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RESEARCH ARTICLE

Tissue- and substrate-dependent mitochondrial responses to acute hypoxia–reoxygenation stress in a marine bivalve (*Crassostrea gigas*)

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ABSTRACT

Hypoxia is a major stressor for aquatic organisms, yet intertidal organisms such as the oyster *Crassostrea gigas* are adapted to frequent oxygen fluctuations by metabolically adjusting to shifts in oxygen and substrate availability during hypoxia–reoxygenation (H/R). We investigated the effects of acute H/R stress (15 min at ∼0% O_2 and 10 min reoxygenation) on isolated mitochondria from the gill and the digestive gland of *C. gigas* respiring on different substrates (pyruvate, glutamate, succinate, palmitate and their mixtures). Gill mitochondria showed better capacity for amino acid and fatty acid oxidation compared with mitochondria from the digestive gland. Mitochondrial responses to H/R stress strongly depended on the substrate and the activity state of mitochondria. In mitochondria oxidizing NADH-linked substrates, exposure to H/R stress suppressed oxygen consumption and generation of reactive oxygen species (ROS) in the resting state, whereas in the ADP-stimulated state, ROS production increased despite little change in respiration. As a result, electron leak (measured as H_2O_2 to O_2 ratio) increased after H/R stress in the ADP-stimulated mitochondria with NADH-linked substrates. In contrast, H/R exposure stimulated succinate-driven respiration without an increase in electron leak. Reverse electron transport (RET) did not significantly contribute to succinate-driven ROS production in oyster mitochondria except for a slight increase in the OXPHOS state during post-hypoxic recovery. A decrease in NADH-driven respiration and ROS production, enhanced capacity for succinate oxidation and resistance to RET might assist in post-hypoxic recovery of oysters mitigating oxidative stress and supporting rapid ATP re-synthesis during oxygen fluctuations, as is commonly observed in estuaries and intertidal zones.

KEY WORDS: Mollusks, Mitochondria, Bioenergetics, Oxidative stress, Metabolism, Reverse electron transport

INTRODUCTION

Oxygen fluctuations are common in estuaries, tidal pools, intertidal and coastal zones of the ocean (Breitburg et al., 2019; Diaz and Rosenberg, 2008; Richards, 2011). Oxygen deficiency (hypoxia) limits energy supply because of a decrease in aerobic respiration and ATP synthesis, whereas reoxygenation can cause a surge in reactive oxygen species (ROS), resulting in oxidative damage to DNA, lipids and proteins (Andrienko et al., 2017). In aerobic organisms, mitochondria represent key targets of hypoxia–reoxygenation (H/R) stress, with mitochondrial damage implicated in the disruption of energy and redox homeostasis, and subsequent cellular injury and death (Honda et al., 2005; Kloner, 2017). However, many hypoxia-tolerant organisms including intertidal bivalves, freshwater reptiles, and fish appear to have evolved mechanisms that support robust mitochondrial respiration and redox homeostasis during H/R stress (Farhat et al., 2021; Pamenter et al., 2016; Sokolova et al., 2019). Mitochondrial adaptations in hypoxia-tolerant organisms include maintenance of high activity of electron transport system (ETS) during oxygen fluctuations (Farhat et al., 2021; Ivanina et al., 2012; 2016; Kurochkin et al., 2008; Pamenter et al., 2016), upregulation of antioxidants and protein quality control mechanisms (Freire et al., 2011; Sokolov et al., 2019; Steffen et al., 2020) and, in some species, transient suppression of the mitochondrial ATPase activity to prevent ATP wastage (Boutilier and St-Pierre, 2002; Ivanina et al., 2016; St-Pierre et al., 2000a). Despite significant advances in comparative mitochondrial physiology of hypoxia-tolerant organisms in recent years (for reviews, see Pamenter, 2014, 2020; Sokolova, 2018; Sokolova et al., 2019), the mechanisms of the mitochondrial adaptations to H/R stress are not fully understood. In particular, the implications of the hypoxia-induced changes in the intracellular milieu including shifts in concentrations of metabolic intermediates require further investigation (Bayne, 2017; Brinkhoff et al., 1983; de Zwaan, 1991; Haider et al., 2020a), in light of the strong substrate-dependence of mitochondrial responses to stress (Leverve and Fontaine, 2001; Quinlan et al., 2013).

Mitochondria are metabolically flexible organelles capable of utilizing a broad range of substrates (including carbohydrates, fatty acids, and amino acids) to generate ATP (Fig. 1). The nature of the respiratory substrate affects the stoichiometry of oxygen consumption to ATP production (Leverve and Fontaine, 2001) and ROS generation rates (Quinlan et al., 2013). Mitochondria from different species as well as from different tissues of the same species show preferential dependence on certain substrates that plays a role in energy metabolism and stress sensitivity. For example, in rodents, the brain mitochondria show higher transport and oxidation rates of complex I (NADH-linked) substrates, whereas liver mitochondria show preference for succinate (Gusdon et al., 2015) and heart mitochondria preferably oxidize fatty acids, amino acids and lactate (Kodde et al., 2007). Substrate preference might change in response to stress as shown in a hypoxia-tolerant goldfish, where brain mitochondria switch from carbohydrate to fatty acid oxidation during hypoxia (Farhat et al., 2021). Furthermore, ROS production is also substrate dependent and regulated by the degree of reduction of the coenzyme Q (CoQ) pool (Guarás et al., 2016). Thus, in

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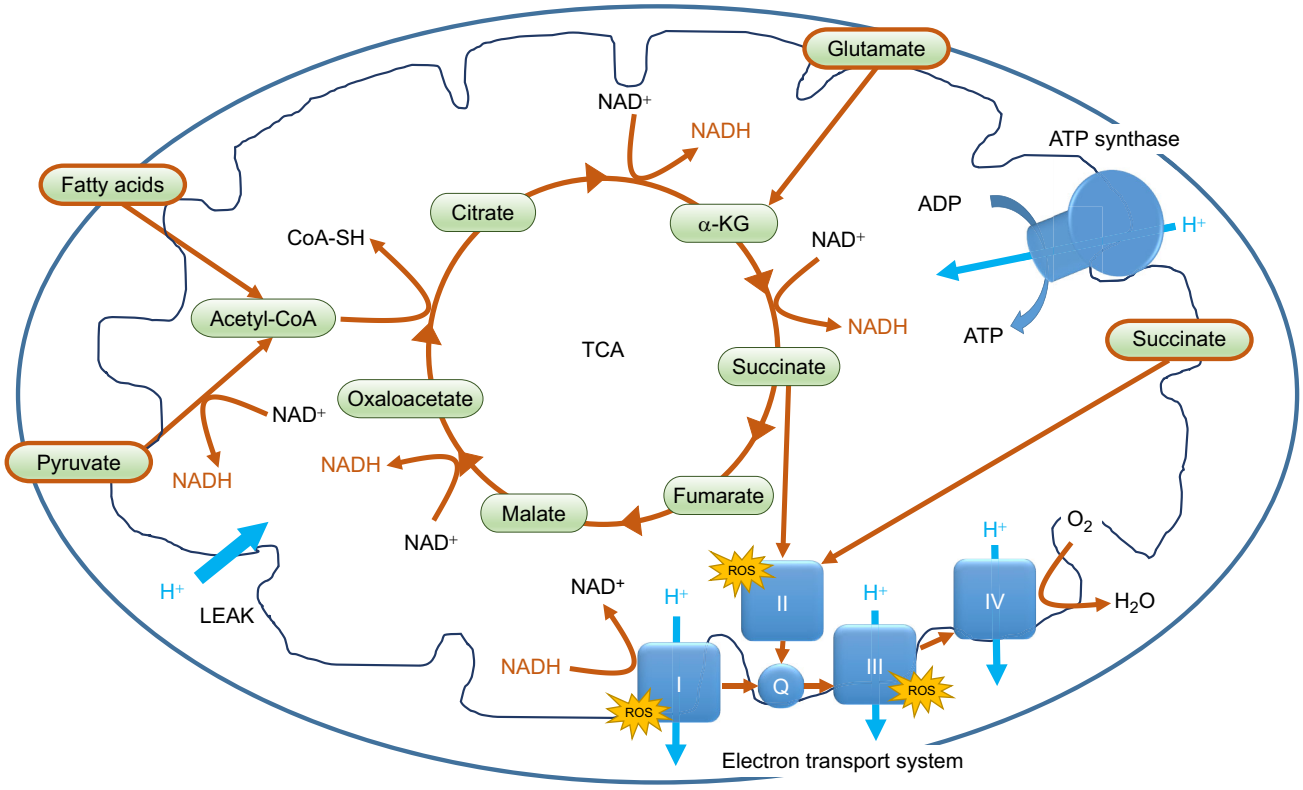

Fig. 1. Schematic representation of the entry points of different substrates (ovals with orange border) into the mitochondrial oxidation. Blue squares indicate electron transport system (ETS) complexes, blue arrows show the direction of the proton transport. NADH donates electron to complex I, and succinate to complex II of the mitochondrial ETS. ROS (superoxide) are generated by electron leak at the complexes I, II and III. Based on Cortassa et al. (2019).

mammalian mitochondria, the redox state of the CoQ pool is maintained near optimum during glucose oxidation but becomes over-reduced during oxidation of fatty acids or succinate, leading to ROS production via reverse electron transport (RET) at complex I (Guarás et al., 2016; Quinlan et al., 2013). ROS generation via RET has been reported in several studies as both a positive and negative regulator of physiological processes and pathogenesis. In *Drosophila*, stimulation of ROS production via RET extended the lifespan by preventing pathogenesis induced by severe oxidative stress (Scialò et al., 2017). In mammals, RET can lead to pathological oxidative damage causing ischemia–reperfusion injury (Chouchani et al., 2016). This dual role of RET renders it a mechanism of interest, especially under stressful conditions.

