05	-0.01150	0.98290	0.9137	-5.440E-06	0.00129	0.03695	0.9676
	AI4 R PL	2.406E-03	-1.41000	275.40000	-17930.00000	0.9771	1.544E-04	-0.05693	5.30200	0.7573	2.940E-06	-0.00137	0.20290	0.7769
	AI7 R PL	6.230E-04	-0.31250	52.26000	-2914.00000	0.9889	5.430E-05	-0.01526	1.13100	0.6728				
	AI8 R PL	3.374E-05	-0.01723	2.94700	-167.90000	0.9951	9.520E-06	-0.00302	0.28210	0.9197	-1.330E-05	0.00376	-0.19260	0.9776
	mean	**5.166E-04**	**-0.29390**	**55.95000**	**-3561.00000**	**1**	**6.370E-05**	**-0.02168**	**1.92500**	**1**	**-5.260E-06**	**0.00123**	**0.01575**	**1**

b)	Sample ID	O_2 consumption					ROS efflux				H_2O_2:O_2 ratio			
		a	b	c	d	R^2	a	b	c	R^2	a	b	c	R^2
Control (C)	AI1 N PS	6.657E-04	-0.33550	56.37000	-3158.00000	0.8195	1.800E-05	-0.00551	0.42770	0.5811	-2.630E-06	0.00054	-0.01020	0.9804
	AI2 N PS	4.102E-04	-0.19210	30.00000	-1563.00000	0.8227	-2.900E-05	0.01032	-0.89870	0.4837	-8.640E-06	0.00214	-0.11010	0.988
	AI3 N PS	2.148E-04	-0.09948	15.35000	-788.90000	0.8446	6.160E-05	-0.01727	1.49500	0.7425	-1.042E-03	0.32760	-25.37000	0.8934
	AI5 N PS						4.890E-05	-0.01285	0.95960	0.692	-2.891E-04	0.08226	-5.66600	0.8083
	AI6 N PS	9.767E-04	-0.45880	71.90000	-3758.00000	0.8225	7.460E-05	-0.02287	1.83300	0.7467	-2.364E-04	0.07379	-5.67800	0.932
	AI7 N PS	8.517E-05	-0.02692	2.82100	-96.75000	0.9398								
	AI8 N PS						2.990E-05	-0.00728	0.53000	0.8949	-2.086E-04	0.05550	-3.53200	0.8841
	mean	**4.705E-04**	**-0.22260**	**35.29000**	**-1873.00000**	**1**	**3.400E-05**	**-0.00924**	**0.72440**	**1**	**-2.979E-04**	**0.09031**	**-6.72800**	**1**
Hypoxia (H)	AI1 A PS	1.350E-04	-0.06281	9.77700	-508.90000	0.8667	8.880E-07	0.00016	-0.00340	0.6557	-2.110E-05	0.00609	-0.40120	0.9433
	AI2 A PS	1.245E-03	-0.54260	78.83000	-3817.00000	0.6452	-7.950E-06	0.00265	-0.19840	0.3264	-1.880E-05	0.00483	-0.28730	0.9359
	AI3 A PS	1.297E-03	-0.54760	77.10000	-3619.00000	0.9437	5.760E-05	-0.01590	1.14900	0.9208	-4.440E-06	0.00010	0.10550	0.9591
	AI4 A PS	2.055E-03	-0.73710	88.13000	-3512.00000	0.9868	2.430E-05	-0.00494	0.30100	0.5764	-2.050E-05	0.00300	-0.02053	0.9059
	AI5 A PS	4.848E-05	-0.02295	3.63300	-191.60000	0.9764	1.250E-05	-0.00381	0.32620	0.854	-2.120E-05	0.00594	-0.34560	0.9641
	AI6 A PS						3.140E-05	-0.00681	0.46720	0.8898	1.280E-04	-0.04392	3.77000	0.974
	mean	**9.561E-04**	**-0.38260**	**51.49000**	**-2330.00000**	**1**	**1.980E-05**	**-0.00477**	**0.34030**	**1**	**7.000E-06**	**-0.00399**	**0.47010**	**1**
Reoxygenation (R)	AI1 R PS	4.439E-04	-0.19380	28.19000	-1367.00000	0.725	9.030E-05	-0.02647	2.02000	0.859	-1.702E-04	0.05061	-3.69400	0.9452
	AI2 R PS						6.640E-05	-0.02047	1.59900	0.3948	-9.560E-05	0.02916	-2.20600	0.9807
	AI3 R PS	1.747E-04	-0.08449	13.65000	-735.70000	0.9132	3.120E-05	-0.00919	0.78640	0.8831	-6.950E-05	0.02028	-1.32800	0.9742
	AI4 R PS	5.635E-04	-0.24990	36.93000	-1819.00000	0.791	2.800E-05	-0.00769	0.56450	0.8753	-4.710E-05	0.01269	-0.79230	0.9785
	AI5 R PS	-1.660E-04	0.08133	-13.12000	699.00000	0.6722	1.630E-05	-0.00327	0.34160	0.4892	-4.925E-04	0.14250	-10.02000	0.9864
	AI6 R PS						8.060E-05	-0.02482	1.95600	0.9229	-4.970E-04	0.15700	-12.34000	0.9781
	mean	**2.540E-04**	**-0.11170**	**16.41000**	**-805.70000**	**1**	**5.210E-05**	**-0.01532**	**1.21100**	**1**	**-2.286E-04**	**0.06871**	**-5.06300**	**1**

c)	Sample ID	O_2 consumption				
		a	b	c	d	R^2
		Substrate oxidation				
Control (C)	AI1 N SO	-9.018E-06	0.00543	-1.08800	76.52000	0.9978
	AI2 N SO	-1.409E-05	0.00709	-1.20800	73.29000	0.9972
	AI3 N SO	3.209E-06	-0.00208	0.42890	-25.97000	0.9854
	AI4 N SO	-9.311E-06	0.00395	-0.55780	28.57000	0.9979
	AI5 N SO	-2.416E-05	0.01317	-2.39300	147.80000	0.9884
	AI6 N SO	-4.649E-05	0.02593	-4.82200	305.10000	0.9928
	AI7 N SO	-3.182E-06	0.00087	-0.08261	4.82400	0.9979
	AI8 N SO	-1.006E-06	0.00016	-0.00767	1.74600	0.9819
	mean	**-1.301E-05**	**0.00682**	**-1.21600**	**76.48000**	**1**
Hypoxia (H)	AI1 A SO	2.211E-05	-0.01069	1.65500	-77.69000	0.9765
	AI2 A SO	-6.295E-05	0.02943	-4.58000	241.00000	0.9937
	AI3 A SO	6.794E-05	-0.03997	7.74200	-489.10000	0.9689
	AI4 A SO	-7.059E-06	0.00240	-0.27420	13.90000	0.9855
	AI5 A SO	-6.342E-06	0.00211	-0.23340	10.89000	0.9798
	AI6 A SO	4.229E-06	-0.00266	0.52410	-30.33000	0.968
	AI7 A SO	-1.024E-06	-0.00011	0.09529	-4.36700	0.9919
	AI8 A SO	-1.934E-06	0.00053	-0.04953	4.29700	0.9988
	mean	**1.871E-06**	**-0.00237**	**0.60990**	**-41.42000**	**1**
Reoxygenation (R)	AI1 R SO	-6.754E-05	0.03419	-5.78200	329.40000	0.9631
	AI2 R SO	-1.812E-04	0.09791	-17.63000	1061.00000	0.8238
	AI3 R SO	-6.868E-06	0.00271	-0.34260	16.03000	0.9943
	AI4 R SO	-1.489E-05	0.00662	-0.97860	50.68000	0.9692
	AI5 R SO	-2.519E-06	0.00071	-0.06790	3.95200	0.9994
	AI6 R SO	1.844E-06	-0.00149	0.28250	-11.78000	0.9976
	mean	**-4.520E-05**	**0.02344**	**-4.08600**	**241.50000**	**1**

2.2 Publication II: Combined effects of salinity and intermittent hypoxia on mitochondrial capacity and reactive oxygen species production in the Pacific oyster, *Crassostrea gigas*

Published in the Journal of Experimental Biology

Contribution letter

The contents of the second publication chapter from this dissertation have been published in 2023 in the Journal of Experimental Biology under the title "Combined effects of salinity and intermittent hypoxia on mitochondrial capacity and reactive oxygen species efflux in the Pacific oyster, *Crassostrea gigas*".

I predominantly contributed to this publication and was involved in experimental design and planning, performance of incubation experiments, tissue collection, isolation of mitochondria for respiratory rates. Assessment of respiration rates and ROS efflux of isolated mitochondria was also done by me. Additionally, I have also performed the complete statistical analysis and prepared all graphical output. I wrote the first draft of the manuscript and contributed to the revisions of the draft in response to the comments of the peer reviewers.

Contribution of the candidate in % of workload:	
Experimental concept and design	95%
Experimental work and data acquisition	100%
Data analysis and interpretation	95%
Preparation of figures and tables	100%
Drafting the manuscript	85%

Signature of consent

Supervisor	Doctoral candidate
_____	_____
Prof. Dr. Inna Sokolova	Jennifer Barbara Maria Steffen

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RESEARCH ARTICLE

Combined effects of salinity and intermittent hypoxia on mitochondrial capacity and reactive oxygen species efflux in the Pacific oyster, *Crassostrea gigas*

Jennifer B. M. Steffen[1], Eugene P. Sokolov[2], Christian Bock[3] and Inna M. Sokolova[1,4,*]

ABSTRACT

Coastal environments commonly experience fluctuations in salinity and hypoxia–reoxygenation (H/R) stress that can negatively affect mitochondrial functions of marine organisms. Although intertidal bivalves are adapted to these conditions, the mechanisms that sustain mitochondrial integrity and function are not well understood. We determined the rates of respiration and reactive oxygen species (ROS) efflux in the mitochondria of oysters, *Crassostrea gigas*, acclimated to high (33 psu) or low (15 psu) salinity, and exposed to either normoxic conditions (control; 21% O_2) or short-term hypoxia (24 h at <0.01% O_2) and subsequent reoxygenation (1.5 h at 21% O_2). Further, we exposed isolated mitochondria to anoxia *in vitro* to assess their ability to recover from acute (~10 min) oxygen deficiency (<0.01% O_2). Our results showed that mitochondria of oysters acclimated to high or low salinity did not show severe damage and dysfunction during H/R stress, consistent with the hypoxia tolerance of *C. gigas*. However, acclimation to low salinity led to improved mitochondrial performance and plasticity, indicating that 15 psu might be closer to the metabolic optimum of *C. gigas* than 33 psu. Thus, acclimation to low salinity increased mitochondrial oxidative phosphorylation rate and coupling efficiency and stimulated mitochondrial respiration after acute H/R stress. However, elevated ROS efflux in the mitochondria of low-salinity-acclimated oysters after acute H/R stress indicates a possible trade-off of higher respiration. The high plasticity and stress tolerance of *C. gigas* mitochondria may contribute to the success of this invasive species and facilitate its further expansion into brackish regions such as the Baltic Sea.

KEY WORDS: Hypoxia tolerance, Mitochondria, Aerobic capacity, High-resolution respirometry, Oxidative stress marker, AmplexRed

INTRODUCTION

Coastal environments are dynamic and highly productive ecosystems, but they are vulnerable to multiple natural and anthropogenic stressors. Sessile benthic organisms that inhabit these dynamic habitats must rely on physiological adaptations to cope with the challenges posed by these stressors (Grieshaber et al., 1994). Hypoxia, a severe oxygen deficiency that can last for hours, weeks or even months, is becoming increasingly prevalent in marine environments worldwide, particularly in coastal waters (Breitburg et al., 2018, 2019). Most marine animals rely on oxygen for mitochondrial metabolism and ATP production, making them highly susceptible to the negative effects of oxygen deficiency (Diaz and Rosenberg, 1995, 2008). Mitochondria are especially vulnerable to hypoxia-induced metabolic stress, and the degree of mitochondrial resilience to hypoxia generally corresponds to an organism's sensitivity to oxygen fluctuations. In hypoxia-intolerant species, hypoxia leads to severe damage to mitochondrial functions, depolarization of the mitochondrial membrane, and ATP deficiency, as well as Ca^{2+} overload (Piper et al., 2003; Solaini et al., 2010). While reoxygenation can restore ATP levels, it typically comes at the cost of the production of reactive oxygen species (ROS), which can cause damage to cellular macromolecules (Paradis et al., 2016). Hypoxia-intolerant organisms, including benthic invertebrates such as scallops, have been reported to suffer from oxidative damage, collapsed mitochondrial membrane potential, and a loss of oxidative phosphorylation (OXPHOS) capacity during hypoxia and reoxygenation (Ivanina and Sokolova, 2016; Ivanina et al., 2016). In contrast, some hypoxia-tolerant species, such as freshwater turtles of the genera *Chrysemys* and *Trachemys*, some fish species such as crucian carp, and intertidal bivalves, are able to preserve mitochondrial respiration, OXPHOS capacity and mitochondrial membrane potential during *in vivo* hypoxia–reoxygenation (H/R) stress (Galli and Richards, 2014; Pamenter, 2014; Sokolova, 2018; Sokolova et al., 2019). Moreover, in some hypoxia-tolerant species, severe oxygen deficiency can induce a transition to anaerobic ATP production, accompanied by the onset of metabolic rate depression that conserves energy and delays the onset of irreversible disruption of cellular homeostasis (Hochachka et al., 1996; Storey, 2002). In facultative anaerobes like marine bivalves, the transition between aerobic and anaerobic pathways of glucose oxidation is regulated at the phosphoenolpyruvate (PEP) branchpoint that plays an important role as a metabolic switch during oxygen deficiency. Pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) compete for a common substrate (PEP) directing it towards aerobic oxidation or anaerobic glycolysis, respectively (Bayne, 2017; Zammit and Newsholme, 1978). Changes in the PK/PEPCK activity ratio thus can indicate the relative activity of aerobic versus anaerobic metabolism and affect substrate provision to the

[1]Department of Marine Biology, Institute of Biological Sciences, University of Rostock, 18059 Rostock, Germany. [2]Leibniz Institute for Baltic Research, Leibniz Science Campus Phosphorus Research Rostock, 18119 Warnemünde, Germany. [3]Integrative Ecophysiology, Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research, 27570 Bremerhaven, Germany. [4]Department of Maritime Systems, Interdisciplinary Faculty, University of Rostock, 18059 Rostock, Germany.

*Author for correspondence (inna.sokolova@uni-rostock.de)

J.B.M.S., 0000-0003-0570-2354; C.B., 0000-0003-0052-3090; I.M.S., 0000-0002-2068-4302

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List of symbols and abbreviations

ASW	artificial sea water
BSA	bovine serum albumin
ETS	electron transport system
FEL	fractional electron leak
H/R	hypoxia/reoxygenation
LEAK I	mitochondrial respiration respiring on substrates for respiratory complex I
LEAK I+II	mitochondrial respiration respiring on substrates for respiratory complex I and II
MDA	malondialdehyde
$\dot{M}_{O_2}$	oxygen consumption rate
OXPHOS	oxidative phosphorylation
OXPHOS CE	OXPHOS coupling efficiency
PBS	phosphate-buffered saline
PC	protein carbonyls
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
psu	practical salinity units
RCR	respiratory control ratio
ROS	reactive oxygen species
SUIT	substrate–uncoupler–inhibitor titration

mitochondria (Bayne, 2017; Ivanina et al., 2016; Zammit and Newsholme, 1978). Hence, gaining insights into the regulation of mitochondrial bioenergetics and redox balance can offer understanding of the mechanisms and constraints associated with the tolerance of benthic species towards oxygen fluctuations in coastal environments.

Besides fluctuating oxygen conditions, shallow habitats such as the Wadden Sea in the North Sea are characterized by fluctuating salinity (McLusky and Elliott, 2004). Salinity changes are usually caused by seasonal alterations in tidal cycle, precipitation, freshwater run off and evaporation. In times of anthropogenic change, altered frequencies of precipitation increase the pressure of salinity fluctuations (Durack et al., 2012). Fluctuating salinity induces shifts in osmotic balance that can negatively affect the cellular processes and integrity of organelles (Berger and Kharazova, 1997; Prosser, 1991). In molluscs, acclimation to different salinities requires regulation of intracellular osmolarity to match the ambient osmolarity and prevent excessive changes in cell volume (Evans, 2009; Shumway, 1977; Yancey, 2005). In the short term, marine bivalves seal their mantle cavity to prevent water–salt exchange with surrounding waters (Berger and Kharazova, 1997). This process relies on the ability of marine bivalves to survive the accumulation of metabolic end products in their tissues (Berger and Kharazova, 1997). In contrast, survival of long-term exposure to salinity stress requires the regulation of cell volume by organic and inorganic osmolytes to achieve the isosmotic state of the cellular environment relative to the habitat. This state is achieved by altered activity of *de novo* amino acid synthesis and protein breakdown (Meng et al., 2013; Zhao et al., 2012), and changes in the transport and retention of inorganic ions, particularly sodium (Podbielski et al., 2022).

The euryhaline Pacific oyster, *Crassostrea* (*Magallana*) *gigas* (taxonomic currently under debate, here the common name *C. gigas* is used), originally hailing from Pacific coastal areas and estuaries with a broad range of environmental conditions, has successfully invaded European waters since its introduction by humans in the mid-20th century (Sigwart et al., 2021; Troost, 2010). The species' success as an invasive organism can be attributed to its ability to tolerate diverse environmental factors. In response to H/R stress, *C. gigas* relies on mitochondrial function rearrangements to maintain energy homeostasis, which may contribute to its adaptability and stress tolerance (Sussarellu et al., 2013). A shifted proteome during H/R stress promotes mitochondrial resilience, leading to upregulation of the electron transport system (ETS) and suppression of pathways channelling electrons to ubiquinone, which may reduce ROS production (Sokolov et al., 2019). These findings suggest that maintenance of mitochondrial integrity and ATP synthesis capacity are crucial for oysters to tolerate H/R stress.

In osmoconformers such as oysters, metabolism is highly sensitive to changes in ambient salinity, which can result in shifts in cellular osmolarity and ion content (Berger and Kharazova, 1997). The resulting osmotic stress caused by ion disbalance and failing redox balance can have direct effects on metabolism in bivalves, leading to electron chain dysfunction, reduced coupling and high ROS efflux, thereby impairing mitochondrial efficiency and causing oxidative stress (Bal et al., 2022; Ballantyne and Moyes, 1987; Rivera-Ingraham et al., 2016a,b; Sokolov and Sokolova, 2019). Thus, high-salinity stress in *C. gigas* leads to respiratory disturbances, stimulates glycolysis, and leads to a depletion of glycogen reserves, indicating disruption of aerobic metabolism and energy deficiency (Chen et al., 2022; Fuhrmann et al., 2018). Conversely, low-salinity stress suppresses glycolysis and increases levels of proteins and energy reserves (carbohydrates and triglycerides) in oysters, indicating positive energy balance (Fuhrmann et al., 2018). These changes are modulated by AMP-dependent protein kinase (AMPK), a major regulator of cellular energy metabolism that directly affects mitochondria (Chen et al., 2022; Fuhrmann et al., 2018). These findings suggest that salinity stress may alter mitochondrial bioenergetics and redox balance, which may have implications for mitochondrial resilience towards other stressors such as H/R, warranting further investigation.

In this study, we investigated the impact of acclimation of different salinities on the resilience of mitochondria to intermittent hypoxia in the Pacific oyster, *C. gigas*, a stress-tolerant marine osmoconformer. Our hypothesis was that acclimation to low salinity would impair mitochondrial resilience to hypoxia and reoxygenation. To test this hypothesis, we acclimated oysters from the tidal habitat of the Wadden Sea to a low salinity of 15±1 psu, while another group was kept at their collection site salinity of 30±1 psu. After acclimation, the oysters were exposed to severe hypoxia for 24 h, followed by a recovery period of 1.5 h under normoxic conditions. To gain further insight into the intrinsic mechanisms of hypoxia tolerance and salinity acclimation, acute *in vitro* H/R exposure of isolated mitochondria was also conducted, as previously done in other studies (Adzigbli et al., 2022; Onukwufor et al., 2017; Sappal et al., 2015; Sokolov and Sokolova, 2019). Mitochondrial respiration, ROS efflux, activity of the PEP branchpoint enzymes and the levels of oxidative damage [malondialdehyde (MDA) protein conjugates and protein carbonyls (PC)] were measured to explore the impacts of acclimation to different salinities on the metabolic resilience to intermittent hypoxia in *C. gigas*.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Carl Roth (Karslruhe, Germany), Sigma-Aldrich (Merck KGaA, Darmstadt, Germany)

or Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise noted and were of analytical grade or higher.

Animal maintenance and experimental exposures

Adult oysters (mean±s.e.m. shell length: 124.76±14.52 mm) were obtained from the low intertidal zone of the German Wadden Sea near List/Sylt (55°01′42″N, 8°26′04″E) and transported within 48 h of collection to the University of Rostock, Germany, in coolers lined with seawater-soaked paper towels. For habituation to laboratory conditions, oysters were kept in recirculated temperature-controlled aquarium systems (Kunststoff-Spranger GmbH, Plauen, Germany) with aerated artificial seawater (ASW) (Tropic Marin®, Wartenberg, Germany) at a salinity of 33±1 psu and temperature of 15±0.5°C for 2 weeks. Salinity and temperature conditions were within the natural range of the oyster's habitat. Oysters were then randomly divided into two groups. One group was placed in a separate tank at 15±1°C and adjusted to a salinity of 15±1 psu at a rate of 2.5 psu per day. After the target salinity was achieved, oysters were transferred to a recirculated temperature-controlled aquarium system (Kunststoff-Spranger GmbH) with aerated ASW at a salinity of 15±1 psu and 15±1°C and acclimated for 3 months. The second (control) group was kept for the same duration at a salinity of 33±1 psu and 15±1°C. During the laboratory habituation and salinity acclimation, bivalves were fed *ad libitum* by continuous addition of a commercial live algal blend (DTs Premium Blend Live Marine Phytoplankton, Coralsands, Mainz Castel, Germany) according to the manufacturer's instructions (daily 80 ml per 500 l ASW) using an automated aquarium feeder.

For experimental hypoxia, bivalves were exposed to 24 h of severe hypoxia (<0.01% O_2) by aeration of ASW with pure nitrogen (Westfalen AG, Münster, Germany) in air-tight glass jars (two oysters per 2 l ASW) at 15±0.5°C and the respective salinity. Oxygen concentration was monitored with an Intellical™ LDO101 Laboratory Luminescent/Optical Dissolved Oxygen (DO) Sensor (HACH, Loveland, CO, USA). During exposure, animals were not fed to prevent bacterial growth in the chambers. Subsequent to hypoxia exposure, a subset of animals was allowed to reoxygenate in normoxic ASW (21% O_2) for 1.5 h. Incubation periods were chosen based on previous studies showing a strong physiological response within the first hours of reoxygenation (Falfushynska et al., 2020a; Haider et al., 2020). The control group was maintained in normoxia (21% O_2) in recirculated temperature-controlled aquarium systems. Throughout experiments, no mortality was observed.

Mitochondrial assays

Mitochondria were isolated from gills as described elsewhere (Ivanina and Sokolova, 2016; Kurochkin et al., 2011). Briefly, 1.1– 1.4 g of gill tissue were homogenized in ice-cold isolation buffer (pH 7.5, 760 mOsm;100 mmol l^{-1} sucrose, 200 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl, 30 mmol l^{-1} Hepes, 8 mmol l^{-1} EGTA, 30 mmol l^{-1} taurine) in the presence of 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF), 2 µg ml^{-1} aprotinin and 2 mmol l^{-1} sodium orthovanadate using a Potter-Elvenhjem homogenizer at 200 rpm. The homogenate was centrifuged at 4°C, 2000 ***g*** for 8 min to remove cell debris and the supernatant was again centrifuged at 4°C, 8500 ***g*** for 8 min to isolate mitochondria. The pellet was resuspended in ice-cold assay buffer, the osmolarity of which corresponded to the respective salinity acclimation of oysters. Assay buffer for oysters acclimated to low salinity (525 mOsm, pH 7.5) contained 165 mmol l^{-1} sucrose, 50 mmol l^{-1} taurine, 10 mmol l^{-1} NaCl, 130 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl{\cdot}6H_2O$, 10 mmol l^{-1} KH_2PO_4 and 1% (w/v) bovine serum albumin (BSA), while assay buffer for oysters maintained at high salinity (750 mOsm, pH 7.5) consisted of 390 mmol l^{-1} sucrose, 50 mmol l^{-1} taurine, 10 mmol l^{-1} NaCl, 130 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes,10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl{\cdot}6H_2O$, 10 mmol l^{-1} KH_2PO_4 and 1% (w/v) BSA.

Oxygen consumption and emission of ROS were measured in parallel by high-resolution respirometry and fluorometry using a 2k Oxygraph (Oroboros GmbH, Innsbruck, Austria). The oxygen electrodes were calibrated to 100% (by fully aerated assay medium) and 0% oxygen (by 30 mmol l^{-1} dithionite solution). ROS efflux was measured by the rate of emission of hydrogen peroxide (H_2O_2) by energized mitochondria as described elsewhere (Ouillon et al., 2021). Assay media contained 5 U ml^{-1} superoxide dismutase to convert superoxide radicals to detectable H_2O_2, 10 µmol l^{-1} AmplexRed as a reporter and 1 U ml^{-1} horseradish peroxidase (HRP) to catalyse the H_2O_2-dependent conversion of AmplexRed to its fluorescent form. Fluorometric sensors were calibrated with 0.2 µmol l^{-1} H_2O_2.

Mitochondrial oxygen consumption and H_2O_2 emission were measured using the following substrate–uncoupler–inhibitor titration (SUIT) protocol: 5 mmol l^{-1} pyruvate and 1 mmol l^{-1} malate to stimulate Complex I (LEAK I), 10 mmol l^{-1} succinate to stimulate Complex II (referred to hereafter as pre-*in vitro* anoxia LEAK I+II), 3.57 mmol l^{-1} ADP to achieve maximum ATP synthesis and OXPHOS activity and 10 µmol l^{-1} cytochrome *c* to assess mitochondrial integrity (referred to hereafter as pre-*in vitro* anoxia OXPHOS). We routinely use >10% increase in the OXPHOS respiration rate upon cytochrome *c* addition as a criterion to exclude the mitochondrial isolate due to a potentially poor quality. However, in the present study, no sample met this exclusion criterion so that all mitochondrial isolates were considered of sufficiently good quality for further analyses. To allow exposure to *in vitro* anoxia, mitochondrial suspensions were allowed to consume all oxygen in the chamber and maintained for 10 min at 0% O_2. Subsequently chambers were reoxygenated and oxygen consumption and ROS efflux were recorded (referred to hereafter as post-*in vitro* anoxia recovery phase OXPHOS). Afterwards, the SUIT was continued with 5 µmol l^{-1} oligomycin to inhibit F_OF_1-ATPase (referred to hereafter as post-*in vitro* anoxia recovery phase LEAK I+II), stepwise titration (2.4 µmol l^{-1} steps) with carbonyl cyanide-chlorophenyl hydrazine (CCCP, final maximum concentration 14.3 µmol l^{-1}) to uncouple ATP synthesis from ETS and thus measure maximum ETS activity, 9.5 µmol l^{-1} antimycin A to inhibit Complex III and 0.5 mmol l^{-1} *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and 2 mmol l^{-1} ascorbate to achieve maximum cytochrome *c* oxidase activity.

Protein concentrations of mitochondrial suspensions were measured using the Bradford assay (Thermo Fisher Scientific) and corrected for the BSA content of the media. Oxygen consumption and ROS efflux rates were standardized to mitochondrial protein content and expressed as nmol O_2 min^{-1} mg^{-1} protein and nmol H_2O_2 min^{-1} mg^{-1} protein, respectively.

Oxidative stress markers

Oxidative damage of mitochondria were assessed by determination of MDA–protein conjugates and protein carbonyls in mitochondrial suspensions from oysters acclimated to different salinities and oxygen regimes using indirect enzyme-linked immunosorbent

assays (ELISA) (Ivanina and Sokolova, 2016; Matoo et al., 2013). Protein concentrations of 0.1 µg µl^{-1} for MDA ELISA and to 0.01 µg µl^{-1} for PC ELISA were obtained by diluting mitochondrial suspensions in phosphate-buffered saline (PBS). To prevent protein aggregation, dilutions were sonicated (Sonicator S-4000, Misonix, Famingdate, NY, USA; amplitude 24, 30 s). An MDA dilution series was prepared from a 1 mg ml^{-1} MDA–BSA–control standard (Cell Biolabs, San Diego, CA, USA) in 10 µg ml^{-1} fatty acid- and immunoglobulin-free BSA suspension. PC standards were prepared by oxidizing fatty acid- and immunoglobulin-free BSA with 30% H_2O_2 for 30 min. PC concentration of the oxidized BSA was determined spectrophotometrically as described elsewhere (Levine et al., 1990). Oxidized BSA standard was diluted to 10 µg ml^{-1} protein content with PBS and used to prepare the standard dilution series. ELISA plates were incubated with mitochondrial protein samples and standards at 4°C overnight and washed with PBS prior to further incubation.

For MDA, 1 mg ml^{-1} fatty acid- and immunoglobulin-free BSA was used to block plates for 2 h at 37°C prior to treatment with anti-MDA antibody (1:1000, ab27642, Abcam, Cambridge, UK). Subsequently, plates were incubated with anti-rabbit antibody conjugated with horseradish peroxidase (1:10,000, 111-035-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). For determination of PC, samples and standards were derivatized by incubation for 45 min with 5 mmol l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in the dark prior to forming dinitrophenylhydrazone-protein carbonyl moiety (DNP). After washing plates with PBS–ethanol mixture (1:1 v/v), plates were blocked with 1 mg ml^{-1} fatty acid- and immunoglobulin-free BSA for 2 h at room temperature. Formation of DNP was assessed by incubation with anti-DNP antibody (1:1000, MAB2223, Merck Millipore, Burlington, MA, USA) followed by anti-mouse antibody (1:10,000, 115-035-03, Jackson Immuno Research Laboratories Inc.). Each antibody incubation was conducted for 1 h at room temperature. Bound antibodies were detected by the addition of horseradish peroxidase substrate TMB/E Ultra Sensitive (Merck Millipore) and 2 mol l^{-1} sulfuric acid to stop the reaction. Subsequently, development of colour was detected at 450 nm (SpectraMax iD3, Molecular Devices, LLC, San José, CA, USA).

Enzyme activity

The activity of PK (EC 2.7.1.40) and PEPCK (EC 4.1.1.31) was spectrophotometrically measured in crude tissue extracts of hepatopancreas as described elsewhere (Simpfendörfer et al., 1995). Briefly, 200 mg tissue were homogenized in 10 mmol l^{-1} Tris-HCl (pH 7.0), 5 mmol l^{-1} Na_2-EDTA, 0.1 mmol l^{-1} PMSF and 1 mmol l^{-1} dithiothreitol (DTT) using a FastPrep24 homogenizer with 6.5 m s^{-1} for 5 times 40 s (MP Biomedical, Santa Ana, CA, USA).

Assay media were as follows. PK: 50 mmol l^{-1} Tris-HCl (pH 7.0), 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgSO_4{\cdot}7$ H_2O, 1 mmol l^{-1} ADP sodium salt, 0.2 mg ml^{-1} NADH, 5.5 U lactate dehydrogenase and 2.5 mmol l^{-1} PEP. PEPCK: 100 mmol l^{-1} Hepes (pH 7.0), 2.3 mmol l^{-1} $MnCl_2{\cdot}4$ H_2O, 5 mg ml^{-1} $KHCO_3$, 0.5 mmol l^{-1} IDP sodium salt, 0.2 mg ml^{-1} NADH, 10 U malate dehydrogenase and 15 mmol l^{-1} PEP. For both enzymes, reactions were started with the addition of PEP and monitored at 340 nm (SpectraMAx iD3, Molecular Devices, LLC). Absorbances was corrected by blank measurement. The activity of both enzymes was standardized to fresh tissue mass and expressed as nmol min^{-1} g^{-1} fresh tissue mass.

Data analysis and statistics

Data were checked for normal distribution using the Shapiro–Wilk test in IBM® SPSS® Statistics (v. 25, IBM, Corp. Armonk, NY, USA) and for homogeneity of variances by the Brown–Forsythe test in Sigma Plot (v. 13.0.0.83, Systat Software Inc., San Jose, CA, USA). Outliers were excluded by box–whisker plots in IBM® SPSS® Statistics. In the case of non-normal distribution and/or non-homogeneity of variances, data were transformed by Box–Cox or Johnson transformation in Minitab (v.19, Minitab LLC., State College, PA, USA). Significant differences were tested by two-way ANOVA in SigmaPlot using *in vivo* oxygen and salinity regime as fixed factors. No significant factor interactions were detected by ANOVA for any of the studied parameters (P>0.05; data not shown). Therefore, the subsequent analyses focused on the effects of salinity and oxygen regime as determined by Tukey's honest significant differences (HSD) *post hoc* tests. Statistical comparison of *in vitro* oxygen treatments was analysed using paired *t*-test in Sigma Plot 13 within each salinity group, with *in vitro* oxygen treatment as a fixed factor. To analyse the *in vitro* data by paired *t*-test, we had to curate the data so that missing values in one of the groups (pre- or post-anoxia *in vitro*) were used as a criterion to remove that sample.

RESULTS

Effects of *in vivo* exposure of oysters to hypoxia and reoxygenation

Mitochondrial oxygen consumption

Acclimation to low salinity (15 psu) generally did not affect the baseline (LEAK) oxygen consumption rate ($\dot{M}_{O_2}$) of the oyster gill mitochondria (except during post-hypoxia recovery) but led to a ∼1.5-fold increase in the OXPHOS $\dot{M}_{O_2}$ compared with that of mitochondria from the high-salinity-acclimated oysters (Fig. 1A–C). In oysters acclimated to high salinity (33 psu), the baseline mitochondrial respiration with a Complex I substrate (LEAK I) or a combination of Complex I and II substrates (LEAK I+II) did not change during H/R stress compared with the normoxic control (Fig. 1A,B). In the oysters acclimated to low salinity (15 psu), exposure to hypoxia had no effect on the mitochondrial LEAK $\dot{M}_{O_2}$, but LEAK I and LEAK I+II $\dot{M}_{O_2}$ increased ∼1.4-fold during reoxygenation (Fig. 1A,B). Exposure to H/R stress showed no effect on OXPHOS $\dot{M}_{O_2}$ regardless of the acclimation salinity (Fig. 1C).

Mitochondrial ROS efflux and oxidative damage

ROS efflux rate and the fractional electron leak (FEL), calculated as the ratio of consumed O_2 released as H_2O_2, were higher in the LEAK state than in the OXPHOS state mitochondria in all experimental groups (Fig. 1D–I). *In vivo* H/R stress and salinity acclimation did not alter H_2O_2 efflux from mitochondria in LEAK I and OXPHOS state (Fig. 1D,F). In the low-salinity-acclimated oysters, mitochondrial ROS efflux increased ∼1.6-fold during post-hypoxic recovery relative to the normoxic control in LEAK I+II state (Fig. 1E). However, FEL (H_2O_2:O_2 ratio) remained unaltered by H/R stress and/or salinity acclimation (Fig. 1G–I).

Levels of the oxidative stress markers (MDA–protein conjugates and PCs) in oyster mitochondria remained unaltered by *in vivo* salinity acclimation and H/R stress (Fig. 2).

Mitochondrial coupling efficiency

Acclimation to low salinity led to a modest but statistically significant increase in the mitochondrial respiratory control ratio (RCR) and OXPHOS coupling efficiency (OXPHOS CE) in the

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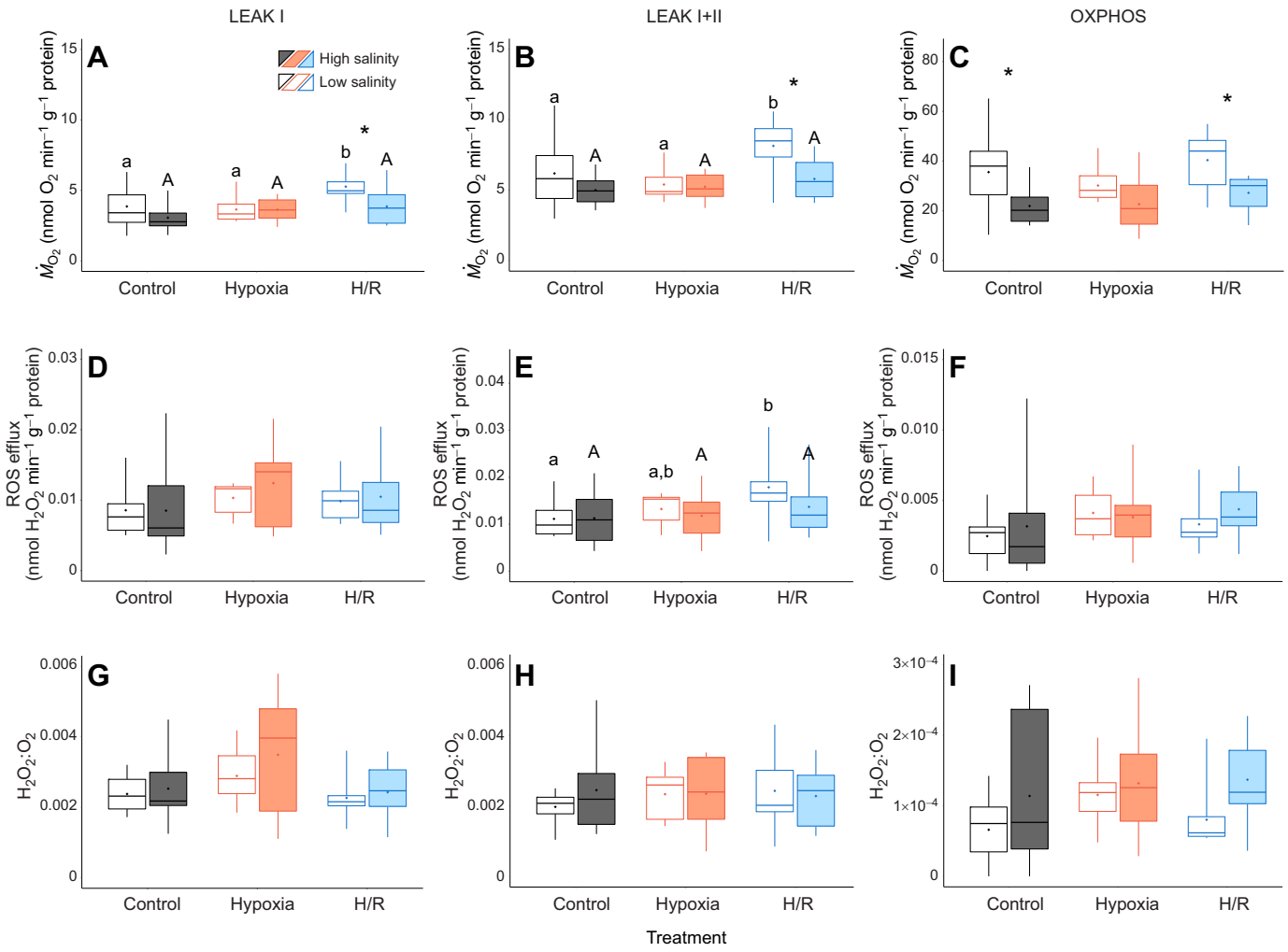

Fig. 1. Effect of salinity acclimation and short-term hypoxia–reoxygenation (H/R) stress on oxygen consumption, reactive oxygen species efflux and fractional electron leak of *Crassostrea gigas* mitochondria in resting (LEAK I and LEAK I+II) and active (OXPHOS) states. Oxygen consumption ($\dot{M}_{O_2}$; A–C), reactive oxygen species (ROS) efflux (D–F) and fractional electron leak (FEL; H_2O_2:O_2 ratio) (G–I) in resting mitochondria respiring on Complex I substrates pyruvate and malate (LEAK I) (left), in resting mitochondria respiring on complex I and II substrates pyruvate, malate and succinate (LEAK I+II) (middle) and active mitochondria (OXPHOS) (right). Experimental groups: control (21% O_2); hypoxia, short-term (24 h) severe (<0.01% O_2) hypoxia; H/R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Box–whisker plots: open, low-salinity (LS; 15 psu) acclimation; filled, high-salinity (HS; 33 psu) acclimation. Means are shown as points within corresponding box–whisker plots. Statistical significance (P<0.05) between oxygen treatments is indicated by different letters above plots and statistical differences (P<0.05) between salinity acclimation within one oxygen treatment are indicated by asterisks above plots. N=9–11, 8–10, 8–10, 8–10, 9–11 and 10–11 in LS control, HS control, LS hypoxia, HS hypoxia, LS H/R and HS H/R, respectively.

oyster mitochondria compared with that of oysters from the high-salinity treatment (Fig. 3). H/R exposure showed no evidence of effect on the RCR and OXPHOS CE within each salinity acclimation group (Fig. 3). Similarly, exposure of isolated mitochondria to *in vitro* anoxia and reoxygenation showed no effect on RCR and OXPHOS CE regardless of the acclimation salinity (Fig. S1).

Activity of enzymes at the aerobic–anaerobic branch point

In the high-salinity acclimation group, PK activity decreased in hypoxia and increased during reoxygenation so that the differences between the hypoxia-exposed and post-hypoxic recovery groups were significant (P<0.05) (Fig. 4A). In the low-salinity group, PK activity remained at the normoxic baseline levels during hypoxia and reoxygenation. PEPCK activity and PK:PEPCK ratios remained unchanged during H/R stress regardless of the acclimation salinity (Fig. 4B,C).

