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RESEARCH ARTICLE

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

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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.

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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

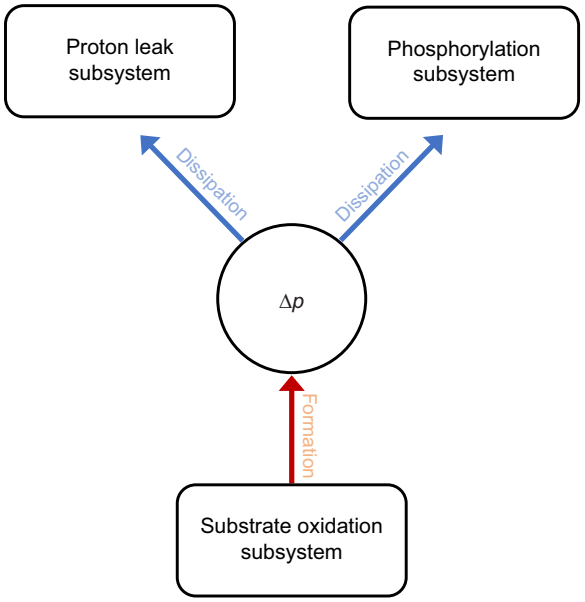

Fig. 1. Mitochondrial subsystems used in top-down metabolic control analysis. Processes of the substrate oxidation subsystem [composed of the TCA cycle, electron transport system (ETS) and metabolite transport] that generates the protonmotive force (Δp) by pumping protons from the mitochondrial matrix 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

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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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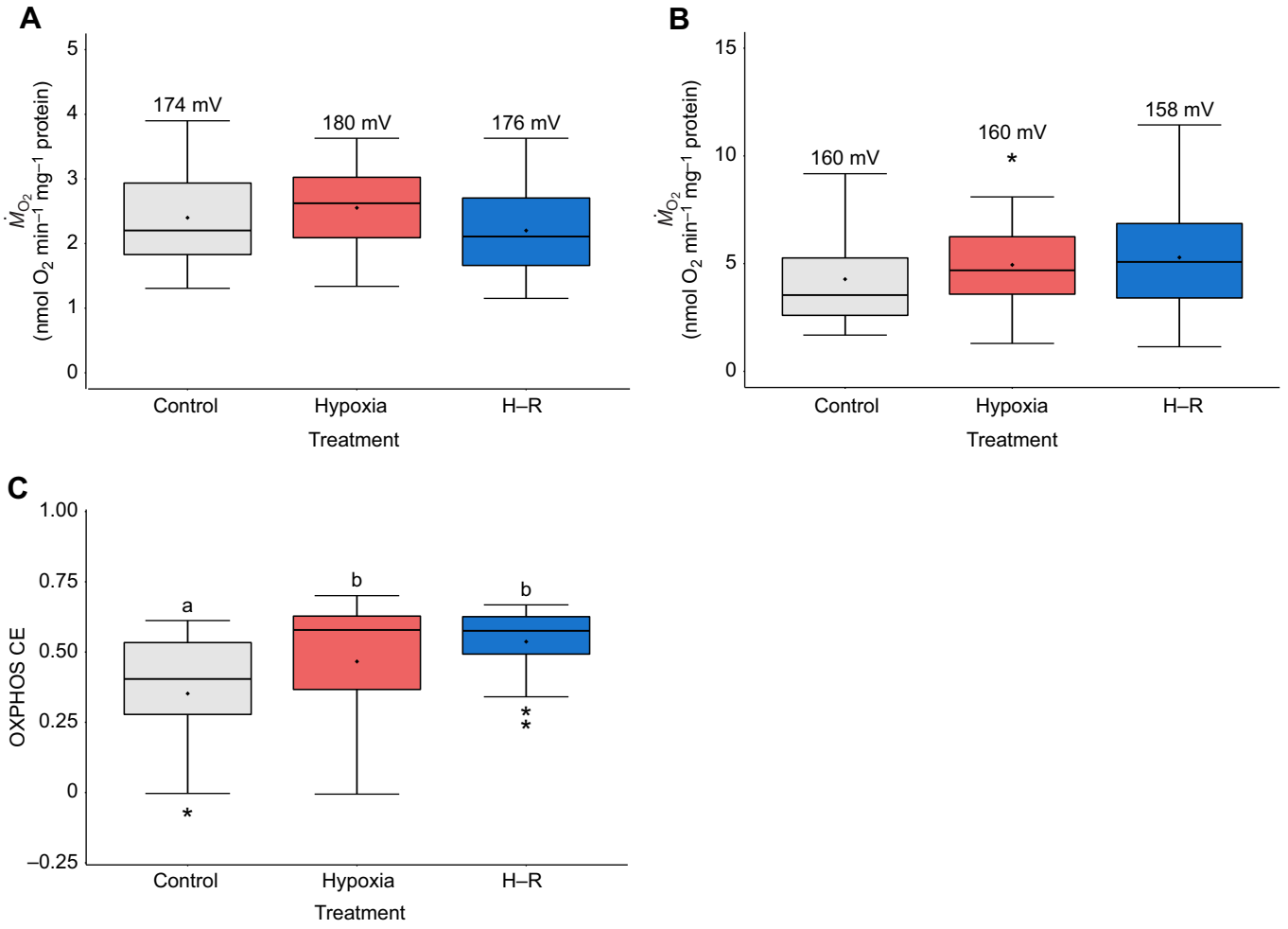