The tissue-specific substrate preferences have not been extensively studied in invertebrate mitochondria, including those of hypoxia-tolerant marine bivalves (Ballantyne, 2004). However, available studies show that the metabolic make-up of invertebrate mitochondria differs from those of vertebrates (Ballantyne, 2004; Doumen and Ellington, 1989). Thus, the heart mitochondria of marine mollusks and horseshoe crabs have limited capacity to oxidize fatty acids and largely depend on proline (mollusks) or carbohydrate (horseshoe crabs) oxidation (Ballantyne, 2004; Doumen and Ellington, 1989). Molluscan mitochondria also commonly show a strong capacity to oxidize succinate (Kurochkin et al., 2009; Tschischka et al., 2000). In facultative anaerobes such as marine bivalves, environmental stressors including H/R can strongly affect intracellular concentrations of metabolic fuels, leading to depletion of carbohydrates, accumulation of succinate and acetate, and a shift in the relative concentrations of different amino acids (Bayne, 2017; Brinkhoff et al., 1983; de Zwaan, 1991; Haider et al., 2020a; Ivanina et al., 2011). These changes might affect the availability of mitochondrial substrates and have major implications for bioenergetics and redox balance. However, the metabolic flexibility in mitochondrial substrate utilization under H/R stress have not been studied in marine bivalves and warrants further investigation.

The aim of this study was to determine the role of utilization of different metabolic substrates (carbohydrates, amino acids and fatty acids) in mitochondrial respiration, ATP synthesis capacity and ROS production, as well as the mitochondrial responses to H/R stress in the Pacific oyster *Crassostrea gigas*. This is a common bivalve species with worldwide distribution in estuaries, intertidal and coastal zones as well as in aquaculture. Oysters possess high tolerance to abiotic stressors and are commonly exposed to fluctuating oxygen levels due to the tidal and diurnal cycles and/or seasonal hypoxia (Kennedy et al., 1996; Zhang et al., 2012) making them an excellent model species to study mitochondrial flexibility under H/R stress. We hypothesized that oyster mitochondria would show tissue-specific preferences for oxidation of different substrates, reflecting the substrate availability in different tissues. Based on the earlier studies reporting concentrations of lipids, free amino acids and succinate in different tissue of marine bivalves (Haider et al., 2020a,b, 2018, 2019), we hypothesized that the digestive gland mitochondria would show a stronger ability to oxidize succinate and fatty acids and that amino acid oxidation would be higher in the gills. Furthermore, we hypothesized that mitochondrial capacity for succinate utilization would be enhanced after H/R stress in oyster mitochondria, facilitating rapid succinate breakdown to prevent RET. To test these hypotheses, we isolated mitochondria from the gills and the digestive gland of *C. gigas*, and

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measured their oxygen consumption and ROS production in the presence of different substrates (pyruvate, succinate, glutamate, palmitate) and their combinations under the control conditions and after exposure to acute H/R stress (15 min of near-anoxia, ~0% O_2 followed by 10 min of reoxygenation). To test for the potential involvement of RET in mitochondrial ROS production, succinate-driven oxygen consumption and ROS production were measured in the presence and absence of a complex I inhibitor, rotenone, which is known to prevent RET (Scialò et al., 2017). Our study showed that the gill and digestive gland mitochondria of oysters demonstrate strong substrate dependence for mitochondrial respiration and ROS production under normal conditions and after H/R stress. Furthermore, assessment of RET as a potential mechanism of ROS production shows that oyster mitochondria have an excellent capacity for succinate oxidation with minimal propensity for RET.

MATERIALS AND METHODS

Chemicals

Chemicals for the experiments were purchased from Sigma Aldrich (Munich, Germany), Fisher Scientific (Schwerte, Germany) or Carl Roth (Karlsruhe, Germany) and were of analytical grade or higher.

Animals

Adult Pacific oysters *Crassostrea gigas* Thunberg 1793 were collected from the island of Sylt in the German Wadden Sea and transported within 24 h of collection to the University of Rostock. On arrival, oysters were acclimated for 3–6 weeks at 15±1°C and salinity 32±1 (practical salinity units) in aerated natural Baltic Sea water adjusted to salinity 32 with Instant Ocean sea salt (Aquarium Systems, Sarrebourg, France). Salinity and temperature conditions were chosen to represent the habitat condition of the studied oyster population during the time of collection. Oysters were fed *ad libitum* with a commercial algal blend (DT's Live Marine Phytoplankton, CoralSands, Wiesbaden, Germany) according to the manufacturer's recommendations.

Mitochondrial isolation

Mitochondria were isolated from gill tissues and digestive glands of oysters. These tissues were chosen because of their involvement in energy metabolism. The gill is the main organ for the oxygen uptake and filter-feeding in marine bivalves, whereas the digestive gland is one of the largest metabolically active organs involved in digestion and energy storage (Kennedy et al., 1996). Briefly, 1–2 g gills or digestive gland tissues were homogenized in an ice-cold isolation buffer containing 30 mmol l^{-1} 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.5, 100 mmol l^{-1} sucrose, 100 mmol l^{-1} NaCl, 200 mmol l^{-1} KCl, 8 mmol l^{-1} ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 50 µg l^{-1} aprotinin, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF), using several passes of a Potter–Elvenhjem homogenizer at 200 r.p.m. The homogenate was centrifuged for 4 min at 2000 ***g*** to remove cell debris. The supernatant was collected and centrifuged for 8 min at 8500 ***g*** to acquire a mitochondrial pellet. The pellet was washed twice with the isolation medium and collected by brief centrifugation (5 min at 8500 ***g***). All centrifugation steps were conducted at 4°C. The mitochondrial pellet was resuspended in an ice-cold assay medium containing 30 mmol l^{-1} HEPES pH 7.2, 185 mmol l^{-1} sucrose, 10 mmol l^{-1} glucose,130 mmol l^{-1} KCl, 10 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KH_2PO_4 and 1% fatty acid free bovine serum albumin (BSA). Gill and digestive gland mitochondria were isolated from the same oysters.

Oxygen consumption rate ($\dot{M}_{O_2}$) and ROS measurements

Oxygen consumption and ROS production of isolated mitochondria were measured at 15°C using an Oxygraph 2k high-resolution respirometer (Oroboros, Innsbruck, Austria) and integrated DatLab 6 software. Oxygen consumption was measured using a Clark-type electrode calibrated with 100% (air-saturated assay buffer) and 0% (saturated solution of sodium dithionite). After stabilization of the oxygen signal (background flux of ±1 pmol O_2 s^{-1} ml^{-1}), mitochondrial suspension was added to the chamber containing 2 ml temperature-equilibrated assay buffer and a substrate-uncoupler inhibitor titration (SUIT) was conducted using following additions: (1) a substrate or substrate mixture to stimulate LEAK oxygen consumption (state 2); (2) 2.5 mmol l^{-1} ADP to achieve ADP-stimulated oxidative phosphorylation (OXPHOS) state; (3) 5 µmol l^{-1} cytochrome *c* as quality control to check the intactness of the mitochondrial membrane. An increase in the mitochondrial respiration due to cytochrome *c* addition was <5%, indicating integrity of isolated mitochondria (data not shown). ADP-stimulated respiration was maintained until all oxygen in the chamber was consumed, and mitochondria were exposed to severe hypoxia (~0% O_2 measured in the Oxygraph 2k chamber) for 15 min. Oxygen tension was then raised to ~80–100% of air saturation, and mitochondria were allowed to recover for 10 min (reoxygenation). After reoxygenation, post-hypoxic OXPHOS respiration rate was recorded and the SUIT continued as follows: (1) 2.5 µmol l^{-1} oligomycin to inhibit mitochondrial F_0, F_1-ATPase and measure post-hypoxic LEAK respiration (state 4); (2) 1 µmol l^{-1} rotenone to inhibit electron flux through complex I; (3) 2.5 µmol l^{-1} antimycin A to inhibit electron flux through complex III; (4) 40 mmol l^{-1} KCN to measure non-mitochondrial respiration (<10% of the total oxygen consumption rate, data not shown). Saturating concentrations of the respective substrates were used, including: (1) 10 µmol l^{-1} palmitoyl-DL-carnitine (PALM), (2) 5 mmol l^{-1} pyruvate with 2 mmol l^{-1} malate to spark respiration (PM), (3) 10 mmol l^{-1} glutamate with 2 mmol l^{-1} malate (G); (4) 10 mmol l^{-1} succinate (S), (5) 10 mmol l^{-1} glutamate, 2 mmol l^{-1} malate and 10 mmol l^{-1} succinate (GS); (6) 10 mmol l^{-1} glutamate, 2.5 mmol l^{-1} pyruvate, 2 mmol l^{-1} malate and 10 mmol l^{-1} succinate (GPS). Palmitoyl-DL-carnitine is a long-chain acylcarnitine that serves as a vector for mitochondrial delivery of palmitate; therefore, mitochondrial respiration in the presence of palmitoyl-DL-carnitine is representative of the rate of palmitate oxidation.