Effects of acute *in vitro* exposure of isolated mitochondria to hypoxia and reoxygenation

Mitochondrial oxygen consumption and ROS efflux

Generally, mitochondria from oysters acclimated to low salinity were more responsive to the acute *in vitro* anoxia that those from the high-salinity-acclimated oysters, regardless of the oxygen conditions (normoxia, hypoxia or post-hypoxic reoxygenation) under which the oysters were collected (Figs 5 and 6). In the high-salinity treatment group, there was a weak tendency for elevated LEAK and OXPHOS respiration of the isolated mitochondria after acute anoxia exposure *in vitro* (significant in the mitochondria isolated from the normoxic oysters for LEAK I+II and from the hypoxic oysters for OXPHOS) (Fig. 5A,B). Acute *in vitro* exposure to anoxia had no effect on the ROS efflux in mitochondria from the high-salinity-acclimated oysters (Fig. 5C,D). FEL in the LEAK state did not respond to acute *in vitro* anoxia exposure but increased in the OXPHOS state in the mitochondria isolated from hypoxic or

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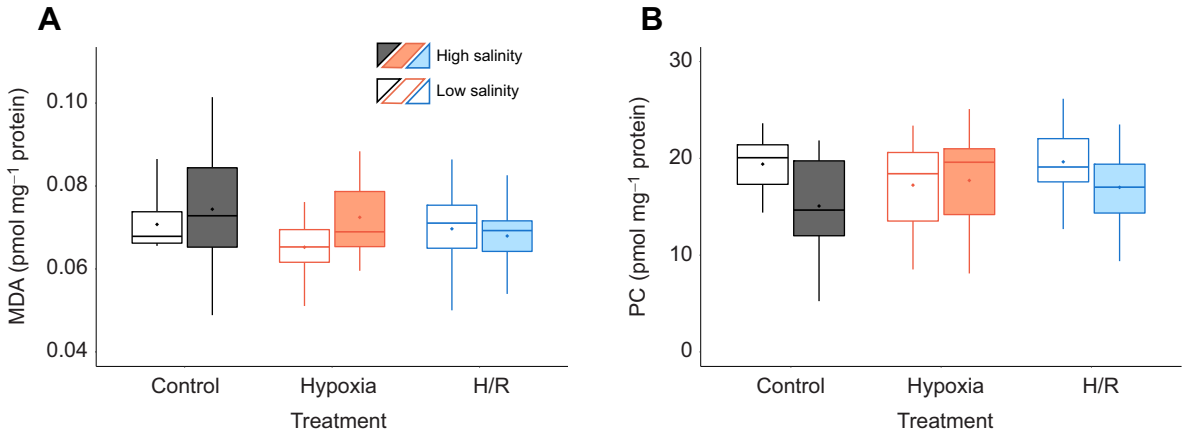

Fig. 2. Assessment of oxidative damage in mitochondrial proteins of oysters exposed to combined salinity and *in vivo* H/R stress. (A) Malondialdehyde (MDA)–protein conjugates; (B) protein carbonyls (PC). Experimental groups: control (21% O_2); hypoxia, short-term (24 h) severe (<0.01% O_2) hypoxia; H/R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Box–whisker plots: open, low-salinity (LS; 15 psu) acclimation; filled, high-salinity (HS; 33 psu) acclimation. Means are shown as points within corresponding box–whisker plots. No significant differences between groups in these traits were found (P>0.05). N=10–11, 10, 10, 10, 10–11 and 10–11 in LS control, HS control, LS hypoxia, HS hypoxia, LS H/R and HS H/R, respectively.

recovering oysters in the high-salinity group (Fig. 5E,F). In the mitochondria of the low-salinity-acclimated oysters, LEAK and OXPHOS respiration, ROS efflux and FEL were stimulated by acute *in vitro* anoxia exposure of the mitochondria regardless of the oxygen conditions (normoxia, hypoxia or post-hypoxic reoxygenation) under which the oysters were collected (Fig. 6). Notably, a ∼1.2-fold increase in the oxygen consumption rates caused by the *in vitro* anoxic exposure was accompanied by ∼2-3-fold increase in the ROS efflux rates, leading to a noticeable increase in the FEL, particularly in the LEAK state (Fig. 6E,F).

DISCUSSION

Effects of salinity and H/R exposure on mitochondrial respiration and ROS efflux in oysters

Our study showed that salinity modulates the mitochondrial responses to long-term and acute hypoxia exposure in an euryhaline intertidal bivalve *C. gigas*. Interestingly, the mitochondrial performance of oysters was improved by acclimation to low (15 psu) salinity relative to the high (33 psu) salinity that corresponds to the salinity of the habitat where the oysters were collected. This was evident in the higher RCR and OXPHOS CE in the mitochondria of oysters from the low-salinity treatment, indicating improved mitochondrial coupling. Furthermore, mitochondria isolated from the gill of the low-salinity-acclimated oysters showed a higher plasticity during acute H/R stress, resulting in higher respiratory flux during post-hypoxic recovery compared with the mitochondria of high-salinity-acclimated oysters. Based on the mitochondrial coupling efficiency, a salinity of 15 psu might be considered closer to the optimum for the metabolic performance in the studied *C. gigas* population than a salinity of 33 psu. An earlier study using the same population of *C. gigas* acclimated to a salinity of 30 psu also showed better coupling, higher rates of ATP synthesis and ETS flux in the gill mitochondria measured at 450 mOsm (corresponding to a salinity of 15 psu) than in 900 mOsm (corresponding to a salinity of 30 psu) (Sokolov and Sokolova, 2019).

Crassostrea gigas was found to survive a wide window of salinity from 12 to 43 psu (Wiltshire, 2007) and can grow well at

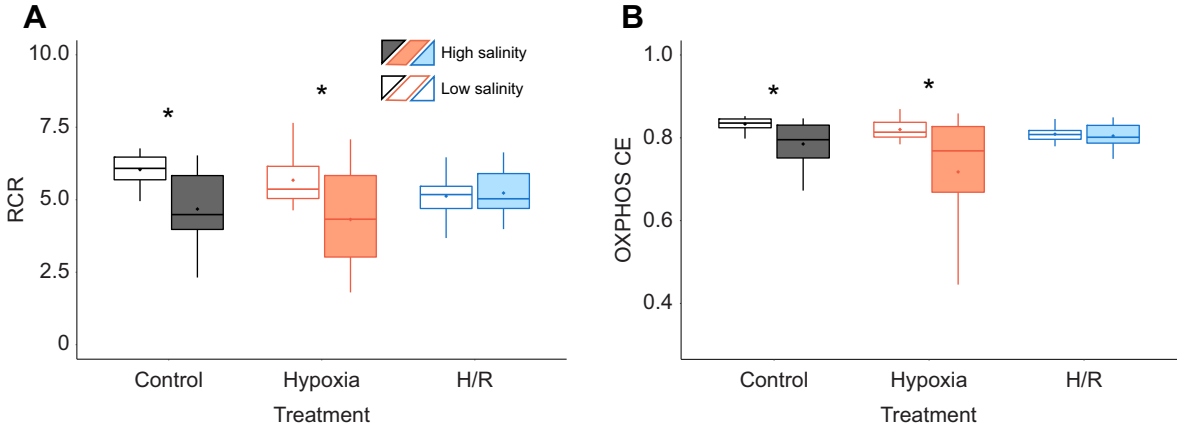

Fig. 3. Effect of salinity acclimation and short-term H/R stress on coupling efficiency of *C. gigas* mitochondria. (A) Respiratory control ratio (RCR); (B) OXPHOS coupling efficiency [OXPHOS CE=1−(LEAK I+II/OXPHOS)]. Experimental groups: control (21% O_2); hypoxia, short-term (24 h) severe (<0.01% O_2) hypoxia; H/R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Box–whisker plots: open, low-salinity (LS; 15 psu) acclimation; filled, high-salinity (HS; 33 psu) acclimation. Statistical differences (P<0.05) between salinity acclimations are indicated by asterisks. N=10, 9–10, 10, 10, 9–10 and 10 in LS control, HS control, LS hypoxia, HS hypoxia, LS H/R and HS H/R, respectively.

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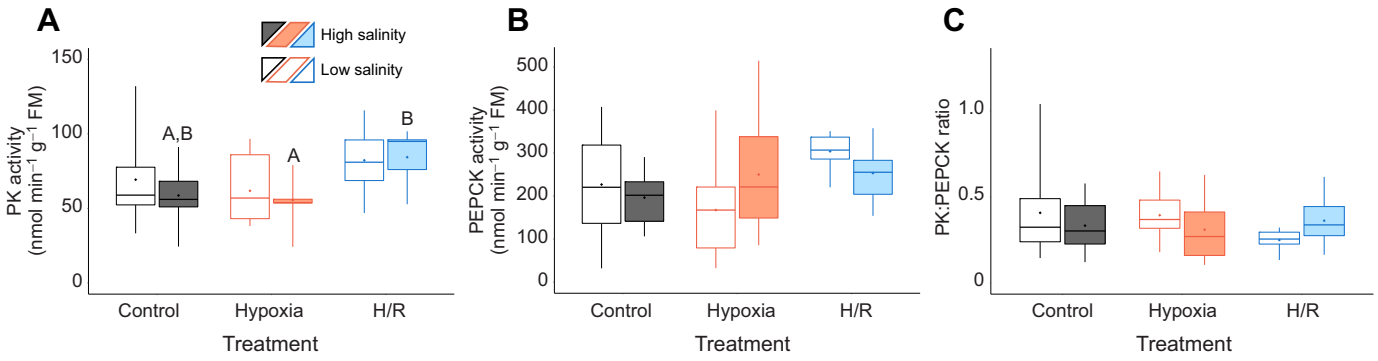

Fig. 4. Metabolic response of pyruvate kinase and phosphoenolpyruvate carboxykinase activity to combined salinity and H/R stress. (A) Activity of pyruvate kinase (PK); (B) activity of phosphoenolpyruvate carboxykinase (PEPCK); (C) PK:PEPCK ratio. Experimental groups: control (21% O_2); hypoxia, short-term (24 h) severe (<0.01% O_2) hypoxia; H/R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Box–whisker plots: open, low-salinity (LS; 15 psu) acclimation; filled, high-salinity (HS; 33 psu) acclimation. Means are shown as points within corresponding box–whisker plots. Statistical significance (P<0.05) between oxygen treatments is represented by different letters above plots. N=11–12, 9–10, 9, 9–10, 8–11 and 9–11 in LS control, HS control, LS hypoxia, HS hypoxia, LS H/R and HS H/R, respectively.

salinities above 15 psu (Calvo et al., 1999). Oysters are marine osmoconformers and thus are required to adjust their intracellular environment to the changing conditions of the surrounding seawater. Consequently, mitochondria need to be adapted to function across a wide range of osmolarities, depending on the habitat range. Mitochondria of euryhaline marine osmoconformers have broad osmotic tolerance as shown in the bivalves *Mercenaria mercenaria*, *Crassostrea virginica*, *Mya arenaria* and *Mytilus edulis* (Ballantyne and Storey, 1983; Ballantyne and Moyes, 1987; Haider et al., 2018; Noor et al., 2021). Outside the osmotic tolerance range, mitochondria of euryhaline marine species show evidence of electron transport chain dysfunction, and high ROS production leading to oxidative stress (Bal et al., 2022; Paital and Chainy, 2012; Rivera-Ingraham et al., 2016a,b). Although the mitochondria of *C. gigas* showed improved performance at a salinity of 15 psu relative to 30 psu (Sokolov and Sokolova, 2019) or 33 psu (this study), no evidence of major ETS dysfunction or elevated ROS efflux was found at the higher acclimation salinity, consistent with notion of the broad salinity tolerance of this euryhaline species.

Mitochondria from *C. gigas* showed high resilience to prolonged (24 h) hypoxia and subsequent reoxygenation, regardless of the acclimation salinity. Respiration rates in the OXPHOS and LEAK state remained unchanged during H/R stress, except for upregulated respiration in post-hypoxic recovery predominantly under low salinity. Elevated mitochondrial oxygen consumption supports sufficient ATP synthesis capacity during reoxygenation. Evidence of a hypoxia-resilient mitochondrial phenotype maintaining high OXPHOS and ATP synthesis capacity during H/R stress was reported earlier in *C. gigas* (Sokolov et al., 2019; Steffen et al., 2020; Sussarellu et al., 2013) and other hypoxia-tolerant bivalves including *C. virginica* and *Arctica islandica* (Ivanina et al., 2012, 2016; Steffen et al., 2021). In contrast, hypoxia-intolerant bivalves such as scallops suffer from downregulation of OXPHOS caused by the loss of ETS capacity when exposed to hypoxia and reoxygenation (Ivanina et al., 2016). The ability to maintain high OXPHOS capacity and mitochondrial coupling during hypoxia and reoxygenation thus appears to be an adaptation to survive hypoxic periods in the intertidal zone (as in oysters and mussels) or anoxic sediments (as in *A. islandica*) not found in highly aerobic subtidal species such as scallops (Ivanina et al., 2012, 2016; Sokolov et al., 2019; Steffen et al., 2021; Sussarellu et al., 2013).

In oysters acclimated to low salinity, the post-hypoxic recovery process was associated with an upregulation of the respiration rate in LEAK state. Mitochondrial LEAK represents the rate of respiration required to counterbalance the depolarization of mitochondria caused by ion cycles that are not linked to ATP generation (Brand et al., 1994). Mildly elevated LEAK respiration is considered an important mitochondrial control mechanism to prevent excessive ROS formation (Brand, 1997; Miwa and Brand, 2003), whereas excessive LEAK rates might be associated with energy wastage leading to impaired OXPHOS efficiency and low coupling (Sokolova, 2023). In this study, we found that the mitochondria from oysters acclimated to low salinity were capable of maintaining normal RCR during post-hypoxic recovery despite the elevated proton leak rates with both Complex I and II substrates. This indicates that the observed modest stimulation of the proton leak during recovery does not result in suppressed mitochondrial efficiency. In contrast, the soft shell clam *M. arenaria* showed elevated LEAK respiration combined with lower OXPHOS respiration rates and a decrease in the OXPHOS coupling efficiency under the fluctuating oxygen regime (Ouillon et al., 2021). Both *C. gigas* and *M. arenaria* are stress-tolerant intertidal species with high invasive potential (Dutertre et al., 2009; Strasser, 1999), but the comparison of mitochondrial responses indicates that mitochondria of *C gigas* might be more resilient to oxygen fluctuations than those of *M. arenaria*.

Modest elevation of proton leak during post-hypoxic recovery might contribute to ROS control, as the FEL remained unchanged during post-hypoxic recovery in the mitochondria of *C. gigas*. Furthermore, no accumulation of oxidative lesions (MDA adducts or protein carbonyls) was found in the mitochondria of *C. gigas* exposed to H/R stress, regardless of the acclimation salinity, consistent with the notion of a resilient mitochondrial phenotype in this species. Hypoxia-tolerant bivalves commonly show stable levels of oxidative lesions during H/R stress compared with their hypoxia-intolerant counterparts (like scallops) (Ivanina and Sokolova, 2016; Ivanina et al., 2016) or hypoxia-intolerant vertebrate species (Cadenas, 2018; Honda et al., 2005; Zorov et al., 2014), which are prone to oxidative injury during H/R stress. This resistance to oxidative damage might be attributed to high levels of antioxidants and other cytoprotective molecules (including osmoprotectants and molecular chaperones) in stress-tolerant

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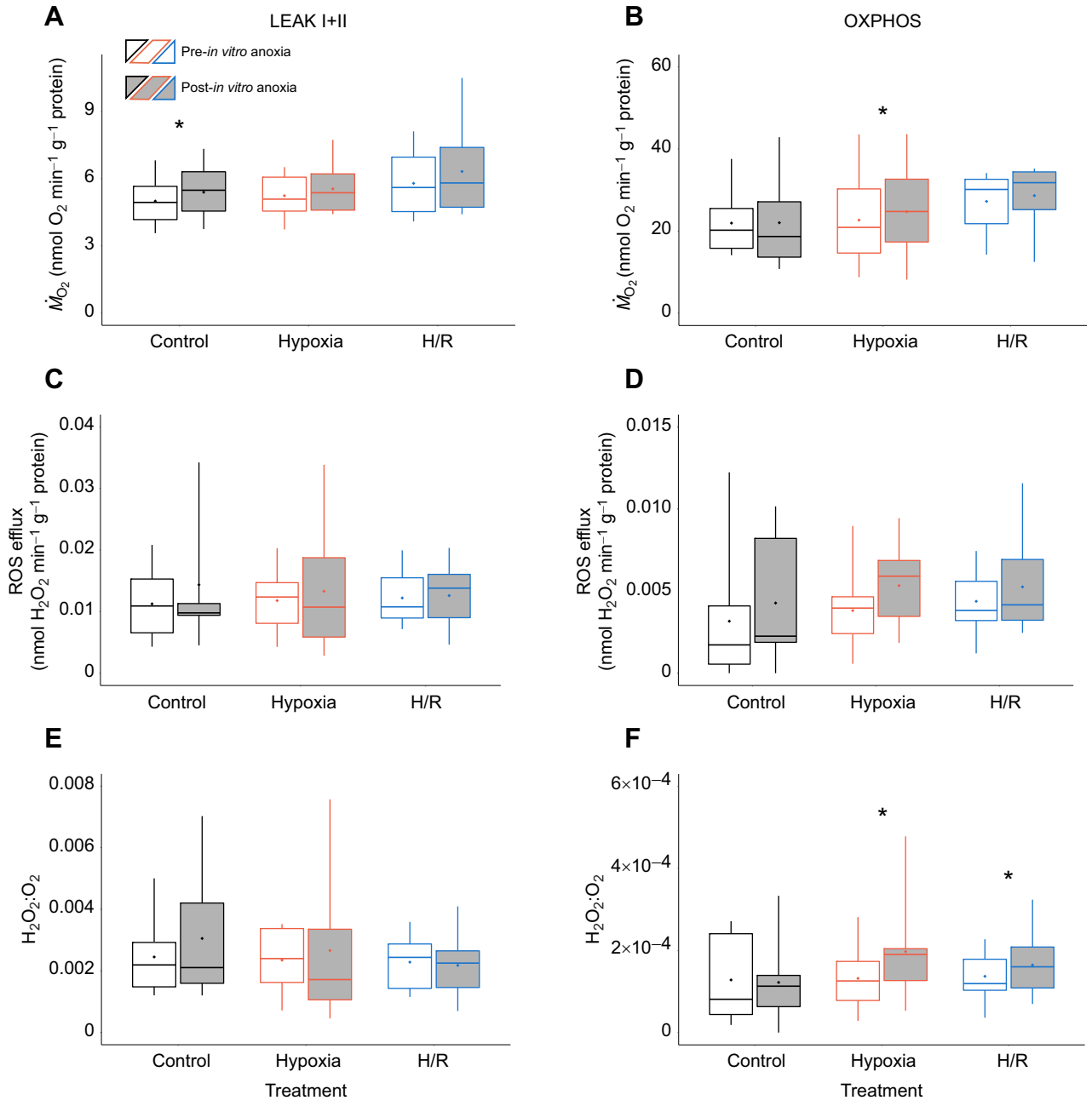

Fig. 5. Effect of *in vitro* H/R stress on oxygen consumption, ROS efflux and FEL of mitochondria in LEAK and OXPHOS states from oysters acclimated to high salinity. Oxygen consumption (A,B), ROS efflux (C,D) and FEL (H_2O_2:O_2 ratio; E,F) of mitochondria in LEAK I+II state (left) and OXPHOS state (right). Experimental groups: control (21% O_2); hypoxia, short-term (24 h) severe (<0.01% O_2) hypoxia; H/R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Box–whisker plots: open, pre-*in vitro* H/R stress; filled, post-*in vitro* H/R stress. Means are shown as points within corresponding box–whisker plots. Statistical differences (P<0.05) between pre- and post-*in vitro* H/R stress are indicated by asterisks above plots. Because of the focus on the effect of *in vitro* H/R stress, statistical results are only shown for paired *t*-tests. Data of open box–whisker plots are based on data of Fig. 1 curated for usage in paired *t*-tests as described in Materials and Methods. N=8–10, 8–10, 8–10, 8–10, 9–11 and 9–11 in control pre-anoxia, control post-anoxia, hypoxia pre-anoxia, hypoxia post-anoxia, H/R pre-anoxia and H/R post-anoxia, respectively.

Crassostrea spp. (Sokolov et al., 2019; Sokolova et al., 2019; Steffen et al., 2020; Wei et al., 2022; Zhang et al., 2012)

Metabolism at PEP branchpoint under variable oxygen and salinity regimes

In facultative anaerobes such as bivalves, breakdown products of glycolysis can be fed into the aerobic mitochondrial respiration (via pyruvate and acetyl-CoA) or anaerobic mitochondrial pathways of ATP production (via oxaloacetate) (Grieshaber et al., 1994; Ivanina et al., 2010, 2012). The PEP branchpoint is an important metabolic junction in the glucose oxidation of facultative anaerobes. PEP can be converted into pyruvate by the enzyme PK or into oxaloacetate by the enzyme PEPCK. The regulation of the PEP branchpoint is complex and is influenced by a variety of factors, including the availability of oxygen, intracellular pH and energy status, and depends on the PK/PEPCK activity ratio (Das et al., 2015; Saz,

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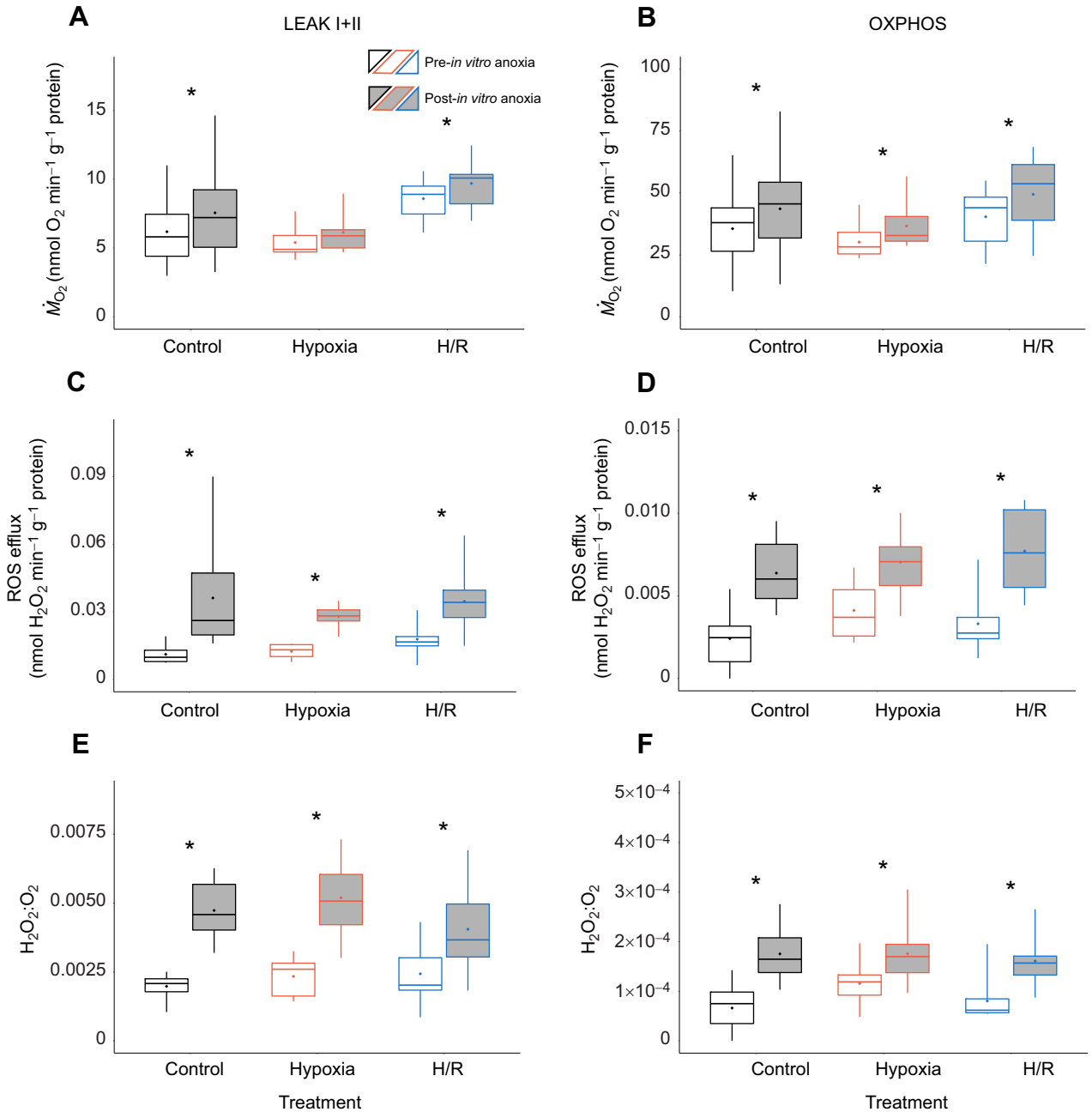

Fig. 6. Effect of *in vitro* H/R stress on oxygen consumption, ROS efflux and FEL of mitochondria in LEAK and OXPHOS states from oysters acclimated to low salinity. Oxygen consumption (A,B), ROS efflux (C,D) and FEL (H_2O_2:O_2 ratio; E,F) of mitochondria in LEAK I+II state (left) and OXPHOS state (right). Experimental groups: control (21% O_2); hypoxia, short-term (24 h) severe (<0.01% O_2) hypoxia; H/R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Box–whisker plots: open, pre-*in vitro* H/R stress; filled, post-*in vitro* H/R stress. Means are shown as points within corresponding box–whisker plots. Statistical differences (P<0.05) between pre- and post-*in vitro* H/R stress are indicated by asterisks. Because of the focus on the effect of *in vitro* H/R stress, statistical results are only shown for paired *t*-tests. Data of open box–whisker plots are based on data of Fig. 1 curated for usage in paired *t*-tests as described in Materials and Methods. N=9–11, 9–11, 8–10, 8–10, 9–11 and 9–11 in control pre-anoxia, control post-anoxia, hypoxia pre-anoxia, hypoxia post-anoxia, H/R pre-anoxia and H/R post-anoxia, respectively.

1971; Zammit and Newsholme, 1978). Generally, high PK/PEPCK activity ratio leads to preferential channelling of PEP into aerobic respiration, and low PK/PEPCK activity ratio favours anaerobic formation of ATP, alanine and succinate (Bayne, 2017; Das et al., 2015; Simpfendörfer et al., 1995). In the present study, PK/PEPCK activity ratio in oyster gills remained unchanged during the H/R cycle. This indicates a lack of metabolic reorganization at the PEP branchpoint and might be due to the relatively short hypoxic exposure (24 h) used in our present study. Although *C. gigas* has been shown to transcriptionally regulate expression of *pk* and *pepck* within the first 6–12 h of hypoxic exposure (Sussarellu et al., 2012), most bivalves including oysters, scallops and clams do not rely heavily on anaerobic metabolism during short-term hypoxia (David et al., 2005; Greenway and Storey, 1999; Ivanina et al., 2010, 2016; Kurochkin et al., 2009). However, in sustained hypoxia, oysters do rely more heavily on anaerobic succinate-producing pathways of

ATP formation indicated by the switch of PK and PEPCK activities (Bayne, 2017; Grieshaber et al., 1994). The green-lipped mussel *Perna viridis*, in contrast, exhibits a different response, with upregulation of PEPCK and elevated production of succinate occurring after only 24 h of hypoxia (Nusetti et al., 2010). Thus, the duration of hypoxic exposure appears to play a crucial role in determining the energy supply pathways utilized by these organisms.

Intrinsic mitochondrial response mechanisms to acute anoxia and reoxygenation *in vitro*

Mitochondria are organelles with semi-autonomous capabilities, possessing their own DNA, protein translation machinery and quality control systems, which allow them to respond directly to H/R transitions independently of the rest of the cell (McBride et al., 2006). Previous research has shown that mitochondria in bivalve species, such as oysters and mussels, have intrinsic mechanisms of metabolic plasticity that involve functional changes in the ETS activity and reorganization of the mitochondrial proteome, regardless of the cellular environment (Adzigbli et al., 2022; Sokolov et al., 2019). When exposed to acute *in vitro* anoxia, isolated mitochondria from oysters and mussels respond by upregulating OXPHOS and modifying multiple proteins involved in ETS, tricarboxylic acid cycle, fatty acid and amino acid metabolism, and protein quality control (Adzigbli et al., 2022; Sokolov et al., 2019). Our study on *C. gigas* revealed that the response of mitochondria to acute short-term anoxia is influenced by acclimation to different salinities, but not by H/R exposure of the entire animal. Specifically, mitochondria from oysters acclimated to high salinity showed minimal changes in oxygen consumption or ROS efflux during acute H/R stress. In contrast, mitochondria from oysters acclimated to low salinity exhibited a strong upregulation of the OXPHOS and LEAK respiration rates after acute anoxia exposure *in vitro*. These findings suggest that acclimation to high salinity reduces the intrinsic mitochondrial plasticity to acute anoxia stress, which is observable in the mitochondria of low-salinity-acclimated oysters. Intrinsic plasticity of isolated mitochondria in response to various stimuli depends on mechanisms such as post-translational protein modifications (Falfushynska et al., 2020b; Mathers and Staples, 2019; Pagliarini and Dixon, 2006; Sokolov et al., 2021; Yang and Gibson, 2019), allosteric regulation of enzyme activity (Acin-Perez et al., 2011; Beauvoit and Rigoulet, 2001; Cherkasov et al., 2007) and proton-motive force-dependent regulation of respiration and ROS production (Brand, 2000; Jastroch et al., 2010; Lambert and Brand, 2004). In our present study, the exact mechanisms responsible for the differences in the intrinsic mitochondrial plasticity between the mitochondria of oysters acclimated to high and low salinity could not be determined requiring further investigation. Furthermore, previous studies on *C. gigas* mitochondria have also shown that lower osmolarity in the mitochondrial environment promotes faster ATP synthesis, higher ETS capacity and improved mitochondrial coupling (Sokolov and Sokolova, 2019). The increase in OXPHOS activity and ATP synthesis capacity in low-salinity-acclimated oysters after acute anoxia exposure may accelerate the restoration of their energy balance compared with high-salinity-acclimated oysters. However, this rapid recovery may come at the expense of redox disturbances, as indicated by higher rates of ROS efflux and elevated FEL rates in the mitochondria of low-salinity-acclimated oysters after acute anoxia stress. Similar findings have been observed in *Danio rerio*, a hypoxia-tolerant zebrafish species, where *in vitro* anoxia increased OXPHOS respiration rates but also ROS efflux (Napolitano et al., 2019). This suggests that the upregulation of oxygen consumption during post-hypoxic recovery may impose costs on mitochondrial functions, if ROS production exceeds the mitochondrial capacity to mitigate redox stress.

It is important to note that ROS play a dual role in cellular responses to H/R, not only causing harmful effects on cellular macromolecules but also acting as signalling molecules that regulate adaptive cellular responses to oxygen fluctuation through the activation of hypoxia-inducible factors and Nrf2-dependent transcriptional regulators (Sies and Jones, 2020; Sies et al., 2022; Zorov et al., 2014). The unexpected increase in ROS levels observed in oyster mitochondria exposed to low salinity and acute anoxia *in vitro* may therefore be linked to adaptive signalling through ROS to activate stress response pathways (Forman et al., 2010; Sokolova, 2018). Additional research is required to elucidate whether the increased release of ROS triggered by acute anoxia stress serves as a beneficial signalling mechanism or represents a trade-off between ATP production and redox balance in oyster mitochondria.

In vivo exposure of oysters to H/R stress did not affect the *in vitro* response of mitochondria during post-hypoxic recovery, regardless of salinity acclimation. However, long-term *in vivo* hypoxia exposure in sablefish resulted in mitochondria becoming more resilient to acute *in vitro* H/R stress, potentially related to reduced ROS production through increased proton conductance via higher LEAK respiration (Gerber et al., 2019). It is possible that the short-term hypoxic exposure in our study was not severe enough to activate adaptive plasticity of oyster mitochondria. Previous studies on vertebrates and invertebrates have found that robust maintenance or even enhancement of OXPHOS and ETS capacity during *in vivo* or *in situ* H/R exposure is a common trait in hypoxia-tolerant species, whereas hypoxia-intolerant species often suffer from loss of OXPHOS and ETS capacity (Ivanina et al., 2016; Paradis et al., 2016; Venditti et al., 2001). Oyster mitochondria displayed strong resilience to hypoxia in our study, which may be associated with a phenotype commonly observed in hypoxia-tolerant bivalve species characterized by unchanged or elevated OXPHOS capacity, ROS mitigation and prevention of ETS collapse during post-hypoxic recovery, partially independent of cellular mechanisms.

Conclusions and outlook

Acclimation to low salinity appears to improve the mitochondrial performance of *C. gigas*, as indicated by higher RCR and better mitochondrial coupling. Oysters acclimated to low salinity also showed higher oxygen consumption during post-hypoxic recovery, indicating higher mitochondrial plasticity in response to H/R stress compared with that of those from high salinity. These results suggest that a salinity of 15 psu might be closer to *C. gigas*' metabolic optimum, even though the studied population originated from a high-salinity (33 psu) habitat. Previous studies have also indicated better mitochondrial performance in low osmolarity for oysters (Sokolov and Sokolova, 2019). However, both high- and low-salinity oysters showed unchanged OXPHOS and LEAK respiration during H/R stress, demonstrating their high resilience to intermittent hypoxia (Ivanina et al., 2016; Sokolov et al., 2019; Sussarellu et al., 2013). Despite better mitochondrial performance in low salinity, high salinity did not cause severe mitochondrial damage or dysfunction during H/R stress, reflecting the broad salinity tolerance of *C. gigas* (Troost, 2010). However, salinity exposure affected the intrinsic response of isolated mitochondria to acute anoxia. Acclimation to low salinity permitted strong upregulation of respiration of mitochondria during post-hypoxic

recovery, which may support the rapid reinstatement of cellular energy homeostasis. This stronger metabolic plasticity, however, came with a higher cost of mitochondrial maintenance, due to elevated ROS production. The characteristic features of a hypoxia-tolerant mitochondrial phenotype (such as elevated OXPHOS capacity and ROS mitigation during H/R stress) can thus be modulated by environmental salinity in euryhaline osmoconformers such as oysters. Mitochondrial performance typically correlates with organismal performance and fitness in ectotherms (Koch et al., 2021). Based on the mitochondrial performance, the studied invasive population of *C. gigas* has the metabolic capacity to perform well in low-salinity habitats, thus potentially spreading further into European brackish waters (Schmidt et al., 2008), provided the salinity barrier for larval recruitment is overcome (Ewers-Saucedo et al., 2020). Whether this high mitochondrial plasticity is indeed one of the adaptive traits of larvae and adult *C. gigas* allowing for the successful invasion of the Baltic Sea requires further investigation.

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Competing interests
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Author contributions
Conceptualization: C.B., E.P.S., I.M.S.; Methodology: J.B.M.S., E.P.S., I.M.S.; Validation: J.B.M.S.; Formal analysis: J.B.M.S.; Investigation: J.B.M.S.; Resources: I.M.S.; Data curation: J.B.M.S.; Writing - original draft: J.B.M.S., I.M.S.; Writing - review & editing: J.B.M.S., C.B., E.P.S., I.M.S.; Visualization: J.B.M.S.; Supervision: E.P.S., I.M.S.; Project administration: I.M.S.; Funding acquisition: C.B., I.M.S.

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Data availability
The metadata of the project are available from the corresponding author upon request.

ECR Spotlight
This article has an associated ECR Spotlight interview with Jennifer Steffen.

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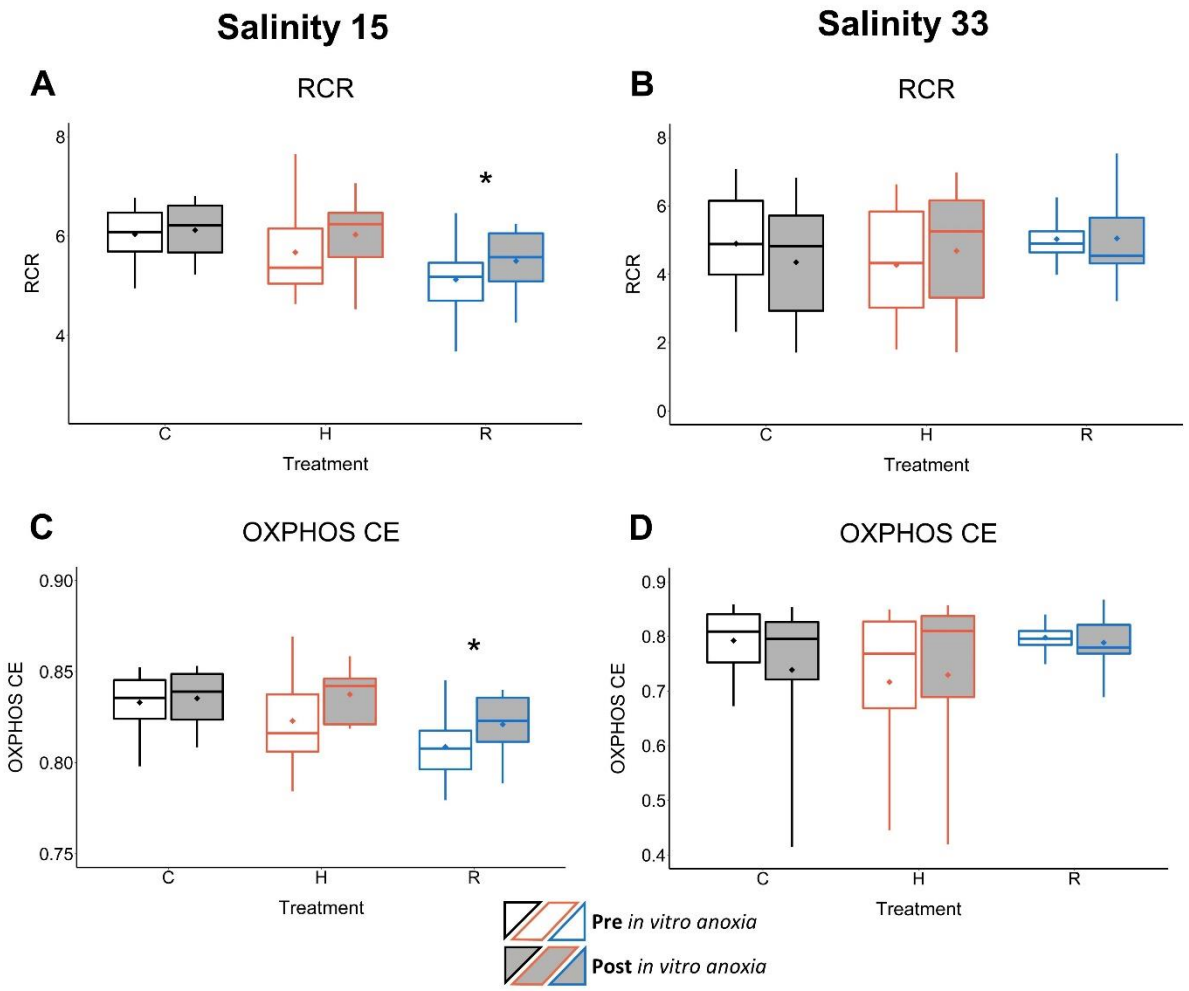

Fig. S1. Effect of *in vitro* H/R stress on coupling efficiency of *C. gigas* mitochondria from oysters exposed to combined *in vivo* salinity and H/R stress.

(A, B) Respiratory control ratio (RCR); (C,D) OXPHOS coupling efficiency (OXPHOS CE); (A,C) mitochondria from oysters acclimated to salinity 15; (B,D) mitochondria from oysters acclimated to salinity 33. Experimental groups: C, control (21% O2); H, short-term (24h) severe (<0.01% O2) hypoxia; R, short-term severe hypoxia (24 h at <0.01% O2) and subsequent 1.5 h reoxygenation (21% O2). Empty Box-Whisker plot – pre in vitro H/R stress; grey-filled Box-Whisker plot – post in vitro H/R stress. Means are shown as points within corresponding BOX-Whisker plots. Statistical differences (P <0.05) between pre and post in vitro H/R stress are represented by asterisk (*) above plots. Plots not marked with a star are not significantly different (P > 0.05). Due to the focus on the effect of in vitro H/R stress, statistical results are only shown for paired t-test. Data of empty Box-Whisker plots are based on data of Fig.2 curated for usage in paired t-tests as described in the Material and Method section. N of LS = 10, 10, 9-10, 9-10, 9-10 and 9-10 in C pre anoxia, C post anoxia, H pre anoxia, H post anoxia, R pre anoxia and R post anoxia, respectively. N of HS = 9-10, 9-10, 10, 10, 10 and 10 in C pre anoxia, C post anoxia, H pre anoxia, H post anoxia, R pre anoxia and R post anoxia, respectively.

2.3 Publication III: Molecular Biomarkers of the Mitochondrial Quality Control Are Differently Affected by Hypoxia-Reoxygenation Stress in Marine Bivalves *Crassostrea gigas* and *Mytilus edulis*

Published in Frontiers In Marine Sciences

Contribution letter

The contents of the third publication chapter from thisdissertation have been published in 2020 in the Journal Frontiers in Marine Science under the title "Molecular Biomarkers of the Mitochondrial Quality Control Are Differently Affected by Hypoxia-Reoxygenation Stress in Marine Bivalves *Crassostrea gigas* and *Mytilus edulis*".

