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Traditio et Innovatio

**MITOCHONDRIAL RESPONSES TO HYPOXIA-REOXYGENATION  
STRESS IN MARINE BIVALVES AND MAMMALS.**

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## Abstract

Oxygen deficiency (hypoxia) is a common stressor in aquatic environments leading to loss in biodiversity and altered ecosystem dynamics. Mitochondria are major targets of hypoxia and reoxygenation stress as hypoxia instigates the collapse of the electron transport system (ETS) leading to decreased ATP production and amplifies reactive oxygen species (ROS) production. Evaluating the response of mitochondria to hypoxic stress is therefore essential in understanding the mechanisms associated with hypoxia tolerance and intolerance. Additionally, the adaptive mechanisms developed by animals to aid survival, boost physiological functions, and mitigate oxidative stress under hypoxia-reoxygenation (H/R) stress need further investigation. This study focused on gaining insight into the physiological and molecular mechanisms of responses to H/R stress in hypoxia-tolerant and intolerant animals and identifying potential adaptive mechanisms associated with hypoxia-tolerant mitochondrial phenotype. To achieve this, we used hypoxia tolerant marine bivalves (the blue mussel *Mytilus edulis* and the Pacific oyster *Crassostrea gigas*) and hypoxia intolerant species (the domestic pig *Sus scrofa* and the king scallop *Pecten maximus*) to investigate the effect of H/R stress on their mitochondrial functional parameters. The study further investigated the transcriptional changes as well as potential molecular mechanisms involved in the response to H/R stress in these species. These mechanisms were measured under different H/R exposure periods (acute for mammal and bivalves and chronic for bivalves only).

## Kurzfassung

Sauerstoffmangel (Hypoxie) ist ein häufiger Stressfaktor in aquatischen Lebensräumen, der zu Biodiversitätsverlust führt. Mitochondrien sind stark von Hypoxie- und Reoxygenationsstress (H/R) betroffen, da Hypoxie den Zusammenbruch des Elektronentransportsystems (ETS) verursacht, was zu verminderter ATP-Produktion und verstärkter Produktion reaktiver Sauerstoffspezies (ROS) führt. Die Untersuchung der Mitochondrienreaktion auf hypoxischen Stress ist entscheidend für das Verständnis der Mechanismen von Hypoxietoleranz und -intoleranz. Weitere Forschung ist erforderlich, um die Anpassungsmechanismen von Tieren zu untersuchen, die das Überleben unterstützen, physiologische Funktionen stärken und oxidativen Stress unter H/R mildern. Diese Studie konzentrierte sich auf Einblicke in physiologische und molekulare Reaktionsmechanismen bei hypoxietoleranten und -intoleranten Organismen und die Identifizierung potenzieller adaptiver Mechanismen im Zusammenhang mit dem hypoxietoleranten mitochondrialen Phänotyp. Dabei wurden hypoxietolerante marine Muscheln (die Miesmuscheln *Mytilus edulis* und die Pazifische Austern *Crassostrea gigas*) und hypoxieintolerante Arten (das Schwein *Sus scrofa* und die Jacobsmuscheln *Pecten maximus*) verwendet, um den Effekt von H/R-Stress auf ihre mitochondrialen Funktionsparameter zu untersuchen. Die Studie analysierte auch transkriptionelle Veränderungen und potenzielle molekulare Mechanismen, die an der Reaktion auf H/R-Stress beteiligt sind, unter verschiedenen H/R-Expositionszeiträumen (akut für Säugetiere und Muscheln, chronisch nur für Muscheln). Unsere Studie ergab, dass die Aufrechterhaltung der ATP-Synthesekapazität mit minimalem ROS-Austritt, insbesondere während der Succinatoxidation, mit dem hypoxietoleranten Phänotyp verbunden ist, während die ATP-Synthese beim hypoxieintoleranten Phänotyp abnimmt. Hypoxie führte zu differenzierter Regulation von Genen, die mit Entzündung, Apoptose, Angiogenese, mitochondrialem ETS und Qualitätskontrolle verbunden sind. Entzündung, Apoptose und Angiogenese reagierten im intoleranten Phänotyp am stärksten, während der antioxidative Schutz und die mitochondriale Qualitätskontrolle für den toleranten Phänotyp relevant waren. Die Studie bietet Einblicke in molekulare und physiologische Mechanismen, die mit der arts- und gewebespezifischen Hypoxietoleranz von tierischen Mitochondrien und dem Schutz vor H/R-Stress verbunden sind.

## List of Abbreviations

H/R – Hypoxia reoxygenation

ROS – Reactive oxygen species

ETS – Electron transport system

RET – Reverse electron transport

C I - Complex I,

C II - Complex II,

C III - Complex III

C IV - Complex IV.

NADH - Nicotinamide adenine dinucleotide

FMN - Flavin mononucleotide

Fe-S - Eight iron-sulfur

TCA - Tricarboxylic acid cycle

FADH<sub>2</sub> - Flavin adenine dinucleotide

ATP – Adenosine triphosphate

OXPPOS – Oxidative phosphorylation

FEL – Fractional electron leak

SCs - Supercomplexes

MPTP - Mitochondrial permeability transition pore

PTM – Post-translational modification

PKA – Protein kinase A

AKAP1 – A-kinase interacting protein 1

HIF – Hypoxia inducible factor

PHDs - Prolyl hydroxylase domain-containing proteins

FIH - Asparagine hydroxylase factor inhibiting HIF

NDUFAB1 - NADH:ubiquinone oxidoreductase subunit AB1

NDUFAB6 - NADH dehydrogenase [Ubiquinone] 1 beta subcomplex subunit 6

NDUFS2 - NADH:ubiquinone oxidoreductase core subunit S2

COX - Cytochrome C oxidase

COX6A1 - Cytochrome C oxidase subunit 6A1

COX6C - Cytochrome C oxidase subunit 6C

COX7A1 - Cytochrome C oxidase subunit 7A1

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# 1 Introduction

## 1.1 Hypoxia as an environmental stressor

Oxygen is crucial for metazoan life, and its limitation poses stress and can be fatal to the survival of aerobic organisms. Low oxygen levels (hypoxia) have been reported both in the terrestrial and aquatic environments. In the terrestrial environment, hypoxia is restricted to high altitudes where the partial oxygen pressure is decreased in proportion to the lower ambient pressure and in underground environments where O<sub>2</sub> consumption by organisms exceeds O<sub>2</sub> influx by air exchange <sup>1</sup>. Functionally, hypoxia usually occur in the organs and tissues of terrestrial organisms due to the restriction of oxygen delivery to the various tissues (ischemia) <sup>2,3</sup> or low oxygen content of the blood. Tissue hypoxia is often associated with pathological and pathophysiological conditions like heart attack, stroke, sleep apnea, surgery, or organ transplantation <sup>4-7</sup>.

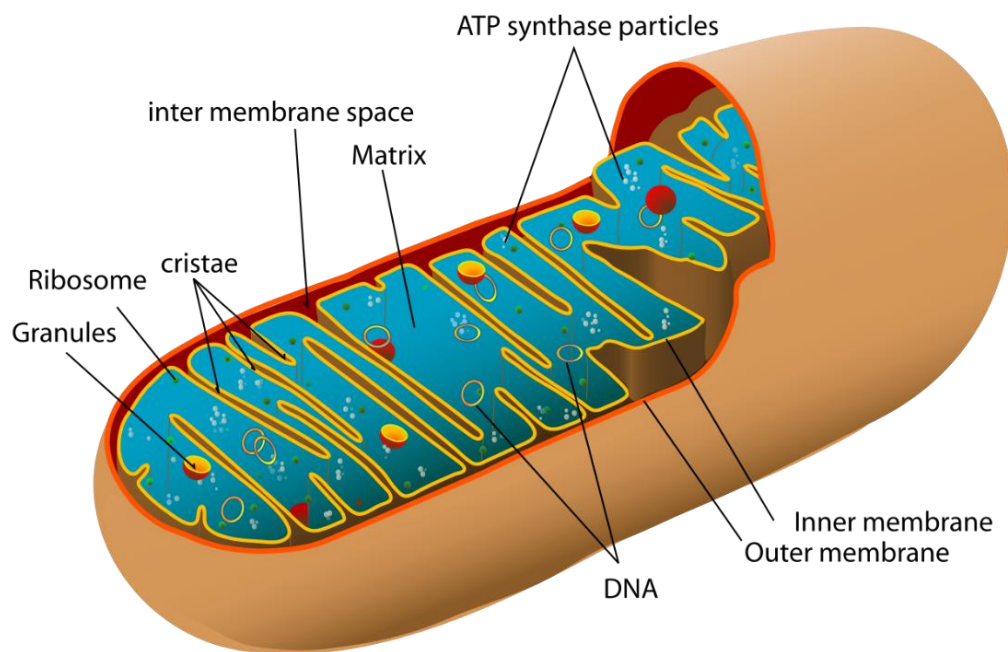
Unlike the terrestrial environment, the aquatic environment is highly vulnerable to hypoxia <sup>8</sup> especially prevalent in estuaries, tidal pools, intertidal, benthic, and coastal zones of the ocean <sup>9,10</sup>. Hypoxia occurs during significant drops in the dissolved oxygen levels of a water body reflecting the dynamics of photosynthesis, respiration, and gas exchange with the atmosphere. In marine ecosystems, the oxygen levels may vary over the time frame from several hours (during diurnal cycles of photosynthesis and respiration) to days and weeks as observed in eutrophicated coastal dead zones <sup>11-13</sup>. Coastal hypoxia arises from a misbalance between oxygen input from the atmosphere and certain biological and chemical processes that consume or produce oxygen. A typical cause of hypoxia is the stratification of the water column that limits the vertical mixing between the water layers. The stratification of the water layers restricts the oxygen transfer between surface and bottom waters creating hypoxic bottom habitats especially during the summer. In the winter season, ice formation cuts off the air exchange at the surface. Hypoxia in the aquatic environments also occur when the water column is enriched with organic matter leading to bacterial decomposition which consumes oxygen <sup>14</sup>. Additionally, nutrients from agricultural sources flowing into aquatic habitats stimulate primary productivity and subsequently bacterial respiration outstripping the oxygen input through photosynthesis, mixing and diffusion <sup>9,15</sup>. Increase in atmospheric

temperature due to the global climate change increases the warming of surface waters reducing O<sub>2</sub> solubility, increasing stratification, and elevating O<sub>2</sub> consumption rates of the resident biota <sup>9</sup>. All these processes contribute to the amplification of coastal hypoxia resulting in a greater spatial extent and a higher frequency and duration of hypoxic events, particularly in eutrophicated coastal environments.

Coastal ecosystems are the most threatened ecosystems in the world. They harbor high biodiversity with valuable economic value. Over the last two decades, coastal hypoxic zones have increased significantly <sup>11</sup> with increasing duration and intensity of hypoxia threatening aerobic organisms. Hypoxia have been reported to alter animal behavior, ecosystem dynamics, disrupt food webs, and cause sublethal stresses like reduced growth and reproduction, and lethal stress like mass mortality in fishes and invertebrates <sup>12,16</sup>. In sea urchins <sup>17</sup>, sea cucumbers <sup>18</sup>, oriental river prawn <sup>19</sup> and croaker fish <sup>20</sup>, hypoxia exposure led to mass mortality resulting in major economic loss. This is a clear indication of the extreme threats of hypoxia to the coastal ecosystems and economy. Despite the many challenges posed by hypoxia, some animals inhabiting the intertidal zones of coastal ecosystems have developed adaptive response mechanisms. Mobile coastal and pelagic species like some fishes are able to escape this threat into less hypoxic zones by swimming <sup>21,22</sup>. However, benthic species like barnacles, mollusks or polychaetes with no or very limited mobility cannot escape hypoxia. These benthic dwellers are the most vulnerable because they are the farthest from atmospheric oxygen and inhabit oxygen depleted coastal sediments. The ecological and behavioral diversity observed in these animals suggests differing responses to hypoxia, especially with bivalve species that have developed adaptive mechanisms to equip them for survival in hypoxic-prone habitats. Hence, investigating these keystone species presents an opportunity to understand their physiology and adaptive mechanisms associated with hypoxic response.