Production of H_2O_2 was measured simultaneously with $\dot{M}_{O_2}$ using Fluorescence-Sensor Green (525 nm) integrated with Oxygraph 2k in an assay buffer containing 10 µmol l^{-1} Amplex Red, 1 U ml^{-1} horseradish peroxidase stock solution, and 5 U ml^{-1} superoxide dismutase (SOD) (Makrecka-Kuka et al., 2015). A two-step calibration was conducted with addition of 0.1 µmol l^{-1} H_2O_2 before and after the addition of the mitochondrial suspension. ROS production was measured as the rate of H_2O_2 efflux in LEAK and OXPHOS states and corrected for the baseline measured in the absence of mitochondria.

Mitochondrial respiratory states and control indices were determined as described elsewhere (Estabrook, 1967; Gnaiger, 2014). OXPHOS flux was determined from the rate of ADP-stimulated mitochondrial respiration reflective of the ATP synthesis capacity and compared between the pre- and post-hypoxic conditions. Pilot studies showed that the LEAK respiration rate in control mitochondria (i.e. not exposed to H/R stress) was similar when measured in state 2 (with substrates but no ADP) and state 4 (in the presence of substrates, ADP and oligomycin) (Δ<5%, P>0.05). Therefore, both LEAK state 2 and LEAK state 4

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respiration were considered representative of the mitochondrial proton leak, reflecting the ETS activity needed to compensate for the futile proton and cation cycles in the absence of ATP synthesis (Rofle and Brand, 1997). Respiratory control ratio (RCR) was calculated as the ratio of OXPHOS to LEAK flux and used as an index of mitochondrial coupling (Estabrook, 1967). To assess electron leak, H_2O_2 production rate was divided by the oxygen consumption rate in the same mitochondrial isolate and expressed as H_2O_2 to O_2 ratio.

Protein concentrations in the mitochondrial suspensions were measured using a Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA, USA) (Bradford, 1976) using BSA as a standard. Protein concentrations of the mitochondrial suspensions were corrected for the BSA content of the assay media. Mitochondrial respiration rates and ROS production were expressed as nmol O_2 min^{-1} mg^{-1} protein and nmol H_2O_2 min^{-1} mg^{-1} protein, respectively. For each experimental group, mitochondria were isolated from the gill and the digestive gland of 12 (for PM), seven (for S) or six (for PALM, G, GS and GPS) oysters.

Statistics

Data on mitochondrial functional traits ($\dot{M}_{O_2}$ and ROS efflux) were subjected to a normality test to identify outliers. To assess the effects of the tissue type, oxidized substrate and their interactions on the mitochondrial oxygen consumption, ROS efflux and electron leak, we used two-way general linearized model ANOVA with repeated measures. Tissue type was treated as a within-subject factor to account for the fact that the mitochondria used in this analysis were isolated from the gills and the digestive gland of the same oyster. The substrate type was used as a between-subject factor since respiration and ROS efflux with different substrates were tested in mitochondrial isolates from different oysters. For the planned comparisons of the group means, the Least Significant Difference test was used. The effects of hypoxia and reoxygenation on mitochondrial $\dot{M}_{O_2}$ and ROS efflux were tested using the paired Student's *t*-test with the paired values measured in the same mitochondrial isolate before and after H/R exposure. Similarly, to test the effects of rotenone addition on the $\dot{M}_{O_2}$ and ROS efflux in the same mitochondrial isolate, paired *t*-test was used. All statistical analyses were conducted using IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v. 7.02 (GraphPad Software Inc., La Jolla, CA, USA) software. Differences were considered significant if the probability of type II error, $P<0.05$. Sample size (N) was 12 for mitochondria respiring on pyruvate, seven for those oxidizing succinate, and six for the mitochondria respiring on glutamate or glutamate-containing mixtures. In some groups, N was lower owing to the removal of 1–2 statistically significant outliers ($P<0.05$) as indicated in figure legends.

RESULTS

Tissue-specific differences in substrate oxidation and ROS production rates

Repeated measures ANOVA showed strong evidence ($P<0.001$) for the effects of the oxidized substrate on the respiration rate of oyster mitochondria (Table 1). There was moderate evidence ($P=0.029$) for the effects of tissue type on the mitochondrial LEAK $\dot{M}_{O_2}$ but only weak evidence ($P=0.058$) for the effects of the tissue on the OXPHOS respiration (Table 1). There was no evidence for the effects of Tissue×Substrate interactions on the mitochondrial oxygen consumption (Table 1).

Gill mitochondria showed the highest LEAK rate with glutamate as a substrate followed by succinate>GPS mixture>GS mixture>palmitate>pyruvate (Fig. 2A). The differences were significant between the LEAK $\dot{M}_{O_2}$ driven by glutamate and pyruvate, but not between other substrates (Fig. 2A). The OXPHOS $\dot{M}_{O_2}$ in the gill mitochondria was highest with GPS mixture followed by glutamate>succinate>GS mixture>pyruvate>palmitate (Fig. 2B). In the gills, the OXPHOS respiration rates with glutamate and succinate were significantly higher than OXPHOS $\dot{M}_{O_2}$ with palmitate or pyruvate (Fig. 2B). The OXPHOS $\dot{M}_{O_2}$ during oxidation of the substrate mixtures were not significantly different from $\dot{M}_{O_2}$ with the corresponding single substrates. In the digestive gland mitochondria, the LEAK respiration rate was higher with succinate compared with pyruvate (Fig. 2A). The OXPHOS respiration in the digestive gland mitochondria were significantly higher with the GPS mixture than with glutamate, pyruvate or palmitate as substrates (Fig. 2B). Gill mitochondria showed higher rates of glutamate oxidation compared to the mitochondria from the digestive gland; the respiration rates with all other substrates and their mixtures were not significantly different between the mitochondria from the gill and the digestive gland (Fig. 2A,B).

Table 1. ANOVA: Effects of the tissue, substrate and their interaction on mitochondrial traits of *Crassostrea gigas*

	Tissue	Substrate	Tissue×Substrate
$\dot{M}_{O_2}$			
LEAK state	**$F_{1,31}$=5.271 P=0.029**	**$F_{5,31}$=6.735 P<0.001**	$F_{5,31}$=2.075 P=0.095
OXPHOS state	$F_{1,34}$=3.844 P=0.058	**$F_{5,34}$=14.981 P<0.001**	$F_{5,31}$=1.616 P=0.182
ROS efflux			
LEAK state	**$F_{1,32}$=46.565 P<0.001**	**$F_{5,32}$=8.891 P<0.001**	**$F_{5,32}$=8.899 P<0.001**
OXPHOS state	**$F_{1,30}$=5.036 P=0.032**	**$F_{5,30}$=4.469 P=0.004**	**$F_{5,30}$=4.365 P=0.004**
H_2O_2:O_2 ratio			
LEAK state	**$F_{1,31}$=50.836 P=0.000**	**$F_{5,31}$=9.075 P=0.000**	**$F_{5,31}$=9.405 P<0.001**
OXPHOS state	**$F_{1,29}$=24.878 P<0.001**	**$F_{5,29}$=6.044 P=0.001**	**$F_{5,29}$=3.869 P=0.008**

Repeated measures ANOVA was used with the tissue as a within-subject factor, and substrate type as a between-subject factor. *F* values with the degrees of freedom for the effect and error (in subscript) and *P* value for each effect are given. Significant effects ($P<0.05$) are highlighted in bold.