Fig. 2. Effect of short-term hypoxia–reoxygenation (H–R) stress on oxygen consumption rate ($\dot{M}_{O_2}$) and membrane potential (Δψ) of *Arctica islandica* mitochondria in active (OXPHOS) and resting (LEAK) state. (A) $\dot{M}_{O_2}$ of mitochondria in the LEAK state. (B) $\dot{M}_{O_2}$ of ADP-stimulated 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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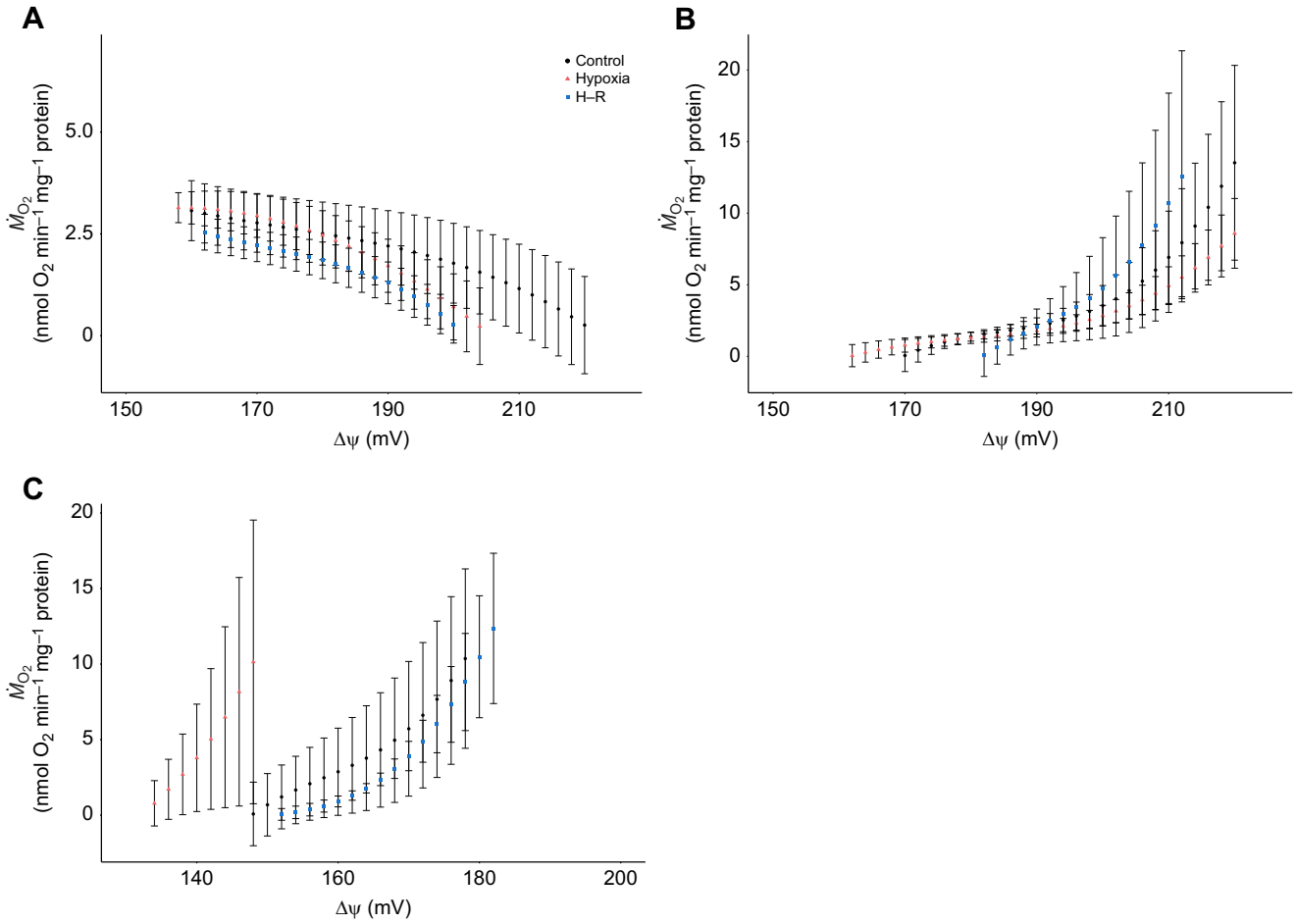