## 1.2 The structure and function of the mitochondria

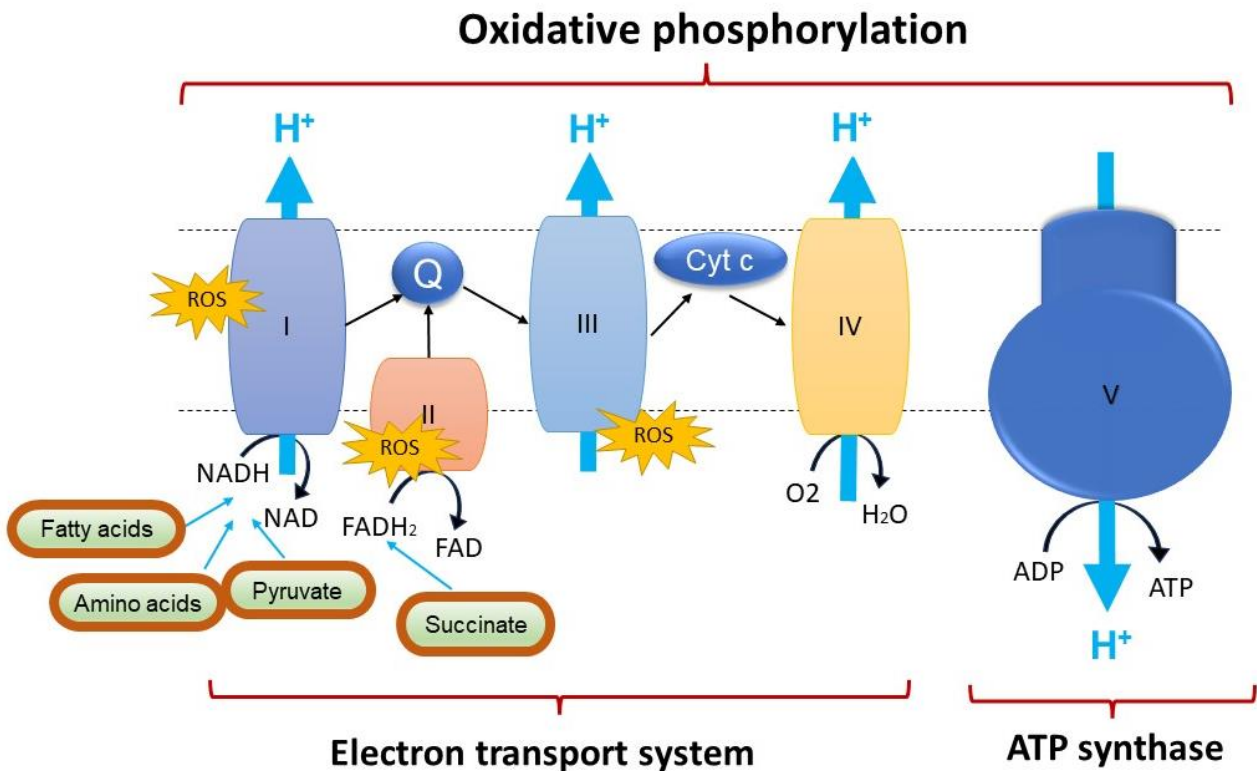
Mitochondria are double membrane layered organelles with their genetic material distinct from the nuclear genome. They possess an outer and inner membrane separated by an intermembrane space (Figure 1). The outer mitochondrial membrane (OMM) is a porous membrane permeable to high molecular weight solutes. The inner mitochondrial membrane (IMM) is less permeable to solutes (except through specific transporters). The IMM creates folds (cristae) that extend into the interior of the mitochondrion known as the matrix. The matrix holds the DNA material of the mitochondria, and a large variety of enzymes including those of the citric acid cycle. The IMM also acts as a major site for electron transport and ATP synthesis and holds the protein components of the electron transport system (ETS). Hence, the IMM and the matrix are the major functional compartments of the mitochondria <sup>23</sup>.



**Figure 1.** Structure of a mitochondrion (Source: Wikimedia commons)

The ETS utilizes reduced equivalents of energy-rich substrates (including carbohydrates, fatty acids, and amino acids) and is primarily made up of four enzyme complexes: Complex I,

Complex II, Complex III and Complex IV. Complex I (C I), NADH:ubiquinone oxidoreductase, is the entry point of electrons from NADH to the respiratory chain. It consists of NADH dehydrogenase, flavin mononucleotide (FMN), and eight iron-sulfur (Fe-S) clusters. C I oxidize NADH generated from glycolysis and the tricarboxylic acid cycle (TCA), and transfers two electrons from NADH to ubiquinone (coenzyme Q) thereby reducing it to ubiquinol <sup>24</sup>. Complex II (C II), succinate dehydrogenase, is another entry point of electrons from reduced substrates to the respiratory chain. C II oxidizes FADH<sub>2</sub> and likewise transfers two electrons to ubiquinone forming ubiquinol <sup>25</sup>. Besides C I and C II, several other dehydrogenases (choline dehydrogenase, dihydroorotate dehydrogenase, mitochondrial glycerol-3-phosphate dehydrogenase, proline dehydrogenase 1/2, sulfide:quinone oxidoreductase<sup>26</sup>) transfer electrons to ubiquinone, hence creating a junction where electrons flow from multiple sources to the ETS. Complex III (C III), cytochrome c reductase, consists of cytochrome b, Rieske subunits (containing two Fe-S clusters), and cytochrome c proteins. C III transfers electron from ubiquinol to the soluble mobile intermembrane space carrier protein cytochrome c. Cytochrome c then transport electrons to the final complex of the ETS, Complex IV. Complex IV (C IV), cytochrome c oxidase, oxidizes cytochrome c and transfers electrons to oxygen, the terminal electron acceptor in aerobic cellular respiration. Electron transfer at the mitochondrial Complexes I, III and IV leads to the protons being pumped across the IMM into the intermembrane space thereby establishing the electrochemical gradient (the protonmotive force) necessary for ATP synthesis via the F<sub>0</sub>, F<sub>1</sub>-ATP Synthase (Complex V, C V). The overall process of electron transfer from C I to C IV coupled to the generation of ATP by C V is called oxidative phosphorylation (OXPHOS) (Figure 2) <sup>27</sup>.



**Figure 2.** Schematic representation of the OXPHOS process in the mitochondria. Black arrows within the ETS complexes show the direction of the electron flow. NADH donates electron to Complex I, and succinate - to Complex II of the mitochondrial ETS. ROS (superoxide) is generated by electron leak at the Complexes I, II and III. Based on Cortossa et al.,<sup>28</sup>.

### 1.3 Mechanisms of response to H/R stress in different organisms

#### 1.3.1 Mitochondrial mechanisms of response to H/R stress

Mitochondria are key players in meeting the energy needs of animal cells by producing ATP. The ATP production process of the mitochondria is highly oxygen-dependent (aerobic) with the mitochondria generating about 90% of cellular ATP. Mitochondrial ETS uses oxygen as a final electron acceptor and generates the protonmotive force to drive ATP synthesis through the OXPHOS process. Insufficient supply of oxygen during hypoxia suppresses OXPHOS resulting in lower rates of ATP production and elevates the production of reactive oxygen species (ROS)<sup>29-31</sup>. Furthermore, hypoxia can lead to calcium overload in the mitochondria

<sup>32</sup>. Despite the negative effects of hypoxia on animals, the return of oxygen after hypoxia can be more damaging. Upon reoxygenation, there is a surge in ROS efflux due to reverse electron transport (RET) and electron leak from the highly reduced metabolic intermediates, which can stimulate mitochondrial damage and lead to collapse of ETS and OXPHOS activity <sup>33,34</sup>. Furthermore, the increased ROS efflux can prompt oxidative damage and injury by targeting DNA, membrane lipids and proteins. For aerobic animals fully reliant on aerobic respiration and the ATP produced by the mitochondria, H/R stress can have lethal and sublethal consequences.

Several studies of model animals such as terrestrial mammals and insects revealed that mitochondria are sensitive to environmental stress including hypoxia and act both as a target of stress and the coordinating center for the adaptive cellular response (reviewed in Sokolova <sup>35</sup>). For instance, in mammals, myocardial ischemia/hypoxia led to damaged ETS <sup>36</sup>. Other studies of these hypoxia-intolerant species (terrestrial mammals, insects, and highly aerobic aquatic invertebrates like scallops) revealed that hypoxia also led to mitochondrial depolarization and oxidative injury after a single H/R cycle <sup>30,37,38</sup>. The suppression of the ETS activity under hypoxia in these intolerant species led to a decreased ATP production related to the loss of C I and C IV activity<sup>38</sup>. In the hypoxia-intolerant terrestrial vertebrates, the suppression of the ETS activity was also partly due to the reversible inactivation of pyruvate dehydrogenase E1 enzyme and oxidative damage to ETS proteins <sup>39-41</sup>. Additionally, hypoxia-intolerant aquatic species like the shovelnose ray *Aptychotrema rostrata* and the bay scallop *Argopecten irradians* showed a suppression in C IV activity <sup>38,42</sup>. Exposure of the hypoxia-intolerant lowland locusts (*Locusta migratoria*) to extreme hypoxia (1.6 kPa P<sub>O2</sub>) suppressed ETS and OXPHOS activity while little or no effect was found in their hypoxia-tolerant high-altitude counterparts<sup>43</sup>. In addition to the major mitochondrial responses observed in hypoxia-intolerant species during hypoxia exposure, reintroduction of oxygen (reoxygenation) also led to a burst in ROS efflux as a result of the fractional electron leak (FEL) from the highly reduced ETS intermediates <sup>30,34</sup>. For instance, in myocardium, reperfusion/reoxygenation induced early mitochondrial-driven injury with a burst in ROS efflux <sup>36</sup>. The burst in ROS efflux further led to oxidative stress and injury thereby stimulating

the mitochondrial membrane permeability transition, cytochrome c release in the cytosol, initiation of the apoptotic cascade, cell death, and organ failure <sup>30</sup>.

Contrary to the hypoxia-intolerant species, hypoxia-tolerant species like intertidal bivalves, freshwater turtles and fish have evolved metabolic mechanisms that enhance survival during periods of hypoxia and facilitate recovery of the physiological (including mitochondrial) functions when oxygen returns <sup>10,44-47</sup>. Most hypoxia survival strategies center on energy conservation such as metabolic rate suppression and use of alternative anaerobic pathways with higher ATP yields and lower toxicity of waste products <sup>48-50</sup>. Investigating the physiological adaptations and mechanisms related to hypoxia tolerance and sensitivity has revealed that the hypoxia-tolerant species such as intertidal invertebrates, anoxia-tolerant fish and reptiles, and naked mole rats have a high metabolic resilience during oxygen fluctuations <sup>51-53</sup>. Some adaptive mechanisms that might contribute to this metabolic resilience include upregulation of the ETS, mitochondrial respiratory flux, antioxidant activity, and protein quality control while suppressing ATP wastage and mitigating oxidative stress <sup>35,54-59</sup>. For instance, the extremely tolerant hard-shell clam *Mercenaria mercenaria* upregulated its ETS capacity after hypoxia exposure while the hypoxia-tolerant turtle *T. scripta*, suppressed ETS activity after exposure to anoxia <sup>38,55</sup>. However, the ETS suppression in the turtle appeared to be restricted to Complex I activity whereas Complexes III and IV activities were stabilized suggesting a complex-specific response to stress. Although both clams and turtles are hypoxia-tolerant species, their mitochondrial response to hypoxia was in opposite patterns. This might be due to the tissue- or species-specific differences in hypoxia tolerance and thus in the adaptive mechanisms. Given this controversy, more studies are needed to understand the tissue-specific response of different species as well as the various adaptive mechanisms to hypoxia stress.

H/R stress has been reported to induce changes in the intracellular milieu including shifts in concentrations of metabolic intermediates <sup>60-62</sup>. With the strong dependence of mitochondrial energy generation on substrate fuels, understanding substrate-dependent mitochondria responses to stress is important. Mitochondria use easily accessible substrate fuels from the breakdown of large organic molecules such as carbohydrates, fatty acids, and

amino acids to generate ATP (Fig. 2). Generally, the nature of the respiratory substrate affects the stoichiometry of oxygen consumption to ATP production<sup>63</sup> and ROS generation rates<sup>64</sup>.

Under pathological, pathophysiological, and stressful conditions, the mitochondria exhibit metabolic flexibility in the differential use of substrate fuels for energy production. In mammals, mitochondria adjust the capacity for different fuel use for respiration depending on the nutrient availability and can alter the fuel use in pathological conditions like type 2 diabetes<sup>65-67</sup>. In invertebrates and some rodents, the metabolic flexibility of mitochondria has been observed under conditions of thermal stress and hypoxia<sup>68-70</sup>. Furthermore, different tissues have different substrate preferences both under the normal physiological conditions and under stress. In mammals, the brain tissues utilize predominantly glucose as energy source<sup>71</sup> and the heart uses fatty acids, despite having the ability to use glucose and ketone bodies<sup>72</sup>. The mammalian kidney and skeletal muscle can use both fatty acids and glucose as energy sources for metabolism. However, in the kidney, the substrate preference depends on nutritional status and physiological conditions while that of the skeletal muscle depends on type and intensity of physical activity<sup>73,74</sup>. The liver is also flexible and has preference for both glucose and fatty acids<sup>75</sup>. In rodents, the brain mitochondria show higher transport and oxidation rates of Complex I (NADH-linked) substrates. Marine mollusks and horseshoe crabs have limited capacity to oxidize fatty acids and largely depend on proline and succinate (mollusks) or carbohydrates (the horseshoe crabs) oxidation<sup>76-78</sup>. Rodent liver mitochondria show preference for succinate<sup>79</sup> and heart mitochondria prefer fatty acids, amino acids, and lactate oxidation<sup>80</sup>. However, the substrate preference of these various tissue mitochondria has the tendency to change under stress. For instance, the brain mitochondria of hypoxia-tolerant goldfish switch from carbohydrate to fatty acid oxidation during hypoxia<sup>58</sup>.