I predominantly contributed to this publication and was involved in experimental design and planning, performance of incubation experiments, tissue collection, isolation of mRNA and assessment by quantitative Realtime PCR. Additionally, I have also performed the complete statistical analysis and prepared all graphical output. I wrote the first draft of the manuscript and contributed to the revisions of the draft in response to the comments of the peer reviewers.

Contribution of the candidate in % of workload:	
Experimental concept and design	85%
Experimental work and data acquisition	100%
Data analysis and interpretation	95%
Preparation of figures and tables	100%
Drafting the manuscript	85%

Signature of consent

Supervisor	Doctoral candidate
_____	_____
Prof. Dr. Inna Sokolova	Jennifer Barbara Maria Steffen

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Molecular Biomarkers of the Mitochondrial Quality Control Are Differently Affected by Hypoxia-Reoxygenation Stress in Marine Bivalves *Crassostrea gigas* and *Mytilus edulis*

*Jennifer B. M. Steffen[1], Halina I. Falfushynska[2], Helen Piontkivska[3] and Inna M. Sokolova[1,4]**

[1] Department of Marine Biology, Institute of Biological Sciences, University of Rostock, Rostock, Germany, [2] Department of Orthopedagogy and Physical Therapy, Ternopil V. Hnatiuk National Pedagogical University, Ternopil, Ukraine, [3] Department of Biological Sciences, Kent State University, Kent, OH, United States, [4] Department of Maritime Systems, Interdisciplinary Faculty, University of Rostock, Rostock, Germany

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Edited by:
Nina Bednarsek,
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Katherine Silliman,
Auburn University, United States

***Correspondence:**
Inna M. Sokolova
inna.sokolova@uni-rostock.de

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Coastal environments commonly experience strong oxygen fluctuations. Resulting hypoxia/reoxygenation stress can negatively affect mitochondrial functions, since oxygen deficiency impairs ATP generation, whereas a surge of oxygen causes mitochondrial damage by oxidative stress. Marine intertidal bivalves are adapted to fluctuating oxygen conditions, yet the underlying molecular mechanisms that sustain mitochondrial integrity and function during oxygen fluctuations are not yet well understood. We used targeted mRNA expression analysis to determine the potential involvement of the mitochondrial quality control mechanisms in responses to short-term hypoxia (24 h at <0.01% O_2) and subsequent reoxygenation (1.5 h at 21% O_2) in two hypoxia-tolerant marine bivalves, the Pacific oysters *Crassostrea gigas* and the blue mussels *Mytilus edulis*. We hypothesized that the genes involved in the mitochondrial quality control will be upregulated during hypoxia, and the less hypoxia-tolerant of the two studied species (*M. edulis*) will show a stronger dependence on transcriptional upregulation of these pathways than *C. gigas*. To test these hypotheses, mRNA expression of 17 (*C. gigas*) and 11 (*M. edulis*) marker genes involved in mitochondrial fusion, fission, proteolysis and mitophagy was analyzed in the digestive gland of *M. edulis* and *C. gigas* in normoxia and during hypoxia-reoxygenation (H/R) stress. In the mussels, the mRNA expression of the transcripts related to mitochondrial dynamics and quality control was strongly altered during H/R stress showing a shift toward fission, suppression of fusion, an increase in mitochondrial proteolysis and onset of mitophagy. These changes indicate that H/R stress induces mitochondrial injury in *M. edulis* requiring upregulation of the protective mechanisms to segregate the dysfunctional mitochondria by fission and degrade the oxidative damaged proteins

and/or organelles. Unlike mussels, the transcript levels of all studied genes in the oysters remained at the baseline (normoxic) levels during H/R stress. This muted transcriptional response of *C. gigas* is in agreement with earlier findings showing better ability to maintain cellular homeostasis and higher resistance to apoptosis during H/R stress in the oysters compared with the mussels. The revealed species-specific differences in the expression of the mitochondrial quality control pathways shed light on the potentially important mechanisms of mitochondrial protection against H/R-induced damage that might contribute to hypoxia tolerance in marine bivalves.

Keywords: mitochondrial fusion and fission, quantitative real time PCR (qPCR), mRNA expression, mollusca, proteolysis, mitophagy

INTRODUCTION

Deoxygenation driven by nutrient pollution and warming is a major stressor in estuarine and coastal oceans worldwide (Breitburg et al., 2018, 2019). The deficiency (hypoxia) or lack (anoxia) of oxygen occurs in the estuarine and coastal habitats where oxygen depletion due to respiration outstrips the oxygen influx from diffusion, water mixing and photosynthesis. As a result, these habitats can experience hypoxic periods from a few hours (such as in a diel cycling hypoxia) to several weeks or months in the case of seasonal hypoxia (Diaz and Rosenberg, 1995, 2008). Most benthic metazoans including important ecosystem engineers and keystone species of bivalves, crustaceans and fish require oxygen to survive and complete their life cycles, and oxygen deficiency can lead to a decrease in growth, performance and survival of marine benthos thereby threatening coastal marine ecosystems (Diaz and Rosenberg, 1995). Low motility of benthic marine invertebrates such as bivalves limits their ability to escape local hypoxia, making them reliant on physiological and biochemical adaptations to survive oxygen deficiency (Grieshaber et al., 1994). Metabolic regulation is at the crux of such adaptations due to the direct dependence of the mitochondria (generating >90% of the cellular ATP) on oxygen availability and the key role of these organelles in the energetic and redox balance of the cell. Investigation of mitochondrial responses to fluctuating oxygen conditions is thus important for understanding the fundamental physiological mechanisms setting limits of hypoxic tolerance in marine organisms.

In benthic invertebrates, severe hypoxia (<4% O_2) and anoxia results in transition to a metabolically suppressed state, where ATP production and consumption are downregulated and anaerobic pathways are engaged to compensate for insufficient aerobic ATP generation (Hochachka et al., 1996; Hochachka and Lutz, 2001; Storey, 2002). Under these conditions, the mitochondrial respiration strongly declines or ceases (Zwaan et al., 1991; van den Thillart et al., 1992). In hypoxia-sensitive organisms such as terrestrial mammals, mitochondria become depolarized and eventually damaged during hypoxia due to the ATP depletion, acidosis and Ca^{2+} overload (Piper et al., 2003; Chen et al., 2007; Solaini et al., 2010). Post-hypoxic reoxygenation restores the aerobic ATP synthesis in mammalian mitochondria but may come at a cost due to a surge of the mitochondrial production of reactive oxygen species (ROS) that can damage the cellular proteins, lipids and DNA (Cadenas and Davies, 2000; Paradis et al., 2016). Similar patterns of mitochondrial responses during hypoxia-reoxygenation (H/R) stress involving oxidative damage, collapse of the mitochondrial membrane potential and loss of the OXPHOS capacity has been reported in hypoxia-sensitive marine mollusks such as the bay scallops (Ivanina and Sokolova, 2016; Ivanina et al., 2016). In hypoxia-tolerant organisms such as freshwater turtles, fish and marine intertidal mollusks, mitochondrial respiration, OXPHOS capacity and membrane potential are preserved and the oxidative damage is mitigated during the H/R stress (Galli and Richards, 2014; Sokolova, 2018; Sokolova et al., 2019). These findings imply that maintenance of the mitochondrial integrity through mitochondrial damage sensing and quality control mechanisms might play an important role in mitochondrial tolerance to H/R stress (Berlett and Stadtman, 1997; Cabiscol et al., 2000; Zorov et al., 2014). However, the mitochondrial quality control mechanisms remain poorly understood in hypoxia-tolerant organisms including benthic marine bivalves and require further investigations.

The mitochondrial quality control is well studied in hypoxia-sensitive terrestrial mammals (such as rodents) and encompasses a complex protective network of proteins regulating mitochondrial dynamics (fission and fusion), as well as proteases and mitophagic pathways degrading oxidatively damaged proteins and defective mitochondria (Eisner et al., 2018; Li and Liu, 2018). Under normal conditions, mitochondria tend to form mitochondrial networks by mitochondrial fusion. Fusion of the outer mitochondrial membrane (OMM) is mediated by mitofusins 1 and 2 (Mfn1 and 2), while dynamin-related GTPase OPA1 induces fusion of the inner mitochondrial membrane (IMM) (Chen et al., 2003; Song et al., 2009). Under stress, membrane depolarization activates the metalloendopeptidase OMA1, which degrades OPA1 (Baker et al., 2014; Xiao et al., 2014) and induces mitochondrial fission through interactions of dynamin-related protein DRP1 and its adaptor proteins (the mitochondrial fission factor Mff and fission protein 1 Fis1) (Otera et al., 2010; Losón et al., 2013). Mitochondrial fission removes the damaged mitochondria and facilitates apoptosis under extreme cellular stress (Youle and van der Bliek, 2012), while the hypoxia upregulated protein (HYOU1) supports the mitochondrial repair by suppressing premature hypoxia-induced cell death (Ozawa et al., 1999).

Mechanisms involved in the degradation of the damaged proteins and organelles play an important role in the cellular responses to hypoxia in mammalian models. Protein quality surveillance in the mitochondria is supported by multiple ATP-dependent and –independent proteases including Lon protease and paraplegin (both selectively degrading oxidatively damaged proteins) (Atorino et al., 2003; Venkatesh et al., 2012; Kuo et al., 2015; Pinti et al., 2015; Shanmughapriya et al., 2015; Sepuri et al., 2017), the IMM protease ATP23, and the caseinolytic matrix peptidase chaperone (Osman et al., 2007; Doyle and Wickner, 2008). On DNA level, the helicase Twinkle is responsible for mtDNA maintenance and replication (Spelbrink et al., 2001). Cells can also degrade complete damaged mitochondria through mitophagy to prevent joining of dysfunctional mitochondria to the network. These pathways involve the PTEN-induced kinase 1 (PINK1) which accumulates under the hypoxia and recruits Parkin to tag damaged mitochondria for degradation (Eiyama and Okamoto, 2015). Mitophagic mediators in the PINK1-Parkin pathway are regulated via reversible phosphorylation by a serine/threonine-protein phosphatase PGAM5 (Chen et al., 2016). Additional to the PINK1-Parkin pathway, an alternative mitophagic pathway degrading unhealthy mitochondria is mediated by the mitochondrial eating protein (MIEAP) (Kitamura et al., 2011; Nakamura and Arakawa, 2017).

The important role of mitochondrial quality control mechanisms (involving the regulation of the mitochondrial fission and fusion, proteolysis and mitophagy) in the responses to H/R stress is well established in the hypoxia-sensitive mammalian models such as rodents (Kulek et al., 2020; Wang and Zhou, 2020). Earlier studies also indicate that high activity and upregulated mRNA expression of mitochondrial proteases correlates with elevated hypoxia tolerance in marine bivalves (Ivanina and Sokolova, 2016; Ivanina et al., 2016). However, involvement of the pathways regulating mitochondrial dynamics and quality control in the response to H/R stress have not been extensively studied in hypoxia-tolerant organisms including bivalves. Here, we explored the molecular mechanisms underlying the mitochondrial responses to H/R stress by measuring the mRNA expression of key marker genes in the mitochondrial quality control pathways in the Pacific oyster *Crassostrea gigas* and the blue mussel *Mytilus edulis*. The two studied species are commonly exposed to fluctuating oxygen conditions in intertidal, coastal and estuarine habitats and are therefore good models to study the mitochondrial adaptations to hypoxia and reoxygenation stress (Sokolova et al., 2019).

Earlier studies found that the Pacific oyster is more tolerant to abiotic stressors (including hypoxia) than the blue mussel (David et al., 2005; Le Moullac et al., 2007; Sokolov et al., 2019). At the cellular level, higher hypoxia tolerance of *C. gigas* was associated with the resistance to apoptosis and muted inflammation response indicating less pronounced mitochondrial and cellular damage during the H/R stress in oysters compared with the mussels (Falfushynska et al., 2020a,b). Therefore, we hypothesized that the less hypoxia-tolerant of the two studied species (*M. edulis*) will more strongly rely on the regulation of the mitochondrial quality control mechanisms to counteract the H/R-induced cellular damage compared with the more tolerant *C. gigas* that experiences less cellular injury (Falfushynska et al., 2020b) and thus might require less protection from the inducible mitochondrial quality control mechanisms. To test this hypothesis, mRNA expression of 17 *(C. gigas)* and 11 (*M. edulis*) key genes in the mitochondrial fusion, fission, proteolysis and mitophagy pathways were analyzed by quantitative RT-PCR in the digestive gland of the two studied species of bivalves exposed to hypoxia and reoxygenation relative to the normoxic controls. We focused on the transcript levels of the following marker genes: for mitochondrial fission and fusion - *mfn*2 (encoding mitofusin 2), *opa*1 (mitochondrial dynamin-like 120 kDa protein), *dnm1l* (dynamin-1-like protein), *mff* (mitochondrial fission factor), *fis*1 (mitochondrial fission protein 1); for protein and DNA quality control - *tsfm* (encoding mitochondrial translation elongation factor Ts), *lonp*1 (mitochondrial Lon protease), *spg*7 (paraplegin), *oma*1 (mitochondrial metalloendopeptidase OMA1), *clpB* (mitochondrial caseinolytic matrix peptidase chaperone subunit B), *atp*23 (mitochondrial inner membrane protease ATP23), *twnk* (mitochondrial twinkle mtDNA helicase); and for mitophagy - *mieap* (encoding mitochondrial eating protein), *hyou*1 (hypoxia upregulated protein 1), *prkn* (parkin), *pink*1 (PTEN-induced kinase 1), and *pgam*5 (mitochondrial serine/threonine protein phosphatase PGAM5).

MATERIALS AND METHODS

Animal Maintenance

Adult oysters [mean shell length ± the standard deviation (SD): 99.3 ± 13.1 mm] were obtained from the low intertidal zone of the German Wadden Sea near List/Sylt (55°01′42″N 8°26′04″E) and transported within 48 h of collection to the University of Rostock, Germany. Adult blue mussels (mean shell length ± SD: 52.5 ± 3.22 mm) were collected at Warnemünde marina "Mittelmole" (54°10′49.4″N 12°05′19.9″E) and brought to the University of Rostock within an hour of collection. All bivalves were transported in coolers lined with seawater-soaked paper towels, and the shells were cleaned from epibionts upon arrival. Two weeks prior to experiments, oysters and mussels were acclimated in recirculated temperature-controlled aquarium systems (Kunststoff-Spranger GmbH, Plauen, Germany) with aerated artificial seawater (ASW) (Tropic Marin®, Wartenberg, Germany) at salinity 33 ± 1 (*C. gigas*) and 15 ± 1 (*M. edulis*), respectively, and temperature 15 ± 0.5°C. These salinity and temperature were within natural range of the respective oysters' and mussels' habitat conditions. Bivalves were fed *ad libitum* by continuous addition of a commercial live algal blend (DTs Premium Blend Live Marine Phytoplankton, Coralsands, Mainz Kastel, Germany) according to the manufacturer's instructions (0.01 ml g^{-1} fresh mass per day) using an automatic aquarium feeder.

Experimental Exposures

Bivalves (three oysters or six mussels in 2 l of ASW) were exposed to 24 h of severe hypoxia (<0.1% O_2) by bubbling the ASW with pure nitrogen (Westfalen AG, Münster, Germany) in

air-tight chambers at 15 ± 0.5°C and respective salinity. Oxygen concentration was monitored with an Intellical™ LDO101 Laboratory Luminescent/Optical Dissolved Oxygen (DO) Sensor (HACH, Loveland, CO, United States). During exposure, bivalves were not fed to prevent bacterial growth in the chambers. It is worth noting that oysters and mussels spontaneously close their shells and cease feeding during hypoxic exposures regardless of the presence of algae in surrounding water. After hypoxia exposure, a subset of bivalves was allowed to recover in normoxic ASW (21% O_2) for 1.5 h. The H/R incubation times were chosen based on tidal and diurnal cycles of oxygen fluctuations and previous findings that show that strongest mitochondrial response to reoxygenation occurs within the first hours of recovery (Kurochkin et al., 2009; Richards, 2011; Ivanina and Sokolova, 2016; Andrienko et al., 2017). The control group was maintained in normoxia (21% O_2) in recirculated temperature-controlled aquarium systems (10 oysters or 30 mussels per 30 l of seawater) with continuous feeding as described in section "Animal Maintenance."

Hepatopancreas tissue was sampled on ice after hypoxia, reoxygenation and in normoxic control, immediately shock-frozen in liquid nitrogen and stored at −80°C until further analysis. We have chosen hepatopancreas because it is one of the largest metabolically active organs in bivalves involved in digestion, energy storage and maintenance of energy homeostasis (Gosling, 1992; Kennedy, 1996). Furthermore, earlier studies in bivalves showed strong hypoxia-induced shifts in the hepatopancreas metabolome (Haider et al., 2020) and induction of apoptotic and inflammatory responses in this tissue during H/R stress (Falfushynska et al., 2020a). This makes hepatopancreas a useful tissue to investigate the pathways that maintain the mitochondrial integrity during H/R exposure. Samples sizes were 10 and 6 per treatment group for oysters and mussels, respectively.

Quantitative Real-Time PCR (qRT-PCR)

RNA extraction and cDNA synthesis was conducted as described elsewhere (Falfushynska et al., 2020a). Briefly, 20 to 50 mg hepatopancreas tissue were extracted in RNA Extracol (EURX Ltd.–molecular biology products, Gdansk, Poland) according to manufacturer's instructions using an automatic homogenizer for 40 s at 6.0 m s^{-1} (FastPrep-24 MP Biomedicals, Valiant Co. Ltd., Yantai, Shandong, China). To avoid potential cross-reaction with residual DNA, RNA extracts were treated with DNase using the TURBO DNA-free kit (ThemoFisher Scientific, Waltham, MA, United States). RNA with a 280/260 absorbance ratio > 2.0 assessed by NanoVue Plus™ spectrophotometer (GE Healthcare, Chicago, IL, United States) was used for further processing. cDNA was synthesized from 2 μg of total RNA using RevertAid RT Kit (Thermo Fischer Scientific, Waltham, MA, United States). Expression levels were quantified by quantitative PCR (qPCR) using the StepOnePlus Realtime-PCR System instrument (Applied Biosystems, Thermo Fisher Scientific Corp., Foster City, CA, United States) and Biozym Blue S'Green qPCR BlueMix Separate ROX kit (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Each reaction comprised of 10 μl 2x qPCR master mix with ROX additive, 6.4 μl DNase/RNase-free water, 1.6 μl gene-specific reverse and forward primer mix (each to a final concentration of 0.4 μM) and 2 μl cDNA. For the genes where species-specific annotated transcript sequences were available, gene-specific primers were designed using published gene sequences of *M. edulis* and *C. gigas* in the NCBI/GenBank database. This was the case for all *C. gigas* target genes (except *lonp*1, *opa*1, *hyou*1 and *fis*1) and for the housekeeping genes from *M. edulis*. For those *Mytilus* sequences where no annotated transcripts were found in the NCBI/GenBank, we have used a transcriptome from a closely related species *Mytilus galloprovincialis* that belongs to the *Mytilus edulis* species complex (Varvio et al., 1988; Gaitán-Espitia et al., 2016). *M. edulis* and *M. galloprovincialis* are closely related, genetically similar and characterized by a high degree of hybridization and introgression in the nature (Gosling and Wilkins, 1981; Varvio et al., 1988; Gardner, 1996; Daguin et al., 2001; Gaitán-Espitia et al., 2016). To identify the candidate regions for primer design in *Mytilus*, we have aligned *M. galloprovincialis* sequences with available homologous sequences from multiple molluscan species, including *Crassostrea*. The regions with the high degree of nucleotide sequence conservation were chosen for primer design, and the qPCR was conducted under stringent conditions to avoid non-specific priming. The relevant fragments of sequence alignments between *Crassostrea* and *M. galloprovincialis* are provided in **Supplementary Table S1**. For *lonp1*, *opa*1, *hyou*1 and *fis*1 genes, for which no transcript from *C. gigas* were publicly available at the time of this study, primers were designed using a sequence from a closely related species, *Crassostrea virginica* (**Supplementary Table S2**). The identified sequence segments were then used to design gene-specific primers (**Table 1**). For primer check, the fragments were amplified using the following cycling parameters: 2 min at 95°C for polymerase activation, 40 cycles of 15 s at 95°C and 30 s at 60°C. Only the primers that produced a single product of expected length were used in the following analyses. All 17 target genes were successfully amplified from *C. gigas*. Six out of 17 target genes (including *tsfm*, *clpB, mieap*, *atp23, hyou1*, and *twnk*) could not be amplified from *Mytilus* due to the low quality or insufficient length of the respective transcript sequences available in the NCBI/GenBank.

To measure mRNA levels of the target and housekeeping genes, the following cycling parameters were used: 2 min at 95°C for polymerase activation, 40 cycles of 15 s at 95°C, 15 s at 57°C (annealing), 20 s at 72°C (extension) and 30 s at a primer specific reading temperature (for acquiring the product signal). For quality control, the melt curve analysis was conducted at the end of each run. To determine the apparent amplification efficiency of the primers and to correct for the possible plate-to-plate variation, a standard dilution series of a single pooled cDNA sample was run on each plate. All measurements were conducted in duplicates. Expression levels of the target samples relative to the reference genes were conducted using the relative standard curve method (Dorak, 2006). Reference genes were chosen as described elsewhere (Falfushynska et al., 2020a). In pilot experiments, we tested five potential housekeeping genes (encoding eukaryotic elongation factor 1, α-tubulin, β-actin,

TABLE 1 | Primers used for qRT-PCR for genes of mitochondrial quality control in *C. gigas* and *M. edulis*.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	NCBI accession No. of targeted sequence
Crassostrea			
mfn2	GCTGGCGGATCATTGCTGTC	ACGACCTCTCCTTGGCCTTG	XM_011450871.2
opa1	CCAGGAGTGCAGGAAAGGATTC	ACTTGTTGTCGGAGAGCGTTAC	XM_022486900.1
dnm1l	GCCCACAGTGTTAAAGGGGT	AGGCGCTCAATGACATCACA	XM_011429781.2
mff	CTTTCCTACCTGGCGACTCC	AGCTTGGCTACTGCGTTTCT	XM_011416039.2
fis1	GGTTGGCTCATCTGAAAGCTCAC	AGGTTGAGGAGGACTGGCTGAG	XM_022482332.1
tsfm	GTTGGGCCAAAGCAGACAAG	CTCCTGCTCTGGACTGCAAA	XM_011429054.2
lonp1	GACAGGAAGTGGCCATGACA	TCCTTGAGTCCACCAATGGC	XM_022478813.1
spg7	GCTGTGGTTTCGTGGTTGAC	GGAGATCCCTCTCCGGCTTA	XM_011448210.2
oma1	CCTCCAGAACTGGTCCTGAATA	GATCGATCGGAGAGCTGCAA	XM_011440234.2
clpB	AGCTGACGGAGTGCCCAAAC	ACCTCACTGGCAAGGTTAGAGG	XM_020071562.1
mieap	GGAACATGAGTGCTCTGGCT	TGGTTCACAAGTGGTGCTGT	LOC105320964
atp23	GCACGGAACATTACGGAGGA	CTGTGACTCGAATGGAGCGA	XM_011425806.2
hyou1	GGCCATGGTGCTGGAAAAAG	TGATCCCCACCATTTCTGCC	LOC111125576
twnk	ACAGAACATTGACCATGTGTAGAGA	GTGTATGTGCACGATTCAGGT	XM_011427422.2
prkn	CTCGGGGTTGGGAACCATAC	TGATCGTGAGTTGGTGGCTC	XM_011439893.2
pink1	ATCGTGCTGCTTTGGGATGTTTAC	GCTTCTTTCACCCTCCTGGCATAAG	XM_011426001.2
pgam5	AACAGGCGGACTACTGTGGAAAG	ACGGCTCTGGTCATTGTAGAAG	XM_020067497.1
*bAct**	TCCGGAATCCATGAAACATCA	TCCTTTTGCATACGGTCAGC	NM_001308859.1
Mytilus			
mfn2	TGACCAATTCTGTCTGGATGCT	ATCGGGAGCTCACTTTGTGG	SRR1598987
opa1	AGGAGACCAGAGTTCTGGGAA	CGCCTGATCCTCTGGGAAAT	SRR1598987
dnm1l	CACAGCAAGAGATGCTCCGA	ACCATTGTGTTAGTTGGAGGT	SRR1598987
mff	GCATCATACCTGAACATAACTCG	AAATCAGCATGATAAATTGTCCCA	SRR1598987
fis1	GAATTTGCAGGCGACATGGG	ATGATTTCCCCACGAAGTGTT	SRR1598987
lonp1	AGAGGTGTTGGAAGAAGACCA	AGCTGGCTTACTGCTATAAATTCAA	SRR1598987
spg7	ACAAGAAGAAGAACAACAGCATTCA	TTTCCCACATCCTGGTCCAC	SRR1598987
oma1	ACAACTGTTTGATGTGTTTGGT	GGAACCGTTCTGGAGGTAGC	SRR1598987
prkn	ACTTTTGTAACTATCTCGATTGCCA	TGCTTCTACGCTTCTTATATTGTTG	SRR1598987
pink1	GAAGAGCCATATTGTTTGGTAATGA	TGATAGCAAGGAGCATCTCG	SRR1598987
pgam5	CAACTTCGCACTCTCGCATC	CTATGGTGTTCCGATGGGGC	SRR1598987
*tsfm**	CAGATCCCAAGTTTGTCGAGT	TTCTGCCATCTTCTCACAGAAT	HQ690240.1

ubiquitin and 18S rRNA) as potential reference genes. Only one out of five tested genes (eukaryotic elongation factor 1 in mussels and actin in oysters) showed no significant change in expression ($p > 0.05$) with ≤1 difference in mean CT values between different experimental treatments. Therefore, target mRNA expression was normalized to the expression of a reference gene (β-actin β*Act* for *C. gigas* and the eukaryotic elongation factor 1 eEF1 *tsfm* for *M. edulis*) and the mean CT of the normoxic group as described elsewhere (Dorak, 2006)

Statistics

Data were tested for normal distribution in IBM® SPSS® Statistics v. 25 (IBM Corp., Armonk, NY, United States) using the Shapiro-Wilk test and for homogeneity of variances by the Brown-Forsythe test in SigmaPlot 13 (v. 13.0.0.83, Systat Software Inc., San Jose, CA, United States). Outliers were removed using box-whisker plots in IBM® SPSS® Statistics. Non-normally distributed data were transformed via Box-Cox transformation by Minitab 19 (Minitab LLC., State College, PA, United States). Significant differences between exposures were tested by a one-way ANOVA within each species using SigmaPlot 13. Oxygen regime was used as a fixed factor with three levels (normoxic control, 1 day hypoxia, reoxygenation). Significant differences between the pairs of means were determined using a Tukey's Honest Significant Differences (HSD) *post hoc* test. Pearson correlation analysis was carried out by RStudio's packages "mixOmics" and "factoextra" [v. 1.2.5033 and R v. 3.6.3 (R Core Team, 2020)] with cut off $R = 0.6$. To reduce the dimensionality of the data, the principal component analysis (PCA) was conducted on transformed data with outliers replaced by group means. PCA and graphs were designed in RStudio. All effects were considered significant at $p < 0.05$.

RESULTS

Mitochondrial Dynamics: Fusion and Fission

In *M. edulis*, severe hypoxia suppressed mRNA levels of *mfn2* encoding mitofusin-2, which were rapidly

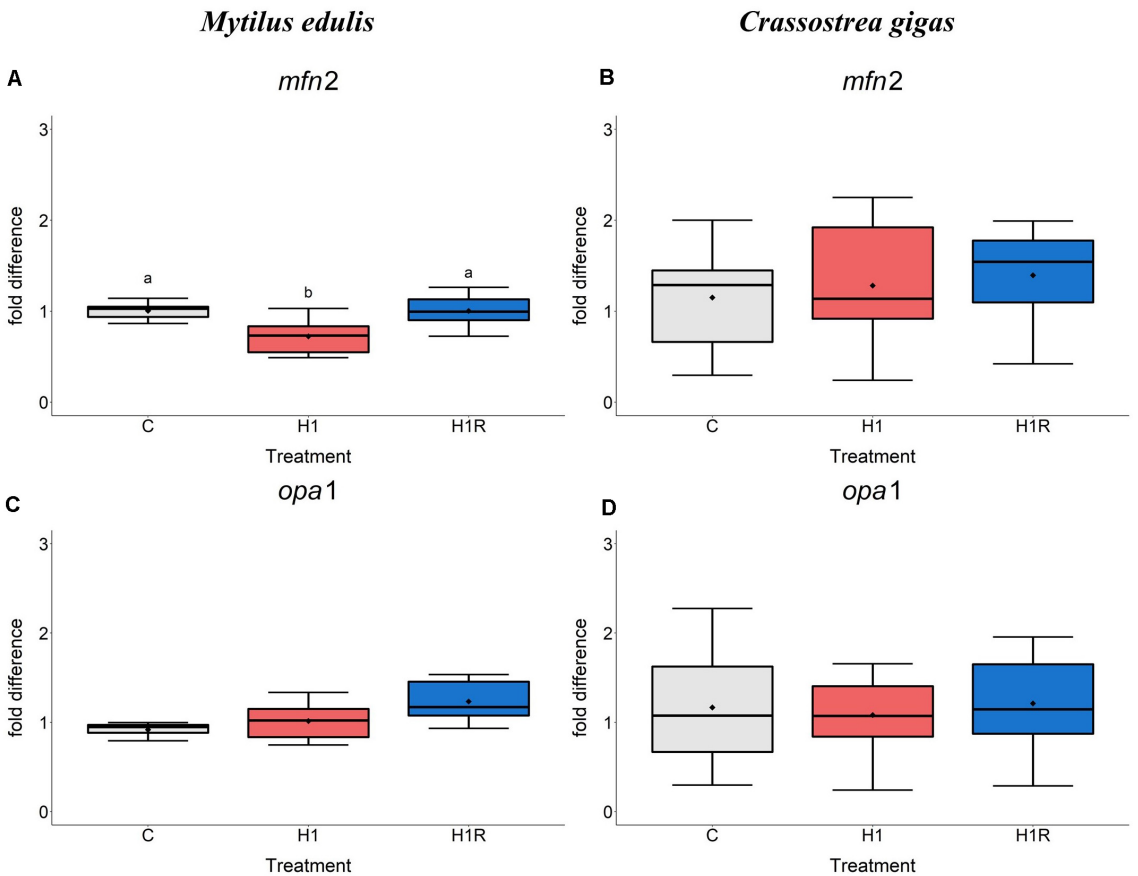

FIGURE 1 | mRNA expression of mitochondrial fusion proteins in *M. edulis* and *C. gigas* hepatopancreas in response to H/R stress. **(A,C)** *M. edulis*; **(B,D)** *C. gigas*; **(A,B)** *mfn*1 mRNA; **(C,D)** *opa*1 mRNA. Experimental groups: C, control; H1, short-term (1 day) severe hypoxia; H1R, short-term (1 day) severe hypoxia and subsequent 1.5 h reoxygenation. Means are depicted as points within corresponding Box-Whisker-plot. Outliers are shown as asterisk (*). Different letters above plots represent values that are statistical significantly different from each other ($p < 0.05$). Plots sharing a letter are not significantly different ($p > 0.05$). $N = 6–10$.

restored during reoxygenation (**Figure 1A**). Transcript levels of *opa*1 encoding dynamin-like 120 kDa protein did not change during H/R exposure in the mussels (**Figure 1C**). In *C. gigas*, tissue levels of *mfn*2 and *opa*1 mRNA did not change in response to H/R stress (**Figures 1B,D**).

Transcript levels of mitochondrial fission mediators *dnm*1*l*, *mff* and *fis*1 were significantly higher in the hepatopancreas of the mussels exposed to hypoxia than the control group (**Figures 2A,C,F**). Reoxygenation led to a further significant increase in the mRNA expression levels of these genes. Levels of *mff* mRNA were ∼4-fold and 6-fold elevated in hypoxia and reoxygenation, respectively, compared to control. Expression of *fis*1 mRNA was ∼2.5- to 3-fold increased in hypoxia and reoxygenation compared to normoxia. The *dnm*1*l* gene was ∼1.5- to 2-fold elevated by hypoxia and reoxygenation in the mussels compared to normoxic control. In *C. gigas*, transcript levels of the mitochondrial fission factors *dnm*1*l*, *mff*, *tsfm* and *fis*1 were not significantly affected by hypoxia and reoxygenation (**Figures 2B,D,E,G**).

Mitochondrial Protein and DNA Quality Control

Transcript levels of the genes encoding mitochondrial proteases involved in the protein quality control including *lonp*1 and *spg*7 were not affected by hypoxia and reoxygenation in *M. edulis* (**Figures 3A,C**). In contrast, transcript levels of metalloendopeptidase OMA1 (*oma*1) increased during hypoxia and reoxygenation by ∼1.5-fold and ∼1.7-fold, respectively, compared to the normoxic controls (**Figure 3E**).

In *C. gigas*, the mRNA level of the genes involved in mitochondrial protein quality control including *lonp*1, *spg*7, *oma*1 (**Figures 3B,D,F**) as well as four additional genes (not measured in *M. edulis* due to the lack of specific primers) including *clp*B, *mieap*, *atp*23 and *hyou*1 did not change in response to H/R stress (**Figures 4A–D**). Transcript levels of the mitochondrial helicase TWINKLE (*twnk*) responsible for mtDNA maintenance, remained unchanged in *C. gigas* throughout experimental exposures (**Figure 4E**) (no data are available for *M. edulis* due to the lack of specific primers).

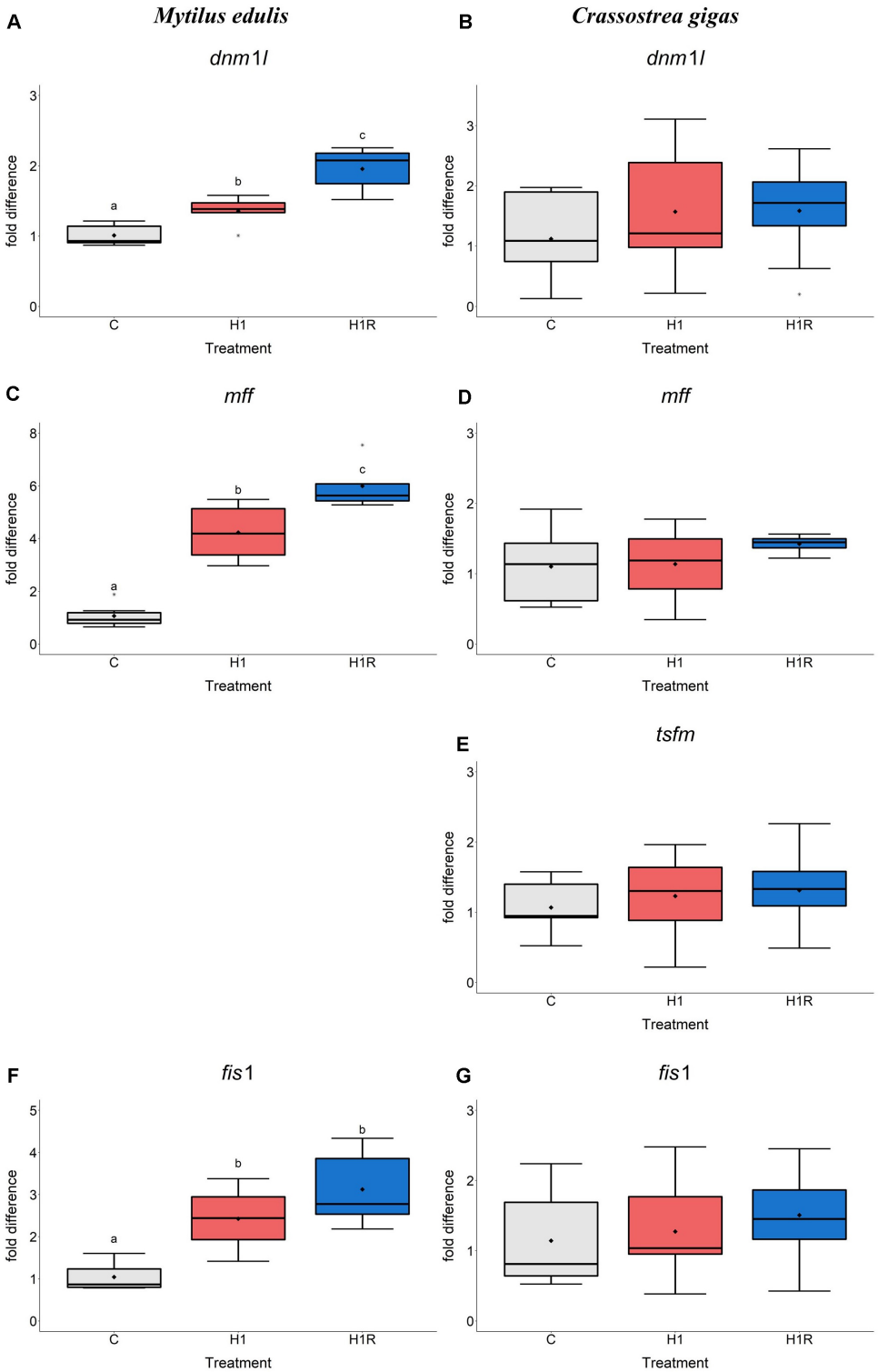

FIGURE 2 | mRNA expression of mitochondrial fission proteins in *M. edulis* and *C. gigas* hepatopancreas in response to H/R stress. **(A,C,F)** *M. edulis*; **(B,D,E,G)** *C. gigas*; **(A,B)** *dnm1l* mRNA; **(C,D)** *mff* mRNA; **(E)** *tsfm* mRNA; **(F,G)** *fis1* mRNA. Experimental groups: C, control; H1, short-term (1 day) severe hypoxia; H1R, short-term (1 day) severe hypoxia and subsequent 1.5 h reoxygenation. Means are depicted as points within corresponding Box-Whisker-plot. Outliers are shown as asterisk (*). Different letters above plots represent values that are statistical significantly different from each other ($p < 0.05$). Plots sharing a letter are not significantly different ($p > 0.05$). $N = 6–10$.

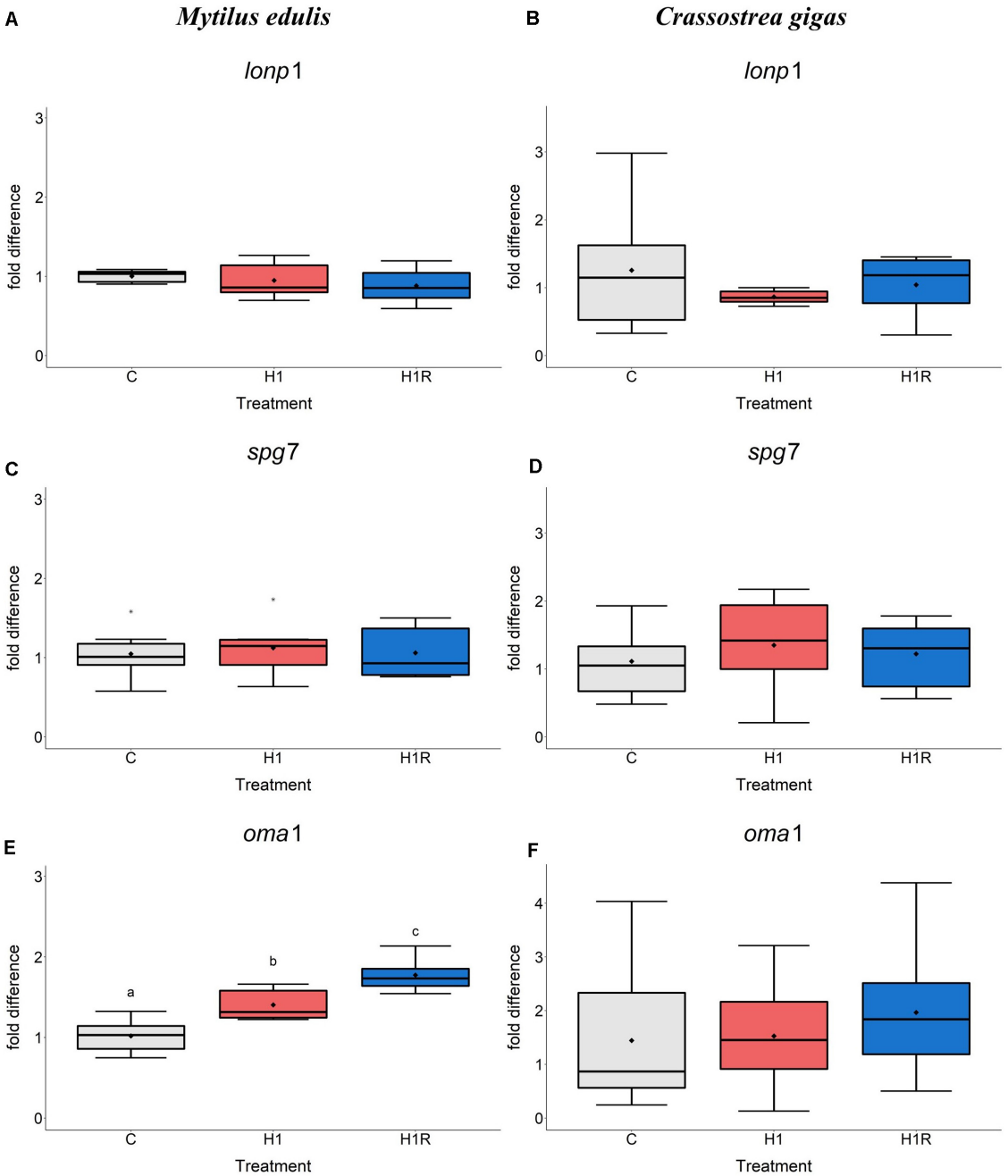

FIGURE 3 | mRNA expression of mitochondrial protein quality control and proteolysis proteins in *M. edulis* and *C. gigas* hepatopancreas in response to H/R stress. **(A,C,E)** *M. edulis*; **(B,D,F)** *C. gigas*; **(A,B)** *lonp*1 mRNA; **(C,D)** *spg*7 mRNA, **(E,F)** *oma*1 mRNA. Experimental groups: C, control; H1, short-term (1 day) severe hypoxia; H1R, short-term (1 day) severe hypoxia and subsequent 1.5 h reoxygenation. Means are depicted as points within corresponding Box-Whisker-plot. Outliers are shown as asterisk (*). Different letters above plots represent values that are statistical significantly different from each other ($p < 0.05$). Plots sharing a letter are not significantly different ($p > 0.05$). $N = 6–10$.