Fig. 3. Kinetic response of the substrate oxidation, proton leak and phosphorylation subsystems to H–R stress. Curves were obtained by averaging the predicted $\dot{M}_{O_2}$ values for a common Δψ (in 2 mV increments based on individual titration curves linking Δψ and $\dot{M}_{O_2}$ for each mitochondrial 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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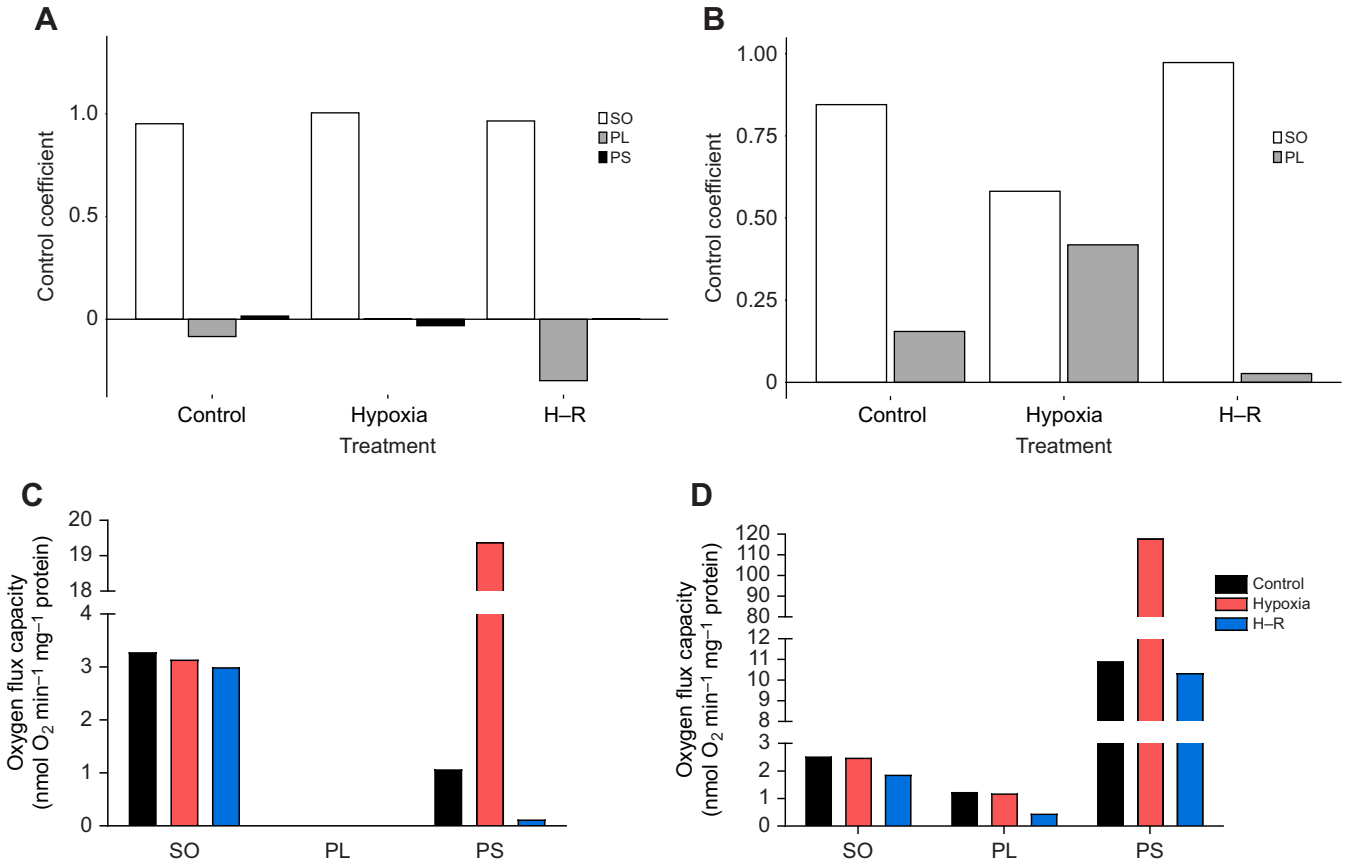