The metabolic flexibility of mitochondria under stress does not only influence mitochondrial oxygen consumption and ATP synthesis but has implications for ROS efflux. ROS production is substrate-dependent and regulated by the degree of reduction of the coenzyme Q (CoQ) pool<sup>81</sup>. In the mitochondrial ETS, C I and CIII are major sites of ROS production<sup>82,83</sup>. Oxidizing C I- linked substrates have been reported to produce ROS both under normal and stressful conditions. In the rat heart, oxidizing C I substrates resulted in ROS production and

inhibiting C I with rotenone did not increase ROS production indicating that most ROS generation occurred via forward electron transport <sup>84</sup>. Although forward electron transport is associated with ROS efflux, reverse electron transport (RET) through C I can greatly enhance the mitochondrial ROS production, particularly under stressful or pathological conditions resulting in succinate accumulation <sup>82,85,86</sup>. Succinate oxidation by C II reduces the CoQ pool thereby reducing NAD<sup>+</sup> to NADH at C I and increasing ROS production via RET at C I <sup>82</sup>. In the mammalian mitochondria, the redox state of the CoQ pool was maintained near optimum during glycolysis but declined during fatty acids or succinate oxidation leading to ROS production via RET at Complex I <sup>64,81</sup>.

### **1.3.2 The role of hypoxia inducible factors in regulating response to hypoxia.**

Hypoxia-inducible factors (HIFs) are essential regulators of oxygen sensing and hypoxic response <sup>87,88</sup>. The HIF family of transcription factors comprises two most common isoforms, HIF-1 and HIF-2, although other isoforms have been identified <sup>89</sup>. HIF-1 is a heterodimer with  $\alpha$  and  $\beta$ -subunits whose regulation is highly dependent on oxygen <sup>90</sup>. The HIF-1 $\alpha$  subunit is continually synthesized but rapidly degraded in the presence of oxygen <sup>91</sup>. HIF regulation is achieved through both oxygen dependent and independent mechanisms. Under normal oxygen conditions, HIF $\alpha$  regulation occurs through the cellular oxygen sensors prolyl hydroxylase domain-containing proteins (PHDs) and the asparagine hydroxylase factor inhibiting HIF (FIH). Under hypoxic conditions, there is limited availability of molecular oxygen to PHDs and FIH. Hence, HIF-1 $\alpha$  stabilizes, accumulates, and binds with the constitutive HIF-1 $\beta$  subunit to form an active HIF-1 that initiates a transcriptional cascade regulating both oxygen supply and oxygen demand of the cell <sup>89,92-94</sup>. HIF-1-dependent cascade involves target genes associated with angiogenesis, energy metabolism, redox homeostasis, cell proliferation, autophagy, and apoptosis <sup>94</sup>. A major group of HIF-1 targets includes genes involved in metabolic rewiring and the shift of cellular metabolism from aerobic OXPHOS to anaerobic glycolysis to meet energy demands under low oxygen conditions <sup>91,95</sup>. HIF-1 $\alpha$  also regulates mitochondrial respiratory capacity limiting ROS production, regulating cytochrome oxidase subunit expression, and suppressing metabolite entry into the tricarboxylic acid (TCA) cycle <sup>92,93</sup>. For instance, in mammalian models, the stabilization of HIF-1 $\alpha$  protected against ischemia-reperfusion injury by enhancing aerobic

glycolysis, downregulating mitochondrial oxidative stress, and inhibiting mitochondrial permeability transition pore (MPTP) opening<sup>96</sup>. Additionally, HIF activation was associated with mitochondrial loss, ischemic damage, and a switch from oxidative phosphorylation to glycolysis<sup>88,97-99</sup>. In the hypoxia-tolerant marine bivalve, the hard-shell clam *Mercenaria mercenaria*, exposure to hypoxia induced an upregulation of HIF-1 $\alpha$  during reoxygenation implying the adaptive role of HIF-1 in post-hypoxic survival<sup>38</sup>

Other molecular and cellular mechanisms are also fully or partially regulated by HIF in response to hypoxia. These includes changes in the epigenome, non-coding RNAs, the metabolome, signaling pathways, and biochemical reactions<sup>100,101</sup>. HIF -1 controls glucose utilization, amino acid metabolism, fatty acid metabolism, oxidative phosphorylation, mitochondrial biogenesis and mitophagy<sup>101</sup>. Under hypoxic conditions, increased HIF activity decreased mitochondrial oxygen consumption by causing a decline in mitochondrial mass through mitophagy, and increased glycolysis, fatty acid synthesis and glutaminolysis (reviewed by Taylor and Scholz<sup>101</sup>). HIF-1 activation reduced mitochondrial biogenesis by increasing the expression of MAX interactor 1 (MXI1)<sup>102,103</sup>. TCA cycle activity was also decreased by repressing levels of enzymes like succinate dehydrogenase subunit A (SDHA), and succinate dehydrogenase subunit B (SDHB)<sup>104-106</sup>. In the blue mussel *M. edulis*, exposure to acute hypoxia induced mitochondrial injury and stimulated the transcriptional regulation of protective genes involved in mitochondrial fission and fusion, proteolysis and mitophagy while those of the hypoxia-tolerant Pacific oyster *C. gigas* remained stable<sup>59</sup>. Furthermore, hypoxia also induced the transcriptionally upregulation of anaerobic glycolysis and protein quality control pathways<sup>44</sup>. Overall, the HIF-dependent regulation of hypoxia is not limited to the cellular level but the mitochondrial level as hypoxia significantly alters mitochondrial function (reviewed by Lee et al.<sup>107</sup>)

Overall, the available data show the importance of mitochondrial mechanisms in the adaptations to H/R stress in animals. However, the understanding of the mechanisms regulating mitochondrial metabolism and their role in adaptive cellular response to oxygen fluctuations is currently incomplete, particularly in the non-model species such as marine invertebrates. While earlier studies showed the importance of the mitochondrial functional

reorganization for maintenance of the mitochondrial integrity and OXPHOS activity during H/R stress <sup>38</sup>, the role of the frequency and duration of the hypoxic events on the mitochondrial metabolic adjustments remains poorly understood. Furthermore, the potential links between the tissue- and species-specific sensitivity to hypoxia and the metabolic flexibility of mitochondrial substrate utilization are not known. To close this gap in our knowledge, we studied the mitochondrial and molecular responses to hypoxia and reoxygenation in animals with different degree of hypoxia tolerance including a hypoxia-intolerant mammal, the domestic pig *Sus scrofa*, and three species of bivalve mollusks: a hypoxia-intolerant king scallop *Pecten maximus*, and two hypoxia tolerant intertidal bivalves, the blue mussel *Mytilus edulis* and the Pacific oyster *Crassostrea gigas*. We focused on the analysis of the effects of hypoxia-reoxygenation regimes of different duration and frequency on mitochondrial respiration rates, coupling efficiency and ROS efflux, and analyzed the implications of use of different substrate fuels for the mitochondrial resilience to H/R stress.

#### **1.4 Bivalves as model organisms for studying the mechanisms of tolerance to hypoxia-reoxygenation stress.**

Bivalves are a class of mollusks characterized primarily by an enclosed shell (left and right shell). These shells prevent foreign materials like mud and sand from entering the mantle as well as protect the bivalves from predators <sup>108</sup>. Bivalves are ubiquitously distributed in aquatic habitats around the globe and inhabit marine, brackish and freshwater ecosystems. Most bivalves are suspension feeders that filter the water column for their food as well as remove inorganic particles and toxins out of the water column. They are also essential ecosystem engineers providing important ecological services like bioturbation, bioirrigation, sediment stabilization, and reef building <sup>61,108</sup>. The bivalves are important for global aquaculture and serve as a good source of protein. Owing to the high ecological and economical importance of bivalves, biology, physiology and genetics of many common species of clams, mussels, oysters, and scallops have been extensively studied <sup>108</sup>. Bivalves are common inhabitants of the intertidal and coastal benthic zones where they are exposed to frequent and strong fluctuations in environmental conditions like temperature, salinity, desiccation, access to food and oxygen, dissolved oxygen concentrations and pH <sup>13,54,109</sup>. Despite the harsh life between the tides, bivalves have successfully colonized the intertidal zones reaching high abundances and biomass. The abundance of bivalves in the coastal

ecosystems, their filter feeding habits and ability to accumulate contaminants make bivalve species important bioindicators of environmental pollution <sup>110-113</sup> and an excellent model system to investigate adaptations to extreme abiotic stress <sup>109</sup>. Studies on physiological and biochemical responses of bivalves to heat stress, toxins, salinity stress, hypoxia <sup>35,57,114</sup> showed that intertidal bivalves are among the animal champions of stress tolerance including the tolerance to hypoxia-reoxygenation stress <sup>115</sup>. Bivalves have a wide species diversity with different degree of hypoxia tolerance, from highly tolerant species like clams, oysters, and mussels to intolerant ones like scallops. Bivalve molluscs therefore serve as an excellent model for investigating the mechanisms of responses and adaptations to hypoxia-reoxygenation stress, which is why they were chosen for this project.

The great scallop *Pecten maximus* (Linnaeus, 1758) is a subtidal bivalve species inhabiting the northeastern coast of the Atlantic Ocean from the northern Norway to the south of Portugal and off the west coast of Africa <sup>116</sup>. Scallops are the only bivalve species with the ability to swim. They swim actively as an escape mechanism to avoid unfavorable environmental conditions and predators <sup>117</sup> distinguishing them from other bivalves <sup>118,119</sup>. Due to their subtidal lifestyle and swimming capacity, scallops have high metabolic activity. *P. maximus* inhabit relatively constant environments and therefore have a relatively high sensitivity to stress. Additionally, due to their vigorous muscle activity during valve clapping, they require oxygenated environments during recovery and hence are intolerant to H/R stress <sup>120</sup>. *P. maximus* represent almost 80% of European wild harvested scallops <sup>121</sup>.

Sedentary bivalves like the blue mussels *Mytilus edulis* (Linnaeus, 1758) and the Pacific oyster *Crassostrea (Magallana) gigas* (Thunberg, 1793), are common inhabitants of the intertidal zone and are characterized by high level of stress tolerance <sup>109</sup>. The mussels of the genus *Mytilus*, including the blue mussel *M. edulis* are dominant bivalve species in rocky intertidal shores around the world. They inhabit high intertidal to subtidal zones of the marine coasts and estuaries <sup>108</sup>. Mussels attach to diverse solid surfaces (like rocks, ships, quay walls and power plant inlets) using their proteinaceous byssus to form mussel beds <sup>108</sup>. The byssus of the blue mussels is vital to their evolution, physiology, and ecology. The mussel beds increase the biomass per unit area of their habitat area thereby creating habitats for other organisms <sup>122</sup>. Pacific Oysters are benthic bivalve species inhabiting marine or brackish habitats. They have sturdy, irregularly shaped shells and strong adductor muscle that ensures effective shell closure during tidal emersion or predator attack <sup>122</sup>. Oysters

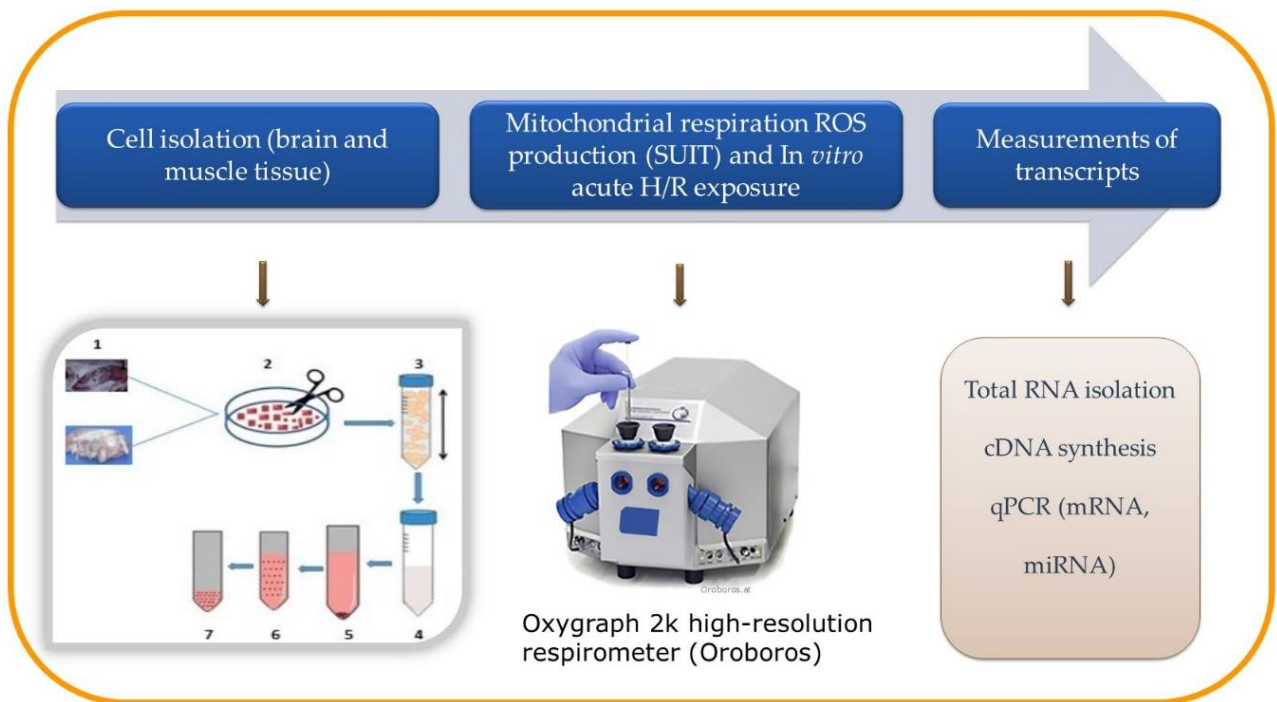
permanently attach to firm substrates like rock and shells. Due to the sedentary life habit of the post-metamorphic (juvenile and adult) mussels and oysters and their inability to escape unfavorable environmental conditions, these species are highly dependent on physiological and molecular adaptations to survive abiotic stress. The mussel *M. edulis* and the Pacific oyster *C. gigas* demonstrate metabolic flexibility and are classified as facultative anaerobes<sup>122</sup>. They can effectively transition between aerobic and anaerobic metabolism, allowing them to survive extended periods without oxygen<sup>9</sup>. Both *M. edulis* and *C. gigas* have been extensively studied with regard to their energy metabolism pathways, and their genomes and transcriptomes are well characterized<sup>108,123-127</sup>. These studies revealed their adaptation to hypoxia therefore rendering them as excellent models to study mitochondrial mechanisms contributing to hypoxia tolerance.