ANOVA showed strong evidence ($P<0.01$) for the effects of Tissue×Substrate interactions on the ROS efflux and electron leak (measured as the ratio of H_2O_2 produced to O_2 consumed) in the oyster mitochondria (Table 1). This indicates that ROS production had different substrate-dependence in the gill and the digestive gland mitochondria. In the gill mitochondria, the ROS efflux rate in the LEAK state was higher with succinate as a substrate than with pyruvate or GPS mixture (Fig. 2C). In the OXPHOS state, ROS efflux was lower in the gill mitochondria respiring on the GPS mixture compared with all other tested substrates (Fig. 2D). In the digestive gland mitochondria, oxidation of palmitate led to significantly higher rates of ROS efflux compared with other tested substrates (increased by ∼2- to 5-fold and ∼1.3- to 3-fold in the LEAK and OXPHOS states, respectively) (Fig. 2C,D). Furthermore, the LEAK state ROS efflux was significantly higher in the digestive gland mitochondria during oxidation of succinate than GPS mixture (Fig. 2C). The rates of the ROS efflux were higher in the digestive gland than in the gill mitochondria respiring with palmitate (in the LEAK state) and with GPS mixture (in the OXPHOS state) (Fig. 2C,D). With all other tested substrates, ROS

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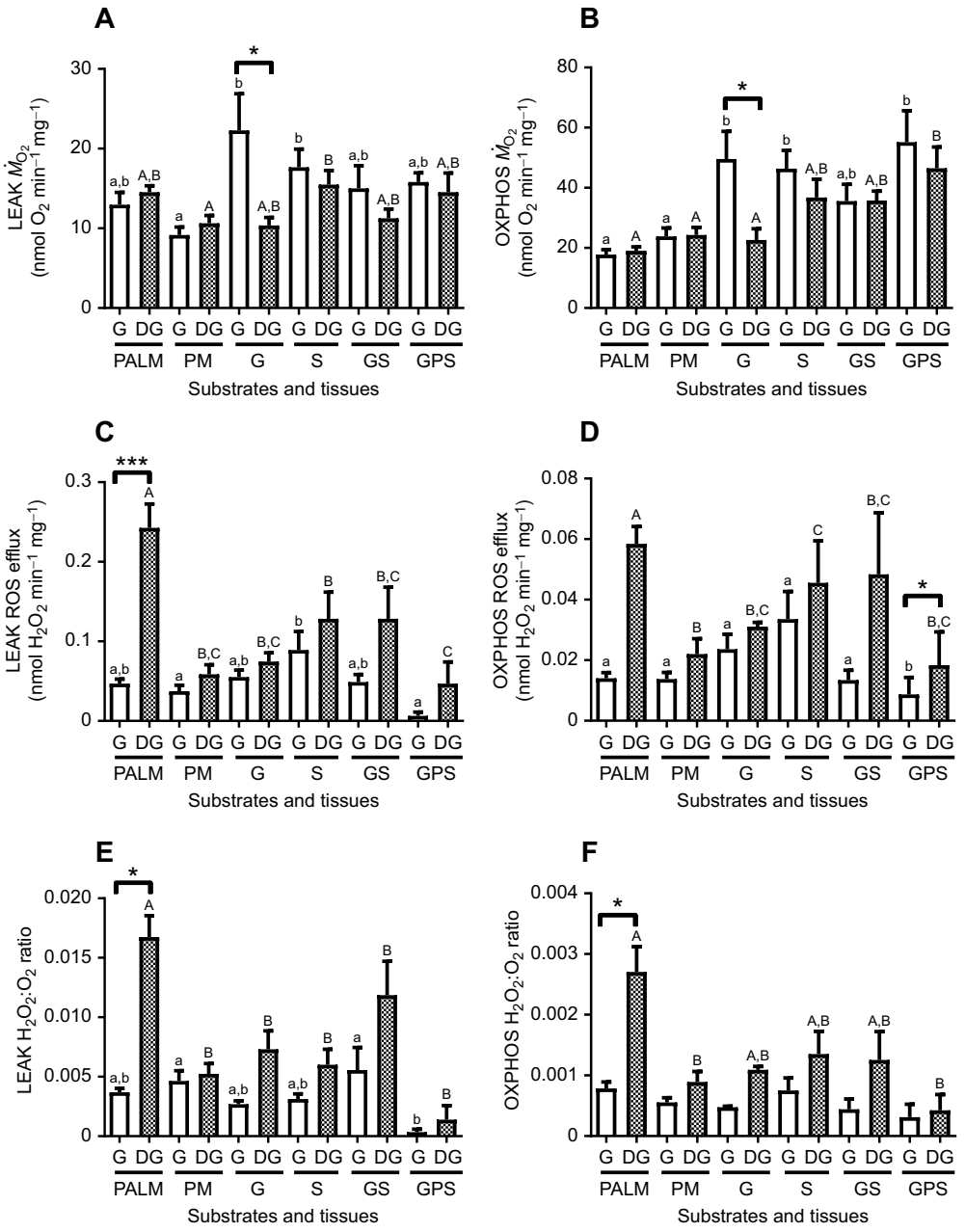

Fig. 2. Oxygen consumption rates ($\dot{M}_{O_2}$) and ROS efflux of *Crassostrea gigas* isolated mitochondria respiring on different mitochondrial substrates under normoxic conditions. Mitochondrial were isolated from the gill (G) or the digestive gland (DG) of *C. gigas* and supplied with palmitate (PALM), pyruvate (PM), glutamate (G), succinate (S), glutamate+succinate (GS) or glutamate, pyruvate and succinate mixture (GPS). (A,C,E) LEAK $\dot{M}_{O_2}$, ROS efflux and H_2O_2:O_2 ratio. (B,D,F) OXPHOS $\dot{M}_{O_2}$, ROS efflux and H_2O_2:O_2 ratio. Within each tissue type, significant differences (P<0.05) between mitochondrial traits measured with different substrates are marked by different letters (capital and lowercase letters denote comparisons within the gills and the digestive gland mitochondria, respectively). Significant differences in a specific mitochondrial trait between the gill and the digestive gland measured with the same substrate are denoted by asterisks (*P<0.05, ***P<0.001). N=4–7 for all substrates except PM, where N=12.

efflux rates were similar in the gill and the digestive gland mitochondria.

The electron leak (measured as the ratio of H_2O_2 produced to O_2 consumed) was significantly lower in the LEAK state gill mitochondria respiring on GPS mixture compared those oxidizing pyruvate or GS mixtures (Fig. 2E). In the OXPHOS state, the electron leak was similar in the gill mitochondria respiring on all tested substrates (Fig. 2F). In the digestive gland mitochondria, the electron leak during palmitate oxidation was considerably higher than with all other tested substrates in the LEAK state, and higher that during oxidation of pyruvate and GPS mixture in the OXPHOS state (Fig. 2E,F). During palmitate oxidation, the electron leak was higher in the digestive gland mitochondria compared with those from the gill (Fig. 2E,F). For other tested substrates and their mixtures, the electron leak was similar in the gill and the digestive gland mitochondria (Fig. 2E,F).

Substrate-dependent mitochondrial responses to H/R stress

Exposure to H/R stress significantly suppressed (by 27–51%) the LEAK respiration in mitochondria from the gills and the digestive gland respiring on palmitate, pyruvate, or glutamate (Fig. 3A,B). The ROS generation rate in the LEAK state also decreased during the post-hypoxic recovery in the gill mitochondria oxidizing palmitate, pyruvate or glutamate (by 20–51%), and in the digestive gland mitochondria respiring on palmitate or pyruvate (by 31–40%) (Fig. 3C,D). The succinate-driven LEAK respiration was stimulated by 56% after H/R stress in the gill (but not the digestive gland) mitochondria (Fig. 3A,B). Notably, an increase in the succinate-driven LEAK $\dot{M}_{O_2}$ was not associated with a significant increase in ROS generation in the gill mitochondria (see Fig. 3A,C,E). The electron leak measured as H_2O_2 to O_2 ratio in the LEAK state mitochondria was not significantly affected by H/R stress (Fig. 3E,F) except for the digestive gland mitochondria

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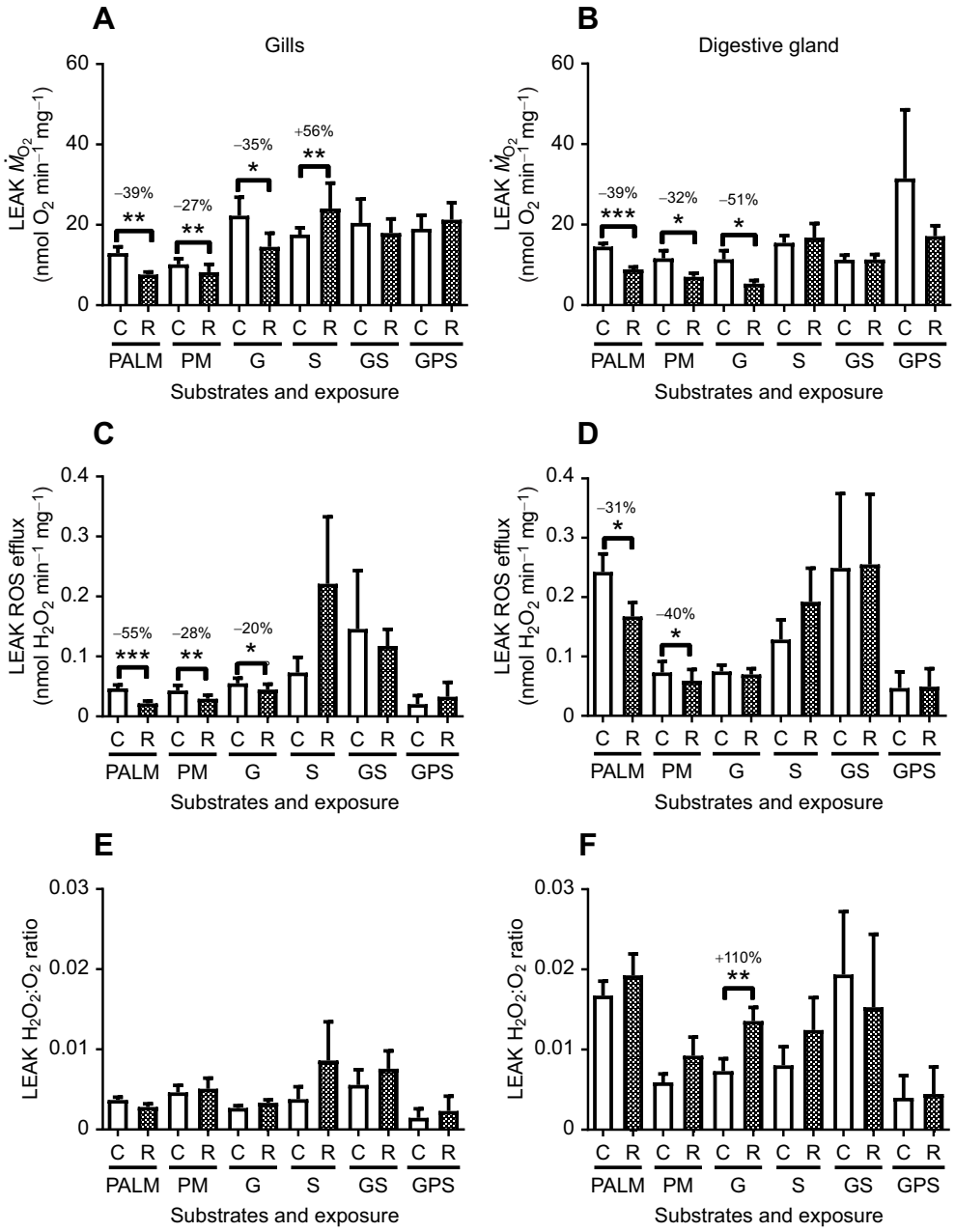