Fig. 4. Control coefficient and oxygen flux capacity of mitochondrial subsystems in OXPHOS and LEAK state in response to H–R stress. (A) Control coefficients over OXPHOS state at 155 mV. (B) Control coefficients over LEAK state at 180 mV. (C) Oxygen flux capacity in the OXPHOS state calculated at a common $\Delta\psi$ 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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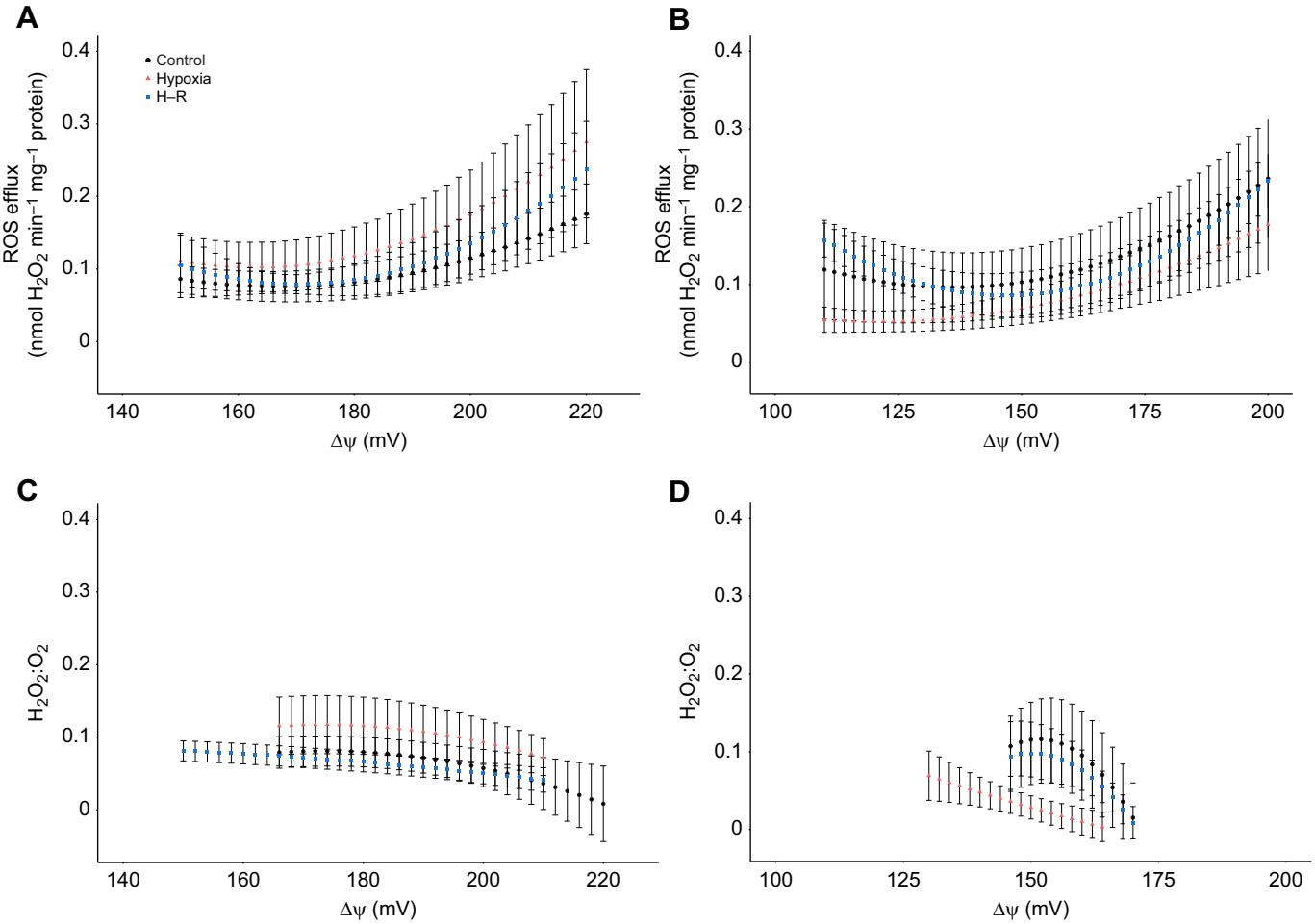