## 1.5 Objectives and experimental design

The goal of this project was to gain insight into the physiological and molecular mechanisms of responses to oxygen fluctuations in animals with different degree of hypoxia tolerance and identify the potential adaptive mechanisms associated with the hypoxia-tolerant mitochondrial phenotype. I focused on marine bivalve species that have different degree of hypoxia tolerance including hypoxia-intolerant scallops and hypoxia-tolerant mussels and oysters. I also used a terrestrial mammal (the domestic pig) as a benchmark species with a low level of hypoxia tolerance to compare the mitochondrial and molecular responses to H/R stress in evolutionarily distant hypoxia-intolerant species (mammals vs. bivalves). To achieve the project's goal, I carried out the following specific aims:

- Determine the effect of H/R stress on the mitochondrial activity and transcriptional profile of two tissues that differ in the degree of hypoxia tolerance in the domestic pig *Sus scrofa* (Publication 1).
- Investigate the impact of H/R stress on the mitochondrial activity, ROS production and mitochondrial substrate preference in tissues of hypoxia-tolerant bivalves, the blue mussel *M. edulis* and the Pacific oyster *C. gigas* (Publications 2, 3).
- Compare the effect of hypoxia duration on the mitochondrial response to H/R stress in a hypoxia-intolerant king scallop *P. maximus* and a hypoxia-tolerant Pacific oyster *C. gigas*.

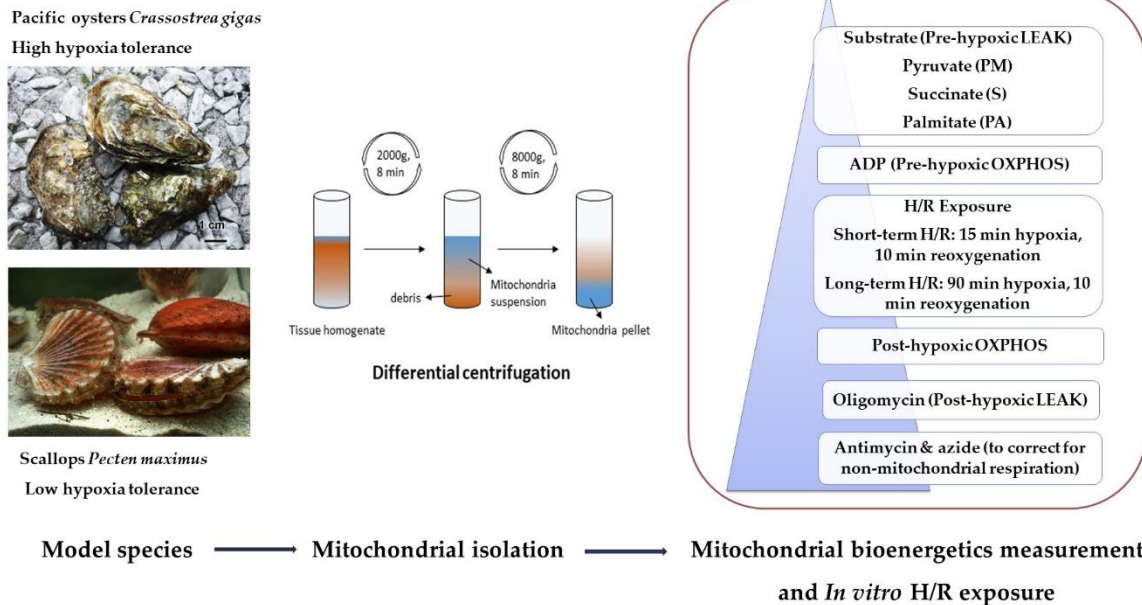
To address these aims, three experiments were conducted. In the first experiment, cells were isolated from two porcine tissues (the brain and the skeletal muscle) with different hypoxia tolerance, and the mitochondrial responses and the changes to the transcriptional profile were measured after acute *in vitro* H/R stress (15 min hypoxia, 10 min reoxygenation) (Fig. 3). Oxygen consumption and ROS efflux were measured with an Oxygraph 2 k high-resolution respirometer (Oroboros, Innsbruck, Austria) and an integrated DatLab 6 software. The cells used for both mitochondrial and transcriptional measurements were permeabilized with saponin (25  $\mu$ M) to allow substrates access into the mitochondrial matrix. For transcript measurements, 88 target genes and 43 miRNAs were selected based on their association with hypoxia response and the vital role in the HIF-1 regulation, apoptosis, redox homeostasis, glycolysis and mitochondrial quality control <sup>91,128,129</sup>. Primers were designed for these transcripts and their mRNA expression was evaluated in the cell samples after H/R exposures. Details of the experimental design and the research methodology are outlined in Adzigbli et al <sup>130</sup>.



**Figure 3.** Graphical summary of the 1<sup>st</sup> experiment for investigating tissue-specific mitochondrial response and transcriptional profile of porcine cells to hypoxia-reoxygenation stress. The hypoxia exposures were conducted *in vitro* with <1% oxygen levels in chamber.

The second experiment focused on the effects of H/R stress on functional mitochondrial responses (including OXPHOS activity, proton leak, mitochondrial coupling and ROS efflux) and the potential molecular mechanisms of the mitochondrial response to H/R stress in two hypoxia-tolerant species: the blue mussel *M. edulis* and the Pacific oyster *C. gigas*. Mitochondria were isolated from the digestive gland of the mussels, or from the gill and the digestive gland of the oysters and subjected to acute short-term H/R stress *in vitro* (15 min hypoxia, 10 min reoxygenation). Mitochondrial functional indices and ROS production were measured at 15°C (in both species), and 25°C (in mussels only). Different substrates and substrate combinations were used to assess the mitochondrial functional traits including: pyruvate with malate (PM), succinate (S), palmitoyl-DL-carnitine (PALM), glutamate with malate (G), glutamate, malate, and succinate (GS) and mixture of glutamate, pyruvate, malate, and succinate (GPS). PM and S were used as mitochondrial substrates for both studied species, and other substrates were only used in the experiments with the oyster mitochondria. In mussels, mitochondrial samples were collected before and after the H/R stress for analyses of the total proteome and phosphoproteome and determination of the abundance and stability of mitochondrial supercomplexes. Details of the experimental design and methodology can be found in Sokolov et al.,<sup>68</sup> and Adzigbli et al.,<sup>131</sup>

The third experiment focused on the effects of different duration of hypoxia on mitochondrial functional characteristics in two bivalve species with different hypoxia tolerance: the Pacific oyster *C. gigas* and the king scallop *P. maximus* (Fig. 4). Mitochondria were isolated from two tissues (the gill and the digestive gland) of scallops and oysters and subjected to different H/R regimes *in vitro*: acute short-term H/R (15 min hypoxia, 10 min reoxygenation), chronic long-term H/R (90 min hypoxia, 10 min reoxygenation) and chronic cyclic H/R (5 cycles of 15 min hypoxia, 10 min reoxygenation; only in oysters). The oxygen consumption rates (MO<sub>2</sub>) and ROS efflux were measured with the Complex I-linked (pyruvate and palmitate) and Complex II-linked (succinate) substrates at 15 °C using an Oxygraph 2k high-resolution respirometer (Oroboros, Innsbruck, Austria) and integrated DatLab 6 software.



**Figure 4.** Graphical summary of the 3<sup>rd</sup> experiment for investigating the substrate- and tissue-specific mitochondrial response to different hypoxia duration in a hypoxia-tolerant (oyster) and -intolerant (scallop) marine bivalve. Both long-term and short-term hypoxia exposure were conducted *in vitro* with <1% oxygen level in the chamber.

## 2 – Summary and discussion of results

The series of experiments conducted unraveled the varied mitochondrial adaptations across different species in response to H/R stress. Interestingly, the degree of tolerance of a particular species varies between different tissues of the species. The details of the findings of the various experiments are presented in the subsections of this chapter.

### 2.1 Tissue-specific mitochondrial and transcriptional response to H/R stress in a hypoxia-sensitive species, the domestic pig *Sus scrofa*

The study of the response of the isolated cells from the benchmark species to H/R stress revealed that the differences in mitochondrial activity and transcriptional regulation between the brain and muscle cells are associated with the different degree of H/R stress tolerance of these tissues. Generally, brain cells are more sensitive to oxygen deprivation in mammals compared with skeletal muscle cells that can tolerate short-term hypoxia<sup>132,133</sup>. At the mitochondrial level, there was a decrease in the ATP synthesis capacity of the brain cells

as compared to a stable ATP synthesis capacity of the muscle cells. The decreased ATP synthesis capacity was further declined during NADH-linked substrate oxidation. No elevated ROS efflux (above the respective tissue-specific baseline) was recorded during H/R stress in either of the two studied cell types suggesting the presence of sufficient cellular antioxidants to maintain the normal ROS levels during acute short-term H/R stress. It is however worth noting that longer H/R exposure could instigate stronger response and damage, which requires further investigation. Finally, C IV activity (COX) decreased significantly after reoxygenation in both studied tissues. This decline was consistent with our finding of downregulated expression of gene transcripts encoding cytochrome C oxidase (COX) subunits (cytochrome C oxidase subunit 6A1 (COX6A1) in the muscle, and cytochrome C oxidase subunit 6C (COX6C) and cytochrome C oxidase subunit 7A1 (COX7A1) in the brain) after reoxygenation in the isolated porcine cells.

The impaired mitochondrial aerobic capacity in the porcine brain cells went hand-in-hand with the transcriptional upregulation of anaerobic glycolysis, likely as a compensatory mechanism to cover ATP deficiency. For Complex-specific regulation, the increase in the mitochondrial C I activity after reoxygenation in the porcine muscle cells was associated with a strong overexpression of NADH:ubiquinone oxidoreductase core subunit S1 (NDUFS1) transcript. NDUFS1 is known to suppress C I activity and increase ROS production in different mammalian cells <sup>134,135</sup>. Therefore, its overexpression during hypoxia might represent an anticipatory response of the porcine muscle cells to support high Complex I activity and mitigate ROS burst during reoxygenation thereby preventing oxidative damage. NDUFS1 overexpression might therefore contribute to the H/R tolerance of the muscle cells. In the porcine brain cells, NDUFAB1 was suppressed and NDUF6A overexpressed after reoxygenation. NDUFAB1 plays a significant role in regulating ETS flux and ROS production in mitochondria and its overexpression mitigates ischemia–reperfusion injury in mammalian cells <sup>136</sup>. Hence, its downregulation might be maladaptive during H/R and contribute to hypoxia sensitivity of the porcine brain cells.

The major elements of oxygen sensing (including HIF-1 and/or prolyl hydroxylase domain protein (PHD)) were transcriptionally modulated by H/R stress in both brain and muscle cell

types. However, the downstream effects differed between the brain and the skeletal muscle cells. In the muscle cells, mitochondrial ETS as well as protective mechanisms (including antioxidants and mitochondrial quality control) were highly expressed, whereas in the brain cells apoptosis and inflammation pathways were the most responsive indicating cell damage. These pathways can be further explored in future studies for a more comprehensive understanding of the mechanisms associated with hypoxia tolerance across functionally different tissues. Furthermore, identification of the pathways modulated in the tolerant but not in the sensitive tissues might open avenues for future clinical interventions that mimic the tolerant molecular phenotype and desensitize tissues to H/R stress.

MicroRNAs (miRNAs) are central posttranscriptional regulators associated with the adaptive and maladaptive hypoxia (ischemia) and reoxygenation responses<sup>137,138</sup>. miRNAs are small non-coding RNA molecules that regulate gene expression by interacting with the 3' untranslated region of target mRNAs to induce mRNA degradation and translational repression. Over 90 hypoxia-inducible miRNAs (hypoxamiRs) have been identified in mammals regulating angiogenesis, cell survival and metabolism<sup>139</sup>. In our study, miRNA expression was differentially affected by H/R in the porcine brain and the muscle cells. In the porcine muscle cells, 4 of the 34 studied miRNAs were suppressed in hypoxia and 9 suppressed in reoxygenation relative to the normoxic controls. In the porcine brain cells, a substantially larger fraction (17 out of 41) of the studied miRNAs were suppressed after H/R stress. This indicates that the hypoxia-induced metabolic reorganization involving hypoxamiRs affects a larger swath of the cellular pathways in the porcine brain than in the muscle cells.<sup>130</sup>

Unexpectedly, we observed transcriptional upregulation of multiple pathways related to metabolism, antioxidant defence and stress survival caused by the procedure of isolation of the muscle and brain cells from the respective tissues. This might be due to the improved nutrient and oxygen delivery to isolated cells suspended in the substrate-enriched media *ex situ*. These findings demonstrate that assessment of the transcriptional shifts between the tissues and isolated cells might serve as a sensitive marker for the deviation of the phenotype of isolated primary cells from the native *in situ* state and can be used in future studies as a

measure of the preservation of the native metabolic phenotype during isolation of the primary cells.