Fig. 3. Effects of hypoxia and reoxygenation (H/R) on LEAK respiration of mitochondria isolated from *C. gigas* respiring on different substrates. Mitochondria were isolated from the gills (A,C,E) or the digestive glands (B,D,F) of oysters and treated with palmitate (PALM), pyruvate (PM), glutamate (G), succinate (S), glutamate+succinate (GS) or glutamate, pyruvate and succinate mixture (GPS). The percentage difference during H/R stress was calculated by standardizing the control values of various substrates to 100%. Significant differences in a specific mitochondrial trait between normoxia (C) and reoxygenation (R) are denoted by asterisks (**P*<0.05, ***P*<0.01, ****P*<0.001). *N*=4–7 for all substrates except PM, where *N*=12.

respiring on glutamate, where a ∼110% increase in the electron leak was observed after H/R stress (Fig. 3F).

Exposure to H/R stress suppressed OXPHOS rate in the gill mitochondria respiring on pyruvate (by 7%) and in the digestive gland mitochondria respiring on palmitate (by 23%) (Fig. 4A,B). In both studied tissues, H/R stress stimulated mitochondrial OXPHOS respiration driven by succinate (by 20–27%) or the mixture of glutamate, pyruvate and succinate (by 16–24%) (Fig. 4A,B). In the gill mitochondria, H/R stress led to an increase in the mitochondrial ROS generation in OXPHOS state with all substrates and their combinations, and this increase (by 114–152%) was significant in the mitochondria oxidizing pyruvate, glutamate, succinate or GS mixture (Fig. 4C). Similarly, H/R stress led to elevated ROS generation in the digestive gland mitochondria in the OXPHOS state, and this increase (by 124–169%) was significant during palmitate, pyruvate, glutamate and succinate oxidation (Fig. 4D). This H/R stress-induced increase in ROS production was reflected in higher H_2O_2 to O_2 ratios in the gill and the digestive gland mitochondria during palmitate, pyruvate or glutamate oxidation (Fig. 4E,F). Notably, the H/R-exposed digestive gland mitochondria showed an extremely high H_2O_2 to O_2 ratio compared with the gill mitochondria when respiring on palmitate in the OXPHOS state (Fig. 4F). H/R stress had no effect on H_2O_2 to O_2 ratios in the gill or the digestive gland mitochondria during oxidation of succinate alone or in combination with other substrates (Fig. 4E,F).

H/R stress led to an increase in the RCR in the gill mitochondria respiring on palmitate, glutamate and GPS mixture (Fig. 5A) and in the digestive gland mitochondria oxidizing pyruvate or glutamate (Fig. 5B).

RET during the succinate-driven mitochondrial respiration

Rotenone addition had no significant effect on the succinate-driven respiration, ROS production or electron leak (measured as H_2O_2 to

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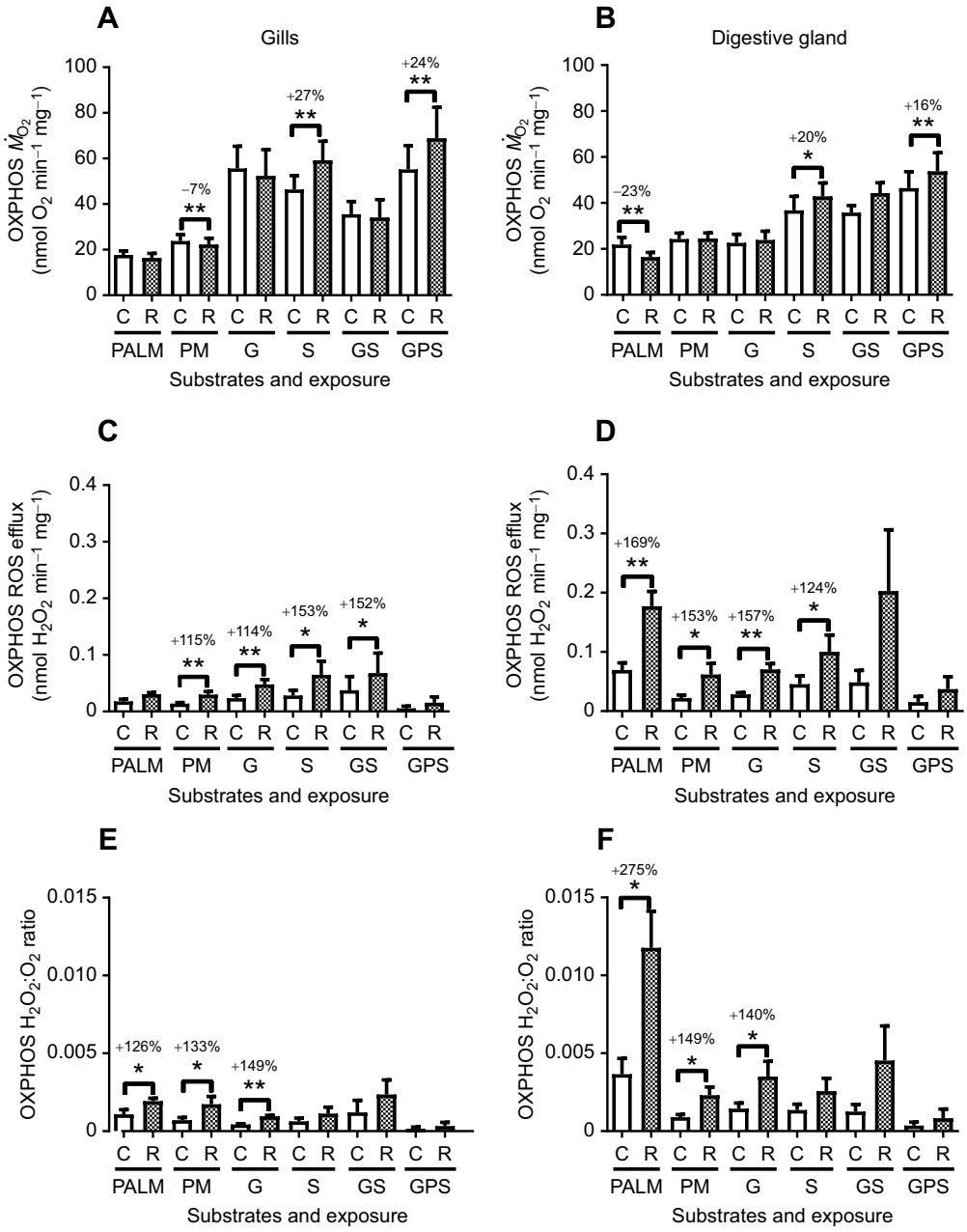

Fig. 4. Effects of hypoxia/reoxygenation (H/R) on OXPHOS respiration of mitochondria isolated from *C. gigas* respiring on different substrates. Mitochondria were isolated from the gills (A,C,E) or the digestive glands (B,D,F) of oysters and treated with palmitate (PALM), pyruvate (PM), glutamate (G), succinate (S), glutamate+succinate (GS) or glutamate, pyruvate and succinate mixture (GPS). The percentage difference during H/R stress was calculated by standardizing the control values of various substrates to 100%. Significant differences in a specific mitochondrial trait between normoxia (C) and reoxygenation (R) are denoted by asterisks (*P<0.05, **P<0.01). N=4-7 for all substrates except PM, where N=12.