Fig. 5. Kinetic response of reactive oxygen species (ROS) efflux and associated H_2O_2:O_2 ratio in OXPHOS and LEAK state mitochondria during H–R stress. Curves were obtained by averaging the predicted ROS efflux and H_2O_2:O_2 ratios for set $\Delta\psi$ values (in 2 mV increments) based on individual titration curves linking $\Delta\psi$ 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 $\Delta\psi$ 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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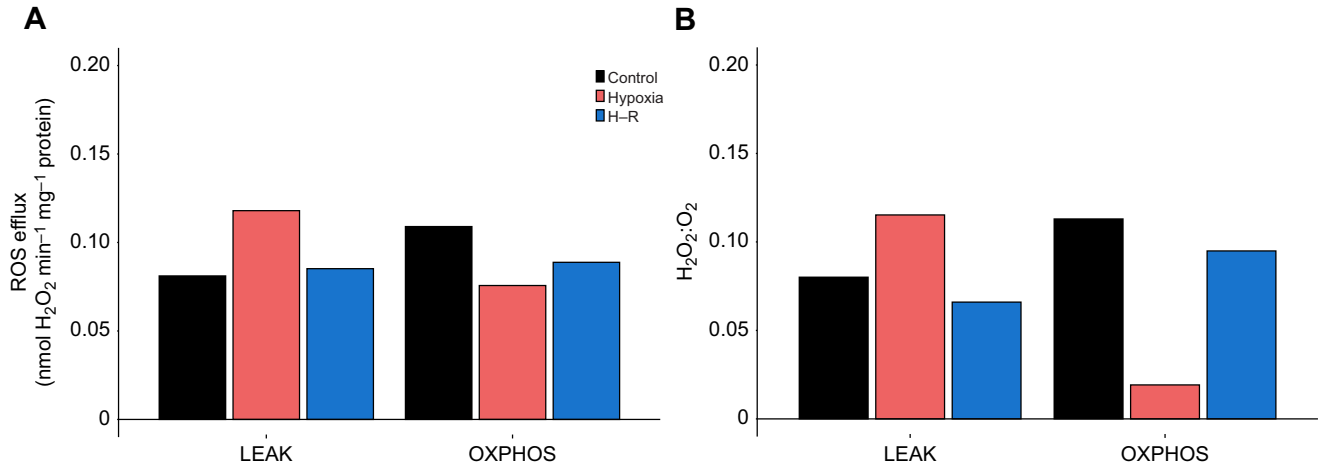