Taken together, the mechanisms of hypoxia response were differentially regulated in hypoxia-tolerant and hypoxia-intolerant tissues of the domestic pig. On the mitochondrial level, the hypoxia-tolerant phenotype in the skeletal muscle cells was associated with stable ATP synthesis capacity while the hypoxia-intolerant phenotype of the brain cells was associated with a decline in ATP synthesis capacity. No effect of H/R on ROS efflux was found in either of the two studied tissues indicating that oxidative stress is unlikely to be involved in the loss of the ATP synthesis capacity of the hypoxia-intolerant brain cells. At the molecular level, target genes associated with apoptosis and angiogenesis were mildly downregulated in the hypoxia-tolerant skeletal muscle tissue while being significantly downregulated in the hypoxia-intolerant brain cells both during hypoxia and reoxygenation. Overall, the muscle cells upregulated protective mechanisms (antioxidants, mitochondrial quality control mechanisms and ETS) and in the brain cells, mechanisms associated with cell damage were elevated after H/R exposure. These observations are in line with that normally observed in hypoxia-tolerant and intolerant animals <sup>36,38,44,57</sup>.

## **2.2 Intrinsic mitochondrial responses to H/R in hypoxia-tolerant marine bivalves involve changes in mitochondrial activity and proteome.**

The regulation of ETS activity and the contributions of the various ETS complexes to the mitochondrial respiration capacity and ROS production under stress potentially play an important role in the (mal-)adaptive response of mitochondria to H/R stress. These regulatory mechanisms may involve the effects of the changes in the intracellular milieu (such as shifts in the levels of metabolic intermediates), shifts in the state of the electron carriers of the ETS and post-translational modifications (PTM) of mitochondrial proteins <sup>63,64</sup>. This study revealed several mechanisms associated with the hypoxia-tolerant mitochondrial phenotype in marine bivalves. Thus, during acute hypoxia exposure, I observed the robustness of both blue mussel and Pacific oyster mitochondria to H/R stress. In both studied species, mitochondria retained the ability to maintain respiratory flux and ATP synthesis while minimizing ROS efflux after acute H/R stress, especially those isolated from the

digestive gland as compared to the gill. Interestingly, the improved ATP synthesis capacity of the mitochondria appears to be largely driven by Complex II substrate (succinate) oxidation, an anaerobic end product that highly accumulates during hypoxia in bivalves. These results suggest the substrate-dependent response of mitochondria to H/R stress and the potential of succinate to be a stress fuel under H/R stress.

Mitochondrial flexibility in utilizing substrate fuels is crucial for regulating metabolism and adjusting functions to physiological conditions. In this study, the findings revealed that the mitochondrial flexibility in substrate utilization in marine bivalves is tissue specific. I observed that the mitochondrial capacity of different substrate utilization varies between the gill and digestive gland tissue of the hypoxia tolerant Pacific oyster, *C. gigas*. In the Pacific oyster, the gill mitochondria generally showed higher OXPHOS respiration rates with glutamate, succinate, and glutamate-and succinate-containing mixtures compared to the digestive gland mitochondria under normal oxygen conditions<sup>131</sup>. The highest OXPHOS rate was achieved with a mixture of glutamate, pyruvate, and succinate, which significantly exceeded the OXPHOS rate with only C I-linked substrates (pyruvate and palmitate). The digestive gland mitochondria showed high palmitate-driven ROS production that was 4-5-fold higher in the digestive gland mitochondria than the gill. This suggests that the digestive gland is metabolically less well adapted for fatty acid oxidation compared to the gills as its usage as a metabolic fuel is associated with high metabolic cost (ROS production). The overproduction of ROS during palmitate oxidation is common in different cell types and have been reported to cause endoplasmic reticulum stress and Ca<sup>2+</sup> overload, leading to cell damage and death<sup>140</sup>. In bivalves, the digestive gland acts as a storage organ and is enriched with fats hence the low efficiency of the digestive gland mitochondria in fatty acid utilization under the normal conditions was unexpected. Our finding thereby suggests that under rapid lipid utilization (for instance during winter starvation or initiation of gametogenesis), there might be consequences on the organism's physiological performance and eventually place the organism under oxidative stress. Additionally, we observed that the gill and the digestive gland mitochondria from oysters have a good capacity for succinate oxidation, both alone and in substrate mixtures. However, succinate oxidation was associated with a higher ROS production compared to mitochondria oxidizing Complex I substrates. That notwithstanding,

we observed that addition of C I substrates like pyruvate or glutamate alleviated the high ROS efflux which could be as a result of NADH production by pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase, increasing the reduced NADH pool in the mitochondria<sup>28,65</sup>. A highly reduced NADH pool can increase the thermodynamic driving force towards the forward electron flow through C I, thereby mitigating RET and associated ROS production. This mitigation of succinate-driven ROS generation in oyster mitochondria may play a protective role *in vivo*. After acute H/R exposure (15 min hypoxia, 10 min reoxygenation) of the isolated mitochondria of the Pacific oyster, we observed suppression of the rate of C I substrates (pyruvate, palmitate) oxidation. This was similar to that recorded in our studies in the blue mussels<sup>56</sup> and other studies<sup>55,58,141</sup>. In turtles<sup>55</sup> and quahogs<sup>141</sup>, the suppression of C I activity was related to damage and hence lower enzymatic activity of C I. In our study, we observed that the suppression of C I activity in the oyster mitochondria was not as a result of damage or inactivation of C I since the suppression was dependent on the physiological state of the mitochondria with a decrease in LEAK respiration and not in OXPHOS. If hypoxia led to the inactivation of C I, OXPHOS respiration rate should be strongly affected as the OXPHOS rate is controlled by the ETS activity in bivalves<sup>38</sup>. Hence, we hypothesize that the decline in C I substrate oxidation might be due to modulation of proton conductance or decrease in resting mitochondrial membrane potential of the Pacific oyster mitochondria. Interestingly, we observed that C I-dependent ROS production declined in the LEAK state and increased in the OXPHOS state despite no change in OXPHOS respiration rate. C I have been reported as the most vulnerable Complex of the ETS to hypoxia<sup>82,142</sup>. Especially in hypoxia-intolerant species, there is a reversible suppression of C I under hypoxia. Although this prevents a burst in reactive species production during reoxygenation, it increases C I susceptibility to oxidative and nitrosative damage<sup>143,144</sup>. Contrary to C I substrate, oxidation of the C II substrate (succinate) was enhanced after acute H/R stress in the oyster mitochondria especially in those from the digestive gland. A similar pattern was found in the mussel mitochondria exposed to acute H/R stress and respiring on succinate. Succinate have been reported to accumulate in bivalves during hypoxia<sup>60</sup> hence the enhanced oxidation capacity observed in our study might be a mechanism facilitating the post-hypoxic recovery by rapidly restoring ATP levels and removing excess succinate. Succinate is known as a pro-oxidant that can strongly stimulate ROS generation due to RET through mitochondrial C I<sup>85</sup>.

Testing the RET mechanism in the hypoxia tolerant Pacific oyster, we observed that under normal conditions, succinate-dependent ROS efflux without rotenone inhibition (blocking C I) was not higher than that observed during the oxidation of NADH-linked substrates. Addition of rotenone to block C I had no effect on the ROS efflux or FEL indicating that RET does not contribute to the succinate-driven ROS generation under normal oxygen conditions. Upon hypoxia exposure, succinate oxidation was associated with heightened ROS efflux. However, this heightened ROS efflux was alleviated by rotenone addition suggesting the functional changes prompted by H/R stress in stimulating the RET mechanism in oysters. Physiologically, ROS production via RET might be low due to the presence of NADH-linked substrates such as pyruvate *in vivo*.

The second mechanism investigated with regard to the hypoxia-tolerant mitochondrial phenotype concerned the changes in the mitochondrial proteome, specifically the reversible protein phosphorylation in response to H/R stress in the blue mussel. Our study revealed changes in the protein phosphorylation patterns in several pathways in response to H/R stress including those involved with ETS and OXPHOS (10 out of 25 differentially phosphorylated proteins), amino acid and fatty acid metabolism (6 proteins), mitochondrial protein and mtDNA homeostasis (5 proteins), and substrate-level phosphorylation (2 proteins). Interestingly, we observed that H/R stress altered the phosphorylation status of several Complex I subunits (e.g. NDUFB1, NDUFB11, NDUFS2, NDUFB6). This observation confirms the vulnerability of C I to H/R stress. Several studies have reported C I as a major target of hypoxia and PTM-dependent regulation during hypoxia <sup>141,145</sup>. Contrarily, Complex IV, another essential regulatory target during H/R stress was not affected by PTM in this study. The differential regulation of the various subunits especially Complex I might contribute to the mussel mitochondria's protection against H/R-dependent damage. This study also found a significant increase in the abundance of A-Kinase Interacting Protein 1 (AKAP1) after H/R stress. AKAP1 functions as a signaling hub regulating metabolic homeostasis and mitochondrial quality control during H/R stress <sup>146,147</sup>. Multiple studies have revealed that functional AKAP1 is essential for cell survival during H/R stress. AKAP1 is also involved in the regulation of protein kinase A (PKA) activity in mitochondria (review in <sup>146,147</sup>). Phosphorylation of AKAP1 in mussel mitochondria might hence indicate an

adaptive response to a decrease in ATP levels during H/R stress and positively modulate mitochondrial respiration and PKA recruitment. Consistent with the putative role of PKA regulation in response to H/R, PKA has been reported to regulate the mitochondrial ETS activity in mussels <sup>141</sup>.

Finally, we evaluated the stability and abundance of mitochondrial supercomplexes (SCs) during H/R stress in the mussels. Earlier studies in model organisms like mammals showed that mitochondrial ETS (mainly C I, C III, and C IV) can transiently assemble into supercomplexes (SCs) <sup>148,149</sup>. Functionally, SCs have been proposed to enhance efficiency of the electron transfer and mitigate ROS production <sup>150</sup>. Although the functions of SCs have been intensely debated, several studies have revealed a link between metabolic adaptations, and stability and plasticity of SCs <sup>141,151,152</sup>. SCs are known to undergo rearrangement under stress like hypoxia. However, in our present study on *M. edulis*, H/R stress did not influence SC stability and abundance. Generally, mussel SCs demonstrated high level of stability regardless of the H/R exposures, a feature that might contribute to the stress-resistant mitochondrial phenotype in this hypoxia-tolerant species. However, the functional consequences of SC formation in marine mollusks including mussels are not known, and further investigation is needed to assess the role of SCs in the maintenance of robust ETS activity and ROS mitigation during hypoxia in hypoxia-tolerant organisms like intertidal marine bivalves.

In summary, our studies lead us to suggest that the adaptive traits linked to the hypoxia-tolerant mitochondrial phenotype in marine bivalves encompass the ability to uphold high mitochondrial ATP synthesis capacity and low ROS efflux under H/R stress, mitochondrial flexibility in substrate utilization (in particular, enhanced capacity to oxidize succinate without a concomitant increase in ROS production through RET), shifts in the mitochondrial phosphoproteome regulating the mitochondrial ETS, electron flux at the Q-junction, and the proteome maintenance machinery during H/R, along with the stability of mitochondrial supercomplexes. Exploring these proposed mechanisms raises intriguing questions about how the use of various substrates to support mitochondrial oxidation might influence gene transcript expressions, mitochondrial proteome, and the phosphorylation status of proteins during H/R stress in hypoxia-tolerant organisms. Furthermore, delving into the specific

functional roles of proteins differentially expressed or modified by PTM during H/R stress expressed in non-model species like mussels and oysters presents an intriguing avenue for future investigation.