Mitophagy

In *M. edulis*, hypoxia induced a ∼2-fold higher expression of *prkn* mRNA (encoding a mitophagy mediator Parkin) compared to normoxic condition (**Figure 5A**). The levels of *prkn* remained elevated during reoxygenation in the mussels (**Figure 5A**). Expression of its co-mediator *pink*1 did not change with hypoxia, while reoxygenation led to a ∼2.5-fold increase of *pink*1 expression compared to normoxic controls (**Figure 5C**). Transcript levels of the protein phosphatase PGAM5 showed a decreasing trend with hypoxia, followed by a significant increase during reoxygenation (**Figure 5E**). In *C. gigas*, the expression

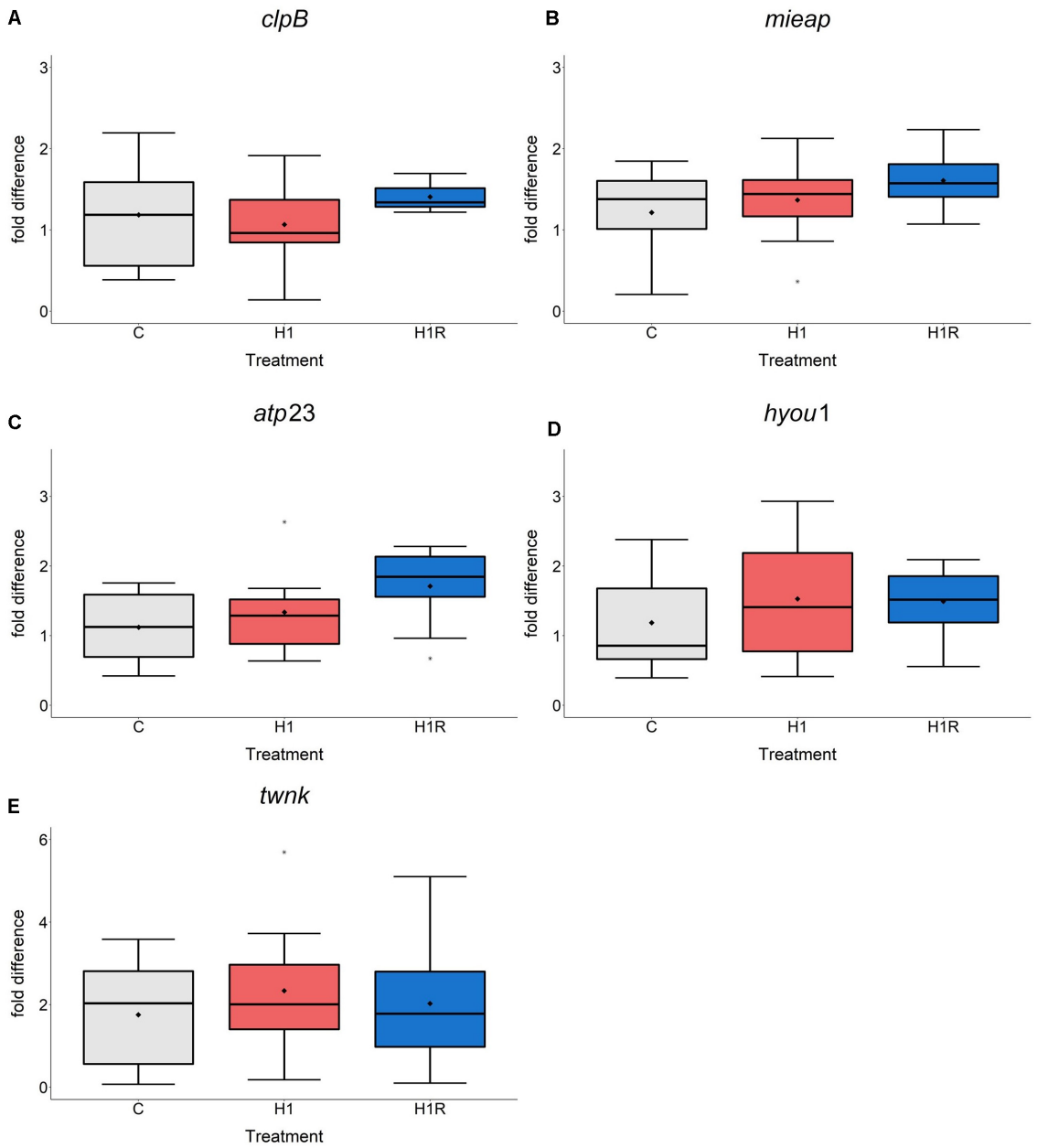

FIGURE 4 | mRNA expression of mitochondrial quality control proteolysis and mtDNA maintenance in *C. gigas* hepatopancreas in response to H/R stress. **(A–D)** *C. gigas*; **(A)** *clpB* mRNA; **(B)** *mieap* mRNA; **(C)** *atp*23 mRNA; **(D)** *hyou*1 mRNA; **(E)** *twnk* mRNA. Experimental groups: C, control; H1, short-term (1 day) severe hypoxia; H1R, short-term (1 day) severe hypoxia and subsequent 1.5 h reoxygenation. Means are depicted as points within corresponding Box-Whisker-plot. Outliers are shown as asterisk (*). Different letters above plots represent values that are statistical significantly different from each other ($p < 0.05$). Plots sharing a letter are not significantly different ($p > 0.05$). $N = 6–10$.

pattern of the studied mitophagy genes did not show pronounced effects of hypoxia and subsequent reoxygenation (**Figures 5B,D,F**).

Data Integration

The principal component analysis in *M. edulis* hepatopancreas revealed two principal components explaining 47.2% (PC1) and 19.1% (PC2) of the data variation (**Figure 6C** and **Supplementary Table S3**). The 1st component showed high positive loadings for genes of mitochondrial fission (*dnm1l*, *mff*, *fis*1) and fusion (*opa1*) and mitophagy (*prkn*, *pink*1) (**Figure 6D** and **Supplementary Table S4**). The 2nd principal component had high positive loadings of genes of mitochondrial quality control (*spg*7, *lonp*1) and a strong negative loading of *mfn*2, mediator of mitochondrial fusion.

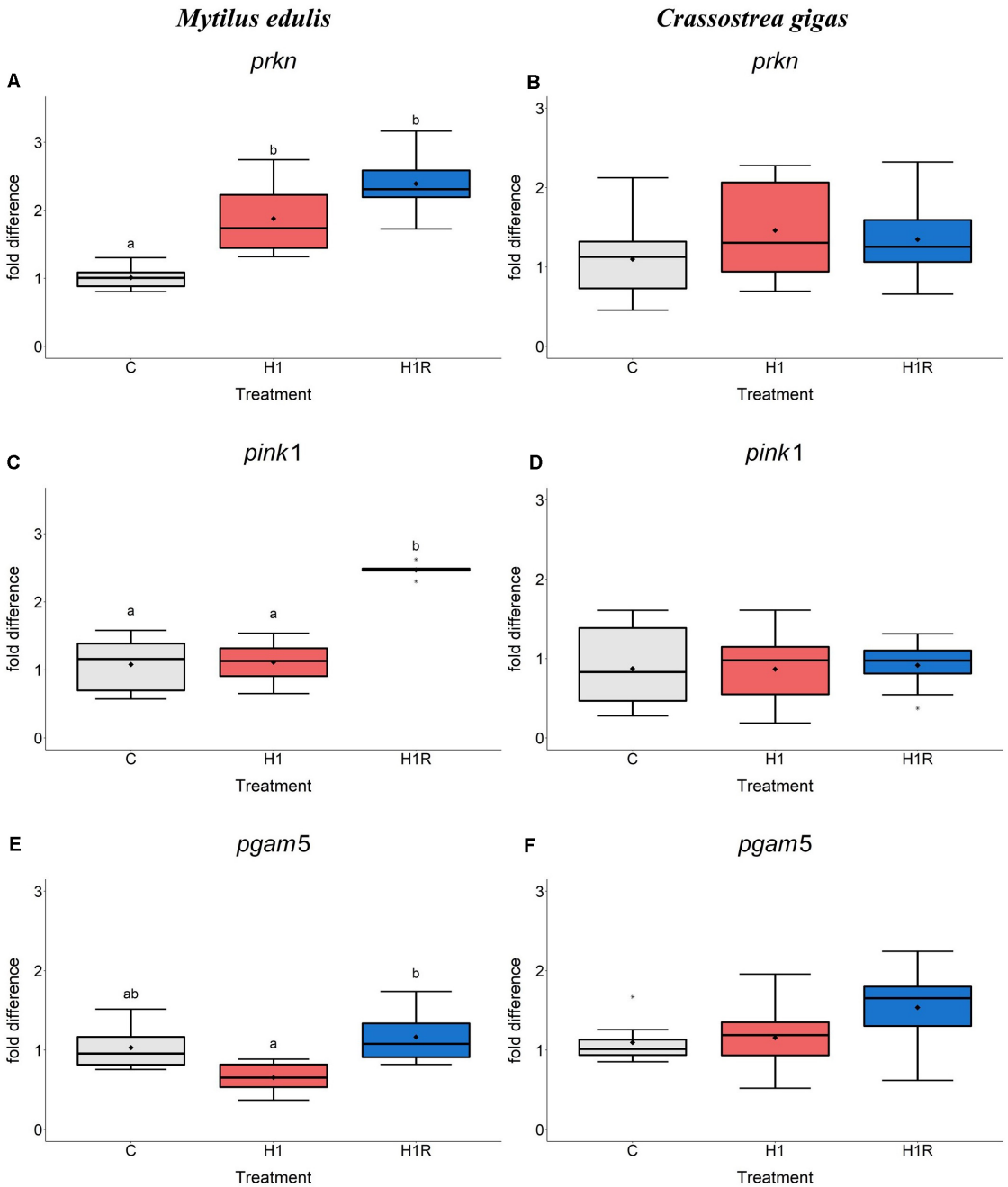

FIGURE 5 | mRNA expression of mitophagy proteins in *M. edulis* and *C. gigas* hepatopancreas in response to H/R stress. **(A,C,E)** *M. edulis*; **(B,D,F)** *C. gigas*; **(A,B)** *pink*1 mRNA; **(C,D)** *prkn* mRNA, **(E,F)** *pgam*5 mRNA. Experimental groups: C, control; H1, short-term (1 day) severe hypoxia; H1R, short-term (1 day) severe hypoxia and subsequent 1.5 h reoxygenation. Means are depicted as points within corresponding Box-Whisker-plot. Outliers are shown as asterisk (*). Different letters above plots represent values that are statistical significantly different from each other ($p < 0.05$). Plots sharing a letter are not significantly different ($p > 0.05$). $N = 6–10$.

In *M. edulis,* control and reoxygenation group were clearly separated along the 1st principal component (**Figure 6C**). Hypoxia and to greater degree reoxygenation induced a shift toward more positive values of the 1st principal component compared to the normoxic control reflecting upregulation of fission genes *mff, fis*1 and *dnm1l* and a gene encoding a mitochondrial protease *oma*1. All experimental treatment groups showed a high variance along the 2nd principal component axis.

Pearson correlation on mRNA expression of *M. edulis* showed a tight connection between the fission mediators *fis*1, *mff* and *dnm1l*, fusion mediator *opa1,* mitophagic inducing genes

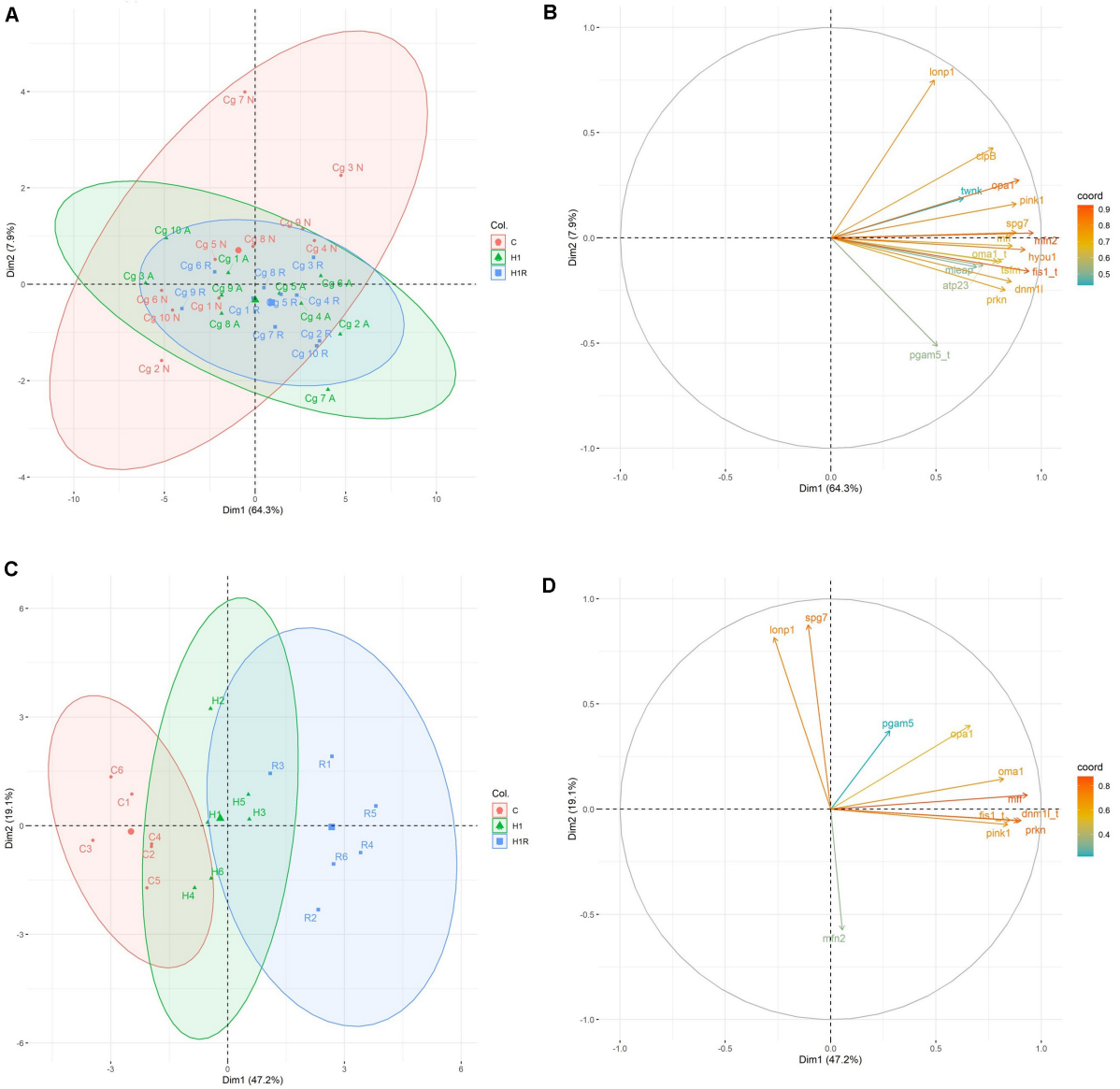

FIGURE 6 | PCA score plots and variable loading plots based on the studied biomarkers from *M. edulis* and *C. gigas* during H/R stress. **(A,B)** *C. gigas*; **(C,D)** *M. edulis*; **(A,C)** PCA score plot, position of the individual measurements from different experimental group within a coordinate system of first and second principal component. Horizontal axis depicts PC1 and vertical axis PC2. Colored ellipse includes all samples within 95% confidence interval. **(B,D)** Variable loading plot, visualization of weight distribution of different biomarkers on first and second principal component based on correlations between variables and dimensions. Close vectors define positive correlation between corresponding biomarkers. Coloring of vectors visualize coordinates. Horizontal axis depicts PC1 and vertical axis PC2. Gene name with appended "t" depicts transformed data to ensure normal distribution. Experimental groups: C, control, red; H1, short-term (1 day) severe hypoxia, green; H1R, short-term (1 day) severe hypoxia and subsequent 1.5 h reoxygenation, blue. Gene abbreviations: *mfn*2, mitofusin 2; *opa*1, mitochondrial dynamin-like 120 kDa protein; *dnm*1*l*, dynamin-1-like protein; *mff*, mitochondrial fission factor; *fis*1, mitochondrial fission protein; *tsfm*, mitochondrial translation elongation factor Ts; *lonp*1, mitochondrial Lon protease; *spg*7, paraplegin; *oma*1, mitochondrial metalloendopeptidase OMA1; *clpB*, mitochondrial caseinolytic matrix peptidase chaperone subunit B protein homolog; *mieap*, mitochondrial eating protein; *atp*23, mitochondrial inner membrane protease ATP23; *hyou*1, hypoxia upregulated protein 1; *twnk*, mitochondrial twinkle mtDNA helicase; *prkn*, E3 ubiquitin-protein ligase parkin; *pink*1, PTEN-induced kinase 1; *pgam*5, mitochondrial serine/threonine protein phosphatase PGAM5; β*Act*, beta Actin

prkn and *pink*1 and mitochondrial dynamics regulator *oma*1 (**Figures 7C,D**).

In *C. gigas*, the 1st and 2nd principal component explained 64.3% and 7.9% of variance, respectively (**Figure 6A** and **Supplementary Table S5**). All genes analyzed in this study had high positive loadings on the 1st principal component (>0.05) (**Figure 6B** and **Supplementary Table S6**).

Gene transcription of *C. gigas* from all three experimental treatments showed considerable overlap in the plane of the

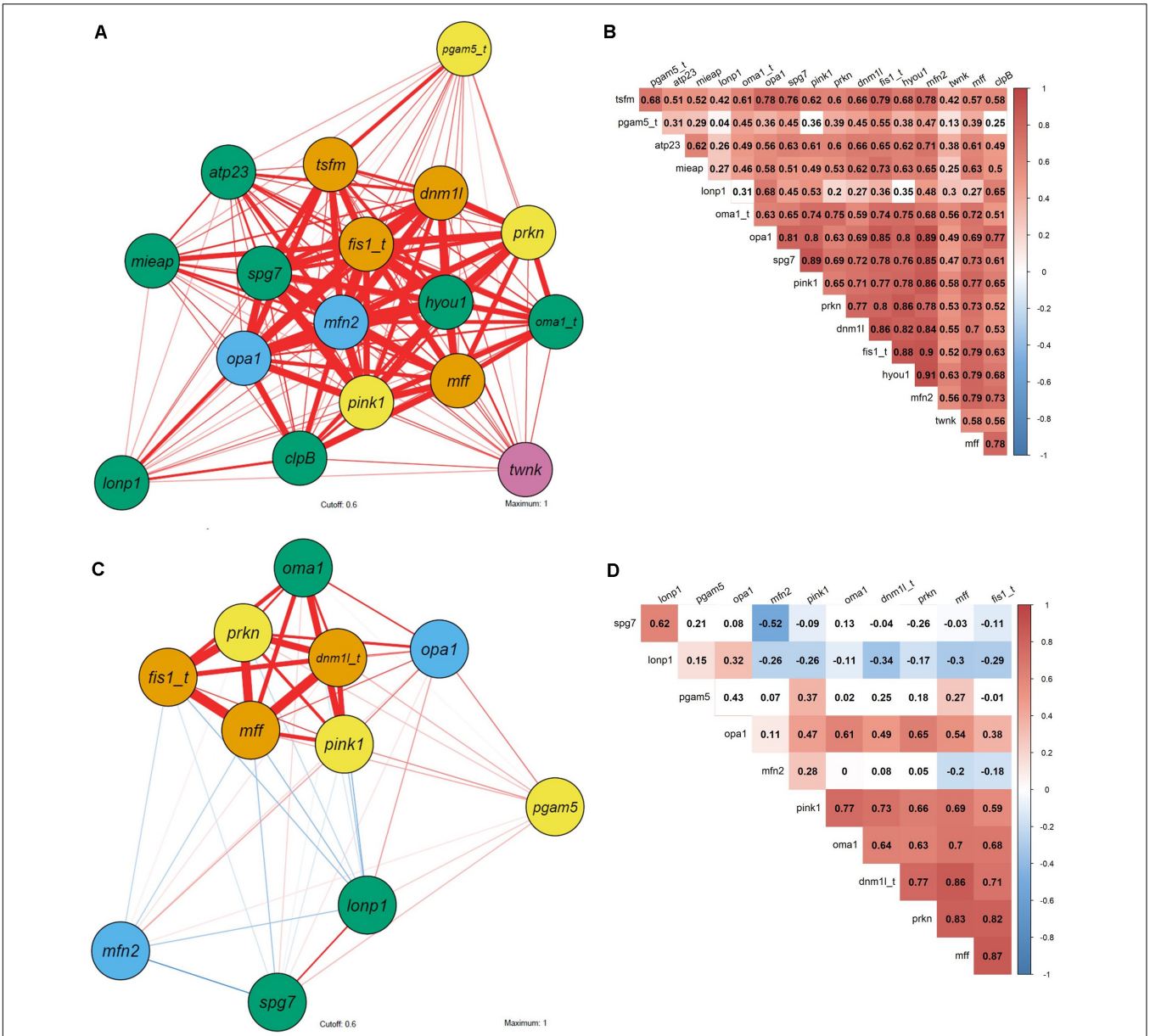

FIGURE 7 | Correlation network and corresponding heatmap of biomarker expression in *C. gigas* and *M. edulis* in response to H/R stress. **(A,B)** *C. gigas*; **(C,D)** *M. edulis*; **(A,C)** correlation network; **(B,D)** heatmap. Functional gene groups: orange, mitochondrial fission; light blue, mitochondrial fusion; green, mitochondrial quality control and proteolysis; yellow, mitophagy; pink, mtDNA maintenance. Pearson correlation coefficient: red, positive correlation; blue, negative correlation. Correlation networks are based on Pearson's correlation with a cutoff of 0.6 and maximum of 1. Non-colored fields of heatmaps and thin shaped correlation network represent non-statistical significant correlations. Thickness of lines represent statistically significant correlations ($p < 0.05$). Underlying Pearson correlation coefficient are depicted in associated heatmap. Gene name with appended "t" depicts transformed data to ensure normal distribution.

two first principal components consistent with the lack of change in the mRNA expression profiles of the studied genes in response to the H/R stress. In *C. gigas*, no apparent correlated gene clusters were found with the tight correlation across all studied genes (**Figures 7A,B**). These data indicate that the observed variability and correlations in the mRNA expression profiles of *C. gigas* mostly reflects individual differences between bivalves rather than the effects of the oxygen regime.

DISCUSSION

Short-term severe hypoxia and subsequent reoxygenation had different effects on mRNA expression of mitochondrial quality control in *C. gigas* and *M. edulis* (**Table 2**). In *C. gigas*, transcript levels were not affected by H/R stress indicating that the mitochondrial injury thresholds needed to induce the protective quality control mechanisms might not have been reached in this hypoxia-tolerant species. In *M. edulis,* mRNA expression of the

TABLE 2 | Summary of the observed changes in mRNA expression patterns of the mitochondrial quality control genes in *Crassostrea gigas* and *Mytilus edulis*.

Gene	*Crassostrea gigas*			*Mytilus edulis*		
	C	H1	H1R	C	H1	H1R
mfn2		↔	↔		↓	↔
opa1		↔	↔		↔	↔
dnm1l		↔	↔		↑	↑
mff		↔	↔		↑	↑
fis1		↔	↔		↑	↑
lonp1		↔	↔		↔	↔
spg7		↔	↔		↔	↔
oma1		↔	↔		↑	↑
clpB		↔	↔	/	/	/
mieap		↔	↔	/	/	/
atp23		↔	↔	/	/	/
hyou1		↔	↔	/	/	/
twnk		↔	↔	/	/	/
prkn		↔	↔		↑	↑
pink1		↔	↔		↔	↑
pgam5		↔	↔		↔	↑

Red fields with upward arrows show upregulated mRNA expression, while blue fields with downward arrows show downregulated mRNA expression (compared to the normoxic baseline). Horizontal arrows – no change. Gray diagonally crossed fields – no data available. Exposure groups: C, normoxia (21% O_2); H1, 24 h hypoxia (<0.01% O_2); and H1R, 24 h hypoxia with subsequent 1.5 h reoxygenation (<0.01% to 21% O_2). Gene abbreviations: mfn2, mitofusin 2; opa1, mitochondrial dynamin-like 120 kDa protein; dnm1l, dynamin-1-like protein; mff, mitochondrial fission factor; fis1, mitochondrial fission protein; tsfm, mitochondrial translation elongation factor Ts; lonp1, mitochondrial Lon protease; spg7, paraplegin; oma1, mitochondrial metalloendopeptidase OMA1; clpB, mitochondrial caseinolytic matrix peptidase chaperone subunit B protein homolog; mieap, mitochondrial eating protein; atp23, mitochondrial inner membrane protease ATP23; hyou1, hypoxia upregulated protein 1; twnk, mitochondrial twinkle mtDNA helicase; prkn, E3 ubiquitin-protein ligase parkin; pink1, PTEN-induced kinase 1; pgam5, mitochondrial serine/threonine protein phosphatase PGAM5.

transcripts related to mitochondrial dynamics and quality control was strongly regulated during H/R stress showing a shift toward the predominance of mitochondrial fission over fusion and an onset of mitophagy.

Effects of H/R Stress on Transcriptional Regulation of Mitochondrial Quality Control in the Mussels

Mitochondrial dynamics (in particular fission and fusion) plays a critical role in maintaining functional mitochondria during stress exposures in animals (Youle and van der Bliek, 2012). In mammalian models, ischemia-reperfusion (I/R) stress regulates transcription and post-translational modifications of fusion and fission proteins reflecting the important role of the mitochondrial dynamics in adjustments to interrupted oxygen supply (Li and Liu, 2018). Cellular stress commonly shifts mitochondrial dynamics toward mitochondrial fission to allow degradation of damaged mitochondria and avoid re-joining of non-functional mitochondria into the mitochondrial network (Chen et al., 2016; Eisner et al., 2018; Sokolova et al., 2019). The GTPase Drp1/Dnm1l mediates mitochondrial fission in response to cellular stress signals such as increase of Ca^{2+} concentration, ROS production or depolarized mitochondrial membrane as shown during H/R (I/R) stress in mammalian models (Yu et al., 2016; Tian et al., 2017; Yang et al., 2017; Li and Liu, 2018). Drp1/Dnm1l binds to the OMM with the help of the adaptor proteins Mff and Fis1 and initializes division of the membrane (Mears et al., 2011; van der Bliek et al., 2013; Osellame et al., 2016). Drp1/Dnm1l is a transcriptional target of p53 and, in rats, it is regulated at the transcriptional level (Li et al., 2010). The finding of transcriptional upregulation of *dnm1l* and its two adapter proteins *mff* and *fis*1 during H/R stress in *M. edulis* is consistent with upregulation of the fission pathways possibly as a mechanism to protect mitochondrial integrity by segregation of dysfunctional mitochondria (Twig et al., 2008; Anzell et al., 2018). mRNA expression of the fission-related proteins remained elevated during reoxygenation indicating that 1.5 h reoxygenation might not be sufficient for the full recovery of the mitochondrial fusion-fission balance in *M. edulis*.

Fusion is an effective strategy to protect against the stress-induced DNA damage, as the damaged mitochondria fuse with the healthy ones and replace the damaged mitochondrial DNA through complementation (Youle and van der Bliek, 2012). In our present study, mRNA levels of a key fusion mediator *mfn*2 (encoding the mitochondrial Mfn 2 protein) were suppressed during hypoxia in *M. edulis* indicating downregulation of the mitochondrial fusion pathways. This lack of induction of *mfn2* might indicate that mitochondrial DNA damage is not critically involved in the H/R stress responses of the blue mussels. An earlier study in the eastern oysters *C. virginica* showed an increase in the levels of DNA damage during exposure to 6 days of hypoxia and subsequent reoxygenation in the oysters exposed to a toxic metal cadmium, but not in their counterparts exposed to H/R stress only (Kurochkin et al., 2009). In the mussels *M. edulis,* hypoxia caused by emersion (72 h) had no effect on the DNA damage assessed by the Comet assay (Singh and Hartl, 2012). In the brown mussels *Perna perna,* 24 h of emersion-induced hypoxia led to the elevated oxidative DNA damage in the gills but not in the digestive gland (Almeida et al., 2005). In these studies, the oxidative lesions were assessed in the total rather than the mitochondrial DNA. To the best of our knowledge, the effects of H/R stress on the mitochondrial DNA damage have not been studied in marine bivalves. It is worth noting that mitofusin 2 requires energy in the form of GTP to mediate the mitochondrial fusion (Filadi et al., 2018), so that the downregulation of *mfn*2 in hypoxia-exposed mussels might be a consequence of the metabolic rate depression, a common energy-saving strategy to survive the oxygen deficiency in hypoxia-tolerant mollusks (Hochachka, 1993; Storey, 1998; Storey and Storey, 2004). During reoxygenation mRNA expression of *mfn*2 returned to the normoxic (baseline) levels in the mussels, which indicates a rapid re-balancing of mitochondrial fusion during post-hypoxic recovery.

The transcript levels of *opa*1 (encoding the mitochondrial dynamin-like GTPase OPA1) were not affected by H/R stress in *M. edulis*. The mitochondrial OPA1 is involved in the regulation of the mitochondrial fusion and the cross-talk between fusion and fission (Cipolat et al., 2004). In mammals, OPA1 activity is

regulated transcriptionally (Ryu et al., 2015; Zheng et al., 2019) as well as post-translationally through proteolytic cleavage by the mitochondrial metalloprotease OMA1 (Chen et al., 2016). Mitochondrial stress such as a decrease in the mitochondrial membrane potential or ATP deficiency stimulates the proteolytic processing of OPA1 generating short isoforms unable to support mitochondrial fusion (Anand et al., 2014; Chen et al., 2016; Ali and McStay, 2018). The posttranslational regulation of OPA1 allows for rapid suppression of mitochondrial fusion and mitochondrial fragmentation but might not be reflected in the changes in mRNA expression (Ali and McStay, 2018). Nevertheless, *opa1* was observed to be transcriptionally regulated during formation of immune cells in mice (Ryu et al., 2015), while disorders related to impaired mitochondrial functions were associated with transcriptional regulation of *opa*1 (Zheng et al., 2019). The observed stability of *opa1* mRNA in the mussels might indicate the lack of involvement of OPA1 in the mitochondrial response to the H/R stress in *M. edulis*. Alternatively, if post-translational regulation plays a predominant role in the OPA1 activation in the mussels, activation of OPA1 might not be reflected at the transcript level. Further studies are needed to distinguish between these alternative explanations.

In *M. edulis*, H/R stress led to a strong upregulation of *oma*1 mRNA encoding the mitochondrial metalloprotease OMA1. As discussed above, OMA1 mediates the proteolytic cleavage of fusion mediator OPA1 during stress thereby suppressing mitochondrial fusion (Anand et al., 2014). OMA1-dependent degradation of OPA1 is thus essential for the selective removal of defective mitochondrial fragments and prevention of incorporation of those into the mitochondrial network (Head et al., 2009; Baker et al., 2014). Consequently, elevated *oma1* expression during hypoxia and subsequent reoxygenation in the mussels might reflect the overall shift in the mitochondrial fusion-fission dynamics toward the predominance of fission (Ali and McStay, 2018) and is consistent with the transcriptional upregulation of the fission regulating proteins *dnm*1*l, mff* and *fis*1 and suppressed expression of a fusion regulator *mfn*2 in *M. edulis* during the H/R stress. Furthermore, OMA1 mediates an ATP-independent proteolytic breakdown of misfolded inner membrane proteins and is complementary to the action of m-AAA proteases (Käser et al., 2003). OMA1 activity is strongly upregulated in response to various stresses in metazoans, plants and yeast (Bohovych et al., 2014; Rainbolt et al., 2015; Migdal et al., 2017) and is required for stabilization of the mitochondrial respiratory supercomplexes and maintenance of the mitochondrial respiration (Bohovych et al., 2015; Richter et al., 2015). Therefore, transcriptional upregulation of *oma*1 during H/R stress in the mussels might assist in the degradation of the damaged mitochondrial proteins and protection of the mitochondrial integrity and function.

Our study shows that unlike *oma*1, two other studied mitochondrial proteases (including the mitochondrial Lon protease and paraplegin, encoded by *lonp*1 and *spg*7, respectively) are not transcriptionally upregulated during H/R stress in *M. edulis*. Lon protease is highly conserved throughout organisms and essential for mitochondrial proteostasis (Pinti et al., 2015). It is activated by ROS, protein carbonylation and lipid peroxidation (Kuo et al., 2015) and specifically targets the oxidatively damaged proteins for ATP dependent degradation (Bota and Davies, 2002; Bayot et al., 2010). Lon protease activity is regulated transcriptionally and post-translationally suggesting a fine tuning by several mechanisms, although stronger regulatory patterns are commonly seen at the protein level (Pinti et al., 2015). Earlier studies in bivalves showed that Lon protease mRNA expression was upregulated during H/R stress in the hypoxia-sensitive scallops *Argopecten irradians* but not in the hypoxia-tolerant hard shell clams *Mercenaria mercenaria* (Ivanina et al., 2016). Upregulation of *lonp1* transcripts in the scallops was associated with accumulation of oxidative lesions (i.e., lipid and protein peroxidation products) in the mitochondria, whereas in the hard shell clams the mitochondrial oxidative stress markers remained at the baseline (normoxic) levels (Ivanina et al., 2016). Upregulation of *lonp1* mRNA expression was also found in the Eastern oyster *C. virginica* during simultaneous exposure to high temperature and a toxic metal cadmium (Sanni et al., 2008), a combination of stressors known to cause strong oxidative stress in oysters (Bagwe et al., 2015). Taken together, these findings indicate that transcriptional upregulation of *lonp1* in bivalves might occur when the oxidative stress reaches a species-specific threshold in the mitochondria, and the lack of transcriptional upregulation of *lonp*1 mRNA levels during H/R stress in *M. edulis* might indicate that this threshold has not been reached under the conditions of our present study. Similar to *lonp1*, transcript levels of *spg*7 gene encoding a mitochondrial metalloprotease paraplegin did not change during H/R stress in the mussels. To the best of our knowledge, expression of *spg*7 has not been studied in other marine bivalves. In model organisms including mammals and yeast, paraplegin is regulated by mitochondrial stress signals such as Ca^{2+} accumulation and elevated ROS levels (Ishihara et al., 2006; Shanmughapriya et al., 2015). If similar ROS-dependent mechanisms exist in bivalves, the lack of *spg*7 (as well as *lonp1*) transcriptional responses to H/R stress might be explained by the relatively low levels of oxidative stress insufficient to trigger transcriptional upregulation of these proteases. Further studies of the ROS production, oxidative injury and expression of mitochondrial proteases in bivalve mitochondria during exposures to different stressors are required to test this hypothesis and shed light on the thresholds of mitochondrial injury required for induction of these protective mechanisms.

Mitophagy is a selective form of autophagy to degrade damaged mitochondria (Pickles et al., 2018). Based on the studies in the model organisms, the PINK1-Parkin pathways plays a key role in the stress-induced mitophagy (Jin et al., 2010). In healthy mitochondria, the constitutively expressed PINK1 is translocated to mitochondria in a membrane-potential-dependent manner, cleaved by proteases and released to cytosol for further degradation. Membrane depolarization in dysfunctional mitochondria causes stabilization and dimerization of PINK1 on mitochondrial surface (Ding and Yin, 2012). Bound PINK1 recruits and phosphorylates parkin (Dagda et al., 2009; Eiyama and Okamoto, 2015), which tags damaged mitochondria for further degradation by mitophagy (Narendra and Youle, 2011).

Activity of the protein phosphatase PGAM5 also contributes to the activation of mitophagy (Chen et al., 2014; Wu et al., 2014) and promotes the mitochondrial fission by activating the Drp1/Dnm1l protein (Wang et al., 2012). In our present study, hypoxia exposure induced elevated mRNA expression of *prkn* in *M. edulis*, while the transcript levels of the associated key genes *pink*1 and *pgam*5 rose during reoxygenation mitochondria. This expression pattern indicates an onset of mitophagy during reoxygenation as the PINK1-parkin pathway is increasingly required to tag dysfunctional mitochondria caused by oxidative stress. Transcriptional upregulation of PGAM5 also indicates activation of the cell death pathways (Wang et al., 2012) during reoxygenation in *M. edulis*, consistent with the earlier findings of upregulated apoptosis in this species during H/R stress (Falfushynska et al., 2020a).

The correlation network analysis showed strong correlation of mRNA expression of all three fission mediators, the PINK1-parkin pathway regulators and *oma*1, underpinning the common regulatory mechanism for these pathways. This is also reflected by the clear separation of mRNA expression profiles of the mussels exposed to hypoxia and (especially) reoxygenation from the normoxic control groups along the 1st principal component axis in the PCA associated with the high loadings of the fission-mitophagy-related transcripts. An earlier study showed a similar major shift of the mRNA expression profiles of the pro-apoptotic and pro-inflammatory genes in *M. edulis* exposed to the short-term H/R stress (Falfushynska et al., 2020a). The metabolic profiling in a related study showed onset of anaerobiosis, downregulation of gluconeogenesis and a shifted balance between protein synthesis and breakdown in *M. edulis* indicating early onset of severe stress response during H/R exposure (Haider et al., 2020).

Muted Transcriptional Response of the Mitochondrial Dynamics and Quality Control Genes During H/R Stress in *C. gigas*

Unlike *M. edulis*, *C. gigas* did not change its mRNA expression in any of the 17 studied genes in response to 24 h hypoxia and subsequent 1.5 h reoxygenation. Furthermore, the overlap of gene transcription found by PCA and the apparent tight correlation across all studied genes supports a conclusion of the lack of a transcriptional shift in the studied pathways during H/R stress in the Pacific oyster. A similar transcriptional stability was found for *lonp1* expression after 6 days of hypoxia and 1–12 h of reoxygenation in the eastern oyster *C. virginica* (Ivanina et al., 2010). Previously, metabolic and mRNA expression profiling on apoptotic and pro-inflammatory genes in *C. gigas* revealed no changes in response to short-term hypoxia (Falfushynska et al., 2020a; Haider et al., 2020). Only 6 days exposure to hypoxia led to a small shift in metabolite profiles indicating metabolic rate depression, maintenance of balanced FAA/metabolite pool and effective detoxification by urea and purine cycles. Furthermore, maintenance of steady activity levels of mitoproteases during the H/R stress was earlier reported in *C. gigas* (Ivanina and Sokolova, 2016). Together with the muted mRNA expression regulation of mitochondrial quality control genes and mitochondrial dynamics, these findings indicate that 24 h of severe hypoxia might not be sufficient to induce strong stress markers in *C. gigas*. However, short-term hypoxia (24 h, <1% O_2) and subsequent reoxygenation affected a reversible phosphorylation pattern of mitochondrial quality control proteins such as PGAM5 in *C. gigas* (Sokolov et al., 2019). This indicates that despite the lack of a strong transcriptional induction of the key genes in the mitochondrial quality control pathways, the activity of these pathways might be modulated by posttranslational protein modifications (PTM) in *C. gigas*. This hypothesis requires further investigation as the role of the PTM in the regulation of the mitochondrial function of marine bivalves has not been extensively studied (Sokolov et al., 2019; Falfushynska et al., 2020b). Notably, a muted transcriptional response to short-term hypoxia and subsequent reoxygenation has been earlier shown for other important pathways involved in cellular bioenergetics of the oysters. Thus, hypoxia induced genes (especially respiratory chain genes) were regulated in *C. gigas* only after 7 days of exposure to 30% O_2 saturation, with no apparent changes at the earlier exposures (David et al., 2005). In the eastern oyster *C. virginica*, key marker genes in the mitochondrial electron chain and Krebs' cycle, as well as the cytosolic glycolytic genes showed stable expression during 6 days of severe hypoxia (< 1% O_2) and subsequent reoxygenation (Ivanina et al., 2010). Like other hypoxia-tolerant mollusks, the Eastern and Pacific oyster rely on metabolic rate depression to cope with oxygen deficiency (Storey and Storey, 2004) and use a reversible global transcription repression to arrest energy consuming transcription (Larade and Storey, 2002). Only specific transcription factors seem to be upregulated to allow transcription of certain hypoxic genes (David et al., 2005). These factors might contribute to the muted transcriptional response during H/R stress in the oysters. Overall, the hypoxia tolerant Pacific oyster seems to maintain its mitochondrial integrity and functions by reversible enzyme regulations sufficient for fast recovery in reoxygenation, which might contribute to the high stress tolerance of this species adapted to the habitat conditions of daily immersion and emersion (Sussarellu et al., 2013). Further investigations in a broader comparative framework (involving multiple species of bivalves with different levels of hypoxia tolerance) are needed to determine whether the hypoxia-tolerant mitochondrial phenotypes generally correlate with the transcriptional stability and stronger dependence on post-translational regulatory mechanisms during H/R stress in marine bivalves.

