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], Inna M. Sokolova[1,4,*]

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

[*]Author for correspondnce: inna.sokolova@uni-rostock.de

Keywords: hypoxia tolerance; mitochondria; aerobic capacity; high-resolution respirometry; oxidative stress marker; AmplexRed

Summary statement: *Crassostrea gigas* shows higher mitochondrial plasticity during H/R stress when acclimated to low salinity contributing to its ecological and invasive success in varying environments.

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 the oysters acclimated to high (33) or low (15) salinity, and exposed to either normoxic conditions (control; 21% O_2) or to 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 salinity 15 might be closer to the metabolic optimum of *C. gigas* than salinity 33. 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 indicate 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 to brackish regions such as the Baltic Sea.

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; Breitburg et al., 2019). Most marine organisms 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. For 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 observed to suffer from oxidative damage, collapsed mitochondrial membrane potential (MMP), 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 like crucian carp, and intertidal bivalves, are able to preserve mitochondrial respiration, OXPHOS capacity, and MMP 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 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 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 vs. anaerobic metabolism and affect substrate provision to the 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 crucial 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 like 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 mollusks, acclimation to different salinities requires regulation of intracellular osmolarity to match the ambient osmolarity and prevent excessive changes in the cell volume (Evans, 2009; Shumway, 1977; Yancey, 2005). Thus, changes of surrounding seawater osmolarity cause changes in cellular osmolarity and ion concentration that can affect cellular processes like ion transport, action potential, protein folding and enzyme activity (Somero and Yancey, 2010). 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 accumulation of metabolic end products in their tissues (Berger and Kharazova, 1997). In contrast, survival of long-term exposure to salinity stress requires mostly the regulation of cell volume by organic and inorganic osmolytes to achieve the isosmotic state of cellular environment relative to the habitat. This state is typically achieved by altered activity of certain pathways providing organic osmolytes such as 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 common name *C. gigas* 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 hypoxia/reoxygenation stress promotes mitochondrial resilience leading to upregulation of the electron transport system (ETS) and suppression of pathways channelling electrons to ubiquinone, which may reduce reactive oxygen species production (Sokolov et al., 2019). These

findings suggest that maintenance of mitochondrial integrity and ATP synthesis capacity are crucial for oysters to tolerate hypoxia/reoxygenation 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; Rivera-Ingraham et al., 2016b; 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 impair mitochondrial bioenergetics and redox balance, which may have implications for mitochondrial resilience towards other stressors such as hypoxia-reoxygenation, 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, while another group was kept at their collection site salinity of 30±1. After acclimation, the oysters were exposed to severe hypoxia for 24 hours, followed by a recovery period of 1.5 hours 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 a stress-tolerant marine osmoconformer, the Pacific oyster *C. gigas*.

Material 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 shell length ± s.e.m: 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 48h after 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 salinity 33±1 and temperature 15±0.5°C for two 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 salinity 15±1 at a rate of 2.5 practical salinity units per day. After the target salinity was achieved, oysters were transferred to a recirculated temperature-controlled aquarium system (Kunststoff-Spranger GmbH, Plauen, Germany) with aerated ASW at salinity 15±1 and 15±1°C and acclimated for 3 months. The second (control) group was kept for the same duration of time at salinity 33±1 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 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 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} 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 8 mmol l^{-1} ethylene

glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (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 with 2000 x g for 8 minutes to remove cell debris and the supernatant was again centrifuged at 4°C with 8500 x g for 8 minutes to isolate mitochondria. The pellet was resuspended in ice-cold assay buffer, which osmolarity corresponded to the respective salinity acclimation of oysters. Assay buffer for oysters acclimated to salinity 15 (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 x $6H_2O$, 10 mmol l^{-1} KH_2PO_4, 1% (w/v) bovine serum albumin (BSA), while assay buffer for oysters maintained at salinity 30 (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 x $6H_2O$, 10 mmol l^{-1} KH_2PO_4, 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 the 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 hydrogen peroxide (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 electrodes were calibrated with 0.2 µmol l^{-1} H_2O_2.

The 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 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 hereafter as pre *in vitro* anoxia OXPHOS). We have used >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 maintained 10 min at 0% O_2 and subsequently chambers were reoxygenated (referred 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 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 (CCO).

Protein concentrations of mitochondrial suspensions were measured using the Bradford assay (Thermo Fisher Scientific, Waltham, USA) 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 precent protein aggregation, dilutions were sonicated (Sonicator S-4000, Misonix, Famingdate, NY, USA, amplitude 24, 30s). 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. Protein carbonyl 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 of 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 Immuno Research 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 form 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 were 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., West Grove, PA, USA). Each antibody incubation was conducted for 1 h at room temperature. Bound antibodies were detected by addition of horseradish-peroxidase substrate TMB/E Ultra Sensitive (Merck Millipore, Burlington, MA, USA) and 2 mol l^{-1} sulfuric acid to stop reaction. Subsequently, development of colour was detected at 450 nm (SpectraMax iD3, Molecular Devices, LLC, San José, CA, USA).

Enzyme activities. Activities of pyruvate kinase (PK, EC 2.7.1.40) and phosphoenol-pyruvate-carboxykinase (PEPCK, EC 4.1.1.31) were 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} dinatrium-ethylenediaminetetraacetic acid (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 secs (MP Biomedical, Santa Ana, CA, USA).

Assay media were the following:

PK: 50 mmol l^{-1} Tris-HCl (pH 7.0); 50 mmol l^{-1} KCl; 5 mmol l^{-1} $MgSO_4$ x 7 H_2O; 1 mmol l^{-1} ADP sodium salt; 0.2 mg ml^{-1} NADH; 5.5 U lactate dehydrogenase (LDH); 2.5 mmol l^{-1} phosphoenolpyruvate (PEP)

PEPCK: 100 mmol l^{-1} HEPES (pH 7.0); 2.3 mmol l^{-1} $MnCl_2$ x 4 H_2O; 5mg ml^{-1} $KHCO_3$; 0.5 mmol l^{-1} IDP sodium salt; 0.2 mg ml^{-1} NADH; 10 U malate dehydrogenase (MDH); 15 mmol l^{-1} PEP

For both enzymes, reactions were started with addition of PEP and monitored at 340 nm (SpectraMAx iD3, Molecular Devices, LLC, San José, CA, USA). Absorbances were corrected by blank measurement. Activities of both enzymes were standardized to fresh tissue weight (FW) and were expressed as nmol min^{-1} g^{-1} fresh tissue weight (FW).

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, United States) and for homogeneity of variances by the Brown-Forsythe test in Sigma Plot (v. 13.0.0.83, Systat Software Inc., San Jose, CA, United States). Outliers were excluded by Box-Whisker plots in IBM® SPSS® Statistics. In case of non-normal distribution and/or non-homogeneity of variances, data were transformed by BoxCox or Johnson transformation in Minitab (v.19, Minitab LLC., State College, PA, United States). 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 the 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 using *in vitro* oxygen treatment as 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) generally did not affect the baseline (LEAK) oxygen consumption rate ($\dot{M}_{O2}$) of the oyster gill mitochondria (except during post-hypoxic recovery) but led to a ~1.5-fold increase in the OXPHOS $\dot{M}_{O2}$ compared to the mitochondria from the high salinity acclimated oysters (**Fig. 1A-C**). In oysters acclimated to high salinity (33), 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 to the normoxic control (**Fig. 1A,B**). In the oysters acclimated to low salinity (15), exposure to hypoxia had no effect on the mitochondrial LEAK $\dot{M}_{O2}$, but LEAK I and LEAK I+II $\dot{M}_{O2}$ increased ~1.4-fold during reoxygenation (**Fig. 1A,B**). Exposure to H/R stress showed no effect on the OXPHOS $\dot{M}_{O2}$ 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 compared to 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. 3)**.

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 oyster mitochondria compared with those from the high salinity treatment (**Fig. 2**). H/R exposure showed no evidence of effect on the RCR and OXPHOS CE within each salinity acclimation group (**Fig. 2**). Similarly, exposure of isolated mitochondria to *in vitro* anoxia and reoxygenation showed no effect on RCR and OXPHOS CE regardless of the acclimation salinity (**Supplementary Figure S1**).