### **2.3 Effects of hypoxia duration on the mitochondrial response to H/R stress in bivalves with varying degree of hypoxia tolerance.**

Coastal hypoxia varies in frequency and duration. In the field, coastal hypoxic episodes can range from several hours (during diurnal cycles of photosynthesis and respiration) to days and weeks in coastal dead zones <sup>12</sup>. The dead zones are incompatible with most metazoan life while areas with periodic hypoxic episodes like the intertidal zones are enriched with high biodiversity. Intertidal species are adapted to hypoxia-reoxygenation stress. Hence, understanding the mitochondrial responses to the variable oxygen regimes with different duration and frequency of hypoxic episodes in hypoxia-adapted intertidal animals can shed light on the mechanisms and limiting factors of hypoxia tolerance. Unfortunately, isolated mitochondria cannot endure extended periods *in vitro*, irrespective of the oxygen conditions. Therefore, investigations into the impacts of varying hypoxia durations on intrinsic mitochondrial responses are unavoidably constrained to shorter periods, typically not surpassing a few hours. In the present work, we exposed the isolated mitochondria from hypoxia-tolerant (oyster) and hypoxia-intolerant (scallop) species of bivalves to different periods of hypoxia - acute short-term (15 min hypoxia and 10 min reoxygenation), constant long-term (90 min hypoxia and 10 min reoxygenation), and cyclic long-term (5 cycles of 15 min hypoxia and 10 min reoxygenation) hypoxia. The H/R exposures mimicked those observed during shell closure and periodic valve gaping during low tide <sup>120</sup>. We investigated the tissue-specific responses (gill and digestive gland tissue) of mitochondria to H/R stress in these two species. Our results revealed that both acute and long-term exposures modulated mitochondrial bioenergetics albeit the modulation was different under the various H/R exposures and in the different species. In the oyster's mitochondria, acute short-term hypoxia resulted in a loss of respiration capacity during pyruvate and palmitate oxidation. Furthermore, long-term cyclic hypoxia led to a progressive inactivation of C I in oyster mitochondria shown by a gradual loss of capacity for pyruvate and palmitate oxidation with increasing hypoxic duration. Palmitate oxidation generated the lowest respiratory flux

under normoxia and the highest decline under both long-term constant and long-term cyclic H/R stress. This further confirms the inability of *C. gigas* mitochondria to effectively utilize fatty acids as a source of energy, especially during intermittent hypoxia. Studies of other marine mollusks also reveal their limited capacity for oxidizing fatty acids<sup>76,77</sup>. Interestingly, succinate oxidation was more robust to long-term hypoxia and reoxygenation in oyster mitochondria. Even though succinate-fueled oxygen consumption rate declined after long-term constant or cyclic H/R stress, the suppression of succinate oxidation rate was 2-3 times lower than that of C I substrates oxidation. Additionally, ROS efflux rate and FEL did not change after H/R relative to the baseline (normoxic) values during succinate oxidation. Findings from this study revealed a similar substrate-specific oxygen consumption rate and ROS efflux in both gill and digestive gland mitochondria of oysters during the long-term constant and cyclic H/R stress. Hence, we focused on the gill mitochondria to investigate the impact of mitochondrial respiration with different substrates on the mitochondrial oxidative damage. We found no evidence of accumulation of oxidative damage to proteins (indicated by protein carbonyls) after long-term constant hypoxia in succinate-energized mitochondria. However, our results suggest that the mitochondrial antioxidant systems might have become overwhelmed under prolonged and frequent oxygen fluctuations as we observed a significant increase in protein carbonylation after the third cycle of the cyclic hypoxia exposure. The mitochondrial coupling efficiency remained either stable or improved following H/R stress during succinate oxidation in isolated mitochondria from both the gill and the digestive gland. In contrast, mitochondrial coupling efficiency decreased during the oxidation of C I-linked substrates, particularly in the digestive gland mitochondria. The robustness of mitochondrial succinate oxidation observed in this study aligns with our prior research on the impact of acute short-term H/R stress on isolated mitochondria from oysters and mussels, as detailed in Chapter 2.3 of this thesis. Taken together, these findings indicate that succinate might serve as a rescue fuel during H/R stress in bivalve mitochondria, bolstering mitochondrial activity and facilitating efficient ATP production while minimizing oxidative stress.

Exposing the mitochondria of the hypoxia-intolerant scallop *P. maximus* to both acute short-term and long-term constant hypoxia revealed a reduction in respiratory capacity in both gill

and digestive gland mitochondria. Notably, when the mitochondria were oxidizing succinate, the reduction in respiratory capacity was 50% less compared to when oxidizing pyruvate and palmitate (NADH-linked substrate). In contrast to mitochondria respiring on succinate, during pyruvate oxidation, the H/R-induced decline in respiratory capacity depended on the respiratory state of the mitochondria, resulting in decreased OXPHOS respiration and increased LEAK respiration. This rise in LEAK respiration after H/R exposure may be attributed to mitochondrial dysfunction and uncoupling of the ETS from ATP synthesis, subsequently increasing energy loss as heat and decreasing the efficiency of OXPHOS, as observed in the present study. In the gill mitochondria of *P. maximus*, the decline in mitochondrial respiratory capacity with different substrates was not associated with changes in ROS efflux and FEL. In contrast, in the digestive gland mitochondria, both short-term and long-term constant H/R exposure led to a decrease in ROS efflux and FEL during LEAK respiration. During OXPHOS respiration, ROS efflux decreased in the digestive gland mitochondria respiring on succinate after short-term and long-term constant H/R. With regard to C I substrates, an H/R-induced decrease on the ROS efflux was found during pyruvate oxidation after short-term H/R and during palmitate oxidation after long-term H/R. However, an FEL decline was only found after long-term constant H/R exposure in the digestive gland mitochondria of scallops. Our findings suggest that H/R stress has a more pronounced effect on the digestive gland mitochondria than on the gill mitochondria of *P. maximus*. C I-linked substrate oxidation uncouples scallop mitochondria, especially in the gill tissue, under both H/R exposures. Notably, H/R stress does not affect the coupling efficiency of the gill mitochondria oxidizing succinate and reduces the coupling efficiency only during chronic H/R stress in the digestive gland mitochondria. Therefore, succinate appears to be better suited as a substrate for scallop mitochondria under H/R stress. In conclusion, it is noteworthy that the H/R-induced changes observed in scallop mitochondria were not associated with oxidative damage, as levels of oxidative stress markers (protein carbonyls and lipid peroxidation products) remained unchanged after H/R stress compared to normoxic values.

## 2.4 Conclusions

The present study revealed mechanisms of response of hypoxia-tolerant and hypoxia-intolerant species to H/R stress. Findings from this study are consistent with those observed in other studies, where H/R stress led to the collapse of the ETS and caused a decline in ATP synthesis in hypoxia intolerant species<sup>30,35,38</sup>. Moreover, a distinctive tissue-specific response to H/R stress emerged, particularly in mammals, where the brain tissue exhibited intolerance to H/R, marked by a more pronounced decline in mitochondrial OXPHOS activity compared to the more resilient skeletal muscle tissue. Tissue-specific patterns of mitochondrial responses to H/R stress were also observed in bivalves, revealing variations in substrate preference and differential sensitivity of Complex I to H/R stress between the gill and the digestive gland tissues. Specifically, the study highlighted that the digestive gland mitochondria of oysters and scallops were more susceptible to hypoxia than gill mitochondria. Additionally, it confirmed Complex I as the most vulnerable complex in both hypoxia-tolerant and intolerant species, while Complex II demonstrated resilience to H/R stress, especially in hypoxia-tolerant bivalves. The type of mitochondrial substrate oxidized played a significant role in determining the mitochondrial response to H/R stress.

Contrary to some previously published studies<sup>82,84</sup>, our present work in hypoxia-intolerant species (including a mammal, the domestic pig (chapter 2.1), and a hypoxia-intolerant bivalve, the king scallop ((chapter 2.3)) showed no evidence of the elevated ROS efflux during the H/R stress. This discrepancy may arise from the relatively short duration of H/R exposure in our present study or from the specifics of the H/R exposure regime, which involved isolated mitochondria *in vitro* rather than *in vivo*. In the hypoxia-tolerant Pacific oyster, acute H/R stress elevated ROS production during both C I- and C II-linked substrate oxidation. The heightened ROS levels during succinate oxidation were attributed to an increased oxygen consumption rate, in contrast to the lower rate observed during C I-linked substrate oxidation. However, co-oxidizing succinate with C I-linked substrates mitigated ROS efflux. Notably, exposure to long-term constant and cyclic H/R stress had no discernible effect on ROS efflux.

Under varying hypoxia exposure periods, distinctions appear between acute short-term and long-term H/R stress in the hypoxia-tolerant oyster compared to the hypoxia-intolerant scallop, particularly during succinate oxidation. Interestingly, there was no significant difference between long-term constant and long-term cyclic H/R stress of the same duration in the hypoxia-tolerant oyster. This suggests that, in the Pacific oyster, the predominant factor influencing the extent of mitochondrial damage is the duration and intensity of hypoxia, with reoxygenation itself not posing as a significant stressor. In contrast, the sensitivity of the king scallop seems independent of the duration and intensity of hypoxia exposure, as both acute short-term and long-term H/R stress led to a similar decline in respiratory capacity. Future studies should investigate the underlying mechanisms contributing to the heightened sensitivity of scallops to H/R stress.

In conclusion, the robustness of mitochondrial succinate oxidation in the face of oxygen fluctuations, along with the minimal succinate-driven RET and the substantial capacity of bivalve mitochondria for succinate oxidation, underscores the significance of succinate as a metabolic fuel during H/R stress. This utilization of succinate ensures adaptability in employing alternative metabolic fuels and contributes to overcoming the limitations associated with Complex I during hypoxia. Similar observations in other ectotherms, such as *Drosophila*, where mitochondrial substrate preference shifts from Complex I-linked substrates to succinate during high temperatures<sup>69,70</sup>, further support the notion that succinate as a rescue fuel during environmental stress exposures may extend beyond molluscan mitochondria to include ectothermic mitochondria in general.

### 3 References

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## 4 Appendix

### Contributions to publications included in this thesis.

Publication 1: **Adzibli L.**, Sokolov P.E., Wimmers K., Sokolova M. I., Ponsuksili S. (2022). Scientific Reports, 12, 19881.

*“Effects of hypoxia and reoxygenation on mitochondrial functions and transcriptional profiles of isolated brain and muscle porcine cells”.*

I performed the mitochondrial experiments and sample collection for the transcript analysis. I analyzed the data and wrote the manuscripts. I contributed 70% to the paper.

Publication 2: Sokolov P.E., **Adzibli L.**, Markert S., Bundgaard A., Fago A., Becher D., Hirschfeld C., Sokolova M. I (2021). Frontiers in Marine Science, 8:773734.

*“Intrinsic mechanisms underlying hypoxia-tolerant mitochondrial phenotype during 1 hypoxia-reoxygenation stress in a marine facultative anaerobe (Mytilus edulis)”.*

I analyzed some data and wrote a part of the manuscript. I contributed 20% to the paper.

Publication 3: **Adzibli L.**, Sokolov P.E., Ponsuksili S., Sokolova M. I (2021). Journal of Experimental Biology, 225(1).

*“Tissue- and substrate-dependent mitochondrial responses to acute hypoxia–reoxygenation stress in a marine bivalve (Crassostrea gigas) (Thunberg, 1793)”.*

I performed all experiments, analyzed the data, and wrote the manuscript. I contributed 80% to the paper.

Manuscript 4: **Adzibli L.**, Ponsuksili S., Sokolova I (2024). Scientific Reports 14, 9658.

*“Mitochondrial responses to long-term and cyclic hypoxia depend on the oxidized fuel in a hypoxia-tolerant marine bivalve Crassostrea gigas”.*

I performed all experiments, analyzed the data, and wrote the manuscript. I contributed 80% to the paper.

Manuscript 5: **Adzibli L.**, Ponsuksili S., Bock C., Mark F., Sokolova I. Under Review

*“Effect of different hypoxia-reoxygenation duration on the mitochondrial bioenergetics of the hypoxia-intolerant king scallop Pecten maximus”.*

I performed all experiments, analyzed the data, and wrote the manuscript. I contributed 80% to the paper.



OPEN

## Effects of hypoxia and reoxygenation on mitochondrial functions and transcriptional profiles of isolated brain and muscle porcine cells

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Oxygen fluctuations might occur in mammalian tissues under physiological (e.g. at high altitudes) or pathological (e.g. ischemia–reperfusion) conditions. Mitochondria are the key target and potential amplifiers of hypoxia-reoxygenation (H-R) stress. Understanding the mitochondrial responses to H-R stress is important for identifying adaptive mechanisms and potential therapeutic solutions for pathologies associated with oxygen fluctuations. We explored metabolic response to H-R stress in two tissue types (muscle and brain) with different degrees of hypoxia tolerance in a domestic pig *Sus scrofa* focusing on the cellular responses independent of the systemic regulatory mechanisms. Isolated cells from the skeletal muscle (masseter) and brain (thalamus) were exposed to acute short-term (15 min) hypoxia followed by reoxygenation. The mitochondrial oxygen consumption, reactive oxygen species (ROS) production rates and transcriptional profiles of hypoxia-responsive mRNA and miRNA were determined. Mitochondria of the porcine brain cells showed a decrease in the resting respiration and ATP synthesis capacity whereas the mitochondria from the muscle cells showed robust respiration and less susceptibility to H-R stress. ROS production was not affected by the short-term H-R stress in the brain or muscle cells. Transcriptionally, prolyl hydroxylase domain protein EGLN3 was upregulated during hypoxia and suppressed during reoxygenation in porcine muscle cells. The decline in EGLN3 mRNA during reoxygenation was accompanied by an upregulation of hypoxia-inducible factor subunit  $\alpha$  (HIF1A) transcripts in the muscle cells. However, in the brain cells, HIF1A mRNA levels were suppressed during reoxygenation. Other functionally important transcripts and miRNAs involved in antioxidant response, apoptosis, inflammation, and substrate oxidation were also differentially expressed between the muscle and brain cells. Suppression of miRNA levels during acute intermittent hypoxia was stronger in the brain cells affecting ~ 55% of all studied miRNA transcripts than in the muscle cells (~ 25% of miRNA) signifying transcriptional derepression of the respective mRNA targets. Our study provides insights into the potential molecular and physiological mechanisms contributing to different hypoxia sensitivity of the studied tissues and can serve as a starting point to better understand the biological processes associated with hypoxia stress, e.g. during ischemia and reperfusion.