CONCLUSION

Our study showed that differential transcriptional regulation of the mitochondrial quality control pathways is involved in the molecular response to H/R stress in the blue mussels *M. edulis* but not in the oysters *C. gigas*. In the mussels, the H/R stress induced the pathways related to the mitochondrial fission,

mitophagy and ATP-independent mitochondrial proteolysis. Interestingly, the transcripts of the proteins encoding ATP-dependent mitochondrial quality control proteins (such as mitofusin 2, Lon protease and paraplegin) remained stable or downregulated during hypoxia, possibly as a consequence of the metabolic rate depression and an associated decrease in the ATP turnover rates. In *C. gigas*, no clear transcriptional response of the mitochondrial quality control pathways was found during H/R stress, with all studied transcripts maintained at control (normoxic) levels. This might indicate that unlike *M. edulis*, other mechanisms (such as post-transcriptional and post-translational regulation of the respective pathways) are involved in the regulation of the mitochondrial integrity in oysters. Alternatively, it is possible that the level of the mitochondrial damage in oysters did not reach the threshold required to trigger the respective transcriptional activation of the mitochondrial quality control pathways. The latter explanation appears plausible, given the relative stability of the metabolomics profile and the transcript levels of other stress genes (such as those involved in apoptosis and inflammation) during H/R stress in oysters compared with the mussels (Falfushynska et al., 2020a; Haider et al., 2020). Our present study and earlier published research (Falfushynska et al., 2020a; Haider et al., 2020) also indicate that the mRNA expression of the stress-related genes may not be a good biomarker of hypoxia-induced stress in an extremely tolerant species such as *C. gigas*. It is worth noting that the natural habitat conditions differ with regard to salinity (ocean vs. brackish, respectively) and the tidal height (intertidal vs. subtidal, respectively) between the two studied populations of the oysters and the mussels. Further studies are needed to determine whether the established links between the mRNA expression profiles of the stress-related mitochondrial genes and hypoxia tolerance reflect the species-specific differences between the oysters and the mussels, or some other aspects of the local adaptation to the divergent habitats in which these organisms were collected.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Zenodo: doi: 10.5281/zenodo.4266408.

AUTHOR CONTRIBUTIONS

JS and HF conducted the experimental exposures and tissue collections with *C. gigas*, and *M. edulis*, respectively. mRNA extraction, cDNA synthesis and quantitative real-time PCR were done by JS and HF, and statistical analysis and data integration was done by JS. HF and JS designed primers according to transcripts provided by bioinformatical analysis from HP. JS, IS, and HP wrote the first draft of the manuscript, and all author contributed to the data interpretation and revisions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.604411/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Supplementary Table 1. BLASTN alignments of the transcript sequences from *M. galloprovincialis* and two *Crassostrea* species (*C. virginica* and *C. gigas*) showing the primer sequences used to amplify the respective transcript fragments from *M. edulis* from the Baltic Sea.

Forward primer sequences are shown in blue font and highlighted in yellow, reverse primer sequences are shown in red font. In all alignments, query is a *Crassostrea* sequence, subject – *M. galloprovincialis* sequence. For *fis*1 and *pink*1, we were unable to reconstruct alignment with relevant *C. gigas* transcript because only partial transcript from *C. gigas* is available. Thus, for these two transcripts we show the relevant alignment of *M. galloprovincialis* and *C. virginica* sequences.

mfn2

```
Query: Cgigas|XM_011450871.2_cds_XP_011449173.1_1_gene_LOC105343476 Length: 2256

Score:203 bits(224), Expect:3e-56,
Identities:169/207(82%),  Gaps:0/207(0%), Strand: Plus/Plus

Query  589  GAAGATGTGGTATTTTTAGACAGTCCTGGGATTGATGTAACTCCAGATTTAGACGACTGG  648
            |||||||| || ||| ||||||| ||||| || ||||| || ||||||||||||  ||||
Sbjct  1    GAAGATGTTGTCTTTGTAGACAGCCCTGGAATAGATGTCACACCAGATTTAGACTCCTGG  60

Query  649  ATTGATAAACATTGCTTGGATGCTGATGTGTTTGTATTGGTGGCTAATGCAGAGTCAACC  708
            |||||  ||   || ||||||||||||||| ||||| || || |  |||||||| || ||
Sbjct  61   ATTGACCAATTCTGTTTGGATGCTGATGTTTTTGTTTTAGTTGGAAATGCAGAATCGACA  120

Query  709  CTTATGCAGACAGAGAAGAACTTCTTTCACAAAGTGAGTTCACGGTTGTCCAAACCCAAC  768
            || ||||| || |||||||| || || |||||||||||| || || || ||||||||||||
Sbjct  121  CTCATGCAAACTGAGAAGAATTTTTTCCACAAAGTGAGCTCCCGATTATCCAAACCCAAC  180

Query  769  ATCTTCATTCTGCAAAACAGATGGGAC  795
            || || ||| ||||||||||||||||
Sbjct  181  ATATTTATTTTGCAAAACAGATGGGAC  207
```

opa1

```
Query: PREDICTED: Crassostrea gigas dynamin-like 120 kDa protein, mitochondrial
(LOC105325345), transcript variant X1, mRNA Query ID: XM_011424861.2 Length: 3686

Score:190 bits(210), Expect:9e-52,
Identities:210/280(75%),  Gaps:0/280(0%), Strand: Plus/Plus

Query  993   GAGGAAGAGCCTGATAGACATGTATTCGGATGTGCTTGATGAGTTAGAACGCTATGATTC  1052
             ||||||   | | ||||| ||||| ||||||||  | ||||| |||  | | ||||||||
Sbjct  1     GAGGAAATCCTTAATAGATATGTACTCGGATGTATTAGATGAATTATCAGGATATGATTC  60

Query  1053  CAGCTACAACACACAGGACAACCTACCCAGGGTTGTTGTGGTGGGAGACCAAAGTTCTGG  1112
             ||  |||||||||||||  || |||| |||||||| || || ||||| || ||||||||
Sbjct  61    CACATACAACACACAGGATTACTTACCGAGGGTTGTAGTTGTAGGAGATCAGAGTTCTGG  120

Query  1113  AAAAACCAGTGTCCTGGAGATGATAGTACAAGCTCGTATATTTCCCAGGGGATCTGGTGA  1172
              ||||| |||    | || |||||||     ||| | |||||||||||| ||||| || ||
Sbjct  121   GAAAACAAGTACTTTAGAAATGATAGCTGGTGCTAGGATATTTCCCAGAGGATCAGGCGA  180

Query  1173  GATGATGACAAGGTCGCCGGTCAAGGTCACCCTCAGTGAGGGTCCCTACCACATTGCATC  1232
              ||||||||| | || || || || || ||  | ||||| || || ||||| || |||
Sbjct  181   AATGATGACACGATCTCCAGTTAAAGTTACATTAAGTGAAGGACCATACCATATAGCAGG  240

Query  1233  ATTCAAAGACAGCTCCAGAGAATTTGATCTCACAAAGGAA  1272
             ||| |||||||    |||||||||||||| || || |||
```

```
Sbjct  241   ATTTAAAGACATGAGCAGAGAATTTGATCTTACTAAAGAA  280
```

dnm1l

```
Query= XM_011429781.2 PREDICTED: Crassostrea gigas dynamin-1-like protein
(LOC105328775), mRNA

 Score = 211 bits (233),  Expect = 9e-57
 Identities = 181/224 (81%), Gaps = 0/224 (0%)
 Strand=Plus/Plus

Query  1358  GAGGAGCCAAGTCTACGATGCGTAGAATTAGCTCACGAGGAAATGCAGAGGATTATACAA  1417
              || ||||||||| |||| ||  ||||||||| ||| || |||||||||| ||||| |||
Sbjct  1357  GAAGAGCCAAGTTTACGTTGTATAGAATTAGTTCATGAAGAAATGCAGAGAATTATTCAA  1416

Query  1418  CACTGTGGAACACAGCAAGAGATGTTGAGGTTTCCAAAATTACATGAGAAGATAGTAGAT  1477
              || ||||||||||||||||||||  |  | |||||||| |||||||||| || || |||
Sbjct  1417  CATTGTGGAACACAGCAAGAGATGCTCCGATTTCCAAAGTTACATGAGAAAATTGTTGAT  1476

Query  1478  GTAGTAACTAACTTATTACGGAAGAGATTGCAACCCACCAACAACATGGTGCAAAATTTA  1537
              ||||| || || || ||||| |  ||||| |  || || ||||  |||||  |||||||
Sbjct  1477  GTAGTCACAAATTTGTTACGAAGAAGATTACCTCCAACTAACACAATGGTAGAAAATTTG  1536

Query  1538  GTAGCAATAGAGTTAGCATATATCAATACCAAACATCCAGATTT  1581
               |  | || ||||||||||||| |||||||||||||||| ||
Sbjct  1537  ATGTCCATTGAGTTAGCATATATAAATACCAAACATCCAGACTT  1580
```

mff

```
BLASTN 2.9.0+Query= XM_011416039.2 PREDICTED: Crassostrea gigas mitochondrial fission
factor-like (LOC105318773), mRNA

 Score = 44.6 bits (48),  Expect = 9e-08
 Identities = 41/51 (80%), Gaps = 1/51 (2%)
 Strand=Plus/Plus

Query  1068  AAAGCATCATACCTGAGAATACTGCTACAATTAAAACAACCTT-ATTTGAT  1117
              ||||||||||||||||  |||   | | ||||||||||  || |||||||
Sbjct  1065  AAAGCATCATACCTGAACATAACTCGATAATTAAAACAATGTTCATTTGAT  1115

 Score = 50.9 bits (55),  Expect = 2e-09
 Identities = 83/118 (70%), Gaps = 7/118 (6%)
 Strand=Plus/Plus

Query  1173  CAAATCAACGATTGATGGTGACTGATTATCATGCTGATTTGATGTCGGCCATAAAGAAAA  1232
              |||||||| ||  ||||| |||   ||||||||||||||                 |||
Sbjct  1171  CAAATCAAGGAG-GATGG-GACAATTTATCATGCTGATTTNNNNNNNNNNNNNNNNNAAA  1228

Query  1233  CCAGTTTTAGCTGATTTATTCTCCATGTAG-TGTTATATTGTGTTTATACATGTTCTA  1289
              | | ||||| | ||||||| |||||||||  | ||| |||||  ||||||||||||||
Sbjct  1229  CAATTTTTA-CAGATTTAT-CTCCATGTAAATTTTACATTGT--TTATACATGTTCTA  1282
```

fis1

```
Query= XM_022482332.1 PREDICTED: Crassostrea virginica mitochondrial fission
1 protein-like (LOC111133712), mRNA

Score = 39.2 bits (42),  Expect = 6e-06
 Identities = 49/65 (75%), Gaps = 2/65 (3%)
 Strand=Plus/Plus

Query  1173  AATTTCAAGCAGATATGGTA-GTCTTTCCATTCATACCATTTTAGTTTC-CAAACTTAAT  1230
              |||||  ||  || |||| | |||||| |||||||  ||  |   |||| ||||||||||
Sbjct  1173  AATTTGCAGGCGACATGGGATGTCTTTTCATTCATTTCAAATAGTTTTCTCAAACTTAAT  1232

Query  1231  ACCAA  1235
              |||||
Sbjct  1233  ACCAA  1237
```

```
 Score = 131 bits (144),  Expect = 1e-33
 Identities = 113/141 (80%), Gaps = 2/141 (1%)
 Strand=Plus/Plus

Query  1672  ACATAAAAAATGACATCAGTGAAAAAAGAAATG-CACTTCGTCAGGAAATCATTTGTCTA  1730
              ||||||||||||||| ||  |||| || ||||  ||||||||  |||||||||||||||
Sbjct  1680  ACATAAAAAATGACAACATCGAAATAAAAAATAACACTTCGTGGGGAAATCATTGTCTA  1739

Query  1731  ATGATATGCCATTAAGGTGGGTCCTACTCTGCATTTTTATGGTTAATATTATGTGTTTTC  1790
              ||| | | ||| |||          |||||||||||| |||| ||| ||| |||| |||||
Sbjct  1740  ATGTTGTTCCACTAANNNNNNNNNNNACTCTGCATTTCTATGTTTA-TATGATGTCTTTTC  1798

Query  1791  TTTTGCAAAAGGTTGATTAAT  1811
              |||||||||||||||||||||
Sbjct  1799  TTTTGCAAAAGGTTGATTAAT  1819
```

lonp1

```
Query= XM_022478813.1 PREDICTED: Crassostrea virginica lon protease homolog,
mitochondrial-like (LOC111131342), mRNA

 Score = 95.1 bits (104),  Expect = 3e-22
 Identities = 100/132 (76%), Gaps = 0/132 (0%)
 Strand=Plus/Plus

Query  1577  TTTACCTTGGGGAAAATTCACAGAGGAAAACCTAGAACTAGCCAAAGCCAAAGAAATATT  1636
              ||||||  |||||  ||||  |  ||  |||||  | |||  |||   ||||  |||||  | ||
Sbjct  1577  TTTACCCTGGGGTAAATATAGTGAAGAAAATTTGGAAATAGAACAAGCAAAAGAGGTGTT  1636

Query  1637  GGAAGAAGACCATTATGGCATGAAAGATGTCAAGGATAGAATCATGGAATTCATTGCAGT  1696
              ||||||||||||||||  |||  |||||||  ||  |||||||  |  |||||  ||  |||||
Sbjct  1637  GGAAGAAGACCATTATGGAATGGAAGATGTTAAAAATAGAATTCTTGAATTTATAGCAGT  1696

Query  1697  GAGTCAACTGAA  1708
               ||  ||  ||  ||
Sbjct  1697  AAGCCAGCTAAA  1708
```

For Lonp1, we were unable to reconstruct BLASTN alignment between *C. gigas* and putative *Mytilus* sequence because of the short predicted *Mytilus* sequence. Therefore, multiple sequence alignment of the relevant sequences from *C. gigas*, *C. virginica* and *M. galloprovincialis* was constructed manually and shown below. Dots indicate identical residues.

Multiple sequence alignment of a relevant conserved segment: C. gigas-M. galloprovinicalis-C. virginica

```
XM_011452094.3     TTTACCATGG GGAAAATTTA CTGAAGAAAA TCTAGAGCTG ACCAAAGCAA
predictedMytilus   ......C... ..T....A.. G......... .T.G..AA.A GAAC......
XM_022478813.1     ......T... ........C. .A..G..... C.....A..A G.......C.

XM_011452094.3     AGGAAATCTT AGAAGAGGAC CACTATGGCA TGAAAGATGT CAAAGACAGA
predictedMytilus   .A..GG.G.. G.....A... ..T.....A. ..G....... T...A.T...
XM_022478813.1     .A.....A.. G.....A... ..T....... .......... ...G..T...

XM_011452094.3     ATCATGGAAT TCATTGCAGT CAGTCAACTG AA
predictedMytilus   ..TC.T.... .T..A..... A..C..G..A ..
XM_022478813.1     .......... .......... G......... ..
```

spg7

```
Query= XM_011448210.2 PREDICTED: Crassostrea gigas paraplegin (LOC105341618),
mRNA

 Score = 41.0 bits (44),  Expect = 3e-06
 Identities = 37/43 (86%), Gaps = 3/43 (7%)
 Strand=Plus/Plus

Query  924  AATGGAAAAAG-CACTAAGCAAGATACAACAGCATTCAAATCA  965
            |||||||||||  ||  ||| |||| ||||||||||||||| ||
Sbjct  931  AATGGAAAAAGACAAGAAG-AAGA-ACAACAGCATTCAAAACA  971

 Score = 44.6 bits (48),  Expect = 3e-07
 Identities = 30/34 (88%), Gaps = 0/34 (0%)
 Strand=Plus/Plus

Query  1177  TTGGTCCACCAGGATGTGGGAAAACACTTTTGGC  1210
              |||||  ||||||||||||||||||  ||||||
Sbjct  1180  TTGGTGGACCAGGATGTGGGAAAACAGCTTTGGC  1213
```

oma1

```
Query= XM_011440234.2 PREDICTED: Crassostrea gigas metalloendopeptidase OMA1,
mitochondrial-like (LOC105336058), mRNA

 Score = 529 bits (586),  Expect = 6e-154
 Identities = 405/489 (83%), Gaps = 8/489 (2%)
 Strand=Plus/Plus

Query  608   TATGTCTATCTTGTTGATTTTTTAAAAACATTTTATGAGAGGttttttttGTGATATCCAT  667
              || |||| ||||||||||||||||||||||||||||||||| |||||||| ||||||||||
Sbjct  608   TAGGTCT-TCTTGTTGATTTTTTAAAAACATTTTATGAGATGTTTTTTTTATGATATCCAT  666

Query  668   GCATTGCCAAATATTTAATATATTATATTTGTTATATGCATGTGTTAGATGTATGTTTTG  727
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  667   GCATTGCCAAATATTTAATATATTATATTTGTTATATGCATGTGTTAGATGTATGTTTTG  726

Query  728   --CAATTTATCAACTTTTGTTTCTAGATGGATGTACAATAGTCTATTGACAA--GGGTTT  783
                ||||||||||||||| ||||||||||      |||| | |||| ||||   | |||
Sbjct  727   ATAAATTTATCAACTTTTATTTCTAGATGGNNNNNNAATACT-TATTCACAAAGAGATTT  785

Query  784   TAACATTTTTGTTATATATTGTACATGTATGGTATTTCATTTGCACACAATTAAGCATAA  843
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||| |
Sbjct  786   TAACATTTTTGTTATATATTGTACATGTATGGTATTTCATTTGCACACAATTAACCATCA  845

Query  844   TTTATTATCt-tttttttttATAAACAAGTCATTGATACACACTTT-GTAGTCAAATGACA  901
              || ||||| | ||||||||||||||||||||||||||||||||| ||| ||
Sbjct  846   TTCATTATATATTTTTTTTATAAACAAGTCATTGATACACACTTTAGTATTCNNNNNNNN  905

Query  902   ACTATAGAAGTGCTGATTGTTTAGATGACATTGTACATGTATTGTACAACTGTTTGATGT  961
                                  ||||||| ||| |||| ||||||||||||||||||||||
Sbjct  906   NNNNNNNNNNNNNNNNNNNTTTAGATAACAATGTATATGTATTGTACAACTGTTTGATGT  965

Query  962   GTTTGGTAAttttttttATATATCATAATTCTTTATAGATTCACCttttttttCACATTTGA  1021
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  966   GTTTGGTAATTTTTTTTATATATCATAATTCTTTATATATTCACCTTTTTTTTCACATTTGA  1025

Query  1022  AAAGATGAGGGGTGTATTTGTTTGCTACCTCCAGAACTGGTCCTGAATATCTCCATTGTC  1081
              ||                  |||||||||||||||| | ||| | |||||| ||  |||
Sbjct  1026  AANNNNNNNNNNNNNNNNNGTTTGCTACCTCCAGAACGGTTCCAGGATATCTACAGCGTC  1085

Query  1082  TACCTGCTA  1090
              ||| |||||
```

```
Sbjct  1086  TACTTGCTA  1094
```

prkn

```
Query= XM_011439893.2 PREDICTED: Crassostrea gigas E3 ubiquitin-protein
ligase parkin (LOC105335799), mRNA

Score = 270 bits (299),  Expect = 2e-75
 Identities = 328/447 (73%), Gaps = 19/447 (4%)
 Strand=Plus/Plus

Query  1728  ACACAATTGACAATTACCGGTAAACTGTGTGATATTTTTTACCTGGGTCTTTTGATTTTG  1787
              ||| |||| ||||||||    ||| ||||||||||||||| |||||| |||| ||||||
Sbjct  1725  ACAAAATTTACAATTACA---AAAATGTGTGATATTTTTTAGCTGGGT-TTTTAATTTTG  1780

Query  1788  ACTTCACTATGCCA--AATTTTGAAATATAATGTGTGTTCAAGGTGttttttttAATGGTT  1845
               ||||| ||   ||  ||||||||||||||| |||  | ||| |||||||||||||||
Sbjct  1781  TCTTCAGTACAGCATAAATTTTGAAATATAATTTGTACT-AAGATGTTTTTTTTAATGGTT  1839

Query  1846  GAGTCATAACAAATAATCTGTGTCATTATGTTGACCGTTTATTTTGTATGATCTAGATTT  1905
              | ||                               |||| |||| ||||  || ||||| |||
Sbjct  1840  GTGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNGACCTTTTAATTTGATTGTTCTAGTTTT  1899

Query  1906  TATTTTGTAAATAGTCTAGTTGAGATCAATGTGATATACTG-TTTGATT-TCAACCATTT  1963
              ||||||||||||||||||||| |||||||||||||||| ||||||| |||| | |||
Sbjct  1900  TATTTTGTAAATAGTCTAGTTGCGATCAATGTGATATACTGTTTTGATTCTCAAAC-TTT  1958

Query  1964  TATGACTATCTTTATTGTGCCATATTTTGTGTA-AAGCAATGATTTTGTCATACTTAACA  2022
              | | |||||||  ||  ||||||||||||||| |||   | |||||||    |||||||
Sbjct  1959  TGTAACTATCTCGAT--TGCCATATTTTGTGTATAAGGTTTTATTTTGTNNNNCTTAACA  2016

Query  2023  CA--AAGATAAACAGACGAG-AGCAGttttttttttACCTTGGCCATCATCCAACCTTGAT  2079
              ||  || ||||  ||   || ||||||||||||||                    ||||
Sbjct  2017  CAACAATATAAGAAGCGTAGAAGCAGTTTTTTTTTTANNNNNNNNNNNNNNNNNNNTTGAA  2076

Query  2080  TTTATCGTTAGGAATCTCTATTTTGTGTAAATGTTACTTTTGTTTCAAACAATTTTATGT  2139
              || ||||||  | |  ||||||||||||||| ||||||||||||||||||||||||||
Sbjct  2077  TTCATCGTTTTGTAATTCTATTTTGTGTAAATTTTACTTTTGTTTCAAACAATTTTATGT  2136

Query  2140  A-ATTTTG-GTTGTAATAT-GTTGTAT  2163
              | |||||| || ||||| | ||| |||
Sbjct  2137  ATATTTTGTGTAGTAATCTAGTTTTAT  2163
```

*pink*1

```
Query= XM_022485570.1 PREDICTED: Crassostrea virginica
serine/threonine-protein kinase PINK1, mitochondrial-like
(LOC111135467), mRNA

 Score = 334 bits (370),  Expect = 1e-94
 Identities = 229/252 (91%), Gaps = 6/252 (2%)
 Strand=Plus/Plus

Query  1956  TATGACACTGGAG-AATTTTTATTTTTATTT-CTTAGAAAAAGAGATTTAGAATTTCCAT  2013
              ||||||| ||||| |||||||||||||||| || ||||| | | ||||||||||||||||
Sbjct  1956  TATGACATTGGAGTAATTTTTATTTTTATTTACTCAGAAAGACACATTTAGAATTTCCAT  2015

Query  2014  GTACCAACTATAAACCTAATATAGtattaatattatactgattcatttttattatttgtt  2073
              || |  | ||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  2016  GT-CTTATTATAAACGTAATATAGTATTAATATTATACTGATTCATTTTTATTATTTGTT  2074

Query  2074  atttgaattaattgttaagtgaaattatGCAAATATAAAGTCTTTTAACATTTAGTTTTA  2133
              |||||||||||||||||||||||||||||||||||||||| ||| |  ||||||||||
Sbjct  2075  ATTTGAATTAATTGTTAAGTGAAATTATGCAAATATAAAGTTTTTGA--ATTTAGTTTTA  2132

Query  2134  GAAGAGGTATAATGCTTGGTAATGACTGAATTTTTGAGTCGGCTCGCAAAGCGAGAAGAC  2193
```

```
             ||||||  ||| || ||||||||||||||||||||||||||||||||| |||||||| | |
Sbjct  2133  GAAGAGCCATATTGTTTGGTAATGACTGAATTTTTGAGTCGGCTCGCGAAGCGAGATG-C  2191

Query  2194  TCCTTGCTATCA  2205
             ||||||||||||
Sbjct  2192  TCCTTGCTATCA  2203
```

pgam5

```
Query= XM_011430031.2 PREDICTED: Crassostrea gigas serine/threonine-protein
phosphatase PGAM5, mitochondrial (LOC105328956), transcript variant
X1, mRNA

 Score = 129 bits (142),  Expect = 2e-33
 Identities = 76/78 (97%), Gaps = 1/78 (1%)
 Strand=Plus/Plus

Query  3   TTGCAACTTCGCACTCTCGCA-CCTCGACCTAAAGGCGAAAGTGCGAATGTCGAAGTGGC  61
           |||||||||||||||||||| |||||||||||||||||||||||||| ||||||||||
Sbjct  3   TTGCAACTTCGCACTCTCGCATCCTCGACCTAAAGGCGAAAGTGCGAAGGTCGAAGTGGC  62

Query  62  CCCATCGGAACACCATAG  79
           ||||||||||||||||||
Sbjct  63  CCCATCGGAACACCATAG  80
```

Supplementary Table 2. BLASTN alignments of the transcript sequences of *lonp*1, *opa*1 and *hyou*1 from two *Crassostrea* species (*C. virginica* and *C. gigas*) showing the primer sequences used to amplify the respective transcript fragments from *C. gigas*.

The NCBI accession numbers for the respective sequences used for the alignment, along with the alignment scores are given. For the sake of brevity, only the fragment of the alignment containing the primers and the immediate flanking regions are shown. Forward primer sequence is shown in blue font and highlighted in yellow, reverse primer sequence is shown in red font. For *fis*1, we were unable to reconstruct alignment with relevant *C. gigas* transcript because only partial transcript from *C. gigas* is available. Query – *C. virginica*, subject – *C. gigas*.

*lonp*1

```
Query: PREDICTED: Crassostrea virginica lon protease homolog, mitochondrial-like
(LOC111131342), mRNA Query ID: XM_022478813.1 Length: 3377

>PREDICTED: Crassostrea gigas lon protease homolog, mitochondrial (LOC105344337), transcript
variant X1, mRNA
Sequence ID: XM_011452094.3 Length: 3421
Range 1: 27 to 3057

Score:2859 bits(3170), Expect:0.0,
Identities:2466/3047(81%),  Gaps:26/3047(0%), Strand: Plus/Plus

Query  2696  CCATCTGCATTCCCCAGAGGGTGCAACGCCAAAAGATGGACCTAGTGCCGGTATCGCCAT  2755
             |||||| || ||||| |||||||| || ||||| |||||||| ||||| || ||||||||
Sbjct  2711  CCATCTCCACTCCCCTGAGGGTGCTACACCAAAGGATGGACCCAGTGCAGGAATCGCCAT  2770

Query  2756  AACAACAGCCCTGATGTCACTGGCTATGAATCGTCCTTGTCGACAGGAAGTGGCCATGAC  2815
             ||| || || ||||||||||| || |||||||| || ||||| ||||||||||| |||||
Sbjct  2771  AACGACTGCACTGATGTCACTAGCCATGAATCGGCCATGTCGGCAGGAAGTGGCGATGAC  2830

Query  2816  AGGAGAGATAACTCTGACTGGAAAGGTGCTGGCCATTGGTGGACTCAAGGAAAAACTTCT  2875
             |||||| || ||||| || ||||| ||  | |||||||||||||||||||||||||| ||
Sbjct  2831  AGGAGAAATTACTCTCACCGGAAAAGTATTAGCCATTGGTGGACTCAAGGAAAAACTCCT  2890

Query  2876  GGCTGCCAAACGAGCCGGAATGAAATGTGTGATAATCCCAGAAGAAAACAGAAGCAAGTT  2935
              || |||||| |||| ||||||||||||||| ||||||||||||| ||||| || || ||
Sbjct  2891  AGCAGCCAAAAGAGCTGGAATGAAATGTGTGTTAATCCCAGAAGAGAACAGGAGTAAATT  2950
```

*opa*1

```
Query: PREDICTED: Crassostrea virginica dynamin-like 120 kDa protein, mitochondrial
(LOC111136213), transcript variant X3, mRNA Query ID: XM_022486900.1 Length: 3538

>PREDICTED: Crassostrea gigas dynamin-like 120 kDa protein, mitochondrial (LOC105325345),
transcript variant X1, mRNA
Sequence ID: XM_011424861.3 Length: 3692
Range 1: 775 to 3132

Score:2362 bits(1279), Expect:0.0,
Identities:2002/2360(85%),  Gaps:13/2360(0%), Strand: Plus/Plus

Query  2648  GAAATTTACAGACCAATGGAATTGAAGTCACCAATGACTTTATCCGGGAGACTTGGTACC  2707
             | ||  | |||||||  ||||| ||||||||||||||||||||  |||| ||||||||||
Sbjct  2694  GGAACCTGCAGACCAGCGGAATAGAAGTCACCAATGACTTTATAAGGGAAACTTGGTACC  2753

Query  2708  ACGTGTACAGGCAGTTCTTCCTAAATAAGGCCCTGGGCCAGTGCCAGGAGTGCAGGAAAG  2767
             | |||||||| ||||||||||| |  ||||||||||| ||||| |||||||| ||||| |
Sbjct  2754  ATGTGTACAGACAGTTCTTCCTGACCAAGGCCCTGGGTCAGTGTCAGGAGTGTAGGAAGG  2813

Query  2768  GATTCTACTACTACCAGAAAGGATTCTCTGATTCAGG---------GCTGGAGTGTAATG  2818
             | |||||||||||||||||||||||| | || |||||         ||||||||||||||
Sbjct  2814  GCTTCTACTACTACCAGAAAGGATTCACCGACTCAGGGGGATTAAAGCTGGAGTGTAATG  2873
```

```
Query  2819  ATGTTGTGCTTTTCTGGAGGTTACAGAGAATGTTGCAAATCACCAGTAACGCTCTCCGAC  2878
             |||||||||| ||||||||||||||||||||| || || |||||||| ||||| ||||
Sbjct  2874  ATGTTGTGCTCTTCTGGAGGTTACAGAGAATGTTACAGATTACCAGTAATGCTCTACGAC  2933

Query  2879  AACAAGTCATTAATAATGAAGCAAGAAGATTAGAAAGAATAATAAAGGAAGTTTTGGATG  2938
             |||||||  | |||||||||||||||||| || ||||||||||||||||||||||||||
Sbjct  2934  AACAAGTTGTAAATAATGAAGCAAGAAGATTGGAGAGAATAATAAAGGAAGTTTTGGATG  2993

Query  2939  AATTTGGTGAAGA-CAGAGAAACTTTGACCAAATTACTGACTGGTCCAAGAGTTCAGTTA  2997
             ||||||| ||||| ||| || ||| |  ||||| | |||||||||| ||||||||||
Sbjct  2994  AATTTGGGGAAGATCAG-GACACTCTAGCCAAACTGCTGACTGGTCCTAGAGTTCAGTTG  3052
```

hyou1

```
Query: PREDICTED: Crassostrea virginica hypoxia up-regulated protein 1-like (LOC111125576),
transcript variant X1, mRNA Query ID: XM_022469531.1 Length: 4627

>PREDICTED: Crassostrea gigas hypoxia up-regulated protein 1 (LOC105325010), transcript
variant X1, mRNA
Sequence ID: XM_011424337.3 Length: 4337
Range 1: 29 to 3285

Score:2638 bits(1428), Expect:0.0,
Identities:2657/3265(81%),  Gaps:25/3265(0%), Strand: Plus/Plus

Query  490   TATAATATAATCAAGGATGAAGAAAGAGGAACACCTTTGTTTAAAGTAGATGATGAGACC  549
             ||| | ||| | || ||||||||||||||||||||| | ||||||||||||||||||||
Sbjct  506   TATGACATAGTTAAAGATGAAGAAAGAGGAACACCTCTTTTTAAAGTAGATGATGAGACT  565

Query  550   ACATACAGTCCAGAGGAACTGTTGGCCATGGTGCTGGAAAAAGCAAAGTTCTCTGCTGAA  609
             || | |||||| || ||  | |||||||||| || |||||||||||| ||| || |||
Sbjct  566   ACCTTCAGTCCTGAAGAGTTATTGGCCATGGTCCTCGAAAAAGCAAAGTCCTCGGCGGAA  625

Query  610   GCTTTTGCTGATACTCCAATCTCTGATGCTGTGATCACAGTTCCTTCTTACTTCACTCAG  669
             || |||||||||| |||||||||||||||||| ||||| || ||||| |||||||| |||
Sbjct  626   GCATTTGCTGATACCCCAATCTCTGATGCTGTTATCACTGTCCCTTCCTACTTCACCCAG  685

Query  670   TCCGAGAGGCGGGCGGTGAGAGAAGCGGCAGAAATGGTGGGGATCAATGTCTTACAACTC  729
             || |||||  | ||||| | ||| || |||||| |||| || || || ||||| || |||
Sbjct  686   TCTGAGAGAAGAGCGGTCAAAGAGGCCGCAGAATTGGTTGGAATTAACGTCTTGCAGCTC  745

Query  730   CTCAGTGATAATGTGGCAGTGGCCCTAAACTATGGTGTGTTCAGGAGGAAGATGTTTAAT  789
              |||||||||| || ||||||||| | ||||| ||||| ||||| |||||||||| ||
Sbjct  746   ATCAGTGATAACGTTGCAGTGGCCTTGAACTACGGTGTATTCAGAAGGAAGATGTTCAAC  805
```

Supplementary Table 3. Principal component analysis (PCA) of the molecular biomarker profiles in the hepatopancreas of *M. edulis.*

Mytilus edulis				
principal component	**eigenvalues**	**% total variance**	**cumulative eigenvalue**	**cumulative %**
1	**2.278755**	0.472066	2.278755	0.472066
2	**1.448551**	0.190755	3,727307	0.662821
3	**1.185734**	0.127815	4,913041	0.790636
4	0.917000	0.076445	5,830041	0.867080
5	0.825459	0.061944	6,655500	0.929024
6	0.539936	0.026503	7,195436	0.955527
7	0.506228	0.023297	7,701664	0.978824
8	0.373124	0.012656	8,074788	0.991480
9	0.232307	0.004906	8,307095	0.996386
10	0.182522	0.003029	8,489617	0.999415
11	0.080220	0.000585	8,569837	1.000000

Supplementary Table 4. Principal component analysis (PCA) of the molecular biomarker profiles in the hepatopancreas of *M. edulis* – loading plot data based on correlation between variables and dimensions and coordinates of variables (loadings*component standard deviation) in R Studio (v. 1.2.5033 and R v. 3.6.3, (R Core Team, 2020)). Gene name with appended "t" depicts transformed data to ensure normal distribution. Variables with high loadings (i.e. with the absolute value of the correlation coefficient >0.5) are highlighted in bold and red.

	Correlation between variables and coordinates		
	PC 1	PC 2	PC 3
mff	**0.9338**	0.0662	-0.2169
***pink*1**	**0.8406**	-0.0728	0.2486
***fis*1_t**	**0.8522**	-0.0522	-0.3953
***spg*7**	-0.1066	**0.8756**	-0.1460
***pgam*5**	0.2804	0.3721	**0.6206**
***oma*1**	**0.8209**	0.1442	-0.0865
***mfn*2**	0.0555	**-0.5746**	**0.7189**
***opa*1**	**0.6621**	0.3975	0.4069
***dnm*1*l*_t**	**0.8910**	-0.0547	-0.0242
prkn	**0.9038**	-0.0544	-0.0221
***lonp*1**	-0.2682	**0.8160**	0.2084

Supplementary Table 5. Principal component analysis (PCA) of the molecular biomarker profiles in the hepatopancreas of *C. gigas* in R Studio

Crassostrea gigas				
principal component	**eigenvalues**	**% total variance**	**cumulative eigenvalue**	**cumulative %**
1	**3.307112**	0.643352	3,307112	0.643352
2	**1.159869**	0.079135	4,466980	0.722487
3	**1.017614**	0.060914	5,484595	0.783401
4	0.919568	0.049741	6,404162	0.833143
5	0.729181	0.031277	7,133343	0.864419
6	0.681143	0.027292	7,814486	0.891711
7	0.663010	0.025858	8,477496	0.917569
8	0.604214	0.021475	9,081710	0.939044
9	0.547787	0.017651	9,629497	0.956695
10	0.477905	0.013435	10,107402	0.970130
11	0.400665	0.009443	10,508067	0.979573
12	0.350574	0.007230	10,858640	0.986802
13	0.289584	0.004933	11,148224	0.991735
14	0.228719	0.003077	11,376943	0.994812
15	0.183731	0.001986	11,560675	0.996798
16	0.182149	0.001952	11,742824	0.998750
17	0.145781	0.001250	11,888605	1.000000

Supplementary Table 6. Principal component analysis (PCA) of the molecular biomarker profiles in the hepatopancreas of *C. gigas* – loading plot data based on correlation between variables and dimensions and coordinates of variables (loadings*component standard deviation) in R Studio (v. 1.2.5033 and R v. 3.6.3, (R Core Team, 2020)). Gene name with appended "t" depicts transformed data to ensure normal distribution. Variables with high loadings (i.e. with the absolute value of the correlation coefficient >0.5) are highlighted in bold and red.

	Correlation between variables and coordinates		
	PC 1	**PC 2**	**PC 3**
dnm1l	**0.8574**	-0.2082	0.1000
prkn	**0.8277**	-0.2491	0.2464
twnk	**0.6293**	0.1884	0.4681
tsfm	**0.8108**	-0.1127	-0.4246
mff	**0.8609**	-0.0380	0.2221
lonp1	0.4914	**0.7500**	-0.3172
spg7	**0.8789**	0.0252	-0.1257
atp23	**0.7228**	-0.1310	0.0998
oma1_t	**0.7909**	-0.1118	0.1346
mieap	**0.6919**	-0.1386	-0.0092
hyou1	**0.9223**	-0.0562	0.1877
fis1_t	**0.9406**	-0.1591	-0.0528
opa1	**0.8931**	0.2754	-0.2002
pgam5_t	**0.5061**	**-0.5145**	**-0.5393**
pink1	**0.8806**	0.1621	0.0356
mfn2	**0.9606**	0.0237	-0.0367
clpB	**0.7707**	0.4270	-0.0062

Annex

3 Annex

3.1 Manuscript IV: Metabolic response of three marine bivalves to intermittent hypoxia correlates with lifestyle and hypoxia tolerance of species and tissue

Planned to be submitted in the Journal of

Contribution letter

The contents of the fourth manuscript chapter from this dissertation is prepared to be submitted in 2024 under the title "Metabolic response of three marine bivalves to intermittent hypoxia correlates with lifestyle and hypoxia tolerance of species and tissue" and authorship Jennifer B. M. Steffen, Gisela Lannig, Christian Bock, Inna M. Sokolova.

I predominantly contributed to this manuscript and was involved in experimental design and planning, performance of incubation experiments, tissue collection and isolation of metabolites. Measurements by ^{1}H-NMR spectroscopy was done by Anette Tillmann, technician of the Integrative Ecophysiology group at the Alfred-Wegener Institute (AWI), Bremerhaven. In collaboration with the AWI, I completely analysed metabolite profiles received from NMR measurements (metabolic profiling). Additionally, I have also performed the complete statistical analysis and prepared all graphical output. I wrote the first draft of the manuscript and contributed to the revisions of the draft in response to the comments of the co-authors.

Contribution of the candidate in % of workload:	
Experimental concept and design	85%
Experimental work and data acquisition	70%
Data analysis and interpretation	95%
Preparation of figures and tables	100%
Drafting the manuscript	80%

Signature of consent

Supervisor	Doctoral candidate
_____	_____
Prof. Dr. Inna Sokolova	Jennifer Barbara Maria Steffen

Metabolic response of three marine bivalves to intermittent hypoxia correlates with lifestyle and hypoxia tolerance of species and tissue

Jennifer B. M. Steffen[a], Gisela Lannig[b], Christian Bock[b], Inna M. Sokolova[a,c]

[a] Department of Marine Biology, Institute of Biological Sciences, University of Rostock, Rostock, Germany

[b] Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany

[c] Department of Maritime Systems, Interdisciplinary Faculty, University of Rostock, Rostock, Germany

Abstract

Strong fluctuations in the dissolved oxygen content are typical for coastal environments. Consequently, hypoxia/reoxygenation stress can affect bioenergetics and redox balance of marine benthic invertebrates and severely impair their performance. Adaptations to fluctuating oxygen conditions are widespread among intertidal bivalves, but so far the metabolic regulation of differently hypoxia-tolerant species is not well understood. We used ^{1}H-NMR spectroscopy to study the metabolic profiles of heart and gill tissue from three bivalve species differing in hypoxia tolerance (*Crassostrea gigas*, *Ostrea edulis*, *Arctica islandica*) in response to short-term severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h 21% O_2). The intertidal species *C. gigas* and *O. edulis* were able to rapidly metabolize hypoxically accumulated succinate during the post-hypoxic recovery phase, while subtidal *A. islandica* might be more susceptible to sudden reoxygenation maintaining anaerobiosis during recovery. Less hypoxia-tolerant species of the three studied species (*O. edulis* and *A. islandica*) indicated stronger dependence on anaerobic pathways and protein breakdown during hypoxia. Additionally, all three studied species strongly relied on glutamate-glutamine metabolism in response to H/R stress. In contrast to metabolic regulation in gill tissue, low metabolic capacities were observed in heart tissue of oyster species. This study indicates specific metabolic regulations depending on adaption to different lifestyles and habitats in marine bivalve species.