Activity of enzymes at the aerobic-anaerobic branch point. In low salinity (15) acclimation group, the 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 high salinity (33) group, PK activity remained at the normoxic baseline levels during hypoxia and reoxygenation. Activity of PEPCK 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 (15) salinity were more responsive to the acute *in vitro* anoxia that those from the high (33) salinity acclimated oysters, regardless of the oxygen conditions (normoxia, hypoxia or post-hypoxic reoxygenation) under which the oysters were collected (**Figs. 5,6**). 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 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) salinity relative to the high (33) acclimation salinity that corresponds to the salinity of the habitat where the oysters have been collected. This was evident in the higher RCR and OXPHOS CE in the mitochondria of the 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 to the mitochondria of high salinity acclimated oysters. Based on the mitochondrial coupling efficiency, a salinity of 15 might be

considered closer to the optimum for the metabolic performance in the studied *C. gigas* population than a salinity of 33. Earlier study using the same population of *C. gigas* acclimated to salinity 30 also showed better coupling, higher rates of ATP synthesis and ETS flux in the gill mitochondria measured at 450 mOsm (corresponding to salinity 15) than in 900 mOsm (corresponding to salinity 30) (Sokolov and Sokolova, 2019).

C. gigas was found to survive a wide window of salinity 12 to 43 (Wiltshire, 2007) and can grow well at salinities above 15 (Calvo et al., 1999). Oysters are marine osmoconformers and thus are required to adjust intracellular environment to changing conditions of 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 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, high ROS production leading to oxidative stress (Bal et al., 2022; Paital and Chainy, 2012; Rivera-Ingraham et al., 2016a; Rivera-Ingraham et al., 2016b). Although the mitochondria of *C. gigas* showed improved performance at a salinity of 15 relative to salinity of 30 (Sokolov and Sokolova, 2019) or 33 (this study), no evidence of the 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 (24h) hypoxia and subsequent reoxygenation, regardless of the acclimation salinity. Respiration rates in the OXPHOS and LEAK state remained unchanged during H/R stress, except from upregulated respiration in post-hypoxic recovery predominantly under low salinity. Elevated mitochondrial oxygen consumption supports sufficient ATP synthesis capacity during reoxygenation. Evidence of hypoxia-resilient mitochondrial phenotype maintaining high OXPHOS and ATP synthesis capacity during H/R stress was earlier reported 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; Ivanina et al., 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 an mitochondrial coupling during hypoxia and reoxygenation thus appears to be an adaptation to survive hypoxic periods in the intertidal zone (like in oysters and mussels) or anoxic sediments (like in *A. islandica*) not found in highly aerobic subtidal species like scallops (Ivanina et al., 2012; Ivanina et al., 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 indicate 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 fractional electron leak (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 to 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), that 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 *Crassostrea* spp. (Sokolov et al., 2019; Sokolova et al., 2019; Steffen et al., 2020; Wei et al., 2022; Zhang et al., 2012)

Metabolism at phosphoenolpyruvate (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; Ivanina et al., 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, 1971; Zammit and Newsholme, 1978). Generally, high PK/PEPCK activity leads to preferential channelling of PEP into the aerobic respiration, and low PK/PEPCK 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 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 to 12 hours 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; Ivanina et al., 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*, on the other hand, exhibits a different response, with upregulation of PEPCK and elevated production of succinate occurring after only 24 hours 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 the 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 mitochondrial 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. Earlier studies showed that isolated mitochondria exhibit intrinsic plasticity in response to various stimuli through mechanisms like 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 activities (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 and require 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 to 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, as ROS production exceeds the mitochondrial capacity to mitigate redox stress caused by possible accumulation of electrons.

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 intrinsic adaptive traits in 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

against 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 to those from high salinity. These results suggest that a salinity of 15 might be closer to *C. gigas*' metabolic optimum, even though the studied population originated from a high salinity (33) 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 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 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 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 like 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 part of the adaptive traits of larvae and adult *C. gigas* allowing for the successful invasion of the Baltic Sea requires further investigation.

Abbreviations

AMPK	AMP dependent protein kinase
ASW	Artificial sea water
BSA	Bovine serum albumin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CCO	Cytochrome c oxidase
DNP	2,4,-dinitrophenyl
DNPH	2,4-dinitrophenylhydrazine
DO	Dissolved oxygen
DTT	Dithiothreitol
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
ETS	Electron transport system
FEL	Fractional electron leak
FW	Fresh tissue weight
H/R	Hypoxia/reoxygenation
HEPES	2-[4-(2-hydroxyethyl)-1-piperazin-1-yl]ethanesulfonic acid
HRP	Horseradish peroxidase
HSD	Honest Significant Differences
LDH	Lactate dehydrogenase
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
MDH	Malate dehydrogenase
MMP	Mitochondrial membrane potential

M_{O2}	Oxygen consumption rate
Na_2-EDTA	Disodium-ethylenediaminetetraacetic acid
OXPHOS	Oxidative phosphorylation
OXPHOS CE	OXPHOS coupling efficiency
PBS	Phosphate buffered saline
PC	Protein carbonyls
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pyruvate kinase
PMSF	Phenylmethylsulfonyl fluoride
RCR	Respiratory control ratio
ROS	Reactive oxygen species
SUIT	Substrate-uncoupler-inhibitor titration
TMPD	*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

Acknowledgments

We thank Mathias Wegner of the Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research, List/Sylt, Germany, for providing oysters.

Funding

This study was funded by the Deutsche Forschungsgemeinschaft (DFG) within the project “MitoBOX: the mitochondrial basis of hypoxia tolerance in marine mollusks” (award number 415984732). The open access is supported by Read & Publish Open Access agreement between the Univeristy of Rostock and The Company of Biologists.

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.

Authors' contribution

Conceptualization: I.M.S., C.B.; Methology: J.B.M.S., E.P.S.; I.M.S.; Validation: J.B.M.S., E.P.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., E.P.S., C.B., I.M.S; Visualization: J.B.M.S.; Supervision: I.M.S; C.B.; Project administration: I.M.S; Funding acquisition: I.M.S., C.B.

Data availability

The metadata of the project are available upon request to the corresponding author.

References

Acin-Perez, R., Gatti, D. L., Bai, Y. and Manfredi, G. (2011). Protein phosphorylation and prevention of cytochrome oxidase inhibition by ATP: coupled mechanisms of energy metabolism regulation. *Cell Metab.* **13**, 712–719.

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

Bal, A., Panda, F., Pati, S. G., Anwar, T. N., Das, K. and Paital, B. (2022). Influence of Anthropogenic Activities on Redox Regulation and Oxidative Stress Responses in Different Phyla of Animals in Coastal Water via Changing in Salinity. *Water*. **14**, 4026.

Ballantyne, J. S. and Storey, K. B. (1983). Mitochondria from the ventricle of the marine clam, *Mercenaria mercenaria*: Substrate preferences and effects of pH and salt concentration on proline oxidation. *Comp. Biochem. Phys. Part B Biochem. Mol. Biol.* **76**, 133–138.

Ballantyne, J. S. and Moyes, C. D. (1987). The Effects of Salinity Acclimation on the Osmotic Properties of Mitochondria from the Gill Of Crassostrea Virginica. *Journal of Experimental Biology*. **133**, 449–559.

Bayne, B. L. (2017). Metabolic Expenditure. In *Biology of Oysters*, pp. 331–415: Elsevier.

Beauvoit, B. and Rigoulet, M. (2001). Regulation of cytochrome c oxidase by adenylic nucleotides. Is oxidative phosphorylation feedback regulated by its end-products? *IUBMB Life*. **52**, 143–152.

Berger, V. J. and Kharazova, A. D. (1997). Mechanisms of salinity adaptations in marine molluscs. In *Interactions and Adaptation Strategies of Marine Organisms* (ed. A. D. Naumov, H. Hummel, A. A. Sukhotin and J. S. Ryland), pp. 115–126. Dordrecht: Springer Netherlands.