O_2 ratio) in the control oyster mitochondria in the LEAK or OXPHOS state (Figs 6 and 7). After H/R stress, the succinate-driven LEAK and OXPHOS $\dot{M}_{O_2}$ were not affected by rotenone addition in either of the two studied tissues (Fig. 6A,B and Fig. 7A,B). However, the succinate-driven ROS production was suppressed by rotenone addition in the mitochondria exposed to H/R stress; this decrease was statistically significant (P<0.05) in the OXPHOS but not in the LEAK state (Fig. 6C,D and Fig. 7C,D). In the H/R-exposed mitochondria, rotenone addition decreased electron leak (measured as H_2O_2 to O_2 ratio) in the digestive gland mitochondria during the OXPHOS state (Fig. 7E). A similar trend for rotenone-induced suppression of electron leak was found in the gill mitochondria in the OXPHOS state and in the digestive gland and gill mitochondria in the LEAK state, but these effects of rotenone were not statistically significant (P>0.05) (Fig. 6E,F and Fig. 7E).

DISCUSSION

Tissue-specific differences in substrate oxidation and ROS production rates

Mitochondrial flexibility in utilizing different substrates is essential for regulating tissue- and organ-specific metabolism and adjustment of the mitochondrial functions to various physiological conditions and metabolic states (Smith et al., 2018). The tissue-specific preferences of mitochondria for different fuels have been extensively studied in vertebrates, including mammals (Cortassa et al., 2019; Smith et al., 2018) and fish (Farhat et al., 2021). In vertebrates, mitochondria from highly aerobic tissues such as brain commonly demonstrate preference for carbohydrates, whereas liver mitochondria show preference for fatty acid oxidation, and mitochondria from the heart and the skeletal muscle rely on both carbohydrates and fatty acids (Farhat et al., 2021; Gusdon et al., 2015; Kodde et al., 2007). Among invertebrates,

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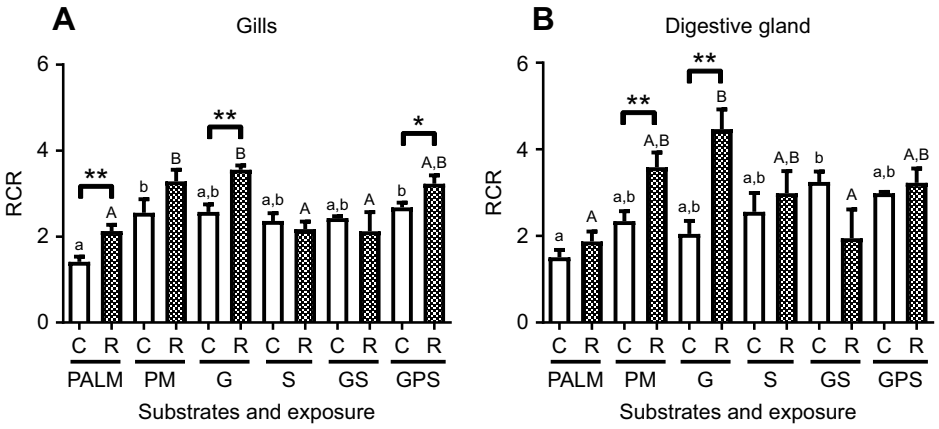

Fig. 5. Effects of hypoxia and reoxygenation (H/R) on respiratory control ratio (RCR) of *C. gigas* mitochondria respiring on different substrates. (A) Mitochondria from gills. (B) Mitochondria from digestive glands. Significant differences in a specific mitochondrial trait between normoxia (C) and reoxygenation (R) are denoted by asterisks (*P<0.05, **P<0.01). N=4–7 for all substrates except PM, where N=12.

mitochondrial substrate preference has been studied in insects and varies depending on the aerobic endurance, with carbohydrates predominantly used by the short-range (flies and beetles) fliers and fatty acids utilized in the long-range (butterflies and locusts) fliers (Bailey, 1975; Krogh and Weis-Fogh, 1951; Soares et al., 2015). Our present study showed that the mitochondrial capacity of different substrate utilization differs between tissues of a marine bivalve *C. gigas*. The gill mitochondria generally showed higher OXPHOS respiration rate with glutamate, succinate as well as glutamate-and succinate-containing mixtures compared with the OXPHOS respiration driven by pyruvate or palmitate. In the digestive gland mitochondria, the highest OXPHOS rate was achieved with the mixture of glutamate, pyruvate and succinate that significantly exceeded the OXPHOS rate with the complex I-linked substrates (palmitate, pyruvate or glutamate). Interestingly, the gill mitochondria of *C. gigas* showed higher glutamate-driven respiration compared with the digestive gland mitochondria. The high oxidation rate of glutamate by the gill mitochondria might be related to high intracellular levels of free amino acids in the gill (Haider et al., 2020a; Haider et al., 2019; Noor et al., 2021). Interestingly, despite higher lipid content of the digestive gland compared with the gills of bivalves (Pernet et al., 2007, 2006), a fatty acid (palmitate) appears to be a less preferable substrate for the digestive gland mitochondria of oysters compared with those from the gills. While the palmitate-driven LEAK and OXPHOS respiration rates were similar in the gill and the digestive gland mitochondria, the palmitate-driven ROS production was 4- to 5-fold higher in the digestive gland mitochondria compared with those from the gill. This was reflected in the differences in the apparent electron leak between the gill and the digestive gland mitochondria oxidizing palmitate. Thus, the gill mitochondria converted 0.08–0.4% of consumed O_2 to H_2O_2 during palmitate oxidation, whereas in the digestive gland this fraction was 0.3–1.7%. Overproduction of ROS during oxidation of palmitate is common in different cell types as shown in mammalian pancreatic cells, heart and skeletal muscle, endothelial cells, hepatocytes and adipocytes (Ly et al., 2017). Palmitate-driven excessive ROS production induces endoplasmic reticulum stress and Ca^{2+} overload, which in turn increases oxidative stress leading to cell damage and death (Ly et al., 2017). Our findings thus indicate that the digestive gland tissue is metabolically less well adapted for amino acid and fatty acid oxidation compared with the gills. Furthermore, utilization of fatty acids as metabolic fuel is associated with high costs due to oxidative stress and elevated ROS production in the digestive gland making this organ potentially susceptible to lipotoxicity. This might have implications for the whole-organism stress and physiological performance during periods of rapid lipid utilization, such as during winter starvation or initiation of gametogenesis (Gosling, 1992; Haider et al., 2020b; Kennedy et al., 1996).

Mitochondria from the gill and the digestive gland of *C. gigas* showed similarly good capacity for succinate oxidation, both alone and in substrate mixtures, especially in the OXPHOS state. There was evidence for higher ROS production in succinate-oxidizing mitochondria of *C. gigas* compared with those respiring on complex I substrates (except for the palmitate in the digestive gland that led to very high ROS production rates). However, succinate-driven ROS production was alleviated by addition of NADH-linked substrates (glutamate and pyruvate) in the mitochondria of *C. gigas*. The protective effect of pyruvate and glutamate might be due to the production of NADH by pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, respectively, which increases the reduced NADH pool in the mitochondria (Fig. 1). A highly reduced NADH pool might increase the thermodynamic driving force towards the forward electron flow through complex I and thus mitigate the RET and associated ROS production (Robb et al., 2018). Furthermore, NADH can directly act as an antioxidant by scavenging ROS in the mitochondria and thereby suppressing the ROS efflux (Kirsch and De Groot, 2001). The mitigation of succinate-driven ROS generation in oyster mitochondria in the presence of NADH-linked substrates might play a protective role *in vivo* where complex mixtures of NADH- and $FADH_2$-linked substrates are available to the mitochondria.

Effects of H/R stress on mitochondrial respiration and ROS production

Mitochondrial ability for alternative fuel utilization is sensitive to mitochondrial stress and might reflect different susceptibility of mitochondrial ETS complexes to metabolic disturbances and external stressors. Among ETS complexes, complex I (CI) is considered the most vulnerable to H/R stress (Cadenas, 2018; Robb et al., 2018; Sharma et al., 2009). In hypoxia-sensitive species such as rodents, exposure to hypoxia induces a reversible suppression of CI activity that prevents oxidative burst during reoxygenation but makes CI more susceptible to oxidative and nitrosative damage (Dröse et al., 2016; Ten and Galkin, 2019). Our present study indicates that H/R stress suppresses the oxidation rate for CI (NADH-linked) substrates (palmitate, pyruvate and glutamate) in *C. gigas* mitochondria. One possible explanation for this decrease might be inactivation and/or damage of CI due to H/R stress leading to lower enzymatic activity (V_{max}) of CI. A decrease in CI activity has been reported in hypoxia-tolerant marine bivalves (Falfushynska et al., 2020), turtles (Galli et al., 2013; Pamenter