Keywords: Hypoxia tolerance - metabolomics, ^{1}H-NMR, Mollusca, *Arctica islandica – Crassostrea gigas – Ostrea edulis*

Corresponding author: Inna M. Sokolova, inna.sokolova@uni-rostock.de

Introduction

Coastal regions are highly challenging habitats, as they are characterized by strong fluctuations in environmental conditions such as temperature, salinity and oxygen saturation. Thus, exposure to environmental fluctuations is common for benthos, particularly for immobile/sessile organisms. Severe oxygen deficiency, called hypoxia, is increasing in marine environments during the last decades, especially coastal waters are predominantly exposed (Breitburg et al., 2018; Breitburg et al., 2019). Hypoxia occurs when oxygen depletion by respiration exceeds oxygen influx from diffusion, water mixing and photosynthesis. Concentration of dissolved oxygen (DO) below 2 ml L^{-1} seawater (=7.4% O_2 at 25°C and salinity 20) defines beginning of hypoxic conditions, while DO down to 0.01 ml L^{-1} seawater (= ~0.03% O_2, at 25°C and salinity 20) determines anoxia (Diaz and Rosenberg, 1995). Naturally, hypoxic events persist daily for few hours during tides or seasonally, but nowadays hypoxic events extend to weeks, even months exacerbated by anthropogenic pressure via climate change and pollution (Diaz and Rosenberg, 1995; 2008). The most extreme climate scenario (RCP 8.5) given by the Intergovernmental Panel on Climate Change (IPCC) predicts an increase in ocean surface temperature by 1.8 to 4°C together with a decline in seawater (SW) pH of 0.3 to 0.4 pH units (IPCC, 2023). As oxygen solubility decreases with increasing temperature, warming of coastal waters, eutrophication and full stratification of the water column result in severe oxygen depletion (Breitburg et al., 2018; Diaz and Rosenberg, 2008; Keeling et al., 2010).

Since changing habitat conditions can interfere with vital cellular functions and homeostasis, sessile life style of benthic organisms requires physiological adaptations to survive in such harsh conditions (Grieshaber et al., 1994). Most essential to maintain cellular functions is sufficient energy provision via aerobic energy metabolism and thus sufficient oxygen supply (Diaz and Rosenberg, 2008). Mitochondria, key organelles in energetic and redox balance of the cell, use oxygen as final electron acceptor generating most ATP (<90%) in aerobic organisms during oxidative phosphorylation (OXPHOS). Thus, insufficient oxygen supply causes severe damage to mitochondrial functions due to depression of electron transport system (ETS), depolarization of mitochondrial membrane resulting in ATP deficiency and subsequently to impaired recovery and cell death (Chouchani et al., 2016; Piper et al., 2003; Solaini et al., 2010). Damage to mitochondria has major systemic consequences resulting in severe inflammation, elevation of reactive oxygen species (ROS) and secondary injury to organs and tissues up to the point of increased mortality (Kalogeris et al., 2012; Kalogeris et al., 2014). Consequently, metabolic regulation plays a key role in physiological adaptations. Tolerance to environmental changes is based on coping with disturbed energy homeostasis, where organisms' requirements are not or not fully met by a constrained aerobic energy supply (Pörtner et al., 2017; Sokolova et al., 2012; Sokolova, 2021). Tolerant species adapted to hypoxia by using metabolic rate depression (MRD), where ATP production and consumption is reduced to conserve energy, anaerobiosis is initiated and toxic waste accumulation is decreased (Hochachka et al., 1996; Storey, 2002). Specifically bivalves, as facultative anaerobes, evolved alternative pathways for substrate-level phosphorylation, mitigation of toxic waste accumulation and low intracellular pH during anaerobiosis (Bayne, 2017). In addition, their mitochondria maintain respiratory function and mitigate oxidative damage during hypoxia-reoxygenation (H/R) stress

(Ivanina et al., 2016; Sokolov et al., 2019; Sokolov et al., 2021; Sokolova et al., 2019). Previous studies focused extensively on energy homeostasis in hypoxia-tolerant species (Bayne, 2017; Grieshaber et al., 1994; Hochachka and Lutz, 2001) and started to underline the importance of homeostasis of intermediary metabolites during hypoxia (Haider et al., 2020). The metabolic branchpoint, where pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) compete for phosphoenolpyruvate (PEP), determines the direction of glycolytic flux towards aerobic or anaerobic mitochondrial metabolism. In early hypoxia, PK remains actively producing pyruvate, that is further metabolized to alanine or aspartate resulting in fumarate and succinate accumulation. In cytoplasm, glycogen fermentation and anaerobic glycolysis leading to formation of lactate and opines are common. Prolonged hypoxia induces activation of PEPCK converting PEP to oxalacetate resulting in succinate, acetate and propionate accumulation (Bayne, 2017). This metabolic pathway yields higher amount of ATP per unit glycose than lactate or opine formation and its low production of metabolic protons or volatile end products minimizes metabolic acidosis (Bayne, 2017; Sokolova et al., 2000b). In addition, high tissue stores of glycogen and amino acids (AAs) support anaerobic ATP production in prolonged survival under hypoxia conditions (Bayne, 2017). During post-hypoxic recovery, additional costs occur due to allocation of energy to restore cellular homeostasis, disrupted by MRD, ATP deficiency and accumulation of anaerobic end products. This can be observed in the so-called oxygen debt, where organismal oxygen consumption typically increase during recovery (Kurochkin et al., 2009; Sokolova, 2021; Vismann and Hagerman, 2008). The ability of hypoxia-tolerant bivalves to reincorporate anaerobic end-products into cellular functions plays an important roles in restoration of energy metabolism (Bayne, 2017). Consequently, accumulation or depletion of end products and intermediates characterize shifts in aerobic and anaerobic metabolic pathways. Thus, analysis of metabolic pathways can highlight cellular mechanisms responding to environmental changes (Götze et al., 2020; Haider et al., 2020). Due to the important role of mitochondria as central hub connecting various cellular pathways, it is most likely that mitochondrial resilience correlates with regulatory patterns in cellular metabolism. However, it is less studied how mitochondrial mechanisms are integrated into cellular metabolism. To better understand how hypoxia and reoxygenation affect cellular responses and metabolic pathways linked to mitochondria, we studied the metabolome of three bivalve species *Crassostrea gigas*, *Arctica islandica* and *Ostrea edulis* that differ in their lifestyle and hypoxia sensitivity. The Pacific oyster, *Crassostrea* (*Magallana*) *gigas* (Thunberg, 1793) is an euryhaline intertidal species originating from the Pacific coast, specifically from estuarine areas. For commercial use and aquaculture, it was introduced into European waters in the 1950s and thrives as an invasive species (Troost, 2010). *C. gigas* is known to be highly resistant to hypoxia, presumably due to its tidal life style and it has been shown to rearrange mitochondrial functions in response to H/R stress to maintain energy homeostasis (Sussarellu et al., 2013). Regulation of proteome/phosphoproteome as well as mitochondrial quality control mechanisms are key for their mitochondrial resilience to H/R stress (Sokolov et al., 2019; Steffen et al., 2020). Furthermore, mitochondria show high metabolic flexibility in the use of substrates to maintain mitochondrial respiration during hypoxia and recovery (Adzigbli et al., 2022). The extreme longevity of the subtidal ocean quahog, *A. islandica* is assumed

to be connected to its exceptional tolerance to hypoxia (Theede et al., 1969; Theede, 1973). *A. islandica* can experience unpredictable hypoxic events in its habitat (Conley et al., 2011; Gustafsson et al., 2012), but it exposes itself to prolonged periods of hypoxia by digging into the substrate, which leads to metabolic rate depression and anaerobiosis (Oeschger and Storey, 1993; Taylor, 1976). Mitochondrial studies revealed that they are highly resistant to hypoxia, but remain sensitive to sudden reoxygenation (Steffen et al., 2021). The comparatively weakest environmental robustness shows the intertidal European flat oyster, *O. edulis*. Although oysters as intertidal and estuarine species are commonly seen as hypoxia-tolerant, their ability to tolerate chemical and environmental stress differ (Lemasson et al., 2018; Perić et al., 2020; Stevens and Gobler, 2018). *O. edulis* is more susceptible to diseases and abiotic stressors compared to the more robust and invasive *C. gigas* (Drinkwaard, 1999; Haelters and Kerckhof, 2009; Lucas, 2012; Perry and Jackson, 2017). In the end of the 19th century *O. edulis* became intensively overfished, causing a decline in oyster population, which has barely recovered to this day (Gercken and Schmidt, 2014; Pogoda, 2019).

Since metabolic adaptations and biochemical pathways of anaerobic and aerobic metabolism are well studied in marine bivalves (Bayne, 2017; Zwaan and Wijsman, 1976), their lifestyle and known environmental robustness (Diaz and Rosenberg, 2008), make them suitable model species for our comparative approach to investigate changes in metabolic pathways during H/R stress.

According to habitat and lifestyle, we hypothesized that regulation of metabolic pathways related to hypoxia tolerance, i.e. anaerobiosis and detoxification, will be different in the three species, and intensify during post-hypoxic recovery. To test this hypothesis, control bivalves were kept in normoxic condition (21% O_2) throughout, while others were exposed to severe hypoxia (<0.01% O_2) for 24 hours and subsequently one group was allowed to recover in normoxia (21% O_2) for 1.5 hours. Effects of H/R stress on cell metabolism were determined by assessing metabolic profiles using ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, which is a state-of-the-art method to analyse changes in a wide range of metabolites associated to different pathways of energy metabolism, macromolecule turnover and detoxification processes. In this study, alternative anaerobic pathways and detoxification processes are considered as important key functions in hypoxia tolerance highlighting the variations in tolerant phenotypes based on species' lifestyle.

Material and Methods

Animal maintenance

Adult oysters, *O. edulis* (mean shell length: 73.8 ± 4.9 mm), that were obtained from the aquaculture facility of the University of Vigo, Spain in June/July 2019, and *C. gigas* (mean shell length: 99.3 ± 3.1 mm), that were collected in the German Wadden Sea near List/Sylt (55°01'42''N 8°26'04''E) in October 2019, were transported within 48 h after collection to the University of Rostock, Germany, in coolers lined with seawater-soaked paper towels. Adult *A. islandica* (mean shell length: 44.4 ± 0.8 mm) were collected off the coast of Kühlungsborn, Germany (54°17.145'N 11°47.143'E) during the Research Cruise Solea 777 in May/June 2020 and transported to the University of Rostock (Germany) within 6 h of collection. During transport, the quahogs were maintained in aerated cooled Baltic Sea water from the site of collection. For acclimation and maintenance, bivalves were kept in recirculated temperature-controlled aquarium systems (Kunststoff-Spranger GmbH, Plauen, Germany) with aerated artificial seawater (ASW) (Tropic Marin®, Wartenberg, Germany) at a salinity of 33±1 (psu) (oysters) and 15 ±1 (quahogs), respectively, and a temperature of 15±0.5°C for two to four weeks prior to the experiments. Bivalves were fed *ad libitum* by continuous addition of a commercial live algal blend (DTs Premium Blend Live Marine Phytoplankton, Coralsands, Wiesbaden, Germany) according to manufacturer's instructions (80 mL per 500 L ASW) by an automatic aquarium feeder.

Experimental exposures

Bivalves were transferred to air tight glass jars (2 oysters or 8 quahogs per 2 L ASW) and exposed to severe hypoxia (<0.01% O_2) for 24 h by aeration of ASW with pure nitrogen (Westfalen AG, Münster, Germany) at 15±0.5°C and the respective salinity. Oxygen concentration was monitored with an Intellical™ LDO101 Laboratory Luminescent/Optical Dissolved Oxygen Sensor (HACH, Loveland, CO, United States). After hypoxia exposure, a subset of bivalves was allowed to recover in normoxic ASW (21% O_2) for 1.5 h. The control group was maintained at normoxic conditions (21% O_2) in the recirculated temperature- controlled aquarium systems. Animals were dissected on ice and gill tissue (in all three species) and heart tissue (in *C. gigas* and *O. edulis*) were sampled immediately, flash frozen in liquid nitrogen and stored at -80°C until further analyses.

Metabolite extraction

All chemicals were purchased from Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Merck KGaA, Darmstadt, Germany)

Metabolites were extracted as described elsewhere (Götze et al., 2020) except for substituting dichloromethane (DCM) instead of trichloromethane for safety reasons (Amiel et al., 2019; Chen et al., 1981). Briefly, 40 to 50 mg tissue (fresh mass, FM) was homogenized in a methanol:DCM (1:1; MeOH-DCM) solution (Bligh and Dyer, 1959). The upper metabolite-MeOH phase was dried overnight in a SpeedVac (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 1550 rpm, 30°C and air pressure of 0.1 mbar.

The dried pellets from the gill tissue extracts were re-suspended in deuterated water (D_2O) containing 0.05 wt. % 3-(trimethylsilyl)proprionic-2,2,3,3-d4 acid sodium salt (TSP), as internal standard to a final concentration of 0.5 g mL^{-1} (gill samples) of the original FM used for extraction. For the heart samples, the dilution factor was increased to obtain sufficient amount of suspension for ^{1}H-NMR spectroscopy (*C. gigas*= 0.16 – 1 g mL^{-1}, *O. edulis*=0.1 – 0.3 g mL^{-1}), which was recalculated to a common final concentration of 1 g mL^{-1} of the original FM used for extraction.

^{1}H-NMR spectroscopy

For metabolite profiling, one-dimensional ^{1}H-NMR spectroscopy was used by means of an ultra-shielded vertical 0.4 T NMR spectrometer (Advance III HD 400 WB, Bruker-BioSpin GmbH, Billerica, MA, USA) and triple tuned ^{1}H-^{13}C-^{31}P-HRMAS NMR probe. Acquisition of metabolic profiles was done using the TOP-SPIN 3.2 software (TopSpin 3.2, Bruker-BioSpin GmbH, Rheinstetten, Germany). For metabolic profiling a Carr-Purcell-Meibook-Gill (CPMG) sequence was selected (as described in (Götze et al., 2020; Schmidt et al., 2017)).

Spectra were corrected by baseline, shim and phase and calibrated to internal standard TSP by means of the Chenomx NMR suite 8.1. Assignment of metabolites were done according to chemical shift of the NMR signal using Chenomx' database. Quantification of metabolites was based on the integration routine of Chenomx by calibration of the TSP signal to a concentration of 3.2 mmol L^{-1}. Signals of adenylates AMP, ADP and ATP were summed as differentiation of their profiles is problematic. Due to technical issues, the detected opine could not be precisely identified and is hereafter referred to as one "opine".

Statistical analysis

Metabolic profiles were statistically analysed using the online platform Metaboanalyst (Metaboanalyst 5.0, Canada). As metabolite data are generally non-normal distributed, values were log transformed to meet requirements of Gaussian normal distribution. Due to constant signal of adenylates across species and treatments, we decided against normalization to adenylate concentration. Metabolite data of heart samples were normalized to their specific dilution factor (as mentioned above).

Statistical comparison of the species was performed using control (normoxic) data to assess differences in metabolic profiles across bivalve species. The subsequent univariate statistical analysis assessed effects of H/R stress on metabolite profiles separately for each of the species studied.

Unsupervised principal component analysis (PCA) evaluated variances of samples and did not detect any outlier. Further, PCA was used to assess distinction of metabolic profiles and separation of experimental groups. Two components that explain the largest number of variances in the profiles are shown as 2D scoreplots, where the distance between samples represents the differences in metabolite patterns. Threshold cut-off for factor loadings was > 0.2 and < -0.2, respectively. Differences in levels of target metabolites due to species or H/R stress, respectively, were tested by one way ANOVA with species or oxygen regime as fixed factor. Tukey's Honestly Significant Differences (HSD) *post hoc* test (p-value < 0.05) was carried out to determine which pairs of means

were significantly different from each other. Statistical comparison of species-specific heart tissue metabolites was carried out by unpaired t-test (p-value < 0.05, FDR). Pearson correlation was used to evaluate correlation between all metabolites during H/R stress (with a maximum of 1). Significant changes in metabolite concentrations are presented in mmol L^{-1} as boxplots by means of RStudio [v. 1.2.5033 and R v 3.6.3, (R Core Team, 2020)].

Pathway enrichment analysis

We assessed metabolic pathways via pathway enrichment analysis (PEA). Data sets of gill and heart tissue were log-transformed as described above. PEA allows only two group comparison; thus we applied PEA on the following pairings: tissue-specific PEA dependent on species was tested against each species studied (*O. edulis* vs *A. islandica*, *C. gigas* vs *A. islandica*, *O. edulis* vs *C. gigas*). PEA of H/R stress was assessed within each species: hypoxic (H) and recovering (R) group were tested against controls (C), as well as against each other (C vs. H; C vs. R; H vs. R). The library of *Drosophila melanogaster* was chosen as reference pathway library to identify single pathways. We decided for this library as most metabolites could be assigned to those library pathways and as arthropoda were phylogenetically closest reference to mollusks from the available reference libraries. PEA was based on a global test of Bayesian generalized linear model to determine metabolite patterns associated to specific pathways. Pathway topological analysis assessed importance of each compound to specific pathway using relative betweenness centrality. Pathways were defined as relevant with a Pathway Impact > 0.4 and -log(p) > 1.3 (corresponds to p > 0.05). Additionally, pathways associated to plant or bacterial metabolism and pathways, where less than 30% of compounds could be assigned, were excluded as possible uncertain or false discovery.

Results

Species specific differences in the baseline profiles of the gill tissue metabolites

PCA of the gill metabolic profiles of the three studied bivalve species collected under the control (normoxic) conditions identified two principal components (PCs) explaining 37.7% and 17.5% of data variation, respectively **(Fig. 1A).** Species-specific metabolite profiles of the gill tissues were mostly separated along the PC1 axis, with *A. islandica* being shifted further to the positive values of PC1 relative to the two Ostreidae species (**Fig. 1A**). The PC1 showed high positive loadings of energy-related metabolite fumarate **(Supplementary Table S1).** PC1 had high negative loadings of organic osmolytes (betaine, homarine, glycine, hypotaurine), trimethylamine-N-oxide, alanine and imidazole. The two oyster species showed high spread of individual metabolite profiles along PC2 compared to the profile of the ocean quahog, where the individual samples were tightly grouped together. PC2 had high negative loadings of proprionate, phenylalanine and pi-methylhistidine (**Supplementary Table S1**). ANOVA revealed significant species-specific differences in osmolytes, metabolites associated with aerobic and anaerobic energy metabolism, signalling-related metabolites and AAs **(Table 1).**

PEA revealed five metabolic pathways differing in the gills of the three studied species under normoxic conditions (**Supplementary Fig. S6).** The taurine-hypotaurine-metabolism was significantly different between all three studied species (**Supplementary Fig. S6A-C).** The glutamine-glutamate-metabolism differed between *O. edulis* and the two other species (**Supplementary Fig. S6B,C).** Biosynthesis of arginine and alanine-aspartate- glutamate-metabolism showed differences in *C. gigas* gills relative to those of either *O. edulis* or *A. islandica* (**Supplementary Fig. S6A,B**). Furthermore, biosynthesis of phenylalanine, tyrosine, and tryptophan differed between the gills of *C. gigas* and *A. islandica.* (**Supplementary Fig. S6A**).

Table 1 ANOVA: Metabolite concentration in gill tissue of *C. gigas*, *O. edulis* and *A. islandica* under normoxic conditions. Superscript letters indicate significant differences in mean values according to Tukey's HSD post hoc test. Values that do not share a letter are significantly different ($p < 0.05$). Sample size was N = 10 for all studied species. Metabolites given in grey were not significantly different between the three studied species.

	Metabolite concentration [mmol L^{-1}]		
	Arctica islandica	***Crassostrea gigas***	***Ostrea edulis***
1,3-Diaminopropane	0.294 ± 0.080 ^{a}	0.873 ± 0.259 ^{b}	0.424 ± 0.089 ^{c}
4-Aminobutyrate (GABA)	0.060 ± 0.036 ^{a}	0.193 ± 0.088 ^{b}	0.110 ± 0.052 ^{b}
Acetate	0.064 ± 0.014 ^{a}	0.053 ± 0.019 ^{ab}	0.041 ± 0.010 ^{b}
Adenylates	0.175 ± 0.041 ^{a}	0.091 ± 0.055 ^{b}	0.211 ± 0.046 ^{a}
Alanine	0.300 ± 0.163 ^{a}	2.679 ± 0.844 ^{b}	0.468 ± 0.267 ^{a}
Aspartate	1.258 ± 0.261 ^{ab}	0.990 ± 0.212 ^{a}	1.629 ± 0.598 ^{b}
β-Alanine	0.197 ± 0.087 ^{a}	0.488 ± 0.135 ^{b}	0.355 ± 0.146 ^{b}
Betaine	1.266 ± 0.336 ^{a}	20.094 ± 2.867 ^{b}	25.236 ± 3.675 ^{c}
Choline	0.058 ± 0.027 ^{a}	0.171 ± 0.060 ^{b}	0.111 ± 0.029 ^{b}
Fumarate	0.005 ± 0.005 ^{a}	0.001 ± 0.002 ^{b}	0.005 ± 0.002 ^{a}

	Metabolite concentration [mmol L^{-1}]		
	Arctica islandica	*Crassostrea gigas*	*Ostrea edulis*
Glutamate	1.167 ± 0.627	1.173 ± 0.370	1.155 ± 0.136
Glutamine	0.344 ± 0.080 [a]	0.359 ± 0.136 [a]	0.170 ± 0.035 [b]
Glycine	0.225 ± 0.058 [a]	1.805 ± 0.406 [b]	0.376 ± 0.075 [c]
Homarine	0.393 ± 0.189 [a]	5.809 ± 0.909 [b]	4.101 ± 0.594 [c]
Homocysteine	0.242 ± 0.052 [a]	0.843 ± 0.286 [b]	0.565 ± 0.231 [c]
Hypotaurine	0.001 ± 0.001 [a]	0.007 ± 0.004 [b]	0.004 ± 0.002 [b]
Imidazole	0.018 ± 0.009 [a]	0.199 ± 0.147 [b]	0.063 ± 0.015 [b]
Isoleucine	0.018 ± 0.006 [a]	0.045 ± 0.019 [b]	0.020 ± 0.006 [a]
Lactate	0.020 ± 0.005 [a]	0.078 ± 0.017 [b]	0.025 ± 0.006 [a]
L-Arginine	0.445 ± 0.086	0.566 ± 0.174	0.457 ± 0.092
Leucine	0.035 ± 0.008 [a]	0.085 ± 0.031 [b]	0.050 ± 0.010 [c]
Lysine	0.076 ± 0.032 [a]	0.176 ± 0.055 [b]	0.111 ± 0.017 [c]
N-Acetylcysteine	0.087 ± 0.043	0.087 ± 0.043	0.101 ± 0.044
N-Acetylglutamine	0.070 ± 0.056 [a]	0.095 ± 0.046 [a]	0.024 ± 0.009 [b]
Nα-Acetyllysine	0.043 ± 0.018 [a]	0.097 ± 0.033 [b]	0.023 ± 0.008 [c]
O-Acetylcholine	0.029 ± 0.008 [a]	0.060 ± 0.021 [b]	0.036 ± 0.005 [a]
O-Phosphocholine	0.703 ± 0.172 [a]	0.154 ± 0.032 [b]	0.177 ± 0.053 [b]
Phenylalanine	0.005 ± 0.001	0.007 ± 0.008	0.008 ± 0.011
Propionate	0.014 ± 0.004	0.021 ± 0.012	0.012 ± 0.004
π-Methylhistidine	0.015 ± 0.005	0.071 ± 0.084	0.017 ± 0.017
Sarcosine	0.178 ± 0.061 [a]	0.197 ± 0.164 [a]	0.034 ± 0.010 [b]
sn-Glycero-3-phosphocholine	0.222 ± 0.074 [a]	0.604 ± 0.176 [b]	0.427 ± 0.109 [b]
Succinate	0.286 ± 0.373	0.032 ± 0.022	0.163 ± 0.227
Taurine	13.587 ± 2.277 [a]	26.571 ± 3.854 [b]	39.211 ± 6.090 [c]
Threonine	0.050 ± 0.010 [a]	0.158 ± 0.098 [b]	0.024 ± 0.007 [c]
Trimethylamine N-oxide	0.351 ± 0.132 [a]	2.568 ± 1.031 [b]	3.001 ± 1.136 [b]
Tyrosine	0.009 ± 0.005 [a]	0.035 ± 0.019 [b]	0.022 ± 0.014 [b]
UDP-glucose	0.250 ± 0.063 [a]	0.132 ± 0.041 [b]	0.338 ± 0.082 [c]
Valine	0.020 ± 0.006 [a]	0.071 ± 0.033 [b]	0.030 ± 0.006 [c]

Species-specific differences in the baseline profiles of the heart tissue metabolites

Heart samples were only available for the two oyster species (*O. edulis* and *C. gigas*). The heart metabolite profiles of the two oyster species collected under the normoxic conditions were well separated in the plane of the two first PCs (**Fig. 1B**). PC1 (34.8% of data variance) had high positive loadings of alanine, one opine, β-alanine, glycine, succinate and threonine, and high negative loadings of sn-glycero-3-phosphocholine. PC2 (22.1% of data variance) had high negative loadings of phenylalanine, hypotaurine, succinate and pi-methylhistidine **(Supplementary Table S1).**

Concentrations of energy-related metabolites (carnitine, alanine and one opine) were significantly lower in the normoxic heart of *O. edulis* compared to that of *C. gigas* (**Table 2**). Interestingly, carnitine and one opine were only detected in the heart tissue of *C. gigas* and *O. edulis*, but not in the gill of any of the three studied species. Concentrations of two osmolytes (glycine and taurine) showed opposite patterns in the two studied oyster species with taurine being higher in *O. edulis* and glycine in *C. gigas.* Concentrations of membrane related-metabolites O-phosphocholine and sn-glycero-3-phosphocholine were significantly higher in the heart of *O. edulis,* whereas choline concentrations were higher in the heart of *C. gigas*. Among the studied AAs and AA intermediates, aspartate and imidazole concentrations were significantly higher in the heart of *O. edulis,* whereas concentrations of β-alanine, threonine, glutamine, glutamate, N-acetylglutamine and sarcosine were significantly higher in *C. gigas* (**Table 2).**

PEA showed significant differences in the activity of arginine biosynthesis, glutamate-glutamine metabolism and alanine-aspartate-glutamate metabolism between the heart tissues of *O. edulis* and *C. gigas* (**Supplementary Fig. 8A**).

Table 2 ANOVA: Metabolite concentrations in heart tissue of *C. gigas* and *O. edulis* under normoxic conditions. Metabolite concentrations in bold, black font are significantly different between the two species according to unpaired t-test ($p < 0.05$).

	Metabolite concentration [mmol L^{-1}]	
	Crassostrea gigas	***Ostrea edulis***
1,3-Diaminopropane	1.594 ± 0.445	1.271 ± 0.445
4-Aminobutyrate (GABA)	0.201 ± 0.057	0.425 ± 0.369
Acetate	0.210 ± 0.099	0.302 ± 0.161
Adenylates	0.870 ± 0.233	0.775 ± 0.177
Alanine	9.751 ± 4.323	2.299 ± 1.796
Aspartate	2.751 ± 1.144	4.692 ± 1.311
β-Alanine	4.141 ± 2.515	1.115 ± 0.543
Betaine	55.591 ± 4.607	52.122 ± 8.651
Carnitine	0.714 ± 0.340	0.383 ± 0.114
Choline	0.323 ± 0.155	0.166 ± 0.047
Fumarate	0.018 ± 0.014	0.017 ± 0.008
Glutamate	4.879 ± 1.252	3.314 ± 1.001
Glutamine	0.609 ± 0.149	0.380 ± 0.099
Glycine	7.764 ± 2.298	1.338 ± 0.520
Homarine	17.266 ± 4.429	13.140 ± 3.501
Homocysteine	1.314 ± 0.531	1.273 ± 0.524
Hypotaurine	0.009 ± 0.006	0.011 ± 0.009
Imidazole	0.054 ± 0.032	0.146 ± 0.059
Isoleucine	0.097 ± 0.050	0.055 ± 0.037
Lactate	0.122 ± 0.025	0.086 ± 0.047
L-Arginine	1.623 ± 0.404	1.486 ± 0.432
Leucine	0.201 ± 0.088	0.124 ± 0.074
Lysine	0.357 ± 0.053	0.357 ± 0.112
N-Acetylcysteine	0.117 ± 0.065	0.077 ± 0.040
N-Acetylglutamine	0.157 ± 0.064	0.092 ± 0.031
Nα-Acetyllysine	0.127 ± 0.047	0.083 ± 0.041
O-Acetylcholine	0.165 ± 0.105	0.135 ± 0.100
O-Phosphocholine	0.230 ± 0.267	0.497 ± 0.338
Opine	0.684 ± 0.337	0.161 ± 0.156
Phenylalanine	0.027 ± 0.023	0.035 ± 0.018
Propionate	0.047 ± 0.009	0.037 ± 0.016
π-Methylhistidine	0.118 ± 0.093	0.183 ± 0.182
Sarcosine	0.147 ± 0.044	0.085 ± 0.029
sn-Glycero-3-phosphocholine	0.203 ± 0.088	0.825 ± 0.406
Succinate	0.479 ± 0.656	1.109 ± 1.357
Taurine	45.233 ± 6.092	75.722 ± 13.929
Threonine	0.478 ± 0.155	0.223 ± 0.123
Trimethylamine N-oxide	5.169 ± 2.702	6.735 ± 2.566
Tyrosine	0.128 ± 0.062	0.168 ± 0.122
UDP-glucose	0.168 ± 0.049	0.193 ± 0.037
Valine	0.184 ± 0.109	0.110 ± 0.070

Impact of H/R stress on metabolic profiles of the gill tissue

C. gigas. In *C. gigas* gills, two first PCs explained, respectively, 36.3% and 19.2% of metabolite variation within and between different oxygen regimes (**Fig. 2A**). PC1 had high negative loadings of energy-related metabolites fumarate and propionate, phenylalanine and pi-methylhistidine. PC2 showed high positive loadings of pi-methylhistidine, and high negative loadings of phenylalanine, succinate and imidazole (**Supplementary Table 1**). There was a considerable overlap of the gill metabolite profiles between the groups (control, hypoxia and reoxygenation), with the control (normoxic) group showing greater individual variation than the groups exposed to hypoxia or post-hypoxic recovery (**Fig. 2A**).

Analysis of individual metabolites in the gill of *C. gigas* showed a significant increase in the levels of fumarate and glutamate during post-hypoxic recovery whereas succinate levels increased under hypoxia and returned back to the baseline levels after 1.5 h reoxygenation (**Fig. 3 A-C**). All other metabolites detected in the present study were not significantly changed by H/R stress in gill tissue of *C. gigas*.

PEA showed alterations of the alanine-aspartate- glutamate-metabolism, and biosynthesis of phenylalanine, tyrosine, and tryptophan in the gills of hypoxia-exposed *C. gigas* compared to the controls (**Supplementary Fig. 7A).** Reoxygenation led to changes in arginine biosynthesis, glutamate-glutamine-metabolism and alanine-aspartate-glutamate-metabolism compared to both control and hypoxia-exposed oysters (**Supplementary Figure 7B**). Furthermore, reoxygenation altered biosynthesis of phenylalanine, tyrosine and tryptophan compared to the control **(Supplementary Fig. 7C**).

Pearson correlation analysis of metabolite concentrations across all oxygen treatments showed three clusters of positively correlated metabolites in *C. gigas* gills (**Supplementary Figure S1**)**.** Thus, tissue levels of osmolytes (glycine, hypotaurine, β-alanine, and alanine), a β-alanine precursor 1,3-diaminopropane, branched chain AAs (BCAAs: valine, leucine and isoleucine), glutamate, and threonine were positively correlated with each other. High positive correlation was also observed among the gill tissue levels of cytoprotective osmolytes taurine, betaine and homarine and aspartate. Choline, O-phosphocholine, N-acylcysteine, and sn-glycero-3-phosphate formed another positively correlated cluster. Strong negative correlations were detected between several AA derivatives (lysine, pi-methylhistidine, sarcosine), trimethylamine N-oxide (TMAO), and anaerobic metabolites (fumarate, lactate and propionate), on one hand, and membrane-associated metabolites (choline and O-phosphocholine), energy-related metabolites (succinate, UDP- glucose, adenylates) and imidazole, on the other (**Supplementary Figure S1**).

O. edulis. PCA analysis of the gill metabolite profiles of *O. edulis* identified the first two principal components explaining 30.8% (PC1) and 26.2% (PC2) of the data variation (**Fig. 2B**). Positive loading of PC1 included fumarate, pi-methylhistidine and phenylalanine, while PC2 had negatively loadings of anaerobic metabolites succinate, alanine and AA phenylalanine. High positive loadings of PC2 included fumarate (**Supplementary Table 1**). Hypoxia exposure led to the shift in the

metabolite profiles of the gills towards more negative PC2 values, which returned to the normoxic values during reoxygenation (**Fig. 2B**).

ANOVA showed that anaerobic intermediates succinate, acetate, L-arginine and alanine were significantly upregulated by hypoxia in *O. edulis* gills (**Fig. 4A, D, I, M**), while aspartate levels decreased (**Fig. 4G**). During reoxygenation, concentrations of succinate, alanine and aspartate returned to normoxic baseline levels (**Fig. 4D, G, M**), while acetate and L-arginine levels remained elevated (**Fig. 4A, I**). Fumarate levels did not change during hypoxia but significantly increased during reoxygenation (**Fig. 4K**). Concentrations of AAs leucine, isoleucine, threonine, lysine and valine as well as glutamate and glutamine significantly increased during hypoxia and remained elevated during reoxygenation in *O. edulis* gills (**Fig. 4B, C, E, F, H, J, L**).

PEA showed shifts in glutamate-glutamine metabolism and alanine-aspartate-glutamate metabolism in the gill of hypoxia exposed *O. edulis* compared with the normoxic controls (**Supplementary Fig. 7D**). Reoxygenation altered arginine biosynthesis and alanine-aspartate-glutamate metabolism relative to both hypoxia and normoxia **(Supplementary Fig. 7E)** and glutamate-glutamine-metabolism relative to the normoxic control (**Supplementary Fig. 7F).**

Pearson correlation analysis of metabolite concentrations across all oxygen treatments revealed three clusters of metabolites showing strong positive correlations with each other: 1) a cluster of proteinogenic amino acids including BCAAs leucine, isoleucine and valine, as well as glutamate, glutamine and threonine; 2) a cluster including L-arginine, glycine, a neurotransmitter 4-aminobutyrate (GABA), acetate, and AA acetyl derivatives Nα-acetyllysine, and N-acetylglutamine; 3) a cluster including osmolytes homarine, betaine, taurine, an osmolyte precursor 1,3-diaminopropane, sarcosine, and membrane-associated metabolites choline and O-phosphocholine (**Supplementary Figure S2**). Additionally, anaerobic end products alanine and succinate were positively correlated. Negative correlations were found between several AAs including BCAAs, glutamine, glutamate, threonine, lysine, as well as N-acetylcysteine, O-acetylcholine and adenylates, on one hand, and energy intermediates (lactate, sn-glycerol-3-phosphate, UDP-glucose), pi-methylhistidine, imidazole and TMAO, on the other (**Supplementary Figure S2**).

A. islandica. . The first two principal components of the PCA explained 31.6% (PC1) and 17.8% (PC2) of the data variation in the metabolite profiles of *A. islandica* gill (**Fig. 2C**). PC1 showed strong negative loadings of aromatic AAs tyrosine and phenylalanine as well as fumarate, while PC2 had strong positive loadings of succinate, glutamate and alanine and AAs lysine and tyrosine (**Supplementary Table 1**). Control group was spread along PC2 axis in the PCA biplot, whereas hypoxic and post hypoxic recovery were mostly scattered along PC1. Samples from the hypoxia and reoxygenation treatments in *A. islandica* showed less overlap with the control group compared to the two oyster species studied, with the H/R-exposed group displaying a shift towards higher positive PC2 values relative to the control (**Fig. 2C**).

Levels of the anaerobic end products succinate and alanine, as well as some proteinogenic AAs (valine, glutamate) and the osmolyte taurine were significantly upregulated in the gills of *A. islandica* exposed to hypoxia and reoxygenation (**Fig. 5A-E**).

PEA showed that hypoxia and reoxygenation affected alanine-aspartate-glutamate-metabolism and glutamate-glutamine-metabolism in the gill of *A. islandica* compared to the normoxic control (**Supplementary Fig. 7G,H**). Reoxygenation also changed taurine-hypotaurine-metabolism in the gill compared to the control (**Supplementary Fig. 7I**). No significant differences in metabolic pathways between hypoxia and reoxygenation were found in the gills of *A. islandica.*

Pearson correlation analysis of metabolite concentrations across all oxygen treatments showed a large cluster of positively correlated metabolites including osmolytic and proteinogenic AAs glycine, taurine, betaine, lysine, glutamate, alanine, valine, leucine, isoleucine, and threonine, as well as succinate (**Supplementary Figure S3**). A smaller cluster of positively correlated metabolites included Nα-acetyllysine, N-acetylcysteine, GABA, and 1,3-diaminopropane. Concentrations of fumarate, acetate, imidazole, aspartate, pi-mehthylhistidine, tyrosine and phenylalanine were negatively correlated with acetyl derivatives Nα-acetyllysine, N-acetylcysteine, N-acetylglutamine), β-alanine and its precursor 1,3-diaminopropane, membrane-associated metabolites (choline and O-phosphocholine), as well as GABA, sarcosine and sn-glycerol-3-phosphate (**Supplementary Figure S3**).

Impact of H/R stress on metabolite profiles of the heart tissues

C.gigas. PCA analysis of the heart metabolite profiles of *C. gigas* identified PC1 and PC2 explaining 22% and 17.5% of variance, respectively (**Fig. 6A**). Unlike in the gills, metabolic profiles of the heart were clearly separated between hypoxia and reoxygenation exposed animals. Negative loadings of both PC1 and PC2 were dominated by succinate, while PC1 also had high negative loadings of O-phosphocholine. Positive loadings of PC1 were from aspartate, hypotaurine and pi-methylhistidine, while positive loadings of PC2 included alanine, carnitine, fumarate, glutamate, O-phosphocholine and one opine (**Supplementary Table 1**).

Similar to findings in the gills of *C. gigas*, heart levels of succinate increased during hypoxia, and those of fumarate during reoxygenation (**Fig. 7B,C**). Aspartate levels significantly decreased in the heart of *C. gigas* exposed to hypoxia and returned to baseline levels after reoxygenation (**Fig. 7A**).

PEA showed that hypoxia and reoxygenation changed activity of arginine biosynthesis and alanine-aspartate-glutamate-metabolism in *C. gigas* heart compared to the normoxic control (**Supplementary Fig. 8B, C**). Transition from hypoxia to reoxygenation was also characterized by alterations in those two pathways (**Supplementary Fig. 8D**).

In the heart of *C. gigas*, strong positive correlations were found between BCAAs and threonine, as well as between osmolytes betaine, taurine, glycine, and homarine (**Supplementary Figure S4**). Glutamine, alanine and adenylates were also strongly positively correlated with each other. No large clusters of metabolites with strong negative correlations were found (**Supplementary Figure S4**).

O. edulis. The biplot of the heart metabolite profiles of *O. edulis* in the plane of the first two principal components (34.9% and 15% of data variation, respectively) showed no clear separation between oxygen treatments (**Fig. 6B)**. PC1 had high positive loadings of alanine, glycine, hypotaurine, phenylalanine, succinate, valine and pi-methylhistidine. Alanine, one opine and succinate had high

negative loadings on PC2. Positive loadings on PC2 included GABA, hypotaurine and UDP-glucose (**Supplementary Table 1**).

Among studied heart metabolites, only aspartate showed significant effects of H/R stress in *O. edulis*, with levels decreasing during hypoxia and being restored after reoxygenation **(Fig. 8).**

According to PEA, hypoxia affected arginine biosynthesis and taurine-hypotaurine-metabolism in the heart of *O. edulis* compared to normoxic control **(Supplementary Fig. 8E**), while reoxygenation altered arginine biosynthesis and glutamate-glutamine-metabolism compared to the normoxic control (**Supplementary Fig. 8F**). Shift from hypoxia to reoxygenation showed effects on arginine biosynthesis, alanine-aspartate-glutamate-metabolism, taurine-hypotaurine-metabolism, glutamate-glutamine-metabolism, and phenylalanine-tyrosine-tryptophan-biosynthesis in the heart of *O. edulis* (**Supplementary Fig. 8G**).

Three clusters with strong positive correlations of metabolite concentrations were identified in the heart of *O. edulis* across all oxygen regimes: 1) AAs and their derivatives, including glutamate, glutamine, tyrosine, threonine, isoleucine, leucine, valine, alanine, and homocysteine, along with O-acetylcholine and lactate; 2) a cytoprotective osmolyte cluster comprising homarine, taurine, betaine, and TMAO, as well as imidazole; 3) a cluster including various acyl derivatives such as N-acetylcysteine and N-acetylglutamine, osmolytes like beta-alanine and glycine, as well as sn-glycero-3-phosphate, carnitine, and GABA (**Supplementary Figure S5**). The concentrations of metabolites in cluster 2 (cytoprotective osmolytes) and cluster 3 were negatively correlated with each other (**Supplementary Figure S5**).

Discussion

Untargeted metabolic profiling of the gills and the heart tissues showed species -specific differences in the baseline levels of different metabolites as well as in the metabolic responses to H/R exposure among three studied hypoxia-tolerant species of marine bivalves, including two species of Ostreidae (*C. gigas* and *O. edulis*) and a species of the Arcticidae family (*A. islandica*).