Brand, M. D. (1997). Regulation analysis of energy metabolism. *J. Exp. Biol.* **200**, 193–202.

Brand, M. D. (2000). Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Experimental Gerontology*. **35**, 811–820.

Brand, M. D., Chien, L.-F., Ainscow, E. K., Rolfe, D. F. S. and Porter, R. K. (1994). The causes and functions of mitochondrial proton leak. *Biochim. Biophys. Acta*. **1187**, 132–139.

Breitburg, D., Levin, L. A., Oschlies, A., Grégoire, M., Chavez, F. P., Conley, D. J., Garçon, V., Gilbert, D., Gutiérrez, D. and Isensee, K. et al. (2018). Declining oxygen in the global ocean and coastal waters. *Science*. **359,** eaam7240, 1–11.

Breitburg, D., Baumann, H., Sokolova, I. and Frieder, C. (2019). 6. Multiple stressors -forces that combine to worsen deoxygenation and its effects. In *Ocean deoxygenation: Everyone's problem. Causes, impacts, consequences and solutions* (ed. D. D. A. Laffoley and J. M. Baxter), pp. 225–247. Gland, Switzerland: IUCN.

Cadenas, S. (2018). Mitochondrial uncoupling, ROS generation and cardioprotection. *Biochim. Biophys. Acta Bioenerg.* **1859**, 940–950.

Calvo, G. W., Luckenback, M. W. and Burreson, E. M. (1999). A Comparative Field Study of *Crassostrea gigas* and *Crassostrea virginica* in Relation to Salinity in Virginia. Special Reports in Applied Marine Science and Ocean Engineering (SRAMSOE).

Chen, L., Yu, F., Shi, H., Wang, Q., Xue, Y., Xue, C., Wang, Y. and Li, Z. (2022). Effect of salinity stress on respiratory metabolism, glycolysis, lipolysis, and apoptosis in Pacific oyster (*Crassostrea gigas*) during depuration stage. *J Sci Food Agric*. **102**, 2003–2011.

Cherkasov, A. A., Overton, R. A., Sokolov, E. P. and Sokolova, I. M. (2007). Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, *Crassostrea virginica*: a role of temperature in oxidative stress and allosteric enzyme regulation. *J. Exp. Biol.* **210**, 46–55.

Das, B., Ramnath, Dutta, A. K. and Tandon, V. (2015). Differential kinetics at PK/PEPCK branch point in the cestode, *Raillietina echinobothrida*. *Exp Parasitol*. **153**, 151–159.

David, E., Tanguy, A., Pichavant, K. and Moraga, D. (2005). Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *FEBS J*. **272**, 5635–5652.

Diaz, R. J. and Rosenberg, R. (1995). Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. *Oceanogr. Mar. Biol.* **33**, 245–303.

Diaz, R. J. and Rosenberg, R. (2008). Spreading Dead Zones and Consequences for Marine Ecosystems. *Science*. **321**, 926–929.

Durack, P. J., Wijffels, S. E. and Matear, R. J. (2012). Ocean salinities reveal strong global water cycle intensification during 1950 to 2000. *Science*. **336**, 455–458.

Dutertre, M., Barillé, L., Beninger, P. G., Rosa, P. and Gruet, Y. (2009). Variations in the pallial organ sizes of the invasive oyster, *Crassostrea gigas*, along an extreme turbidity gradient. *Estuarine, Coastal and Shelf Science*. **85**, 431–436.

Evans, D. H. (2009). *Osmotic and ionic regulation: Cells and animals / edited by David H. Evans*. Boca Raton: CRC Press.

Ewers-Saucedo, C., Heuer, N., Moesges, Z., Ovenbeck, K., Schröter, N. and Brandis, D. (2020). First record of the Pacific oyster *Magallana gigas* (Thunberg, 1793) in the Baltic Sea proper. *Mar Biodivers Rec*. **13**.

Falfushynska, H. I., Piontkivska, H. and Sokolova, I. M. (2020a). Effects of intermittent hypoxia on cell survival and inflammatory responses in the intertidal marine bivalves *Mytilus edulis* and *Crassostrea gigas*. *J. Exp. Biol.* **223,** jeb217026, jeb217026.

Falfushynska, H. I., Sokolov, E., Piontkivska, H. and Sokolova, I. M. (2020b). The Role of Reversible Protein Phosphorylation in Regulation of the Mitochondrial Electron Transport System During Hypoxia and Reoxygenation Stress in Marine Bivalves. *Front. Mar. Sci.* **7,** 467.

Forman, H. J., Maiorino, M. and Ursini, F. (2010). Signaling functions of reactive oxygen species. *Biochemistry*. **49**, 835–842.

Fuhrmann, M., Delisle, L., Petton, B., Corporeau, C. and Pernet, F. (2018). Metabolism of the Pacific oyster, *Crassostrea gigas*, is influenced by salinity and modulates survival to the Ostreid herpesvirus OsHV-1. *Biol Open*. **7**.

Galli, G. L. J. and Richards, J. G. (2014). Mitochondria from anoxia-tolerant animals reveal common strategies to survive without oxygen. *J. Comp. Physiol. B*. **184**, 285–302.

Gerber, L., Clow, K. A., Katan, T., Emam, M., Leeuwis, R. H. J., Parrish, C. C. and Gamperl, A. K. (2019). Cardiac mitochondrial function, nitric oxide sensitivity and lipid composition following hypoxia acclimation in sablefish. *J. Exp. Biol.* **222**.

Greenway, S. C. and Storey, K. B. (1999). The effect of prolonged anoxia on enzyme activities in oysters (*Crassostrea virginica*) at different seasons. *J. Exp. Mar. Biol. Ecol.* **242**, 259–272.

Grieshaber, M. K., Hardewig, I., Kreutzer, U. and Pörtner, H.-O. (1994). Physiological and Metabolic Responses to Hypoxia in Invertebrates. *Rev. Physiol. Biochem. Pharmacol.* **125**, 43-147.

Haider, F., Falfushynska, H. I., Timm, S. and Sokolova, I. M. (2020). Effects of hypoxia and reoxygenation on intermediary metabolite homeostasis of marine bivalves *Mytilus edulis* and *Crassostrea gigas*. *Comp. Biochem. Phys. Part A Mol. Integr. Physiol.* **242,** 110657, 110657.

Haider, F., Sokolov, E. P. and Sokolova, I. M. (2018). Effects of mechanical disturbance and salinity stress on bioenergetics and burrowing behavior of the soft-shell clam *Mya arenaria*. *J. Exp. Biol.* **221**.

Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: Molecular/metabolic defence and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9493–9498.

Honda, H. M., Korge, P. and Weiss, J. N. (2005). Mitochondria and ischemia/reperfusion injury. *Ann. N. Y. Acad. Sci.* **1047**, 248–258.

Ivanina, A. V., Kurochkin, I. O., Leamy, L. and Sokolova, I. M. (2012). Effects of temperature and cadmium exposure on the mitochondria of oysters (*Crassostrea virginica*) exposed to hypoxia and subsequent reoxygenation. *J. Exp. Biol.* **215**, 3142–3154.

Ivanina, A. V., Nesmelova, I., Leamy, L., Sokolov, E. P. and Sokolova, I. M. (2016). Intermittent hypoxia leads to functional reorganization of mitochondria and affects cellular bioenergetics in marine molluscs. *J. Exp. Biol.* **219**, 1659–1674.

Ivanina, A. V., Sokolov, E. P. and Sokolova, I. M. (2010). Effects of cadmium on anaerobic energy metabolism and mRNA expression during air exposure and recovery of an intertidal mollusk *Crassostrea virginica*. *Aquat. Toxicol.* **99**, 330–342.

Ivanina, A. V. and Sokolova, I. M. (2016). Effects of intermittent hypoxia on oxidative stress and protein degradation in molluscan mitochondria. *J. Exp. Biol.* **219**, 3794–3802.

Jastroch, M., Divakaruni, A. S., Mookerjee, S., Treberg, J. R. and Brand, M. D. (2010). Mitochondrial proton and electron leaks. *Essays Biochem.* **47**, 53–67.