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**Abbreviations**

ALDOC	Aldolase, fructose-bisphosphate C
ATP5B	ATP synthase F1 subunit beta
ATP5G2	ATP synthase membrane subunit C locus 2
ATP5J2	ATP synthase membrane subunit F
ATP5L	(ATP synthase membrane subunit G
ATP6V0D1	ATPase H + transporting V0 subunit D1
ATP6V1B2	ATPase H + transporting V1 subunit B2
ATP6V1C1	ATPase H + transporting V1 subunit C1
ATP6V1E1	ATPase H + transporting V1 subunit E1
ATP6V1F	ATPase H + transporting V1 subunit F
ATP5G1	ATP synthase membrane subunit C locus 1
CAT	Catalase
CCS	Copper chaperone for superoxide dismutase
COX10	Cytochrome C oxidase subunit 10
COX15	Cytochrome C oxidase subunit 15
COX4-1	Cytochrome C oxidase subunit 4 isoform 1
COX4-2	Cytochrome C oxidase subunit 4 isoform 2
COX5B	Cytochrome C oxidase subunit 5B
COX6A1	Cytochrome C oxidase subunit 6A1
COX6C	Cytochrome C oxidase subunit 6C
COX7A1	Cytochrome C oxidase subunit 7A1
COX7A2	Cytochrome C oxidase subunit 7A2
CS	Citrate synthase
DNMT1	DNA Methyltransferase 1
EGLN1	Egl-9 family hypoxia inducible factor 1
EGLN2	Egl-9 family hypoxia inducible factor 2
EGLN3	Egl-9 family hypoxia inducible factor 3
ENO1	Enolase 1
GPI	Glucose-6-phosphate isomerase
GPX3	Glutathione peroxidase 3
GPX4	Glutathione peroxidase 4
HIF1A	Hypoxia inducible factor 1 subunit alpha
HIF1AN	Hypoxia inducible factor 1 subunit alpha inhibitor
HIF2A	Hypoxia inducible factor 2 subunit alpha
HK2	Hexokinase 2
HMOX1	Heme oxygenase 1
LDHB	Lactate dehydrogenase B
MCL1	MCL1 apoptosis regulator, BCL2 family member
NDRG4	NDRG family member 4
NDUFA10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10
NDUFA11	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11
NDUFA12	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12
NDUFA13	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13
NDUFA3	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3
NDUFA4	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4
NDUFA5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5
NDUFA6	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6
NDUFA8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8
NDUFA9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9
NDUFAB1	NADH:ubiquinone oxidoreductase subunit AB1
NDUFB7	NADH dehydrogenase [Ubiquinone] 1 beta subcomplex subunit 7
NDUFB8	NADH dehydrogenase [Ubiquinone] 1 beta subcomplex subunit 8
NDUFS1	NADH:ubiquinone oxidoreductase core subunit S1
NDUFS5	NADH:ubiquinone oxidoreductase core subunit S5
NDUFS6	NADH:ubiquinone oxidoreductase core subunit S6
NDUFS7	NADH:ubiquinone oxidoreductase core subunit S7
NDUFS8	NADH:ubiquinone oxidoreductase core subunit S8
NDUFV1	NADH dehydrogenase (Ubiquinone) Flavoprotein 1
PDK1	Pyruvate dehydrogenase kinase 1
PFKM	Phosphofructokinase, muscle
PGC-1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGK1	Phosphoglycerate Kinase 1
PYGM	Glycogen phosphorylase, muscle associated
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
SDHB	Succinate dehydrogenase complex flavoprotein subunit B
SDHC	Succinate dehydrogenase complex flavoprotein subunit C
SDHD	Succinate dehydrogenase complex flavoprotein subunit D
SOD1	Superoxide dismutase [Cu-Zn]

SOD2      Superoxide dismutase 2, mitochondrial  
 TXN        Thioredoxin

Oxygen is essential for the survival of animals owing to its role in aerobic ATP production in the mitochondria. In most terrestrial habitats (except underground or at high altitudes), ambient oxygen levels are not limiting for aerobic metabolism, and hypoxic episodes are rare and transient followed by reoxygenation. However, low oxygen levels might occur in the tissue when oxygen demand exceeds oxygen supply (e.g. in exercising muscle) or under pathological conditions that restrict oxygen delivery to the tissue (ischemia)<sup>1,2</sup>. Hypoxia-reoxygenation (H-R) stress can severely damage organs and tissues as shown in human pathologies caused by heart attack, stroke, respiratory failure, sleep apnea, surgery, or organ transplantation<sup>3–6</sup>. There is considerable variability in the tolerance to oxygen fluctuations between different organs, tissues and different species of animals<sup>7</sup>. The mechanisms underlying this variability are not yet fully understood but likely involve variation in the expression of protective mechanisms that support ATP production and prevent mitochondrial injury<sup>8–10</sup>.

Mitochondria are important targets and potential amplifiers of H-R stress in animal cells. Insufficient oxygen supply suppresses ATP generation via oxidative phosphorylation (OXPHOS) and can result in energy deficiency<sup>11,12</sup>. Furthermore, hypoxia can lead to the overproduction of reactive oxygen species (ROS) and calcium overload in the mitochondria<sup>13</sup>. Paradoxically, reoxygenation can amplify mitochondrial stress and is a major source of tissue damage during oxygen fluctuations. Reactivation of mitochondrial OXPHOS results in a burst in ROS production as a result of electron leak from the highly reduced metabolic intermediates and reverse electron transport<sup>11,14</sup>. Excessive ROS levels might result in oxidative injury to the proteins, DNA and membrane lipids, suppress mitochondrial OXPHOS capacity and impair the recovery of energy homeostasis in the cell<sup>3,6,8</sup>. In extreme cases, mitochondrial damage can lead to metabolic collapse, the release of cytochrome c and initiation of the apoptotic cascade resulting in tissue damage and organ failure<sup>11,15</sup>. Studies of the species evolutionarily adapted to hypoxic environments (such as intertidal invertebrates, anoxia-tolerant fish, reptiles and naked mole rats) indicate that tolerance to H-R is associated with the ability to maintain high mitochondrial respiratory flux and mitigate oxidative stress during oxygen fluctuations<sup>4,7,16,17</sup>. To date, most studies of mitochondrial respiration and ROS production during oxygen fluctuations involve comparisons of species with different degrees of hypoxia tolerance<sup>7,16,17</sup>. The direct comparisons of the mitochondrial responses between tissues with different degree of hypoxia tolerance within the same organisms are, to the best of our knowledge, unavailable.

Multiple molecular and cellular mechanisms have evolved to sense oxygen and protect against stress induced by oxygen fluctuations. An essential regulator of hypoxia response in animals is the hypoxia-inducible factor transcription factor HIF-1<sup>18</sup>. The HIF-1 $\alpha$  subunit is continually synthesized but rapidly degraded in the presence of oxygen<sup>19</sup>. Under hypoxic conditions, HIF-1 $\alpha$  accumulates and binds with the constitutive HIF-1 $\beta$  subunit to form an active HIF-1 that initiates a transcriptional cascade regulating both oxygen supply and oxygen demand of the cell<sup>20,21</sup>. HIF-1-dependent cascade involves target genes associated with angiogenesis, energy metabolism, redox homeostasis, cell proliferation, autophagy and apoptosis<sup>19,22</sup>. A major group of HIF-1 targets includes genes involved in metabolic rewiring and the shift of cellular metabolism from aerobic OXPHOS to anaerobic glycolysis to meet energy demands under low oxygen conditions<sup>19,23,24</sup>. HIF-1 $\alpha$  also regulates mitochondrial respiratory capacity limiting ROS production, regulating cytochrome oxidase subunit expression and suppressing metabolite entry into the tricarboxylic acid (TCA) cycle<sup>25,26</sup>.

In recent years, microRNAs (miRNAs) have emerged as important regulators of hypoxic response in animals<sup>27,28</sup>. Small non-coding miRNAs regulate gene expression by interacting with the 3' untranslated region of target mRNAs to induce mRNA degradation and translational repression. Currently, over 90 hypoxia-inducible miRNAs (hypoxamiRs) have been identified in mammals regulating angiogenesis, cell survival and metabolism<sup>29</sup>. Many hypoxamiRs including a master regulator of hypoxia response miR-210 are transcriptionally controlled by HIF-1 ensuring coordination of the transcriptional upregulation and gene silencing in hypoxia<sup>28,30</sup>. Several hypoxamiRs directly regulate the mitochondrial function and thus might play an important role in the mitochondrial responses to hypoxia<sup>31,32</sup>. This includes the regulation of mRNA levels of OXPHOS-related proteins by miR-338, miR181c and miR-210<sup>29,33,34</sup>, mitochondrial fatty acid oxidation (miR-214 and miR-27b) and biogenesis (miR27b, miR25 and miR-696)<sup>29,35,36</sup>.

Our study aims at gaining insight into the mammalian metabolic stress response to H-R by investigating the effects of acute short-term hypoxia and reoxygenation on functional mitochondrial responses (oxygen consumption and ROS production) and transcriptional changes in mRNA and miRNA profiles of the skeletal muscle and brain cells of the pig *Sus scrofa*. The two tissues were chosen based on their different dependence on aerobic metabolism and hypoxia tolerance. The mammalian brain is an organ with high dependence on oxygen availability, low glycolytic capacity and low hypoxia tolerance<sup>37</sup>. In this study, we focused on the thalamus as one of the most hypoxia-sensitive brain regions<sup>38</sup>. Unlike the brain, the skeletal muscle tissue is well adapted to hypoxic conditions owing to its metabolic and structural plasticity<sup>39</sup>. The skeletal muscle might experience hypoxia under physiological conditions such as vigorous physical exercise and can sustain on anaerobic glycolysis<sup>40</sup>. Here we focused on the masseter muscle from the masticatory system of pigs that consists of ~75% type IIA-fibers with high myosin ATPase activity and high oxidative and glycolytic capacity<sup>41,42</sup>. We hypothesized that the cells from the more hypoxia-tolerant tissue (masseter) will maintain stable respiration and ROS production during acute hypoxia-reoxygenation stress, whereas the more hypoxia-sensitive brain cells might respond with a decrease in respiration and elevated ROS production indicating mitochondrial damage. We also hypothesized that the transcriptional and miRNA response to H-R will differ between the two cell types, with a stronger upregulation of glycolysis and protective mechanisms (such as antioxidants, apoptosis, inflammation, angiogenesis, or mitochondrial fusion) in the more hypoxia-tolerant muscle cells. To test these hypotheses, we isolated cells from the muscle and brain tissues and conducted an in vitro H-R exposure (15 min hypoxia and 10 min of reoxygenation)

for determination of mitochondrial functional traits. We also analyzed the expression of 88 hypoxia-sensitive mRNAs and 43 miRNA in isolated cells exposed to normoxia, hypoxia and post-hypoxic recovery focusing on the HIF signalling, mitochondrial OXPHOS, glycolysis, apoptosis, autophagy and inflammation pathways<sup>19</sup>.

## Materials and methods

**Animal care and tissue sampling.** Animal care and tissue collection procedures were approved by the Animal Care Committee of the Institute for Farm Animal Biology and followed the approved guidelines for good scientific practice by the European Communities Council Directive of 24 November 1986 (86/609/EEC). All experimental procedures are reported according to the ARRIVE guidelines<sup>43</sup>. All the necessary and required measures were taken to minimize pain and discomfort. The animals were used for meat production and underwent no experimental treatment, diagnostic sampling or other intervention. Therefore, a specific ethical approval was not required. Animal handling and humane killing were in accordance with the applicable ethical laws, guidelines and provisions. In total, 14 female pigs with an average age of  $175 \pm 11$  days and an average mass of  $105 \pm 9$  kg were used. Eight pigs were used for the respiration measurements and six for mRNA and miRNA studies. The muscle (masseter) and brain (thalamus) tissues were dissected. The subsamples of each tissue were immediately frozen in the liquid nitrogen and stored at  $-80$  °C for mRNA and miRNA analyses. The remaining tissue was used for cell isolation, and the isolated cells were exposed to H-R in vitro. Oxygen consumption and expression of the target mRNA and miRNA were measured in the isolated cells exposed to normoxia, hypoxia and post-hypoxic recovery. The normoxic cells were collected directly after cell isolation. The hypoxic and recovering cells were collected from the respirometer chamber after exposure to hypoxia and reoxygenation, respectively (see below “Oxygen consumption rate ( $\dot{M}O_2$ ) and ROS measurements”).

**Cell isolation.** The muscle and brain tissues ( $\sim 1$  g) were separately placed on a plastic Petri dish containing 3–4 volumes of ice-cold biopsy preservation solution BIOPS (50 mM K-MES, 20 mM taurine, 0.5 mM dithiothreitol, 6.56 mM  $MgCl_2$ , 5.77 mM adenosine triphosphate (ATP), 15 mM phosphocreatine, 20 mM imidazole, 10 mM Ca-ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) buffer, 0.1  $\mu$ M free calcium, pH 7.1), transferred to the laboratory on ice and processed within 10 min of collection. Tissues were sliced into strips (2–5 mm long and about 1 mm in diameter, 5–7 mg wet mass) and digested for 10–15 min in 0.05% trypsin solution with gentle shaking at room temperature. The digestion was stopped by adding 5–10% fetal calf serum. The cell suspension was diluted with phosphate buffered saline (PBS) and centrifuged at  $200 \times g$  for 5 min at 25 °C. The cell pellets were washed twice with PBS and resuspended in 0.5–1 ml of fresh PBS. The cell suspensions of the masseter mostly contained myocytes, whereas thalamus isolates were likely heterogeneous (relay cells, interneurons and thalamic reticular nucleus cells)<sup>44</sup>. However, because the entire thalamus was used in all isolations, the relative composition of the cell isolates from different animals is expected to be similar.