Species specific differences in metabolite tissue metabolite profiles

The gills in marine bivalves are the primary organs for respiration, osmoregulation, feeding, and AA uptake, showing high metabolic flexibility (Adzigbli et al., 2022; Gosling, 1992; Kennedy, 1996). The gill metabolite profiles of *C. gigas* and *O. edulis* showed more similarity with each other, than with those of *A. islandica*. Species-specific differences in the normoxic metabolite profiles of the gills were mostly related to the concentrations of osmolytes, energy-related metabolites and a membrane-associated compound O-phosphocholine. With regard to the osmolytes, these species-specific differences might reflect acclimation to different salinities in oysters (salinity 33) and quahogs (salinity 15). Organic osmolytes including free AAs and TMAO play an important role in isosmotic cell volume regulation of bivalves, and their intracellular concentrations are regulated to match the shifts in ambient salinity (Podbielski et al., 2022; Yancey, 2005). Across invertebrate taxa, taurine, betaine, glycine, proline, serine, and alanine are key osmolytes, with taurine, betaine, and glycine being the most quantitatively significant in mollusks (Podbielski et al., 2022). Therefore, lower levels of osmolytes - including taurine, betaine, glycine, β-alanine, homarine, and TMAO – in the gills of *A. islandica* compared to *C. gigas* and *O. edulis*, likely reflect acclimation to a lower osmolarity environment in the former species. The role of osmoregulation in interspecific metabolic differences is further supported by significant variations in the taurine-hypotaurine metabolism in the gills of the three bivalve species.

While salinity acclimation likely plays a key role in the observed interspecific differences in the gill tissue metabolome, we also found significant differences in metabolite profiles in both the gills and heart between the two oyster species, despite their exposure to the same salinity conditions. Additionally, there were species-specific shifts in several metabolic pathways among all three studied species that did not correlate with salinity acclimation. These findings suggest the presence of intrinsic metabolic variability, reflecting the ecological and evolutionary differences between the two oyster species and the more distantly related *A. islandic*a (Guo et al., 2018). Thus, *O. edulis* gills and heart showed lower concentrations of glutamine and glutamate and significant differences in glutamine-glutamate metabolism compared with *C. gigas* and *A. islandica*. This may indicate that *O. edulis* is less reliant on glutamate metabolism for ammonia detoxification and nitrogen excretion than the other two species under normoxic conditions. Additionally, differences in alanine-aspartate-glutamate metabolism and arginine biosynthesis were observed in the gills of *C. gigas* compared to the other two species, suggesting differences in gluconeogenesis and substrate-level phosphorylation pathways. Species-specific metabolic differences were also found among closely related species of bivalves, as was shown for mytilids *Mytilus edulis* and *Mytilus trossulus* (Sokolova et al., 2024), the venerid clams *Venerupis decussata* and *Venerupis philippinarum* (Rocha et al., 2013), and

freshwater unionid clams *Lampsilis cardium* and *Lampsilis siliquoidea* (Waller et al., 2023). These findings highlight that, while the core intermediate metabolism of bivalves is generally conserved, species-specific differences are common reflecting both metabolic adaptations and inherent constraints and warranting further investigation to fully understand their implications.

Interestingly, two metabolites - carnitine and one opine - were detected exclusively in the heart tissue, but not in gill tissue of the studied bivalve species. Carnitine functions as a shuttle for fatty acids into mitochondria facilitating their β-oxidation to produce energy equivalents ($FADH_2$ and NADH) and acetyl-CoA (Moyes et al., 1990a). In vertebrates, mitochondria in highly aerobic tissues like the brain prefer carbohydrates, while liver mitochondria rely more on fatty acid oxidation, and heart and muscle mitochondria use both pathways (Farhat et al., 2021; Gusdon et al., 2015; Kodde et al., 2007). In bivalve mitochondria, capacity for β-oxidation varies between species and tissues but is generally low (Ballantyne and Storey, 1983; Guderley et al., 1995). Thus, mitochondria from the hearts of squid *Loligo opalescens* and clam *Mercenaria mercenaria* lack the capacity to oxidize palmitoyl carnitine; however, it remains unknown whether they can oxidize fatty acids in general (Moyes et al., 1990b). In contrast, mitochondria isolated from the gill and digestive gland of *C. gigas* were able to oxidize palmitoyl carnitine, though this oxidation in the digestive gland resulted in extremely high ROS production (Adzigbli et al., 2022). Our present findings suggest that, despite the ability of *C. gigas* mitochondria to oxidize palmitoyl carnitine *in vitro*, this pathway may be less relevant *in vivo* due to the lack of carnitine. The presence of carnitine in the hearts of both studied oyster species indicates that this organ may utilize carnitine-dependent fatty acid oxidation for ATP production, albeit likely at a low rate due to the mitochondria's limited capacity for β-oxidation (Moyes et al., 1990b). Despite tissue-specific limiting factors - such as the absence of carnitine in the gills and the low activity of mitochondrial β-oxidation in the heart - the overall low capacity for fatty acid oxidation in bivalve tissues may help mitigate the toxic effects of fatty acids and their oxidation intermediates, especially under hypoxic conditions (Moon et al., 1985). The metabolic role of opines in normoxic oyster hearts warrants further investigation, as these metabolites are typically produced anaerobically in bivalves under conditions that demand high ATP flux (Livingstone, 1991).

H/R effects on aerobic and anaerobic energy metabolism

Metabolites involved in energy metabolism showed a strong response to H/R stress in all studied bivalves, with the magnitude of this response varying between species and tissues. As expected, severe hypoxia (24 h at <0.01% O_2) activated anaerobic metabolism in all three studies species. However, the anaerobic flux, as indicated by the extent of accumulation of anaerobic end products, varied among the three studied species. The European oyster *O. edulis* showed the highest degree of accumulation of anaerobic end products including alanine, succinate and acetate in the gill during hypoxia (increased by 0.674, 1.023 and 0.030 mmol L^{-1}). Alanine is an early indicator of anaerobiosis in marine bivalves, with its accumulation typically preceding that of succinate (Bayne, 2017). During the initial stages of anaerobiosis, alanine is formed from pyruvate and aspartate through the coupled activities of alanine aminotransferase and aspartate aminotransferase (Müller et al., 2012). Similar pattern of accumulation of alanine and depletion of aspartate was found during hypoxia in tissues of

other mollusks including *M. edulis* (Haider et al., 2020) and *Littorina saxatilis* (Sokolova and Pörtner, 2001). The depletion of the aspartate pool in *O. edulis* gills observed in our study is thus consistent with the activation of this early pathway of anaerobic ATP production. Furthermore, accumulation of succinate and acetate in the gill of hypoxia-exposed *O. edulis* indicates onset of mitochondrial fumarate dismutation typical for prolonged anaerobiosis (Müller et al., 2012). Interestingly, in the heart of *O. edulis*, hypoxia led to aspartate depletion but no accumulation of alanine, succinate or acetate indicating that the heart tissue did not activate anaerobic pathways to the same extent as the gills. Given the small biomass of the bivalve heart, it is possible that the energy needs of this organ under hypoxia could be supplied by export of phosphagens or ATP from other tissues (Chaudry, 1982; McMahon et al., 2021; Yi-Dan et al., 2021).

In *A. islandica* gills, an increase in alanine levels was also found in hypoxia, but it was relatively mild (increased by 0.170 mmol L^{-1} relative to the normoxia) and not associated with a significant depletion of aspartate. Succinate was the main anaerobic end product accumulating in *A. islandica* gills during hypoxia with 0.442 mmol L^{-1} increase over the normoxic baseline. In *C. gigas*, no significant depletion of aspartate or accumulation of alanine was observed during hypoxia. Instead, succinate was the only anaerobic end product that accumulated in both the gill (increased by 0.184 mmol L^{-1} relative to the normoxia) and the heart (increased by 1.148 mmol L^{-1} relative to the normoxia). This suggests that *C. gigas* and *A. islandica* may rely more heavily on the more efficient mitochondrial anaerobiosis, which yields higher ATP per unit of glucose compared to cytosolic alanine production (Zwaan, 1983-1988). Succinate is a common anaerobic end product of marine bivalves (Bayne, 2017), and its accumulation has been reported in oysters *C. virginica* (Ivanina et al., 2010) and *C. gigas* (Haider et al., 2020; Sokolov et al., 2019), soft-shell clams *Mya arenaria* (Ouillon et al., 2022) and mussels *M. edulis* (Haider et al., 2020). The relatively low levels of succinate accumulation in *C. gigas* and *A. islandica* compared to *O. edulis* found in our present study could indicate a more effective suppression of metabolic rate in the former two species. This notion is supported by an earlier study on three closely related *Littorina* species with varying hypoxia tolerance, which found that greater hypoxia tolerance was associated with a better ability to suppress ATP turnover and slower accumulation of anaerobic end products, including succinate (Sokolova et al., 2000a). Additionally, acetate, a late-stage anaerobic end product (Bayne, 2017; Müller et al., 2012), did not accumulate in the gill or heart of *C. gigas* and even decreased in the gills of *A. islandica*. This is consistent with the hypothesis of strongly suppressed ATP turnover under hypoxia in these two species and could suggest a limitation in the availability of precursors such as acetyl-Coenzyme A (CoA).

The alterations in concentrations of anaerobic end products (alanine, succinate, and acetate) and an anaerobic substrate (aspartate) during reoxygenation differed between the two Ostreidae species and *A. islandica*. In oysters, tissue concentrations of these anaerobic end products and substrates rapidly returned to normoxic baseline levels within 1.5 hours of reoxygenation in both the gills and the heart, with the exception of acetate levels in the gills of *O. edulis*, which remained elevated. Studies on intertidal bivalves, including oysters (*C. gigas*), soft-shell clams (*M. arenaria*), and blue mussels (*M. edulis*), have shown that their mitochondria possess a high capacity for succinate oxidation,

which is further enhanced by H/R stress (Adzigbli et al., 2022; Ouillon et al., 2021; Sokolov et al., 2021). This post-hypoxic upregulation of succinate oxidation may be part of an adaptive mitochondrial phenotype in hypoxia-tolerant species, facilitating the recovery of metabolic homeostasis disrupted by oxygen deficiency (Bundgaard et al., 2019a; Bundgaard et al., 2019b). This notion is supported by the observed increase in fumarate levels during post-hypoxic recovery in the gill tissue of the two oyster species, indicating activation of succinate metabolism through the forward tricarboxylic acid (TCA) cycle. The delayed recovery of acetate levels in *O. edulis* gills might be due to rapid succinate oxidation, which could inhibit succinate thiokinase activity and limit CoA regeneration, essential for acetate metabolism (Czumaj et al., 2020). A shift of the TCA cycle during the recovery period was earlier shown in *C. gigas* exposed to short-term (12 h) intermittent hypoxia indicating the key role of this metabolic pathway in post-hypoxic recovery of oysters (Bruhns et al., 2023).

Unlike in the two oyster species, tissue levels of anaerobic end products alanine and succinate continued rising during the post-hypoxic recovery in the gill of *A. islandica* indicating continuing reliance on anaerobic pathways of ATP production. Similarly, high succinate accumulation during hypoxia and delayed post-hypoxic recovery of succinate levels was reported for *M. edulis* following exposure to 1 and 6 days of severe hypoxia (Haider et al., 2020). Hypoxia-tolerant ocean quahogs are known to have high anaerobic capacities in their tissues (Oeschger, 1990; Oeschger and Storey, 1993; Taylor, 1976) and able to sustain prolonged periods of hypoxia inducing MRD (Strahl et al., 2011). However, while the mitochondria of *A. islandica* are robust under hypoxia, they are sensitive to sudden reoxygenation, as shown by a decrease in OXPHOS capacity driven by succinate (Steffen et al., 2021). This weakened ability for succinate oxidation may explain the delayed restoration of baseline succinate levels in *A. islandica*. Elevated alanine levels further suggest that major alanine consumers, such as gluconeogenesis and protein synthesis, remain slow during reoxygenation in this species. Overall, a 1.5-hour aerobic recovery period appears insufficient for *A. islandica* to fully recover from MRD and reintegrate anaerobic end products to restore metabolic homeostasis. This is also evident in the lack of any pathway-level shifts in the gill metabolism of *A. islandica* between hypoxia and reoxygenation (**Supplementary Fig. 7H**). This lack of capacity for rapid aerobic recovery may reflect adaptations specific to *A. islandica*, which, unlike intertidal species regularly exposed to predictable cycles of hypoxia and reoxygenation, typically encounters short-term, self-imposed hypoxia by burying into sediments (Strahl et al., 2011) and only rarely and unpredictably experiences prolonged hypoxia, such as that caused by seasonal oxygen depletion in its benthic habitats.

Both hypoxia exposure and post-hypoxic recovery modulated gluconeogenesis in the gills and heart of the three studied bivalve species, as indicated by shifts in alanine-aspartate-glutamate metabolism across all hypoxia-and reoxygenation-exposed groups compared to normoxic controls, with the exception of the hypoxic heart of *O. edulis.* During hypoxia, this shift likely reflects the activation of the aspartate-alanine anaerobic pathway for ATP generation (Bayne, 2017; Müller et al., 2012), while during post-hypoxic recovery, gluconeogenesis is stimulated to restore energy reserves depleted during hypoxia (Ouillon et al., 2022; Picard et al., 2014). Furthermore, post-hypoxic

recovery led to an alteration of arginine biosynthesis pathways in the gills and the heart of the two studied oyster species but not in the gill of *A. islandica*. Arginine is an important intermediate in the urea cycle; however, since urea contributes only minimally to nitrogen excretion in bivalves (Bayne, 2017), changes in arginine biosynthesis are unlikely related to the urea cycle. Alternatively, shifts in arginine biosynthesis may reflect changes in the synthesis and breakdown of phospho-L-arginine (PLA), a phosphagen that serves as a rapid ATP source to maintain cellular ATP levels (Bayne, 2017). Since we measured a total phosphagen and aphosphagen pool (L-Arg + PLA), we cannot tell if PLA was indeed broken down. Taken together, these findings are consistent with a previous study on the combined effects of temperature and hypoxia on the gill metabolome of *C. gigas*, which demonstrated enhanced carbohydrate metabolism, altered arginine biosynthesis, and changes in the TCA cycle during 12-hour emersion/immersion cycles (Bruhns et al., 2023).

H/R effects on amino acid metabolism

Hypoxia and reoxygenation affected glutamate-glutamine metabolism in the gills and heart of *O. edulis* and the gills of *A. islandica*. In *O. edulis*, both glutamine and glutamate levels increased in the gills during hypoxia and reoxygenation, whereas *A. islandica* gills showed elevated glutamate levels specifically during hypoxia. In the heart of *O. edulis*, metabolic alterations were more subtle, with no significant changes in individual AA concentrations. In contrast, *C. gigas* showed no significant changes in the glutamate-glutamine pathway in the gills or the heart, although glutamate levels in the gills rose during post-hypoxic recovery. Similarly, earlier studies reported elevated glutamate levels in *C. gigas* gills during post-hypoxic recovery, while both glutamine and glutamate were upregulated during hypoxia in *M. edulis* (Haider et al., 2020). Upregulation of glutamate-glutamine metabolism may indicate its role in the detoxification of ammonia, a toxic byproduct of metabolism (Limón et al., 2021). Glutamate dehydrogenase catalyses the conversion of ammonia and α-ketoglutarate into glutamate, which can then be used in the synthesis of other AAs or enter the glutamine synthesis pathway (Voss et al., 2021). Glutamine synthetase further detoxifies ammonia by combining it with glutamate to form glutamine (Hakvoort et al., 2017). Upregulated glutamate levels during recovery in *C. gigas* might reflect increased glutamate formation via this process in order to deplete toxic ammonia. The increased glutamate levels during hypoxia (in *O. edulis* and *A. islandica*) and recovery (in both oyster species) together with increased glutamine levels during hypoxia (only *O. edulis*) suggests activation of ammonia detoxification mechanisms. Elevated glutamate may also have protective effects, as *in vitro* studies on *C. gigas* mitochondria showed that glutamate oxidation has mitigating effects on ROS production during H/R stress (Adzigbli et al., 2022).

The concentrations of most AAs studied in the gills and heart of the three bivalve species remained stable during hypoxia and reoxygenation, indicating robust regulation of AA homeostasis. This stability may reflect a downregulation of protein turnover, serving as an energy-saving mechanism during hypoxia. Suppression of protein synthesis is a common response to hypoxia in intertidal mollusks (Sokolova, 2018; Storey and Storey, 2004). Regulation of the protein breakdown during hypoxia and reoxygenation is more complex due to the need to balance energy conservation with the

removal of damaged proteins (Sokolov et al., 2019; Sokolov et al., 2021; Steffen et al., 2020). The two exceptions to the general stability of AA profiles were the shifts in BCAAs in the gills of *O. edulis* and taurine levels in the gills of *A. islandica* during hypoxia and reoxygenation. The levels of essential branched-chain AAs isoleucine, leucine and valine were elevated during hypoxia and reoxygenation in gills of *O. edulis.* Two other essential AAs, lysine and threonine, were also upregulated in the gills of *O. edulis* during H/R stress. Since these AAs cannot be synthesized by mollusks and must be obtained through their diet (Fitzgerald and Szmant, 1997), and considering that the bivalves were not fed during hypoxia exposure, the observed increase likely indicates either increased protein breakdown or reduced utilization of these AAs. In *A. islandica* gills, valine was the only essential AA to show a modest increase during H/R stress, whereas *in C. gigas*, no proteinogenic AAs exhibited elevated levels under these conditions. Earlier studies showed that H/R stress suppresses protein breakdown in hypoxia-tolerant bivalves like *M. mercenaria* and *C. gigas*, whereas hypoxia-intolerant species such as *M. edulis, Anadara inaequivalvis*, and *Argopecten irradians* show increased protein breakdown (Bruhns et al., 2023; Haider et al., 2020; Ivanina et al., 2016; Soldatov et al., 2009). Thus, elevated levels of essential AAs during H/R stress in the gill of *O. edulis* may indicate that this species' protein homeostasis might be more sensitive to oxygen fluctuations due to its greater reliance on protein breakdown for energy maintenance compared with *C. gigas* and *A. islandica*.

Hypoxia/reoxygenation (H/R) stress altered taurine-hypotaurine metabolism in the gills of *A. islandica*, as shown by pathway enrichment analysis. This was marked by a significant increase in taurine concentration during hypoxia, which partially returned to baseline during reoxygenation. Taurine, a key osmolyte (Yancey, 2005) is known for its mitoprotective effects, enhancing mitochondrial function and reducing ROS production in marine bivalves (Sokolov and Sokolova, 2019). Elevated taurine levels are linked to greater hypoxia tolerance in marine bivalves; thus, hypoxia-tolerant species like *C. gigas* maintain high and relatively stable taurine levels during hypoxia, while taurine depletion is observed in less tolerant species like *M. edulis* (Bruhns et al., 2023; Haider et al., 2020; Zhang et al., 2012). The upregulation of taurine in *A. islandica* gills may contribute to the cytoprotection of this highly hypoxia-tolerant species. The increase in taurine in hypoxia is less likely due to its osmoregulatory properties, as no osmotic stress was experienced by the quahogs during our experimental exposures. Interestingly, tissue levels of different osmolytes exhibited distinct patterns in oysters and the quahog, reflecting species-specific roles of compatible osmolytes in cellular osmotic homeostasis (Podbielski et al., 2022). In *A. islandica* gills, taurine levels were positively correlated with glycine, and glycine levels also tended to correlate positively with betaine. Conversely, in the gills of oysters, taurine and glycine levels showed negative correlations, while glycine was positively correlated with betaine, homarine, and β-alanine. In *C. gigas* heart glycine, taurine, betaine, homarine, and β-alanine levels were positively correlated, while in *O. edulis* heart, glycine was only positively correlated with β-alanine, and negatively – with other osmolytes. These findings suggest tissue- and species-specific differences in the mechanisms of intracellular osmoregulation of the studied bivalves.

Conclusion

Metabolic profiling of gill and heart tissue of three marine bivalve species demonstrated differences in regulatory abilities of important metabolic pathways to maintain and recover energy homeostasis during and after oxygen deficiency. Clear differences between metabolic profiles of the species indicate key roles of regulation of metabolic pathways in hypoxia tolerance in marine bivalves.

Species-specific differences were caused mainly by osmolytes indicating the different salinity acclimation of oysters (salinity 33) and ocean quahogs (salinity 15). However, further evidence of intrinsic metabolic variability demonstrates a conserved core intermediate metabolism of marine bivalves, which is modulated by species-specific metabolic adaptation and inherent constraints.

Aerobic and anaerobic metabolism showed strong response to H/R stress in all studied species. As expected, hypoxia induced anaerobic metabolism in gill of all three studied species, but metabolic evidence (accumulation of anaerobic end products) varied between species and was strongest in *O. edulis*. In contrast, *C. gigas* and *A. islandica* relied more on mitochondrial anaerobiosis, which yields more ATP than classic cytosolic alanine production. This indicates a more efficient MRD in both, *C. gigas* and *A. islandica,* during H/R stress. Furthermore, metabolic regulation differed between the three studied species during reoxygenation. *C. gigas* and *O. edulis* showed high capacity to reintroduce succinate accumulated during hypoxia into energy metabolic pathways to recover energy homeostasis during post-hypoxic recovery. This might be associated to the strong ability of bivalve mitochondria for succinate-driven respiration during H/R stress (Adzigbli et al., 2022; Ouillon et al., 2021). Despite of its strong hypoxia tolerance, *A. islandica* remained highly reliant on MRD and anaerobiosis during post-hypoxic recovery. Its weak ability of succinate oxidation results in low capacity for rapid aerobic recovery in *A. islandica*, which resembles its susceptibility to sudden reoxygenation seen previously in mitochondrial functions (Steffen et al., 2021).

Besides energy metabolism, H/R stress affected concentrations of AAs in all three studied species. Strong regulation of glutamate-glutamine metabolism indicated activation of ammonia detoxification processes during H/R stress in all three studies species to deplete toxic ammonia. Elevated glutamate levels might also play an important role in mitochondrial protection, as glutamate oxidation seemed to mitigate ROS production in mitochondria of *C. gigas* during H/R stress (Adzigbli et al., 2022). Furthermore, levels of most studied AAs remained stable indicating robust regulation of AA homeostasis in *A. islandica* and *C. gigas*. Stable AA levels may reflect downregulation of protein turnover to save energy during hypoxia, which is typical seen in marine bivalves during hypoxia (Sokolova, 2018). However, metabolic profiling showed that protein homeostasis in *O. edulis* was more sensitive due to higher reliance on protein breakdown during H/R stress. In contrast to gill tissue, heart tissue showed low metabolic response, while it remains to be investigated if heart tissue lacks metabolic capacity in response to hypoxia.

Our study demonstrates the importance of metabolic regulation and its clear association to mitochondrial functions in hypoxia tolerance of marine bivalves. Further research is needed to unravel how interaction of cellular metabolic pathways and mitochondrial functions are integrated into systemic support of whole organism during H/R stress.

Abbreviations

AA	Amino acid
ANOVA	Analysis of Variance
ASW	Artificial sea water
ATP	Adenosine triphosphate
BCAA	Branched chain amino acids
CoA	Coenzyme A
DCM	Dichloromethane
DO	Dissolved oxygen
ETS	Electron transport system
FDR	False discovery rate
FM	Fresh mass
GABA	4-Aminobutyrate
H	Hypoxia
H/R	Hypoxia and reoxygenation
HSD	Honestly significant difference
IPCC	Intergovernmental Panel on Climate Change
MeOH	Methanol
MRD	Metabolic rate depression
NMR	Nuclear magnetic resonance
OXPHOS	Oxidative phosphorylation
PC	Principal component
PCA	Principal component analysis
PEA	Pathway enrichment analysis
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pyruvate kinase
PLA	Phospho-L-arginine
R	Reoxygenation
ROS	Reactive oxygen species
TCA cycle	Tricarboxylic acid cycle
TMAO	Trimethylamine N-oxide
TSP	3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt
UDP	Uridindiphosphate

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Conflict of interest

We declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure Captions

Figure 1 Principal component analysis based on the studied metabolites from gill and heart of *C. gigas, O. edulis* and *A. islandica.* Gill tissue (A); heart tissue (B)

PCA score plots, where position of single measurement from different experimental groups is placed in a coordinate system of first and second principal component based on variance in data set. Distance of samples represent differences in metabolite patterns. Horizontal axis depicts PC1, while vertical axis represents PC2. Coloured ellipse shows all samples within 95% confidence interval.

Experimental groups: CG – *C. gigas,* green; OE – *O. edulis*, blue; AI – *A. islandica,* red; C – control, grey; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Figure 2 Principal component analysis based on the studied metabolites from gill tissue of *C. gigas, O. edulis* and *A. islandica.* *C. gigas* (A); *O. edulis* (B); *A. islandica* (C).

PCA score plots, where position of single measurement from different experimental groups is placed in a coordinate system of first and second principal component based on variance in data set. Distance of samples represent differences in metabolite patterns. Horizontal axis depicts PC1, while vertical axis represents PC2. Coloured ellipse shows all samples within 95% confidence interval.

Experimental groups: C – control, grey; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Figure 3 Significant effect of short-term H/R stress on metabolite concentration in gill of *C. gigas*. Tissue concentration of fumarate (A), glutamate (B) and succinate (C).

Experimental groups: C – control, black; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Means are shown as yellow points within Box-Whisker plots. Different letters above plots represent statistical significances ($p < 0.05$) between oxygen treatments. Plots with the same letter or not marked with a letter are not significantly different ($p > 0.05$). N = 10, 10, 9 in C, H and R, respectively.

Figure 4 Significant effect of short-term H/R stress on metabolite concentration in gill of *O. edulis*. Tissue concentration of energy metabolites (A,D,G,I,K,M), AAs (B,C,E,F,H,J,L).

Experimental groups: C – control, black; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Means are shown as yellow points within Box-Whisker plots. Different letters above plots represent statistical significances ($p < 0.05$) between oxygen treatments. Plots with the same letter or not marked with a letter are not significantly different ($p > 0.05$). N = 10, 9, 9 in C, H and R, respectively.

Figure 5 Significant effect of short-term H/R stress on metabolite concentration in gill of *A. islandica*. Tissue concentration of energy metabolites (A,B), AAs (C,D,E).

Experimental groups: C – control, black; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Means are shown as yellow points within Box-Whisker plots. Different letters above plots represent statistical significances ($p < 0.05$) between oxygen treatments. Plots with the same letter or not marked with a letter are not significantly different ($p > 0.05$). N = 10, 10, 10 in C, H and R, respectively.

Figure 6 Principal component analysis based on the studied metabolites from heart tissue of *C. gigas* and *O. edulis*. *C. gigas* (A); *O. edulis* (B).

PCA score plots, where position of single measurement from different experimental groups is placed in a coordinate system of first and second principal component based on variance in data set. Distance of samples represent differences in metabolite patterns. Horizontal axis depicts PC1, while vertical axis represents PC2. Coloured ellipse shows all samples within 95% confidence interval.

Experimental groups: C – control, grey; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Figure 7 Significant effect of short-term H/R stress on metabolite concentration in heart of *C. gigas*. Tissue concentration of energy metabolites aspartate (A), fumarate (B) and succinate (C) in heart tissue of *C. gigas*.

Experimental groups: C – control, black; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Means are shown as yellow points within Box-Whisker plots. Different letters above plots represent statistical significances ($p < 0.05$) between oxygen treatments. Plots with the same letter or not marked with a letter are not significantly different ($p > 0.05$). N = 9, 10, 10 in C, H and R, respectively

Figure 8 Significant effect of short-term H/R stress on Aspartate levels in heart of *O. edulis*. Tissue concentration of energy metabolites aspartate in heart tissue of *O. edulis* .

Experimental groups: C – control, black; H – severe hypoxia (24 h at <0.01% O_2), red; R – severe hypoxia (24h <0.01% O_2) and subsequent reoxygenation (1.5h at 21% O_2), blue.

Means are shown as yellow points within Box-Whisker plots. Different letters above plots represent statistical significances ($p < 0.05$) between oxygen treatments. Plots with the same letter or not marked with a letter are not significantly different ($p > 0.05$). N = 10, 10, 10 in C, H and R, respectively

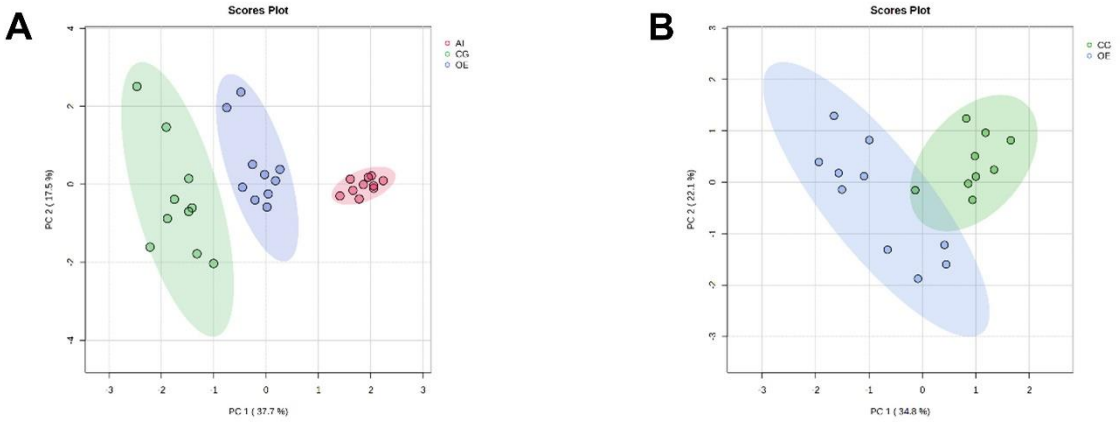

A
Scores Plot
AI
CG
OL
PC 2 (17.5 %)
PC 1 (37.7 %)
B
Scores Plot
CC
OE
PC 2 (22.1 %)
PC 1 (34.8 %)

Figure 1

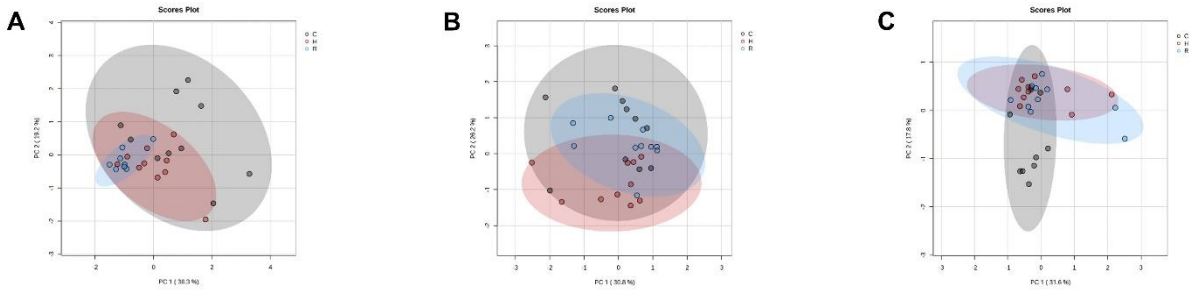


Figure 2

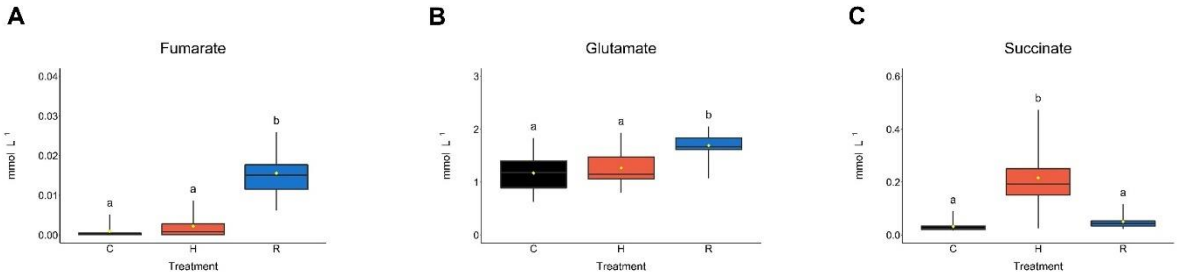

A
Fumarate
mmol L⁻¹
0.04
0.03
0.02
0.01
0.00
a
a
b
C
H
R
Treatment
B
Glutamate
mmol L⁻¹
3
2
1
0
a
a
b
C
H
R
Treatment
C
Succinate
mmol L⁻¹
0.6
0.4
0.2
0.0
a
b
a
C
H
R
Treatment

Figure 3

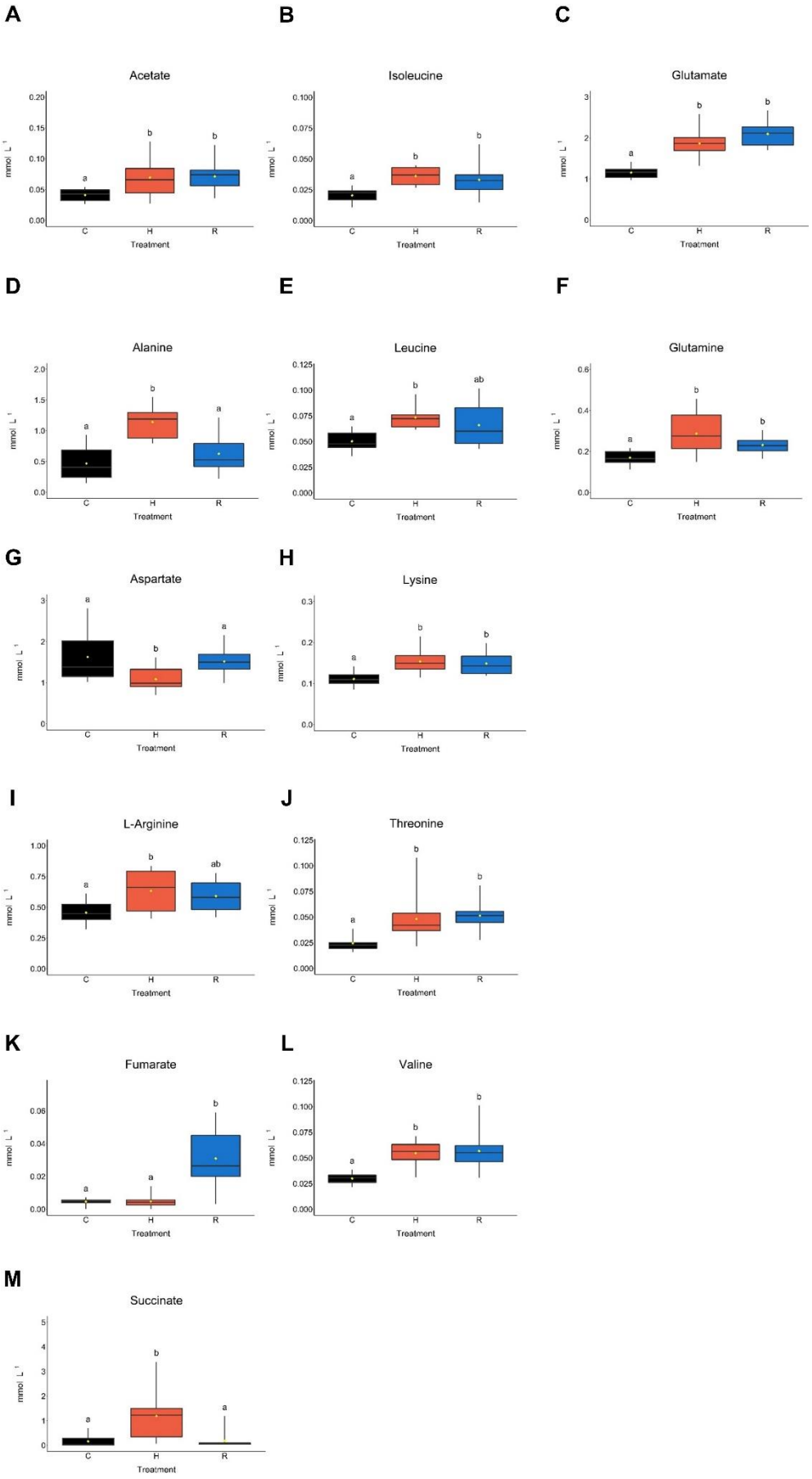

A
Acetate
B
Isoleucine
C
Glutamate
D
Alanine
E
Leucine
F
Glutamine
G
Aspartate
H
Lysine
I
L-Arginine
J
Threonine
K
Fumarate
L
Valine
M
Succinate
mmol L⁻¹
Treatment
C
H
R

Figure 4

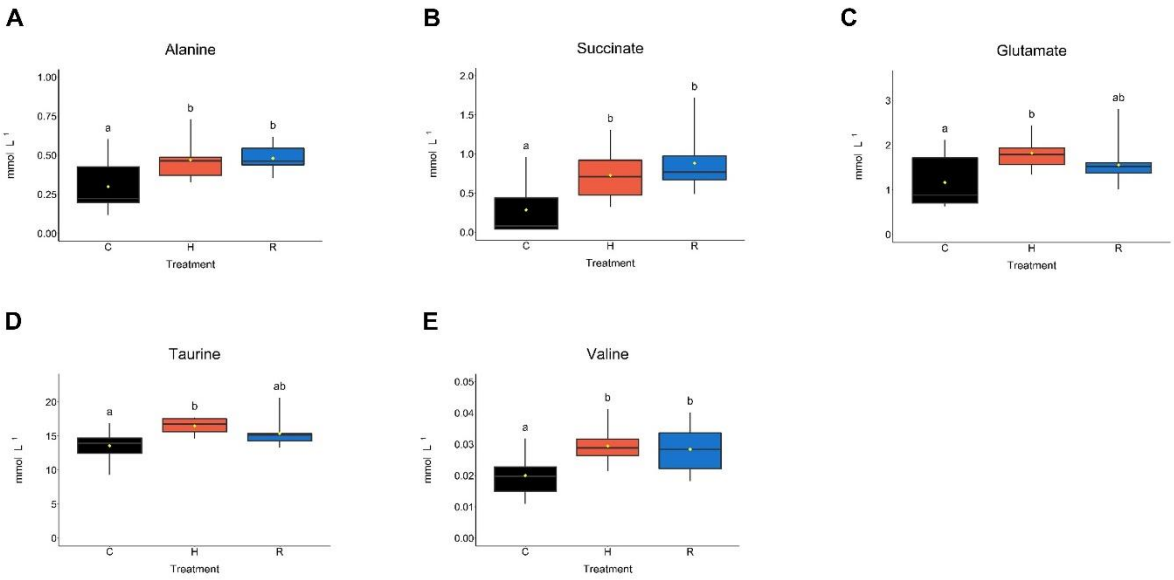

A
Alanine
mmol L⁻¹
1.00
0.75
0.50
0.25
0.00
a
b
b
C
H
R
Treatment
B
Succinate
mmol L⁻¹
2.0
1.5
1.0
0.5
0.0
a
b
b
C
H
R
Treatment
C
Glutamate
mmol L⁻¹
3
2
1
0
a
b
ab
C
H
R
Treatment
D
Taurine
mmol L⁻¹
20
15
10
5
0
a
b
ab
C
H
R
Treatment
E
Valine
mmol L⁻¹
0.05
0.04
0.03
0.02
0.01
0.00
a
b
b
C
H
R
Treatment

Figure 5

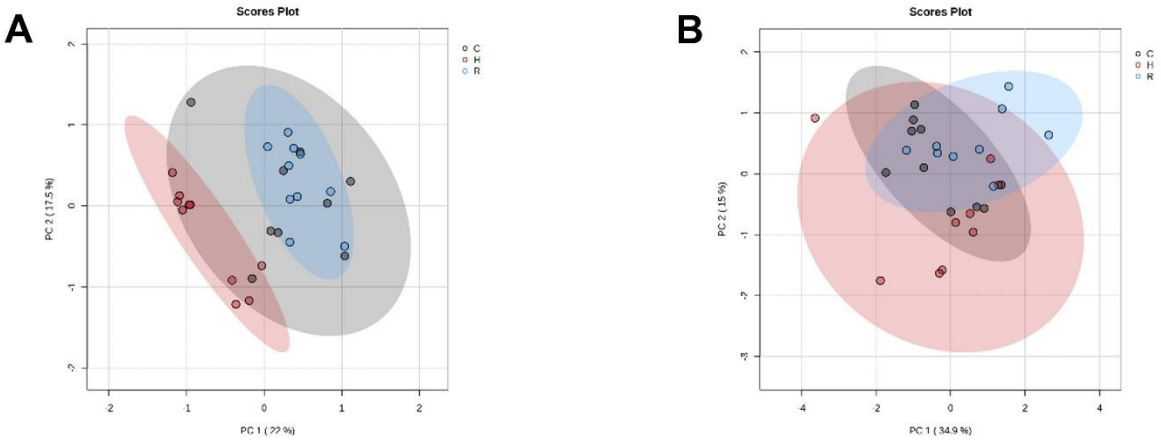

A
Scores Plot
C
H
R
PC 2 (17.5 %)
PC 1 (22 %)
B
Scores Plot
C
H
R
PC 2 (15 %)
PC 1 (34.9 %)

Figure 6

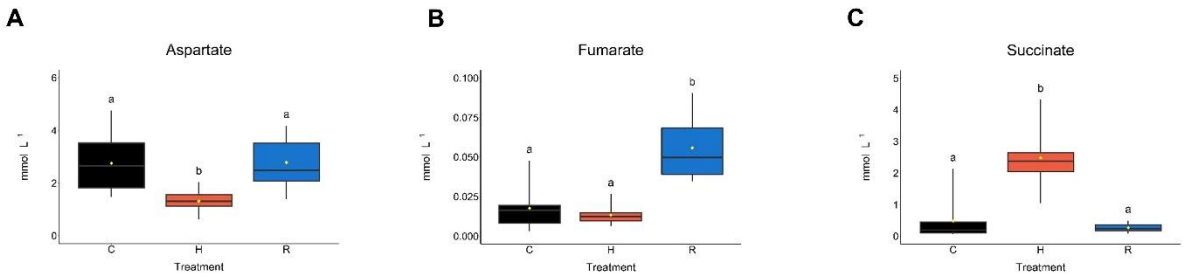


Figure 7

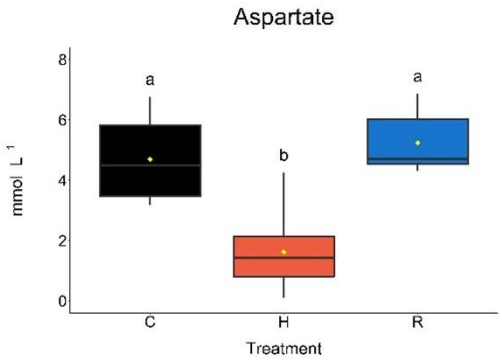


Figure 8

Supplementary material for ”Metabolic response of three marine bivalves to intermittent hypoxia correlates with lifestyle and hypoxia tolerance of species and tissue.”