Koch, R. E., Buchanan, K. L., Casagrande, S., Crino, O., Dowling, D. K., Hill, G. E., Hood, W. R., McKenzie, M., Mariette, M. M. and Noble, D. W. A. et al. (2021). Integrating Mitochondrial Aerobic Metabolism into Ecology and Evolution. *Trends Ecol Evol* [Epub ahead of print].

Kurochkin, I. O., Etzkorn, M., Buchwalter, D., Leamy, L. and Sokolova, I. M. (2011). Top-down control analysis of the cadmium effects on molluscan mitochondria and the mechanisms of cadmium-induced mitochondrial dysfunction. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R21-31.

Kurochkin, I. O., Ivanina, A. V., Eilers, S., Downs, C. A., May, L. A. and Sokolova, I. M. (2009). Cadmium affects metabolic responses to prolonged anoxia and reoxygenation in eastern oysters (*Crassostrea virginica*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R1262-R1272.

Lambert, A. J. and Brand, M. D. (2004). Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem. J.* **382**, 511–517.

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A.-G., Ahn, B.-W., Shaltiel, S. and Stadtman, E. R. (1990). [49] Determination of carbonyl content in oxidatively modified proteins. In *Oxygen radicals in biological systems. Pt B, Oxygen radicals and antioxidants* (ed. L. Packer and A. N. Glazer), pp. 464–478: Elsevier.

Mathers, K. E. and Staples, J. F. (2019). Differential posttranslational modification of mitochondrial enzymes corresponds with metabolic suppression during hibernation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **317**, R262-R269.

Matoo, O. B., Ivanina, A. V., Ullstad, C., Beniash, E. and Sokolova, I. M. (2013). Interactive effects of elevated temperature and CO_2 levels on metabolism and oxidative stress in two common

marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). *Comp. Biochem. Phys. Part A Mol. Integr. Physiol.* **164**, 545–553.

McBride, H. M., Neuspiel, M. and Wasiak, S. (2006). Mitochondria: more than just a powerhouse. *Curr Biol*. **16**, R551-60.

McLusky, D. S. and Elliott, M. (2004). *The estuarine ecosystem: Ecology, threats, and management*. Oxford: OUP.

Meng, J., Zhu, Q., Zhang, L., Li, C., Li, L., She, Z., Huang, B. and Zhang, G. (2013). Genome and transcriptome analyses provide insight into the euryhaline adaptation mechanism of *Crassostrea gigas*. *PLoS One*. **8**, e58563.

Miwa, S. and Brand, M. D. (2003). Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochem. Soc. Trans.* **31**, 1300–1301.

Napolitano, G., Venditti, P., Fasciolo, G., Esposito, D., Uliano, E. and Agnisola, C. (2019). Acute hypoxia/reoxygenation affects muscle mitochondrial respiration and redox state as well as swimming endurance in zebrafish. *J. Comp. Physiol. B.* **189**, 97–108.

Noor, M. N., Wu, F., Sokolov, E. P., Falfushynska, H., Timm, S., Haider, F. and Sokolova, I. M. (2021). Salinity-dependent effects of ZnO nanoparticles on bioenergetics and intermediate metabolite homeostasis in a euryhaline marine bivalve, *Mytilus edulis*. *Sci Total Environ*. **774**, 145195.

Nusetti, O., Tovar, M. and Zapata-Vívenes, E. (2010). Pyruvate Kinase, Phosphoenolpyruvate Carboxykinase, Cytochrome C Oxidase and Catalase Activities in Cadmium Exposed *Perna viridis* Subjected to Anoxic and Aerobic Conditions. *Journal of Shellfish Research*. **29**, 203–208.

Onukwufor, J. O., Stevens, D. and Kamunde, C. (2017). Combined effects of cadmium, temperature and hypoxia-reoxygenation on mitochondrial function in rainbow trout (Oncorhynchus mykiss). *Aquat. Toxicol.* **182**, 129–141.

Ouillon, N., Sokolov, E. P., Otto, S., Rehder, G. and Sokolova, I. M. (2021). Effects of variable oxygen regimes on mitochondrial bioenergetics and reactive oxygen species production in a marine bivalve *Mya arenaria*. *J. Exp. Biol.* **224**, jeb237156.

Pagliarini, D. J. and Dixon, J. E. (2006). Mitochondrial modulation: reversible phosphorylation takes center stage? *Trends Biochem Sci*. **31**, 26–34.

Paital, B. and Chainy, G. B. N. (2012). Effects of salinity on O_2 consumption, ROS generation and oxidative stress status of gill mitochondria of the mud crab *Scylla serrata*. *Comp. Biochem. Phys. Part C Toxicol. Pharmacol.* **155**, 228–237.

Pamenter, M. E. (2014). Mitochondria: a multimodal hub of hypoxia tolerance. *Can. J. Zool.* **92**, 569–589.

Paradis, S., Charles, A.-L., Meyer, A., Lejay, A., Scholey, J. W., Chakfé, N., Zoll, J. and Geny, B. (2016). Chronology of mitochondrial and cellular events during skeletal muscle ischemia-reperfusion. *Am. J. Physiol. Cell Physiol.* **310**, C968-C982.

Piper, H. M., Meuter, K. and Schäfer, C. (2003). Cellular mechanisms of ischemia-reperfusion injury. *Ann. Thorac. Surg.* **75**, S644-S648.

Podbielski, I., Schmittmann, L., Sanders, T. and Melzner, F. (2022). Acclimation of marine invertebrate osmolyte systems to low salinity: A systematic review & meta-analysis. *Front. Mar. Sci.* **9,** 934378.

Prosser, C. L. (1991). *Comparative animal physiology. Environmental and metabolic animal physiology*: John Wiley & Sons.

Rivera-Ingraham, G. A., Barri, K., Boël, M., Farcy, E., Charles, A.-L., Geny, B. and Lignot, J.-H. (2016a). Osmoregulation and salinity-induced oxidative stress: is oxidative adaptation determined by gill function? *J. Exp. Biol.* **219**, 80–89.

Rivera-Ingraham, G. A., Nommick, A., Blondeau-Bidet, E., Ladurner, P. and Lignot, J.-H. (2016b). Salinity stress from the perspective of the energy-redox axis: Lessons from a marine intertidal flatworm. *Redox Biol.* **10**, 53–64.

Sappal, R., MacDougald, M., Fast, M., Stevens, D., Kibenge, F., Siah, A. and Kamunde, C. (2015). Alterations in mitochondrial electron transport system activity in response to warm acclimation, hypoxia-reoxygenation and copper in rainbow trout, *Oncorhynchus mykiss*. *Aquat. Toxicol.* **165**, 51–63.

Saz, H. J. (1971). Facultative Anaerobiosis in the Invertebrates: Pathways and Control Systems. *Am. Zool.* **11**, 125–135.

Schmidt, A., Wehrmann, A. and Dittmann, S. (2008). Population dynamics of the invasive Pacific oyster *Crassostrea gigas* during the early stages of an outbreak in the Wadden Sea (Germany). *Helgol Mar Res*. **62**, 367–376.

Shumway, S. E. (1977). Effect of salinity fluctuation on the osmotic pressure and Na^+, Ca^{2+} and Mg^{2+} ion concentrations in the hemolymph of bivalve molluscs. *Mar. Biol.* **41**, 153–177.

Sies, H., Belousov, V. V., Chandel, N. S., Davies, M. J., Jones, D. P., Mann, G. E., Murphy, M. P., Yamamoto, M. and Winterbourn, C. (2022). Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology. *Nat Rev Mol Cell Biol*. **23**, 499–515.

Sies, H. and Jones, D. P. (2020). Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. *Nat Rev Mol Cell Biol*. **21**, 363–383.

Sigwart, J. D., Wong, N. L. W. S. and Esa, Y. (2021). Global controversy in oyster systematics and a newly described species from SE Asia (Bivalvia: Ostreidae: Crassostreinae). *Mar. Biodivers.* **51**.