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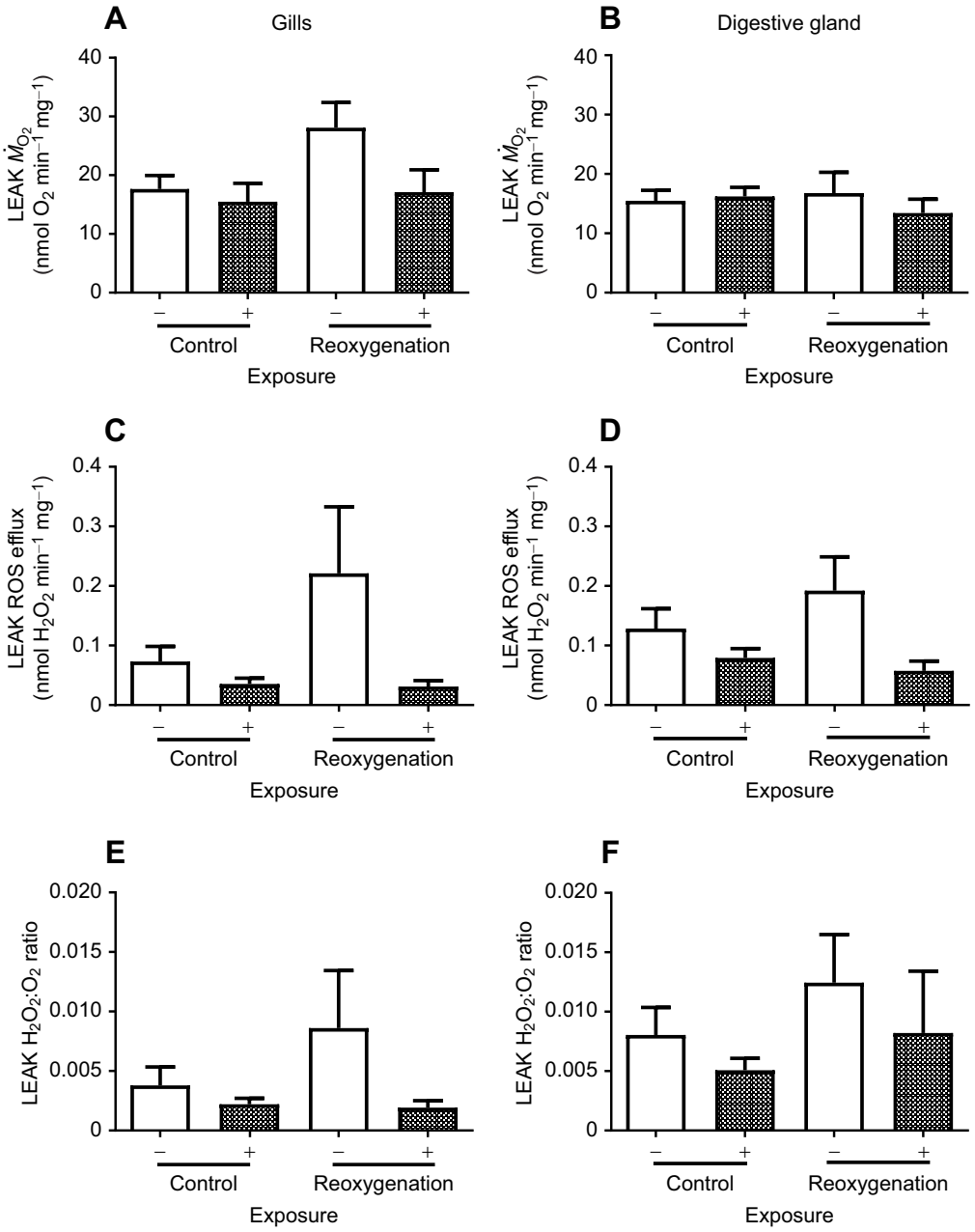

Fig. 6. Effect of complex I inhibitor (rotenone) on succinate-driven respiration and ROS efflux in the LEAK state of *C. gigas* mitochondria. Mitochondria were isolated from the gills (A,C,E) or the digestive glands (B, D,F) of oysters. Mitochondrial traits were assessed immediately after isolation (control) or after H/R stress (reoxygenation) in the absence (–) or presence (+) of rotenone. No significant differences were found between the values measured in the presence or absence of rotenone (P>0.05). N=7.

et al., 2016) and frogs (St-Pierre et al., 2000b) exposed to hypoxia and reoxygenation *in vivo*. However, this mechanism does not appear to be a likely explanation for the observed change in CI-dependent substrate oxidation during acute *in vitro* H/R exposure in *C. gigas* mitochondria. Thus, the H/R-induced suppression of the respiratory flux with CI substrates was particularly strong (by ∼30–50%) in the resting (LEAK state) mitochondria of oysters, whereas the ADP-stimulated OXPHOS respiration generally remained stable (except for palmitate oxidation that decreased by ∼20% in the digestive gland mitochondria). In bivalves including oysters, the OXPHOS respiration is controlled by ETS activity (Ivanina et al., 2012; 2016; Kurochkin et al., 2011) and is 2- to 3-fold faster than the LEAK state respiration (this study). Thus, if the CI activity were rate-limiting owing to H/R-induced inactivation or damage, this limitation should primarily affect OXPHOS respiration rate. Consequently, the observed strong suppression of the LEAK (but not OXPHOS) state respiration after H/R stress implies alternative mechanisms such as the modulation of the proton conductance (e.g. via activity of uncoupling proteins or substrate transporters) (Divakaruni and Brand, 2011; Jastroch et al., 2010) and/or decrease in the resting mitochondrial membrane potential (Brown, 1992; Hafner et al., 1990). Interestingly, a similar pattern has been described in hypoxia-tolerant naked mole rats where acute hypoxia led to a decrease in ETS activity, lower resting membrane potential and lower proton conductance of mitochondrial membrane potential without any change in the V_{max} of individual ETS complexes (Pamenter et al., 2018).

Stress-induced inactivation of the mitochondrial complex I can affect not only the rate of utilization of NADH-linked substrates but also the propensity for the reverse electron transport and associated ROS generation (Emmerzaal et al., 2020; Robb et al., 2018; Sharma et al., 2009). Our present study shows the impact of the H/R-induced

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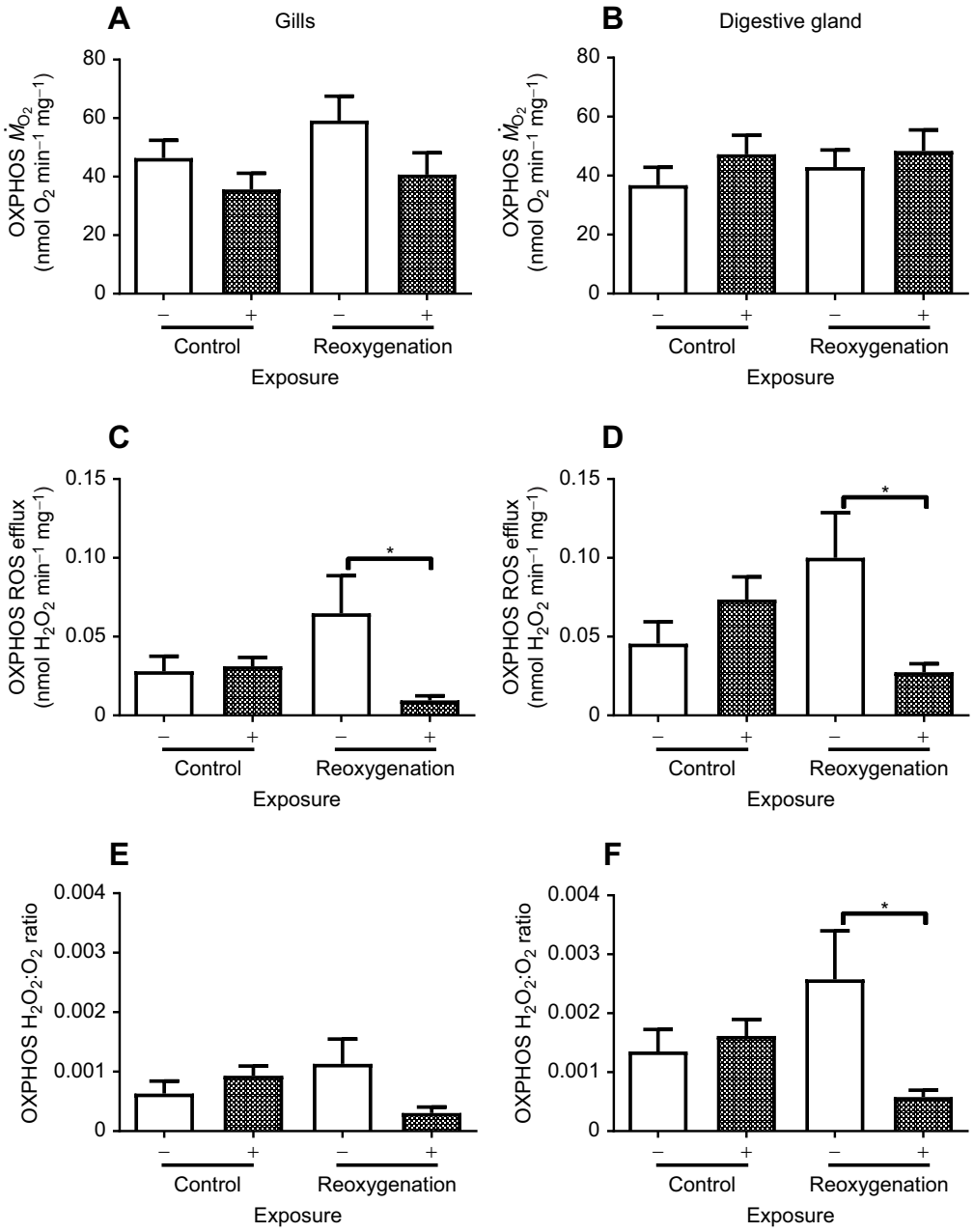