Jennifer B. M. Steffen[a], Gisela Lannig[b], Christian Bock[b], Inna M. Sokolova[a,c]

[a] Department of Marine Biology, Institute of Biological Sciences, University of Rostock, Rostock, Germany

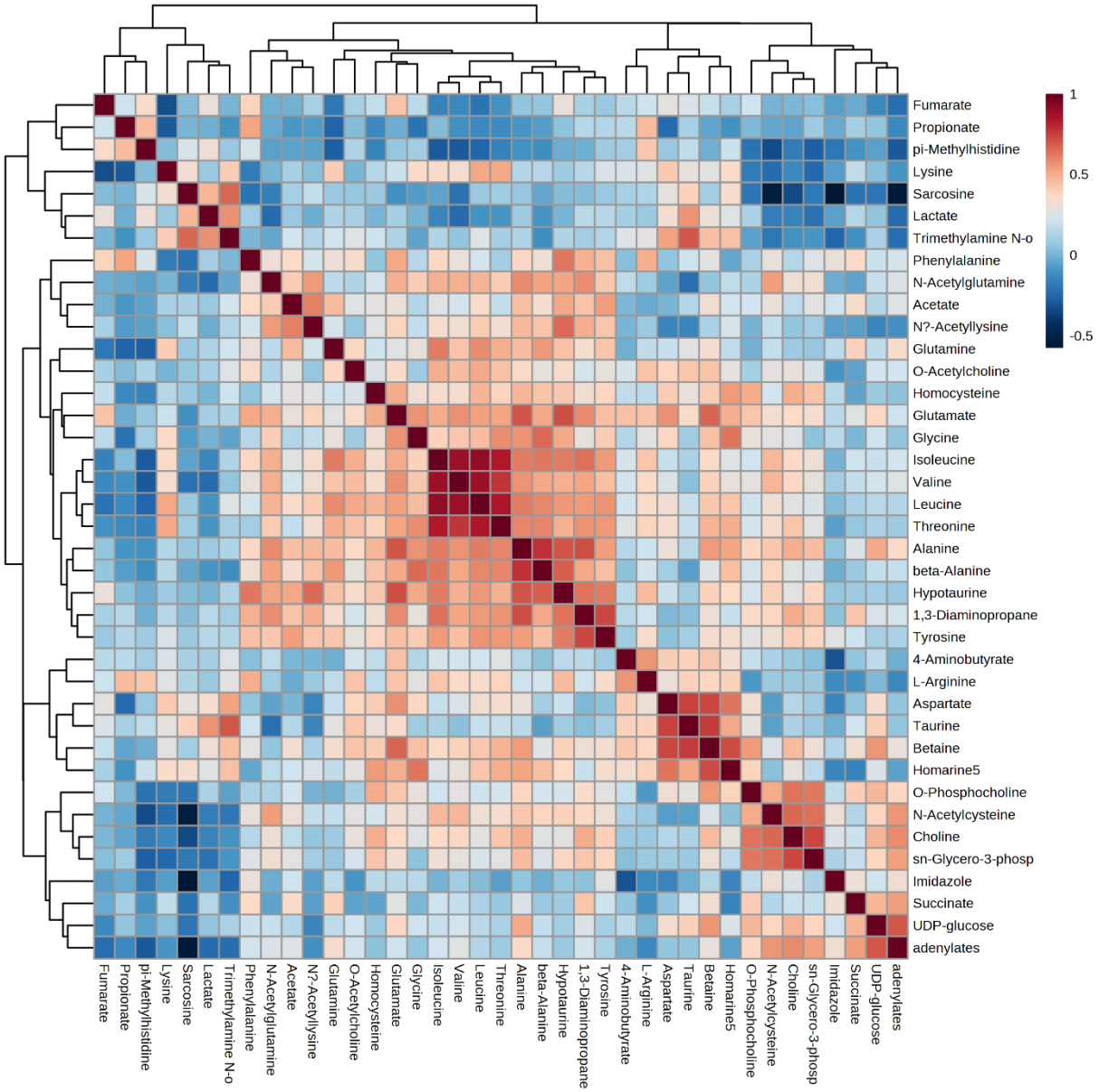
[b] Department of Integrative Ecophysiology, Alfred-Wegener-Institute, Bremerhaven, Germany

[c] Department of Maritime Systems, Interdisciplinary Faculty, University of Rostock, Rostock, Germany

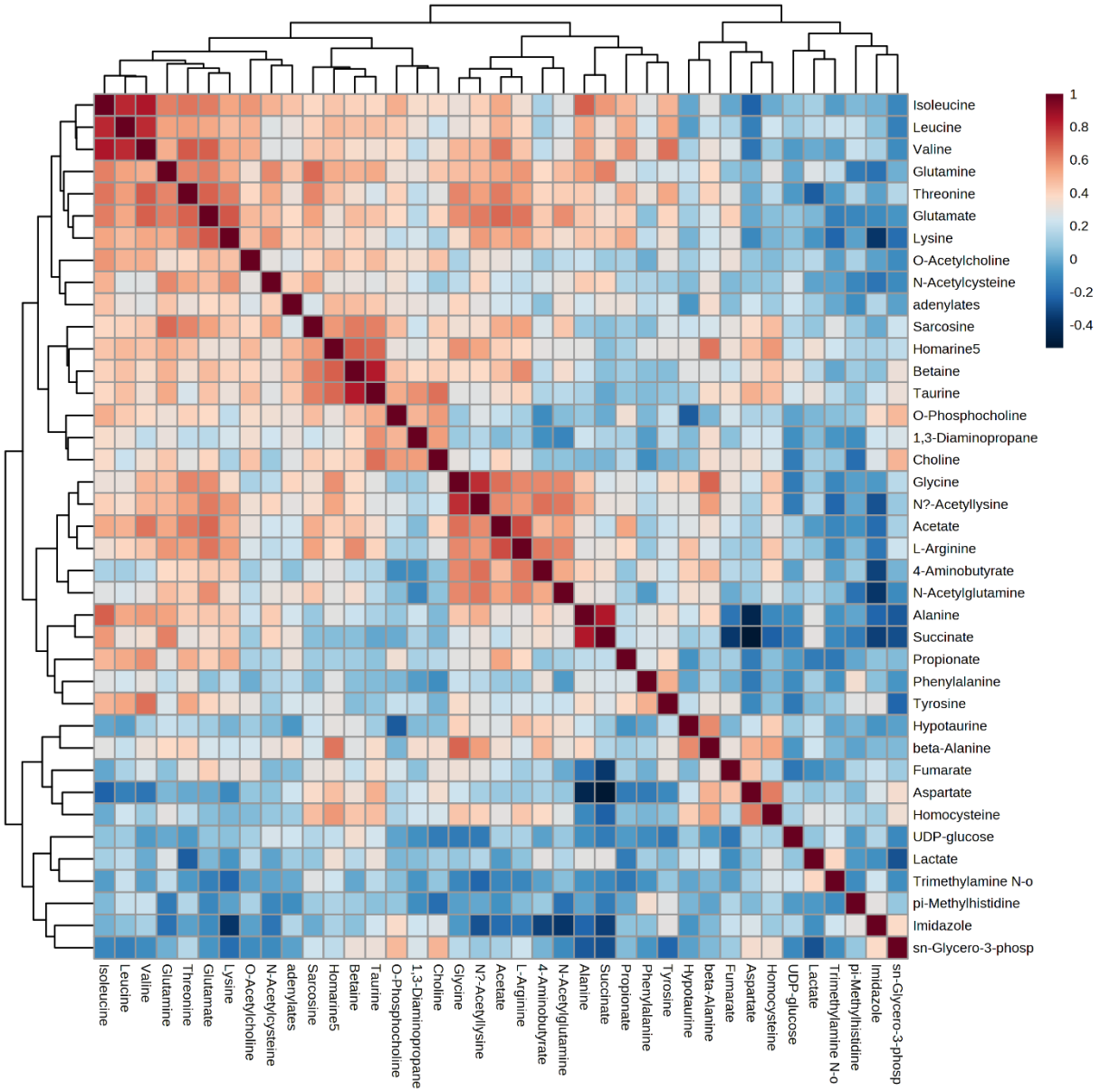
Supplementary table 1 Factor loadings of first two principal components of metabolite biomarkers of gill and heart from *C. gigas, O. edulis* and *A. islandica* exposed to hypoxia-reoxygenation stress. Compounds with high positive loadings (> 0.2) are shaded in bold and red, while compounds with high negative loadings (<-0.2) are shaded in bold and blue. PC – principal component; H/R – hypoxia/reoxygenation

	Control gill species differences		H/R on *C. gigas* gill		H/R on *O. edulis* gill		H/R on *A. islandica* gill		Control heart species difference		H/R on *C. gigas* heart		H/R on *O. edulis* heart	
Compound	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
1,3-Diaminopropane	-0.130	-0.045	-0.022	-0.068	-0.009	-0.015	0.054	0.048	0.075	-0.009	0.011	0.157	0.075	0.009
4-Aminobutyrate	-0.156	-0.085	-0.020	0.004	0.036	-0.100	0.057	0.146	-0.076	0.049	0.027	-0.002	0.153	**0.278**
Acetate	0.023	0.021	-0.010	-0.072	-0.002	-0.053	0.002	0.006	-0.066	0.050	-0.025	0.056	-0.012	-0.106
Alanine	**-0.263**	-0.096	-0.014	-0.092	-0.015	**-0.250**	0.025	**0.230**	**0.390**	-0.004	-0.057	**0.233**	**0.307**	**-0.201**
Aspartate	0.023	0.022	-0.026	-0.005	0.018	0.076	-0.070	-0.035	-0.102	-0.040	**0.210**	0.146	0.161	0.192
Betaine	**-0.343**	0.029	-0.016	-0.042	0.009	-0.008	-0.027	0.081	0.027	-0.019	-0.011	0.080	-0.021	0.012
Carnitine									0.113	0.054	0.019	**0.213**	0.098	0.089
Choline	-0.142	0.028	0.004	-0.108	-0.018	0.003	0.034	0.042	0.146	0.005	-0.153	0.109	0.047	-0.015
Fumarate	**0.328**	-0.118	**-0.698**	-0.071	**0.209**	**0.260**	**-0.608**	0.126	-0.024	-0.071	0.138	**0.287**	-0.027	-0.143
Glutamate	-0.019	0.002	-0.050	-0.077	-0.005	-0.046	0.017	**0.242**	0.121	-0.024	-0.015	**0.211**	0.193	0.019
Glutamine	-0.001	-0.011	0.020	-0.088	-0.018	-0.092	-0.003	0.058	0.121	-0.020	-0.072	0.083	0.094	0.031
Glycine	**-0.244**	-0.069	-0.019	-0.029	0.012	-0.061	-0.015	0.132	**0.330**	0.153	-0.019	0.192	**0.207**	0.071
Homarine	**-0.337**	-0.015	-0.011	0.009	0.022	-0.016	-0.026	0.126	0.079	-0.008	-0.011	0.132	0.014	0.016
Homocysteine	-0.153	-0.006	-0.007	-0.041	0.035	0.027	-0.033	0.006	0.052	-0.091	0.096	0.030	0.142	0.089
Hypotaurine	**-0.244**	-0.031	-0.063	-0.131	0.004	-0.019	-0.104	0.025	0.013	**-0.244**	**0.361**	-0.169	**0.234**	**0.203**
Imidazole	**-0.251**	0.102	0.015	**-0.297**	0.075	0.065	-0.067	-0.043	-0.190	-0.141	0.134	0.003	-0.034	-0.094
Isoleucine	-0.106	-0.033	0.010	-0.091	0.011	-0.118	-0.087	0.134	0.197	-0.039	-0.112	0.052	0.171	-0.084
Lactate	-0.153	-0.091	-0.024	0.016	-0.009	-0.043	-0.062	0.036	0.113	0.008	-0.061	-0.016	0.182	-0.050
L-Arginine	-0.024	-0.060	-0.043	-0.006	0.007	-0.041	-0.023	0.041	0.063	-0.066	-0.007	0.068	0.060	0.008
Leucine	-0.107	-0.030	0.015	-0.056	0.028	-0.051	-0.052	0.114	0.168	-0.075	-0.034	0.049	0.185	-0.019
Lysine	-0.113	-0.039	0.024	0.017	0.003	-0.044	0.005	**0.239**	0.023	-0.017	-0.026	0.005	0.054	0.128
N-Acetylcysteine	-0.012	0.048	0.011	-0.185	-0.013	-0.067	0.064	0.160	0.114	0.032	-0.004	0.107	0.183	0.135
N-Acetylglutamine	-0.051	-0.072	-0.012	-0.091	-0.036	-0.061	0.074	0.101	0.120	0.078	-0.057	0.157	0.136	0.008
N-α-Acetyllysine	-0.095	-0.082	-0.016	-0.043	0.009	-0.060	0.018	0.069	0.127	0.026	-0.007	0.179	0.053	-0.051
O-Acetylcholine	-0.080	-0.052	-0.032	-0.041	0.021	-0.015	0.024	0.056	0.135	-0.063	-0.151	0.175	0.160	0.028
O-Phosphocholine	0.178	0.029	-0.009	-0.069	0.017	-0.003	0.012	0.066	-0.077	-0.173	**-0.212**	**0.346**	0.061	-0.017

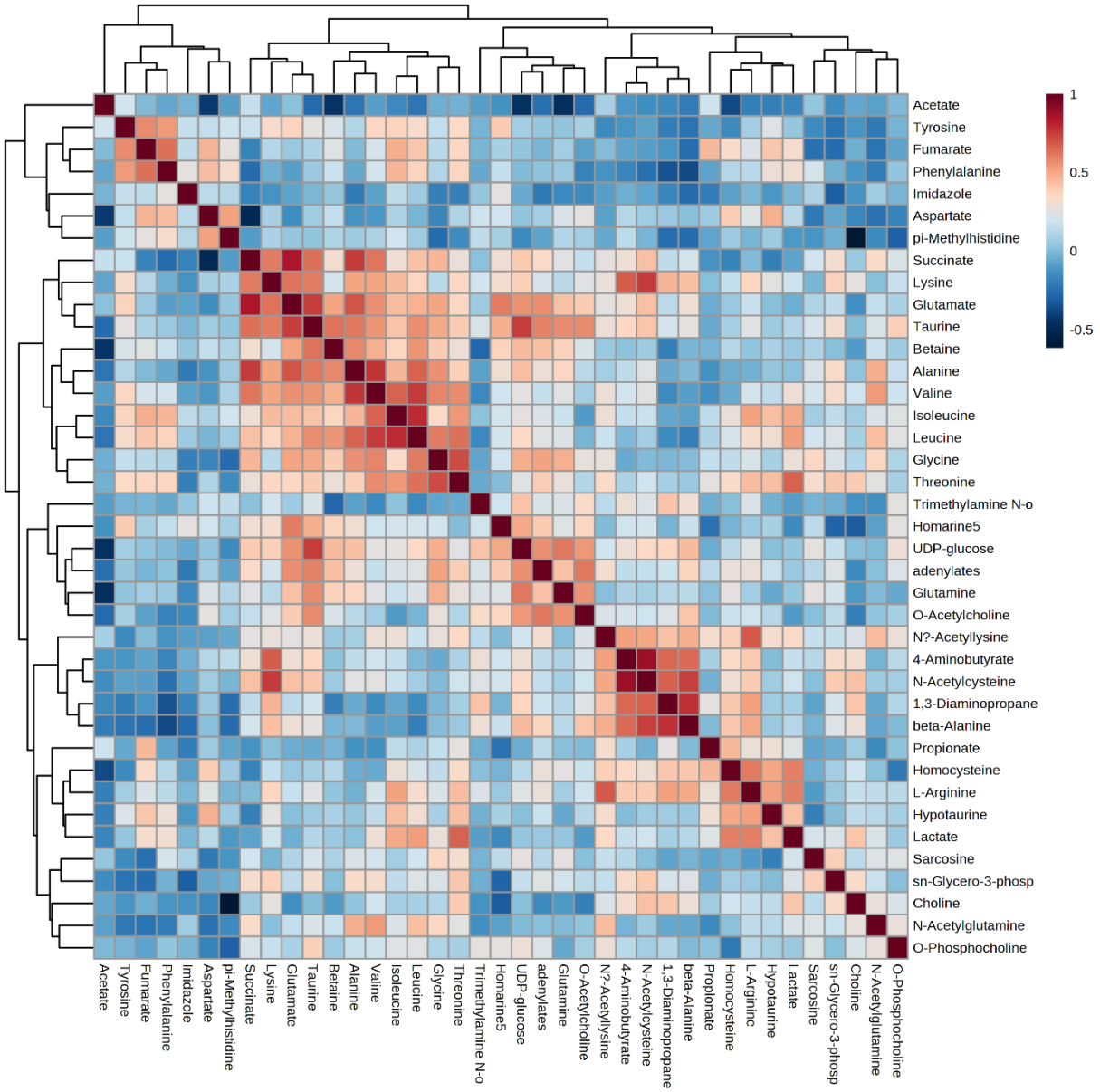
	Control gill species differences		H/R on *C. gigas* gill		H/R on *O. edulis* gill		H/R on *A. islandica* gill		Control heart species difference		H/R on *C. gigas* heart		H/R on *O. edulis* heart	
Compound	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Phenylalanine	0.123	**-0.335**	**-0.413**	**-0.550**	**0.383**	**-0.436**	**-0.637**	0.024	-0.074	**-0.325**	-0.111	-0.171	**0.213**	-0.146
Propionate	0.031	**-0.255**	**-0.227**	-0.011	0.017	-0.070	-0.101	-0.022	0.067	0.040	-0.085	-0.158	0.177	-0.099
Sarcosine	0.050	-0.176	-0.019	0.171	0.022	-0.014	0.021	0.073	0.129	0.032	0.010	0.091	0.095	0.059
sn-Glycero-3-phosphocholine	-0.125	0.003	0.001	-0.114	0.017	0.041	0.048	0.086	**-0.209**	-0.195	0.022	-0.052	0.179	0.119
Opine									**0.377**	0.182	-0.148	**0.252**	0.124	**-0.229**
Succinate	0.147	0.090	-0.021	**-0.263**	-0.169	**-0.757**	0.197	**0.715**	**0.252**	**-0.568**	**-0.703**	**-0.356**	**0.273**	**-0.675**
Taurine	-0.086	0.014	-0.021	-0.006	0.009	-0.001	0.000	0.079	-0.054	-0.077	-0.032	0.129	-0.004	0.022
Threonine	-0.113	-0.115	0.014	-0.063	0.048	-0.087	-0.051	0.098	**0.204**	-0.029	-0.056	0.073	0.167	-0.051
Trimethylamine N-oxide	**-0.239**	-0.029	-0.009	0.036	-0.022	0.030	0.025	0.044	-0.002	-0.070	-0.105	0.150	-0.007	-0.021
Tyrosine	-0.174	-0.078	-0.038	-0.096	0.091	-0.097	**-0.303**	**0.264**	0.060	-0.194	0.057	-0.006	0.174	0.021
UDP-glucose	0.065	0.074	0.004	-0.061	-0.008	0.013	0.006	0.083	-0.020	-0.022	-0.024	0.112	0.115	**0.247**
Valine	-0.150	-0.033	0.014	-0.091	0.046	-0.080	-0.026	0.160	0.188	-0.072	-0.058	0.052	**0.236**	-0.026
β-Alanine	-0.123	-0.004	-0.009	-0.063	0.014	-0.042	0.119	0.098	**0.270**	0.101	-0.148	0.184	0.196	0.158
π-Methylhistidine	0.065	**-0.808**	**-0.520**	**0.546**	**0.868**	-0.004	-0.079	0.000	0.119	**-0.501**	**0.268**	-0.117	**0.326**	0.190
adenylates	0.077	0.140	0.049	-0.189	-0.003	-0.031	0.005	0.075	0.062	-0.029	-0.067	0.157	0.079	0.091



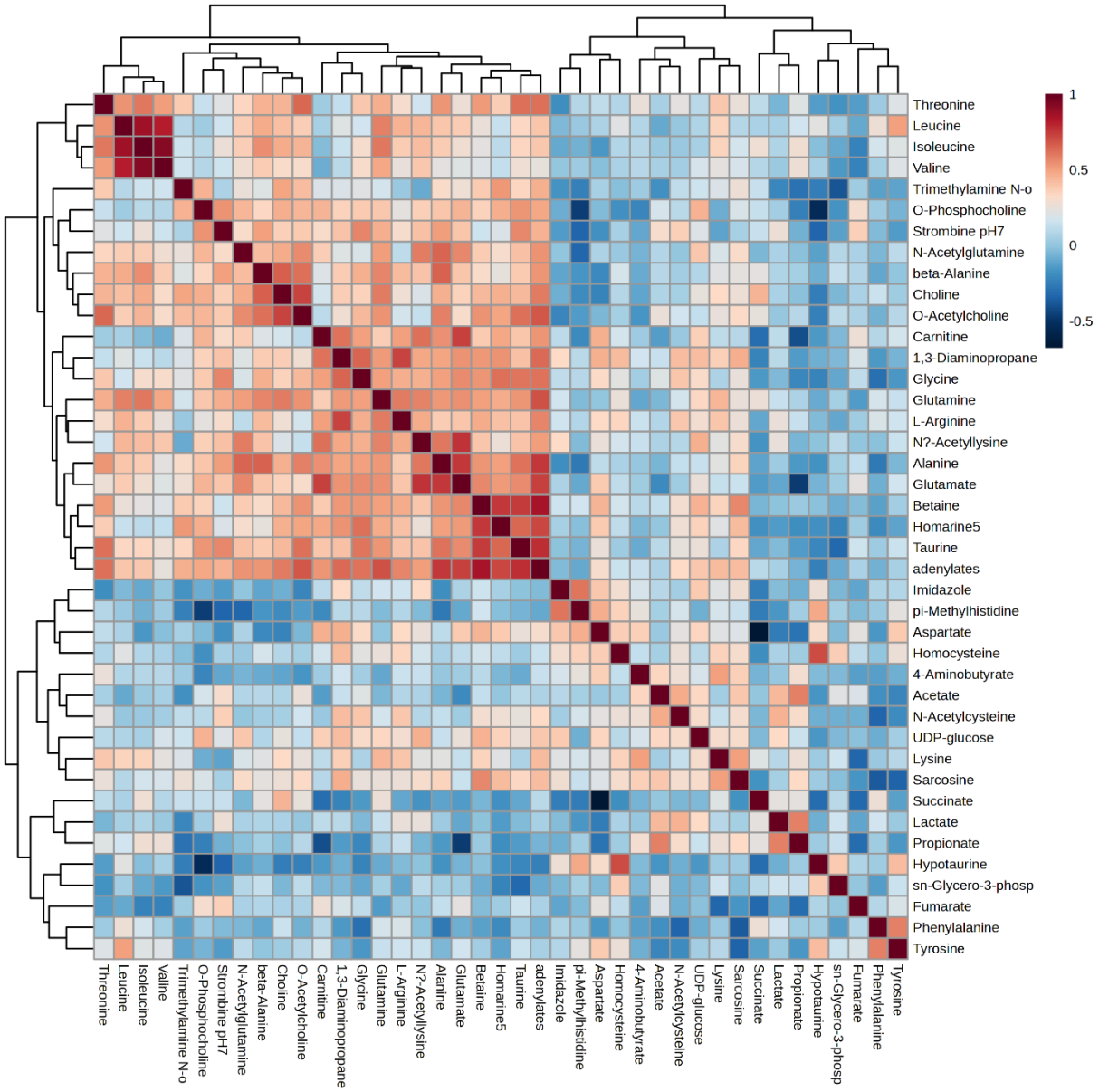
Supplementary Figure S1 Correlation heatmap of metabolite data in gill tissue of *C. gigas* across all oxygen treatments. Correlation analysis is based on Pearson's correlation ranging from -1 to 1. The scale is shown in the top right corner, with shades of red indicating positive correlations and shades of blue indicating negative correlations.



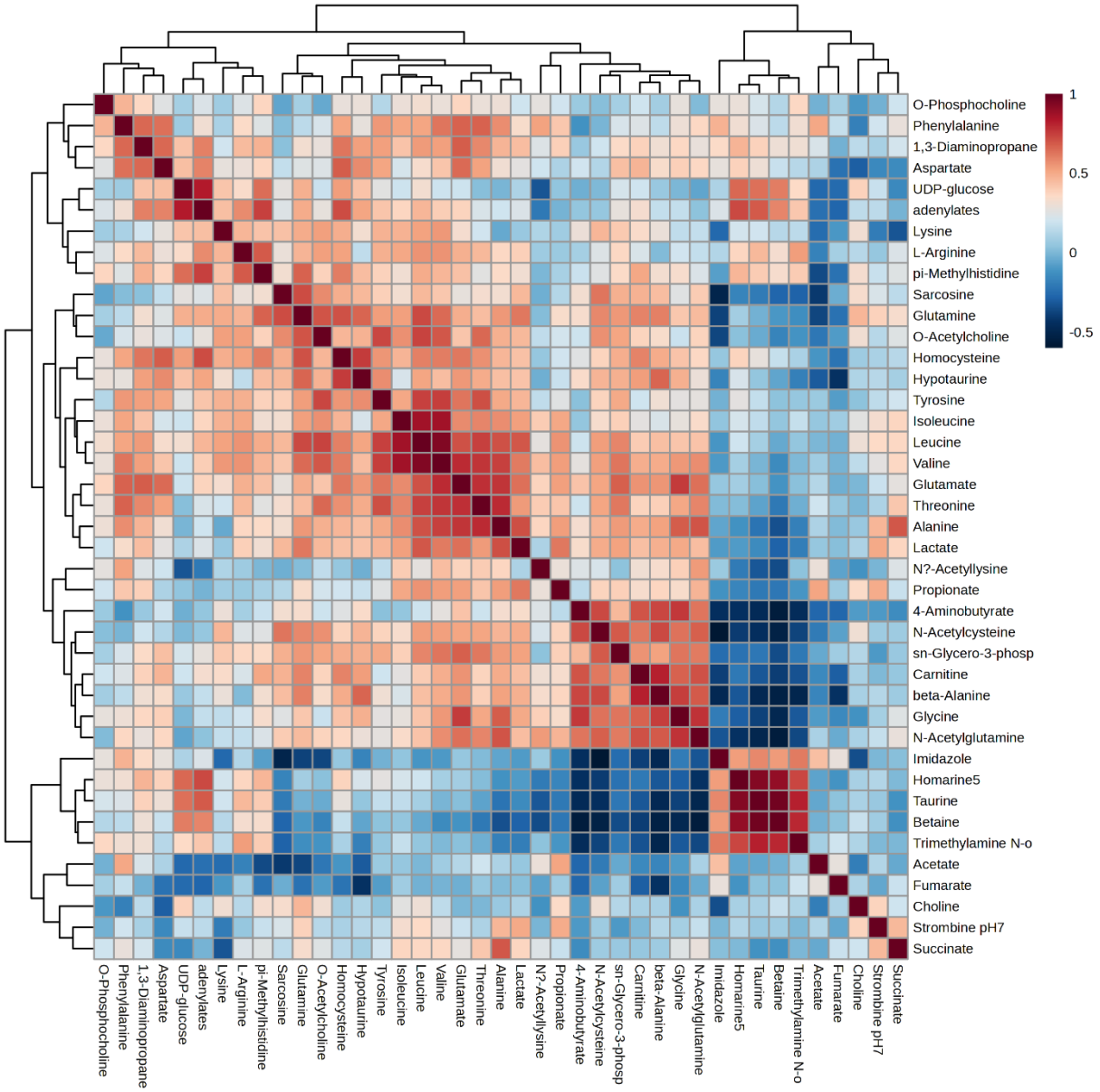
Supplementary Figure S2 Correlation heatmap of metabolite data in gill tissue of *O. edulis* across all oxygen treatments. Correlation analysis is based on Pearson's correlation ranging from -1 to 1. The scale is shown in the top right corner, with shades of red indicating positive correlations and shades of blue indicating negative correlations.



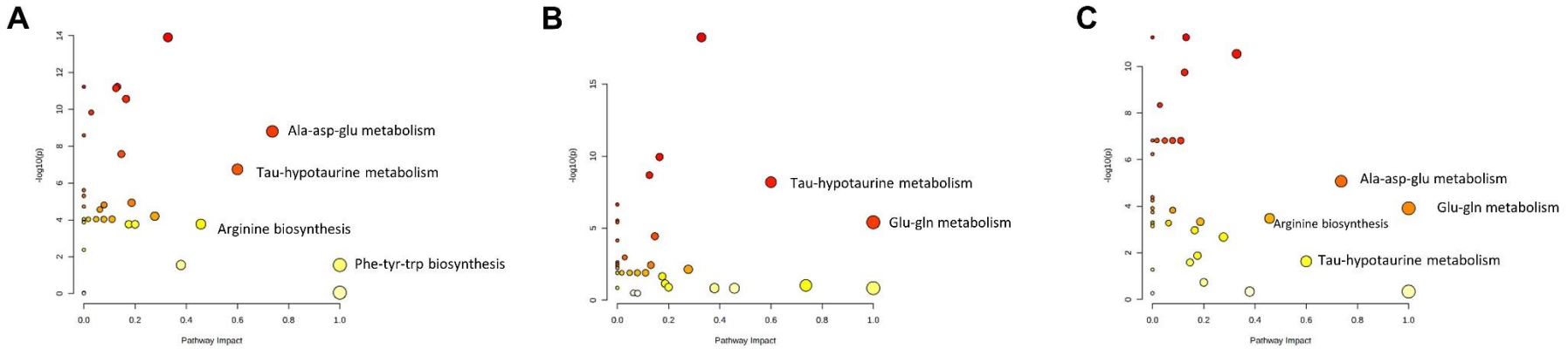
Supplementary Figure S3 Correlation heatmap of metabolite data in gill tissue of *A. islandica* across all oxygen treatments. Correlation analysis is based on Pearson's correlation ranging from -1 to 1. The scale is shown in the top right corner, with shades of red indicating positive correlations and shades of blue indicating negative correlations.



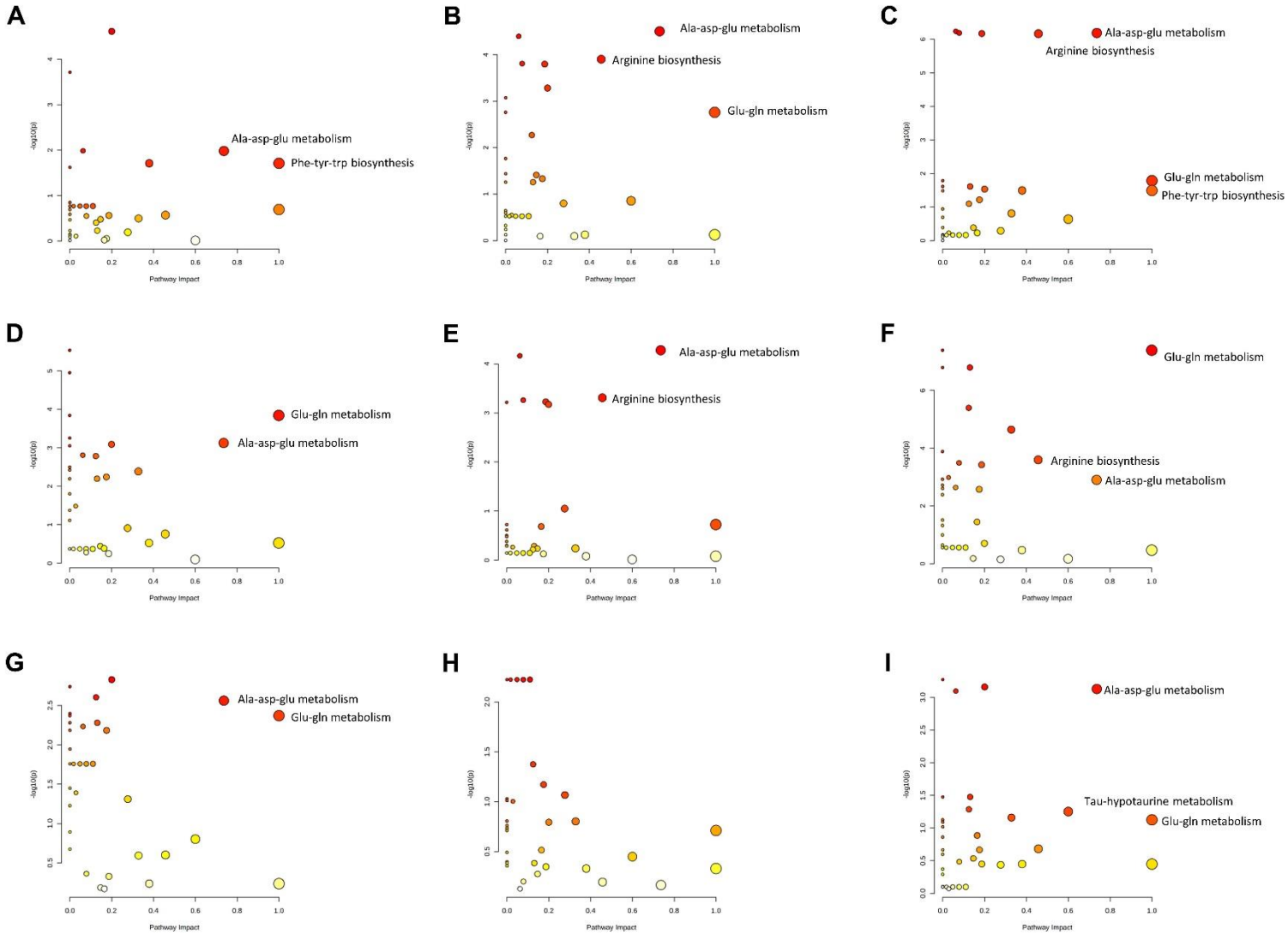
Supplementary Figure S4 Correlation heatmap of metabolite data in heart tissue of *C. gigas* across all oxygen treatments. Correlation analysis is based on Pearson's correlation ranging from -1 to 1. The scale is shown in the top right corner, with shades of red indicating positive correlations and shades of blue indicating negative correlations.



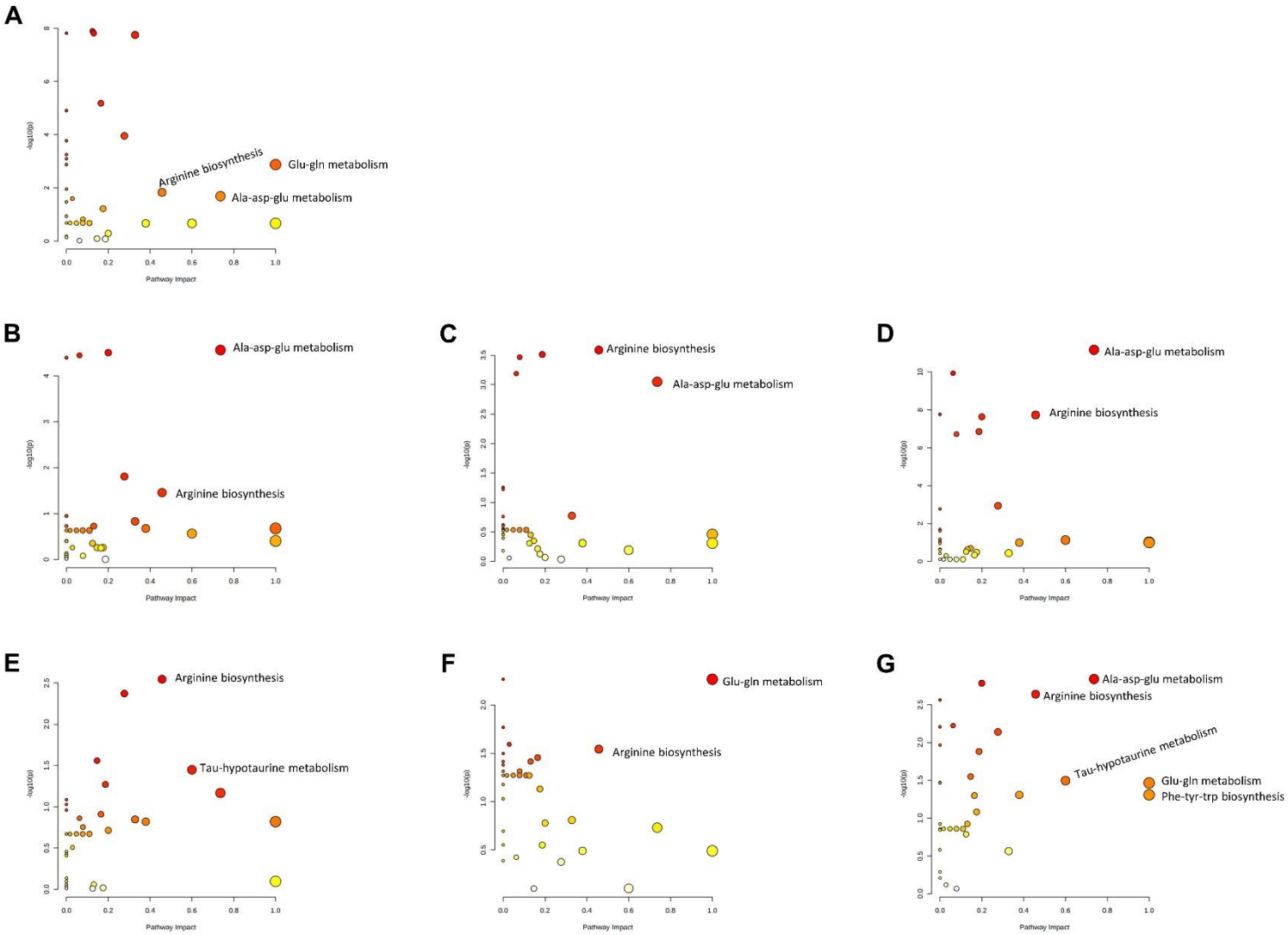
Supplementary Figure S5 Correlation heatmap of metabolite data in heart tissue of *O. edulis* across all oxygen treatments. Correlation analysis is based on Pearson's correlation ranging from -1 to 1. The scale is shown in the top right corner, with shades of red indicating positive correlations and shades of blue indicating negative correlations.



Supplementary Figure S6 Pathway enrichment analysis (PEA) of ^{1}H-NMR metabolite data in gill tissue of three different marine bivalves *C. gigas, O. edulis, A. islandica.* Metabolite data were compared between species: *A. islandica* with *C. gigas* (A), *O. edulis* with *A. islandica* (B), *O. edulis* with *C. gigas* (C). Y. axis: -log10(p); X axis: pathway impact factor. Colour shading shows relative significance with red determining highest significance. Size of circles corresponds to pathway impact factor. A false discovery rate (FDR) of 1.3 (corresponds to threshold p value of 0.05) after adjustments for multiple comparisons and impact factor threshold of 0.4 were used.



Supplementary Figure S7 Pathway enrichment analysis (PEA) of ^{1}H-NMR metabolite data in gill tissue of *C. gigas, O. edulis, A. islandica* exposed to H/R stress. Metabolite data were compared between oxygen treatments (Normoxic control – C, hypoxia (24h <0.01% O_2) – H, reoxygenation (24h <0.01% O_2 and subsequent 1.5h 21% O_2) – R) within each species (*C. gigas* (A-C), *O. edulis* (D-F), *A. islandica* (G-I)). Within each species, the following comparisons were made based on PEA: C vs. H(A,D,G), H vs. R(B,E,H), C vs. R(C,F,I). Y. axis: -log10(p); X axis: pathway impact factor. Colour shading shows relative significance with red determining highest significance. Size of circles corresponds to pathway impact factor. A false discovery rate (FDR) of 1.3 (corresponds to threshold p value of 0.05) after adjustments for multiple comparisons and impact factor threshold of 0.4 were used.



Supplementary Figure S8 Pathway enrichment analysis (PEA) of ^{1}H-NMR metabolite data in heart tissue of *C. gigas and O. edulis* exposed to H/R stress. Metabolite data were compared between species (A) and between oxygen treatments (Normoxic control – C, hypoxia (24h <0.01% O_2) – H, reoxygenation (24h <0.01% O_2 and subsequent 1.5h 21% O_2) – R) within each species (*C. gigas* (B-D), *O. edulis* (E-G)). Within each species, the following comparisons were made based on PEA: C vs. H(B,E), H vs. R(C,F), C vs. R(D,G). Y. axis: -log10(p); X axis: pathway impact factor. Colour shading shows relative significance with red determining highest significance. Size of circles corresponds to pathway impact factor. A false discovery rate (FDR) of 1.3 (corresponds to threshold p value of 0.05) after adjustments for multiple comparisons and impact factor threshold of 0.4 were used.

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Ort, Datum, Unterschrift

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