Simpfendörfer, R. W., Vial, M., López, D. A., Verdala, M. and González, M. (1995). Relationship between the aerobic and anaerobic metabolic capacities and the vertical distribution of three intertidal sessile invertebrates: *Jehlius cirratus* (Darwin) (Cirripedia), *Perumytilus purpuratus* (Lamarck) (Bivalvia) and *Mytilus chilensis* (Hupé) (Bivalvia). *Comp. Biochem. Phys. Part B Biochem. Mol. Biol.* **111**, 615–623.

Sokolov, E. P., Markert, S., Hinzke, T., Hirschfeld, C., Becher, D., Ponsuksili, S. and Sokolova, I. M. (2019). Effects of hypoxia-reoxygenation stress on mitochondrial proteome and bioenergetics of the hypoxia-tolerant marine bivalve *Crassostrea gigas*. *J. Proteomics*. **194**, 99–111.

Sokolov, E. P. and Sokolova, I. M. (2019). Compatible osmolytes modulate mitochondrial function in a marine osmoconformer *Crassostrea gigas* (Thunberg, 1793). *Mitochondrion*. **45**, 29–37.

Sokolov, E. P., Adzigbli, L., Markert, S., Bundgaard, A., Fago, A., Becher, D., Hirschfeld, C. and Sokolova, I. M. (2021). Intrinsic Mechanisms Underlying Hypoxia-Tolerant Mitochondrial Phenotype During Hypoxia-Reoxygenation Stress in a Marine Facultative Anaerobe, the Blue Mussel Mytilus edulis. *Front. Mar. Sci.* **8,** 773734.

Sokolova, I. M. (2018). Mitochondrial Adaptations to Variable Environments and Their Role in Animals' Stress Tolerance. *Integr. Comp. Biol.* **58**, 519–531.

Sokolova, I. M., Sokolov, E. P. and Haider, F. (2019). Mitochondrial Mechanisms Underlying Tolerance to Fluctuating Oxygen Conditions: Lessons from Hypoxia-Tolerant Organisms. *Integr. Comp. Biol.* **59**, 938–952.

Sokolova, I. M. (2023). Ectotherm mitochondrial economy and responses to global warming. *Acta Physiol (Oxf)*, e13950.

Solaini, G., Baracca, A., Lenaz, G. and Sgarbi, G. (2010). Hypoxia and mitochondrial oxidative metabolism. *Biochim. Biophys. Acta*. **1797**, 1171–1177.

Somero, G. N. and Yancey, P. H. (2010). Osmolytes and Cell-Volume Regulation: Physiological and Evolutionary Principles. *Compr Physiol*, 441–484.

Steffen, J. B. M., Falfushynska, H. I., Piontkivska, H. and Sokolova, I. M. (2020). Molecular Biomarkers of the Mitochondrial Quality Control Are Differently Affected by Hypoxia-Reoxygenation Stress in Marine Bivalves *Crassostrea gigas* and *Mytilus edulis*. *Front. Mar. Sci.* **7,** 604411.

Steffen, J. B. M., Haider, F., Sokolov, E. P., Bock, C. and Sokolova, I. M. (2021). Mitochondrial capacity and reactive oxygen species production during hypoxia and reoxygenation in the ocean quahog, *Arctica islandica*. *J. Exp. Biol.* **224**.

Storey, K. B. (2002). Life in the slow lane: molecular mechanisms of estivation. *Comp. Biochem. Phys. Part A Mol. Integr. Physiol.* **133**, 733–754.

Strasser, M. (1999). *Mya arenaria* — an ancient invader of the North Sea coast. *Helgoländer Meeresuntersuchungen*. **52**, 309–324.

Sussarellu, R., Dudognon, T., Fabioux, C., Soudant, P., Moraga, D. and Kraffe, E. (2013). Rapid mitochondrial adjustments in response to short-term hypoxia and re-oxygenation in the Pacific oyster, *Crassostrea gigas*. *J. Exp. Biol.* **216**, 1561–1569.

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

Troost, K. (2010). Causes and effects of a highly successful marine invasion: Case-study of the introduced Pacific oyster *Crassostrea gigas* in continental NW European estuaries. *Journal of Sea Research*. **64**, 145–165.

Venditti, P., Masullo, P. and Di Meo, S. (2001). Effects of myocardial ischemia and reperfusion on mitochondrial function and susceptibility to oxidative stress. *Cell Mol Life Sci*. **58**, 1528–1537.

Wei, S., Xie, Z., Liu, C., Sokolova, I., Sun, B., Mao, Y., Xiong, K., Peng, J., Fang, J. K.-H. and Hu, M. et al. (2022). Antioxidant response of the oyster *Crassostrea hongkongensis* exposed to diel-cycling hypoxia under different salinities. *Mar Environ Res*. **179**, 105705.

Wiltshire, K. (2007). Ecophysiological tolerances of the Pacific oyster, *Crassostrea gigas*, with regard to the potential spread of populations in South Ausralian waters. **no. 222**. West Beach, SA: SARDI Aquatic Sciences.

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.* **208**, 2819–2830.

Yang, Y. and Gibson, G. E. (2019). Succinylation Links Metabolism to Protein Functions. *Neurochem Res*. **44**, 2346–2359.

Zammit, V. A. and Newsholme, E. A. (1978). Properties od Pyruvate Kinase and Phosphoenolypyruvate Carboxykinase in Relation to the Direction and Regulation of Phosphoenolpyruvate Metabolism in Muscles of the Frog and Marine Invertebrates. *Biochem. J.* **174**, 979–987.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X. and Qi, H. et al. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature*. **490**, 49–54.

Zhao, X., Yu, H., Kong, L. and Li, Q. (2012). Transcriptomic responses to salinity stress in the Pacific oyster *Crassostrea gigas*. *PLoS One*. **7**, e46244.

Zorov, D. B., Juhaszova, M. and Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol. Rev.* **94**, 909–950.