Fig. 6. Effect of short-term H–R stress on ROS efflux and H_2O_2:O_2 ratio in LEAK and OXPHOS state at a common Δψ. (A) ROS efflux at a common Δψ. (B) H_2O_2:O_2 ratio at a common Δψ. The common Δψ was 180 mV for LEAK state and 155 mV for OXPHOS state. ROS efflux was measured as emission of hydrogen 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

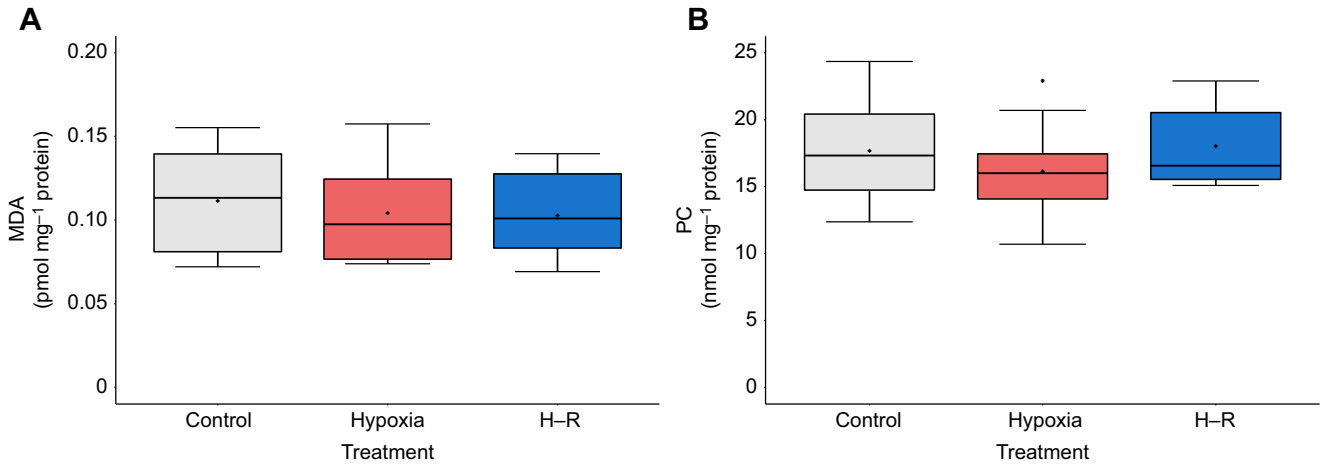

Fig. 7. Oxidative damage in mitochondrial proteins of quahogs exposed to short-term H–R stress. (A) Malondialdehyde (MDA)–protein conjugates. (B) Protein carbonyls (PC). Experimental groups: control (21% O_2); short-term (24 h) severe (<0.01% O_2) hypoxia; short-term severe hypoxia (24 h at <0.01% O_2) 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
The authors declare no competing or financial interests.

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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