Fig. 7. Effect of complex I inhibitor (rotenone) on succinate-driven respiration and ROS efflux in the OXPHOS state of *C. gigas* mitochondria. Mitochondria were isolated from the gills (A,C,E) or the digestive glands (B,D,F) of oysters. Mitochondrial traits were assessed immediately after isolation (control) or after H/R stress (reoxygenation) in the absence (–) or presence (+) of rotenone. Significant differences the values measured in the presence or absence of rotenone (P<0.05) are marked with an asterisk. N=7.

changes in CI-dependent ETS activity on ROS generation strongly depends on the activity state of oyster mitochondria. Thus, in the resting (LEAK state) mitochondria, H/R stress led to a suppression of the ROS generation with CI substrates (pyruvate, glutamate and palmitate). This decrease in ROS generation was roughly proportional to the decline in the CI-driven LEAK respiration, so that electron leak did not change, with ∼0.4% of consumed oxygen converted to H_2O_2 in the gill and ∼1.4% in the digestive gland. In contrast, in the ADP-stimulated (OXPHOS state) mitochondria, the ROS generation rate with NADH-linked substrates increased after H/R stress despite little or no change in the oxygen consumption rates. Unlike oysters, mitochondria from the brain and the heart of anoxia-tolerant freshwater turtles showed a concomitant decrease in CI-driven respiration and ROS production after anoxia exposure as a result of the cumulative effect of inhibition of multiple ETS complexes (Bundgaard et al., 2018). In oysters, increase in ROS generation with CI substrates after H/R stress reflected elevated electron leak. Thus, fraction of O_2 converted to H_2O_2 almost doubled after H/R stress in oyster mitochondria respiring on CI substrates, from 0.07 to 0.14% in the gill and from 0.20 to 0.59% in the digestive gland. It is worth noting that electron leak was generally much lower in the OXPHOS than the LEAK state in oyster mitochondria. This is typical for mitochondria of both ecto- and endotherms and is related to the differences in the mitochondrial membrane potential in the resting and actively phosphorylating mitochondria (Hou et al., 2021; Miwa and Brand, 2003). Overall, the opposing effects of H/R stress on ROS generation in the resting versus actively phosphorylating oyster mitochondria make it difficult to predict physiological consequences of the H/R stress for oyster mitochondria respiring on CI substrates *in vivo*. However, it is likely that an increase in the electron leak and ROS production in the OXPHOS state is at least partially compensated by the

suppression of ROS generation in the resting state serving as a protective mechanism to mitigate oxidative stress during oxygen fluctuations.

Unlike NADH-linked substrates, oxidation of a $FADH_2$-linked substrate (succinate) was enhanced after H/R stress in oyster mitochondria. The digestive gland mitochondria appear particularly well adapted to metabolize succinate as they maintain relatively low succinate-driven proton leak but enhanced OXPHOS activity after H/R stress. In the gill mitochondria utilizing succinate, both the proton leak and OXPHOS rates increased after H/R stress. Succinate is an important metabolic intermediate that accumulates in high concentrations during hypoxia in marine bivalves including oysters (Bayne, 2017; Brinkhoff et al., 1983; de Zwaan, 1991; Haider et al., 2020a; Ivanina et al., 2011). Enhanced mitochondrial capacity for succinate oxidation might be adaptive during post-hypoxic recovery in oysters helping to rapidly restore the ATP levels and remove excess succinate. It is worth noting that enhanced succinate oxidation rates in response to H/R stress observed here in a cell-free system reflect intrinsic mitochondrial mechanisms independent of the retrograde signaling or cytosolic regulatory mechanisms. Previous studies in oysters exposed to H/R stress *in vivo* also reported stimulation of succinate-driven LEAK and OXPHOS respiration in the gill mitochondria (Kurochkin et al., 2008; Sokolov et al., 2019), albeit the effects were generally weaker than those observed in our present study. It is worth noting that earlier *in vivo* exposure studies (Kurochkin et al., 2008; Sokolov et al., 2019) used longer post-hypoxic recovery time (1 h) so that some of the immediate effects of H/R stress on mitochondrial succinate oxidation observed in our present study might have remained undetected.

Besides being an important metabolic fuel, succinate is considered a potential pro-oxidant that can strongly stimulate ROS generation due to the reverse electron transport (RET) through mitochondrial CI (Scialò et al., 2017). Albeit the relevance of this mechanism to ROS generation *in vivo* has been disputed (Andrienko et al., 2017; Murphy, 2009), elevated ROS production with succinate is commonly observed in vertebrate mitochondria (Bundgaard et al., 2018; Pell et al., 2016; Quinlan et al., 2013). In rodents, inhibition of CI with rotenone suppressed ROS production in the brain and skeletal muscle mitochondria energized with succinate supporting the notion that reverse electron flow to CI is a major source of ROS production in these systems (Quinlan et al., 2013; Gusdon et al., 2015). A similar RET mechanism has been reported in hypoxia-tolerant reptiles (freshwater turtles and frogs), albeit succinate accumulation during hypoxia in reptiles was much lower than in mammals and not considered conducive of increased ROS production (Bundgaard et al., 2019). In *C. gigas*, ROS production in control mitochondria energized with succinate was not markedly higher than with NADH-linked substrates such as glutamate or pyruvate, and (in the case of the digestive gland mitochondria) considerably lower than palmitate-driven ROS generation. Furthermore, inhibition of CI with rotenone had no effect on the ROS efflux or electron leak indicating that RET does not contribute to the succinate-driven ROS generation under the control conditions. Interestingly, elevated capacity for succinate oxidation induced by the H/R stress was associated with higher ROS production in oyster mitochondria that was partially alleviated by rotenone. This indicates that functional changes induced by the H/R stress stimulate RET in oyster mitochondria. However, the RET contribution was only significant in the ADP-stimulated mitochondria when the overall ROS production rate and H_2O_2 to O_2 ratios were low. The resting (LEAK) state mitochondria showed no evidence for RET involvement in succinate-driven ROS production after the H/R stress. Furthermore, addition of pyruvate attenuated succinate-driven ROS efflux and electron leak in the H/R-stressed mitochondria confirming the positive effects of NADH-linked substrates on mitochondrial ROS production in oysters. Taken together, these findings indicate that contribution of RET to the mitochondrial ROS production is likely to be low under the physiological conditions due to the presence of NADH-linked substrates such as pyruvate and the minimal RET contribution to the ROS generation of the resting mitochondria.

Conclusions and outlook

Mitochondria of a hypoxia-tolerant marine bivalve *C. gigas* showed metabolic flexibility in utilizing different substrates during stress exposures. The gill mitochondria showed evidence for a stronger capacity for glutamate oxidation and a better control over the ROS efflux and electron leak during palmitate-driven respiration compared with the digestive gland mitochondria. This metabolic flexibility might be related to the multifunctional role of the gill as a respiratory, osmoregulatory and feeding organ as well as a major site for uptake of dissolved nutrients such as amino acids (Gosling, 1992; Kennedy et al., 1996; Manahan, 1983; Siebers and Winkler, 1984). Our data indicate that oxidation of fatty acids (palmitate) in the digestive gland mitochondria might be associated with fitness costs because of high ROS production, further enhanced by the H/R stress. Low efficiency of the digestive gland mitochondria in fatty acid utilization under the normal condition and particularly after the H/R stress was unexpected in view of the role of the digestive gland as the main site of fat storage in marine bivalves (Gosling, 1992; Kennedy et al., 1996). This might indicate that lipids stored by the digestive gland are predominantly utilized elsewhere and requires further investigation.

Oyster mitochondria showed excellent ability to tolerate acute H/R stress and maintain high respiratory flux with low ROS production when respiring on succinate, whereas the organelles oxidizing NADH-linked substrates showed a decline in respiration and elevated electron leak after H/R stress. Succinate addition to the NADH-linked substrates alleviated the negative effects of H/R stress, supporting the notion of positive metabolic effects of succinate in bivalve mitochondria. High capacity for succinate oxidation combined with the low propensity of ROS generation via RET might be considered an adaptive mitochondrial phenotype found in hypoxia-tolerant species such as marine bivalves (this study) or freshwater turtles (Almeida-Val et al., 1994; Bundgaard et al., 2019). In turtles, the RET is prevented by low succinate accumulation in hypoxia (Bundgaard et al., 2019), whereas in oysters, the RET is not observed even at saturating succinate concentrations. This might reflect adaptations of bivalve mitochondria to cope with high accumulation of succinate that serves as the main anaerobic end product during hypoxia in bivalves (Bayne, 2017; de Zwaan, 1991) and indicates coadaptation of aerobic and anaerobic pathways. Interestingly, studies in insects have also reported an increased capacity for succinate utilization at the expense of NADH-linked substrates during thermal stress (Jørgensen et al., 2021). These findings raise the possibility that succinate can serve as a potential stress fuel in ectotherm mitochondria and are consistent with the beneficial effects of high succinate oxidation capacity for mitochondrial stress tolerance (Huang and Lemire, 2009; Walker et al., 2006). Given the important metabolic and signaling roles of succinate (Guo et al., 2020), the role of this important metabolic intermediate in regulating the stress response in ectotherms warrants further investigation.

Competing interests
The authors declare no competing or financial interests.

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

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