Figures

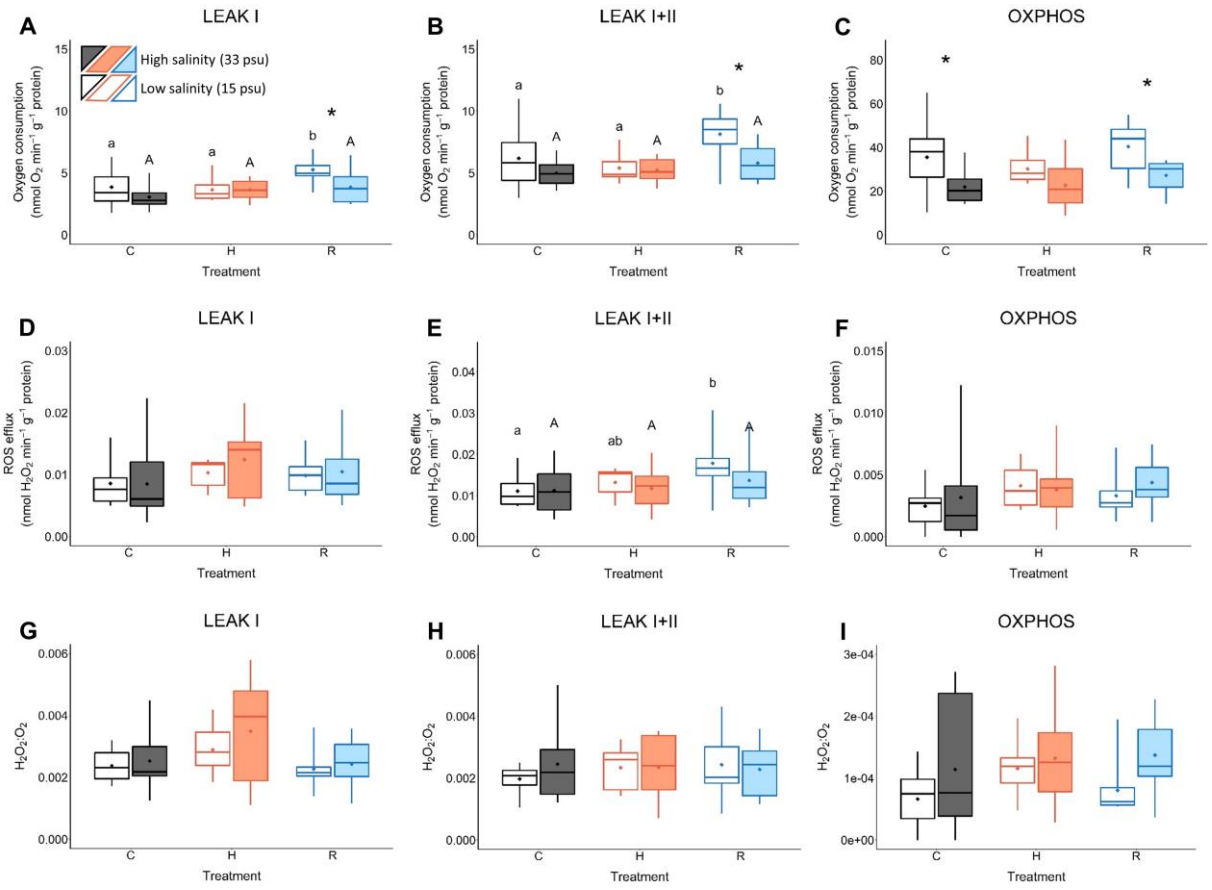

Fig. 1. Effect of salinity acclimation and short-term H/R stress on oxygen consumption (MO_2), ROS efflux and H_2O_2:O_2 ratio of *C. gigas* mitochondria in resting states (LEAK I and LEAK I+II) and active (OXPHOS) state. Oxygen consumption (A,B,C), ROS efflux (D,E,F) and H_2O_2:O_2 ratio (G,H,I) in resting mitochondria respiring on complex I substrates pyruvate (P) and malate (M) (LEAK I) (A,D,G), in resting mitochondria respiring on complex I and II substrates P, M and succinate (S) (LEAK I+II) (B,E,H) and active mitochondria (OXPHOS) (C,F,I). Experimental groups: C, control (21% O_2); H, short-term (24h) 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). Empty Box-Whisker plot – low salinity (LS) acclimation (salinity 15); filled Box-Whisker plot – high salinity (HS) acclimation (salinity 33). Means are shown as points within corresponding Box-Whisker plots. Statistical significances (P < 0.05) between oxygen treatments are represented by different letters above plots and statistical

differences ($P < 0.05$) between salinity acclimation within one oxygen treatment are depicted as asterisk (*) above plots. Plots with the same letter or not marked with a letter or star are not significantly different ($P > 0.05$). N = 9-11, 8-10, 8-10, 8-10, 9-11 and 10-11 in LS C, HS C, LS H, HS H, LS R and HS R, respectively.

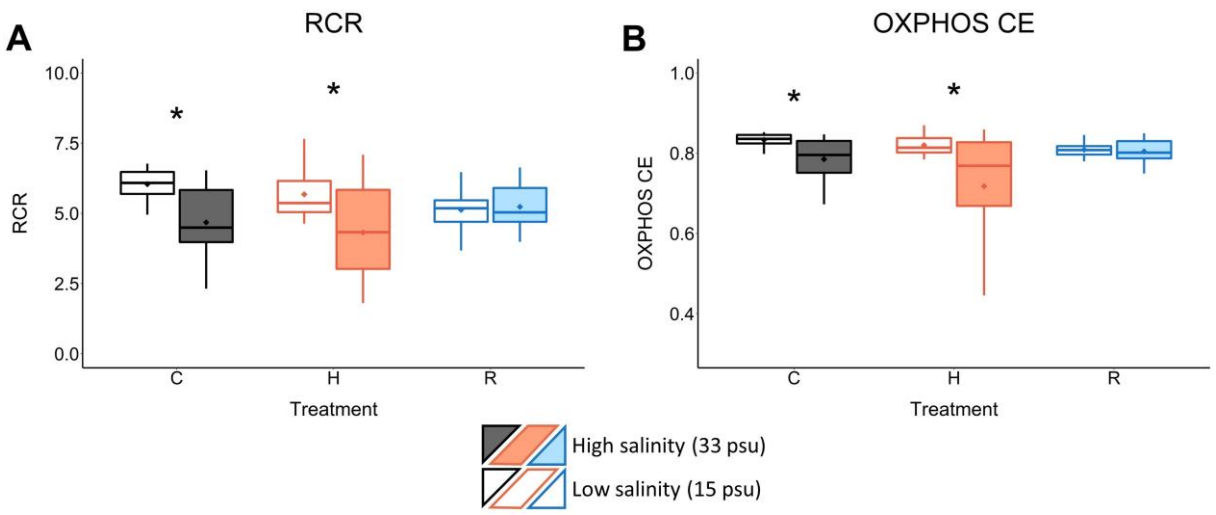

Fig. 2. Effect of salinity acclimation and short-term H/R stress on coupling efficiency of *C. gigas* mitochondria. (A) Respiratory control ratio (RCR); (B) OXPHOS coupling efficiency (OXPHOS CE) = 1-(LEAK I+II/OXPHOS)). Experimental groups: C, control (21% O_2); H, short-term (24h) severe (<0.01% O_2) hypoxia; R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Empty Box-Whisker plot – low salinity (LS) acclimation (salinity 15); filled Box-Whisker plot – high salinity (HS) acclimation (salinity 33). Statistical differences ($P < 0.05$) between salinity acclimations are depicted as asterisk (*). Plots without stars are not significantly different ($P > 0.05$). N= 10, 9-10, 10, 10, 9-10 and 10 in LS C, HS C, LS H, HS H, LS R and HS R, respectively.

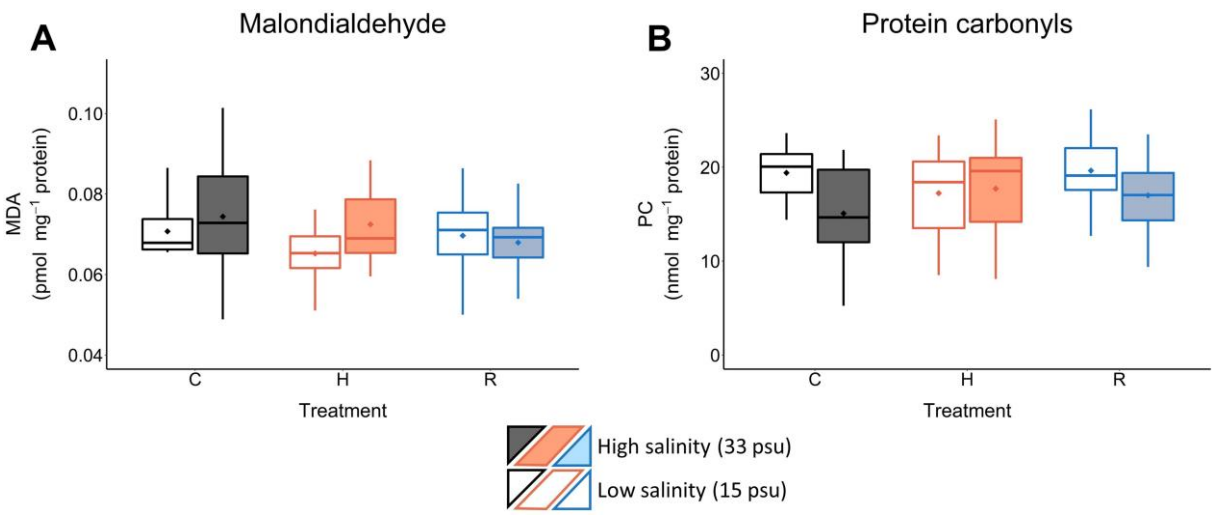

Fig. 3. Assessment of oxidative damage in mitochondrial proteins of oysters exposed to combined salinity and *in vivo* H/R stress. (A) Malondialdehyde (MDA)-protein conjugates; (B) protein carbonyls. Experimental groups: C, control (21% O_2); H, short-term (24h) severe (<0.01% O_2) hypoxia; R, short-term severe hypoxia (24 h at <0.01% O_2) and subsequent 1.5 h reoxygenation (21% O_2). Empty Box-Whisker plot – low salinity (LS) acclimation (salinity 15); filled Box-Whisker plot – high salinity (HS) acclimation (salinity 33). 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 C, HS C, LS H, HS H, LS R and HS R, respectively.

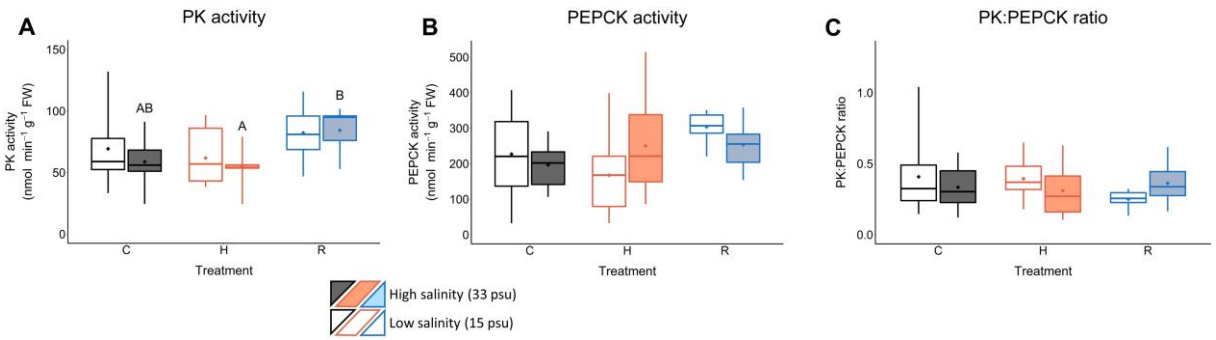

Fig. 4. Metabolic response of enzyme activity of pyruvate kinase and phosphoenolpyruvate carboxykinase to combined salinity and H/R stress. (A) Activity of pyruvate kinase (PK); (B) activity of Phosphoenolpyruvate carboxykinase (PEPCK); (C) PK:PEPCK ratio. Experimental groups: C, control (21% O_2); H, short-term (24h) 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). Empty Box-Whisker plot – low salinity (LS) acclimation (salinity 15); filled Box-Whisker plot – high salinity (HS) acclimation (salinity 33). Means are shown as points within corresponding BOX-Whisker plots. Statistical significances (P < 0.05) between oxygen treatments are represented by different letters above plots. Plots with the same letter or without a letter are not significantly different (P > 0.05). N= 11-12, 9-10, 9, 9-10, 8-11 and 9-11 in LS C, HS C, LS H, HS H, LS R and HS R, respectively.

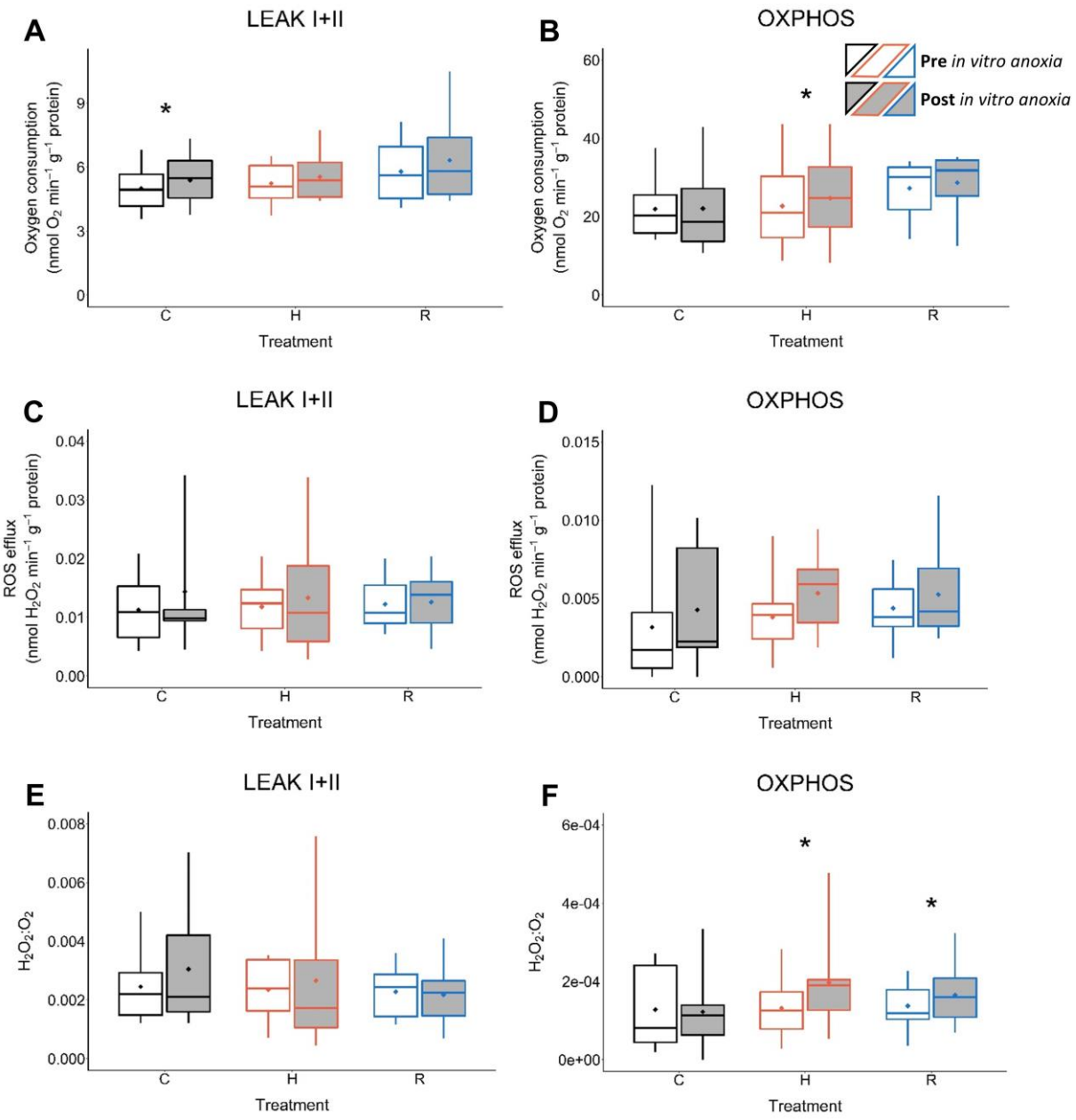

Fig. 5. Effect of *in vitro* H/R stress to oxygen consumption, ROS efflux and H_2O_2:O_2 ratio of mitochondria in LEAK and OXPHOS state from oysters acclimated to high salinity (HS). (A) Oxygen consumption of mitochondria in LEAK I+II state; (B) oxygen consumption in OXPHOS state; (C) ROS efflux of mitochondria in LEAK I+II state; (D) ROS efflux in OXPHOS state; (E) H_2O_2:O_2 ratio of mitochondria in LEAK I+II state; (F) H_2O_2:O_2 ratio in OXPHOS state. Experimental groups: C, control (21% O_2); H, short-term (24h) 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). 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.1 curated for usage in paired t-tests as described in the Material and Method section. N= 8-10, 8-10, 8-10, 8-10, 9-11 and 9-11 in C pre anoxia, C post anoxia, H pre anoxia, H post anoxia, R pre anoxia and R post anoxia, respectively.

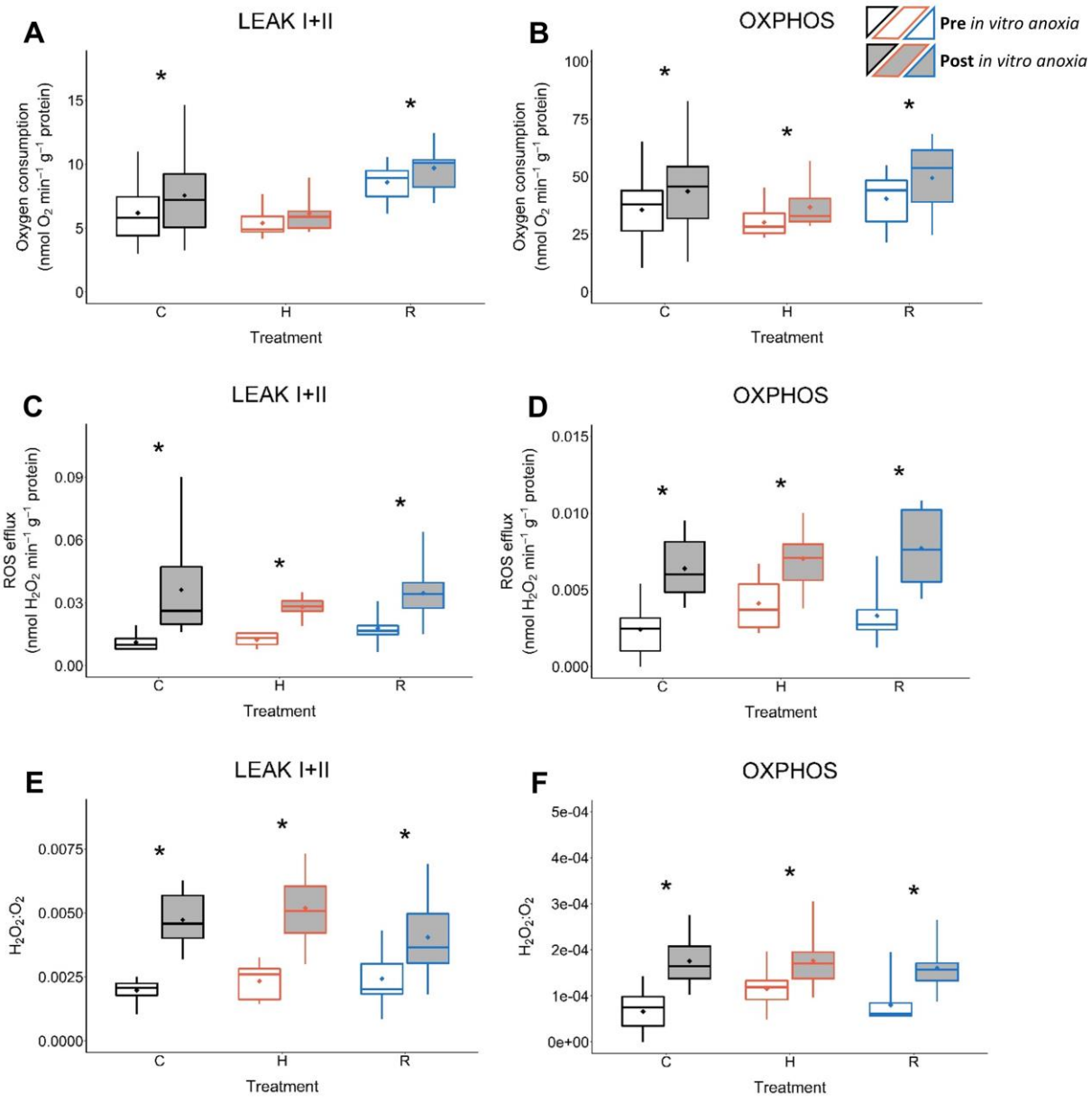

Fig. 6. Effect of *in vitro* H/R stress to oxygen consumption, ROS efflux and H_2O_2:O_2 ratio of mitochondria in LEAK and OXPHOS state from oysters acclimated to low salinity (LS). (A) Oxygen consumption of mitochondria in LEAK I+II state; (B) oxygen consumption in OXPHOS state; (C) ROS efflux of mitochondria in LEAK I+II state; (D) ROS efflux in OXPHOS state; (E) H_2O_2:O_2 ratio of mitochondria in LEAK I+II state; (F) H_2O_2:O_2 ratio in OXPHOS state. Experimental groups: C, control (21% O_2); H, short-term (24h) 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). 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.1 curated for usage in paired t-tests as described in the Material and Method section. N= 9-11, 9-11, 8-10, 8-10, 9-11 and 9-11 in C pre anoxia, C post anoxia, H pre anoxia, H post anoxia, R pre anoxia and R post anoxia, respectively.

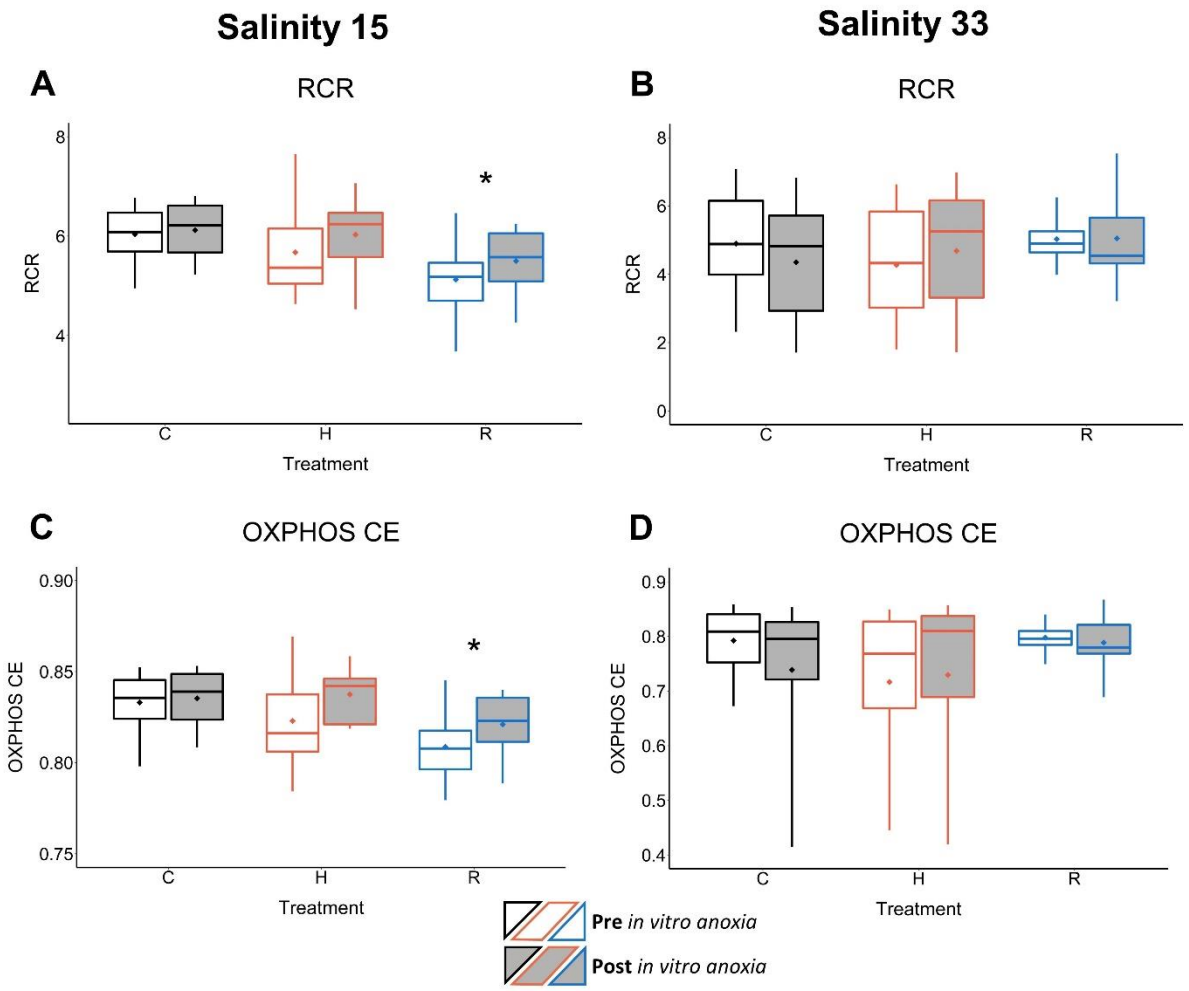

Fig. S1. Effect of *in vitro* H/R stress on coupling efficiency of *C. gigas* mitochondria from oysters exposed to combined *in vivo* salinity and H/R stress.

(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.