**Oxygen consumption rate ( $\dot{M}O_2$ ) and ROS measurements.** Oxygen consumption and ROS production were measured in isolated permeabilized skeletal muscle and brain cells at 37 °C using an Oxygraph 2 k high-resolution respirometer (Oroboros, Innsbruck, Austria) and an integrated DatLab 6 software<sup>45</sup>. Oxygen consumption was measured using a Clark-type electrode calibrated with 100% (air-saturated assay buffer) and 0% (saturated solution of sodium dithionite) solutions. After the oxygen signal stabilized (background flux of  $\pm 1$  pmol  $O_2$   $s^{-1}$   $ml^{-1}$ ), cell suspensions were added to the respiration chambers containing 2 ml of temperature-equilibrated MiR05 solution (110 mM sucrose, 60 mM K lactobionate, 0.5 mM EGTA, 3 mM  $MgCl_2$ , 20 mM taurine, 10 mM  $KH_2PO_4$ , 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 1 g  $l^{-1}$  essentially fatty acid free bovine serum albumin (BSA), pH 7.1). The cells were permeabilized by addition of saponin (25  $\mu$ M) to the respiration chamber. Two independent substrate-uncoupler inhibitor titrations (SUITs) were conducted to measure the effect of H-R stress on respiration of the resting (LEAK) and phosphorylating (OXPHOS) mitochondria (SUIT1) and on cytochrome c oxidase (COX) activity (SUIT2). The effect of H-R stress on LEAK and OXPHOS was measured in SUIT1 with the following sequential additions to the same assay media: 1) 5 mM pyruvate with 2 mM malate to spark Complex I (NADH-linked) respiration (LEAK I; state 2); 2) 10 mM succinate to additionally stimulate the electron flow through Complex II (LEAK I + II; state 2); 3) 2.5 mM ADP to achieve ADP-stimulated OXPHOS state (state 3); 4) 5 mM 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 mitochondria (data not shown). The permeabilized cells were then exposed to hypoxia ( $\sim 0\%$   $O_2$ ) by maintaining ADP-stimulated respiration until the oxygen in the chamber was depleted. The cells were maintained in hypoxia for 15 min, after which the oxygen tension was raised and mitochondria allowed to recover for 10 min (reoxygenation). After reoxygenation, post-hypoxic OXPHOS respiration rate was recorded in the assay containing Complex I and II substrates (OXPHOSreox) and the SUIT1 continued as follows: 1) 2.5  $\mu$ M oligomycin to inhibit mitochondrial  $F_0F_1$ -ATPase and measure post-hypoxic LEAK respiration (LEAKI + IIreox, state 4); 2) 1  $\mu$ M rotenone to inhibit the electron flux through Complex I and determine LEAKIIreox (state 4); 3) 7.5 mM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to uncouple the mitochondrial electron transport system (ETS) (ETSreox); 4) 2.5  $\mu$ M antimycin A to inhibit electron flux through Complex III; 5) 6) 40 mM KCN to measure non-mitochondrial respiration ( $< 10\%$  of the total oxygen consumption rate, data not shown).

SUIT2 to measure the effect of H-R stress on COX involved the following sequential titration steps: 1) 7.5 mM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to uncouple the mitochondrial ETS; 2) 2.5  $\mu$ M antimycin A to inhibit electron flux through Complex III; 3) 0.5 mM N,N,N',N',-tetramethyl-p-phenylenediamin (TMPD) and 2 mM ascorbate to stimulate the activity of Complex IV (COX). The permeabilized cells were then exposed

to H-R stress as described above. Pre- and post-hypoxia COX activity was recorded, and 40 mM KCN was used to measure non-mitochondrial respiration (<10% of the total oxygen consumption rate, data not shown).

Efflux of H<sub>2</sub>O<sub>2</sub> was measured simultaneously with MO<sub>2</sub> using Fluorescence-Sensor Green (525 nm) integrated with Oxygraph 2 k in an assay buffer containing 10 μM Amplex UltraRed, 1 U ml<sup>-1</sup> horseradish peroxidase and 5 U ml<sup>-1</sup> superoxide dismutase (SOD)<sup>45</sup>. A two-step calibration was conducted with 0.1 μM H<sub>2</sub>O<sub>2</sub> before and after the addition of the mitochondrial suspension. ROS production was measured as the rate of H<sub>2</sub>O<sub>2</sub> efflux in LEAK and OXPHOS states and corrected for the baseline measured in the absence of cells.

Protein concentrations in the isolated cells were measured using a Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA, USA)<sup>46</sup> using BSA as a standard. Protein concentrations of the cell suspensions were corrected for the BSA content of the assay media. The cellular protein content in the respirometry chambers (assay volume 2.1 ml) was ~50–500 μg ml<sup>-1</sup>, depending on the isolation. Respiration rates and ROS production were expressed as nmol O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> cellular protein and nmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> cellular protein, respectively. Trypan Blue exclusion assay did not reveal any loss of cell viability after H-R stress in the muscle or brain cell isolates (data not shown) reflecting short H-R exposure times.

Mitochondrial respiratory states and control indices were determined as described elsewhere<sup>47,48</sup>. 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) ( $\Delta < 5\%$ ,  $P > 0.05$ ). Therefore, both state 2 and state 4 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<sup>49</sup>. Respiratory control ratio (RCR) was calculated as the ratio of OXPHOS to LEAK flux, and P-L control efficiency was calculated as the ratio of net to total OXPHOS capacity ( $1 - \text{LEAK I} + \text{II}/\text{OXPHOS}$ )<sup>47,48</sup>. To assess the rate of the electron leak, H<sub>2</sub>O<sub>2</sub> efflux rate was divided by the oxygen consumption rate in the same cell isolate and expressed as H<sub>2</sub>O<sub>2</sub> to O<sub>2</sub> ratio.

**Quantitative real-time PCR.** Total RNA was extracted from approximately 50 mg of tissue or isolated cells using TRIzol Reagent (Invitrogen) and the RNeasy Mini kit (Qiagen) with DNase I treatment according to the manufacturer's recommendations. The quantity and quality of total RNA were determined using NanoDrop ND-2000 (Peqlab, Erlangen, Germany) and Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The average tissue-specific RNA integrity number (RIN) was similar in all treatments (~6 in the muscle and ~8 in the brain).

For PCR analysis, 88 target genes and 43 miRNAs were selected (Supplementary Tables 1 and 2). The targets were chosen based on their important role in the HIF-1 regulation, apoptosis, redox homeostasis, glycolysis and mitochondrial OXPHOS<sup>19</sup> and their previously reported association with hypoxia<sup>31,50–52</sup>. Due to the tissue-specific expression of the target transcripts, 67 mRNA transcripts and 41 miRNA were analyzed in the thalamus, and 41 mRNA transcripts and 34 miRNA were used in the masseter.

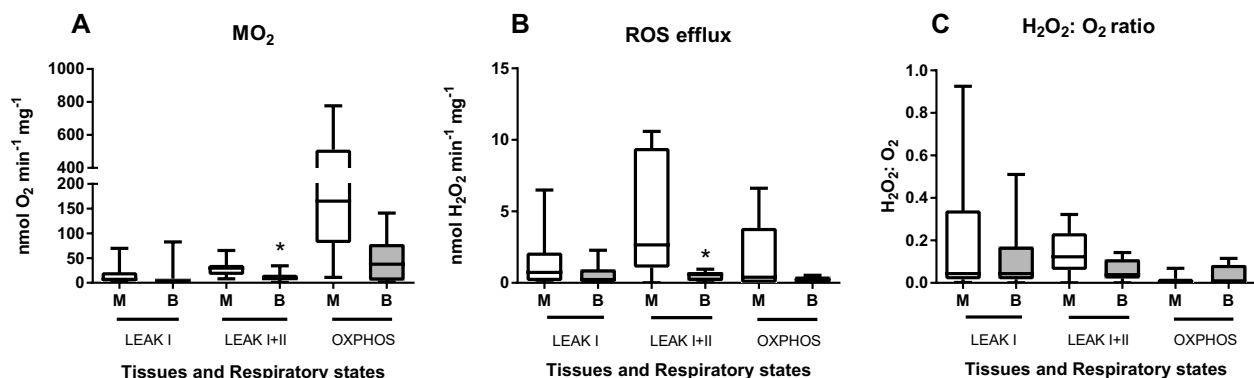
For cDNA synthesis, 200 ng of total RNA was mixed with 1 μL Reverse Transcription Master Mix (Fluidigm PN 100–6297) in 5 μL volume. The reaction was incubated at 25 °C for 5 min, 42 °C for 30 min followed by 85 °C for 5 min. The cDNA synthesis of miRNA was performed as described elsewhere<sup>53</sup>. In brief, 100 ng of total RNA were poly(A) tailed and reverse transcribed using 1 U poly(A) polymerase (BioLab), 0.1 mM of NTPs, RT-primers (CAGGTCCAGTTTTTTTTTTTTTTVN where V is A, C or G and N is A, C, G or T) and 100 U MuLV reverse transcriptase (Invitrogen). The reaction was incubated at 42 °C for 1 h followed by 95 °C to inactivate the enzyme.

The cDNA obtained from mRNA and miRNA was used for qPCR with the Fluidigm BioMark HD System (Fluidigm Corporation, CA, USA). Specific target amplification (STA) was done per the manufacturer's recommendations. Pre-amplification sample mixtures were prepared using PreAmp Master Mix (Fluidigm PN 1,005,581) containing 1.25 μL of cDNA, 1 μL PreAmp Master Mix, 0.5 μL Pooled Delta Gene Assay Mix (500 nM) containing DNA-suspension buffer and primers in 5 μL total volume. The pre-amplification reaction was incubated at 95 °C for 2 min, followed by 10 cycles at 95 °C for 15 s and 60 °C for 4 min. The pre-amplification reaction was cleaned up using exonuclease I and 10× diluted with DNA suspension buffer (TEKnova, PN T0221). Fluidigm quantitative measurement runs were carried out with 96.96 dynamic arrays (Fluidigm Corporation, CA, USA) according to manufacturer's instructions. In brief, 2.5 μL of 2× SsoFast Evagreen Supermix with Low ROX, 0.25 μL 20× sample-loading reagent, and 2.25 μL of treated samples were prepared. Separately, an assay mixture was prepared for each primer pair including 2.25 μL of DNA Suspension buffer, 0.25 μL of 100 μM forward and reverse primer (Supplementary Tables 1 and 2) and 2.5 μL of 2× assay-loading reagents. The dynamic arrays were primed with control line fluid and loaded with the sample and assay mixtures via appropriate inlets using an IFC controller. The array chips were placed in the BioMark Instrument for PCR at 95 °C for 10 min, followed by 30 cycles at 95 °C for 15 s and 60 °C for 1 min. The data were analyzed with real-time PCR analysis software in the BioMark HD instrument (Fluidigm Corporation, San Francisco, CA). For normalization, the internal controls of cel-miR-39-3p, SSC\_5S, SSC\_Met\_tRNA and SSC\_U6 were used for miRNA and 5 housekeeping genes (GAPDH, U6, ACTB, RPL11 and RPL32) were used for mRNA (Supplementary Tables 1 and 2). Data analysis was done by 2 – ΔCt method. The sample size was 6 for all treatment groups.

**Data analysis.** Data on mitochondrial functional traits (MO<sub>2</sub> and ROS production) were tested for normality and homoscedasticity. All data were normally distributed, and most had equal variances except for two set of data before and after the H-R stress (ROS efflux in the LEAK I + II state of brain cells and H<sub>2</sub>O<sub>2</sub> to O<sub>2</sub> ratio in the OXPHOS state of muscle cells). We therefore used non-parametric tests for these two datasets. To test the tissue-specific variability in oxygen consumption rates and ROS production during different respiratory states

	Tissue	Respiratory state	Tissue × Respiratory state
MO <sub>2</sub>	$F_{1,39}=4.879$ , $P=0.033$	$F_{2,39}=6.895$ , $P=0.03$	$F_{2,39}=3.823$ , $P=0.30$
ROS efflux	$F_{1,39}=9.595$ , $P=0.004$	$F_{2,39}=2.052$ , $P=0.142$	$F_{2,39}=1.894$ , $P=0.164$
H <sub>2</sub> O <sub>2</sub> :O <sub>2</sub> ratio	$F_{1,39}=1.251$ , $P=0.270$	$F_{2,39}=2.918$ , $P=0.066$	$F_{2,39}=0.553$ , $P=0.580$

**Table 1.** ANOVA: Effects of tissue type and respiratory states (LEAK I, LEAK I+II, OXPHOS) on the oxygen consumption rates (MO<sub>2</sub>), ROS efflux and H<sub>2</sub>O<sub>2</sub>:O<sub>2</sub> ratio of permeabilized muscle and brain cells under the control (normoxic) conditions. *F* values with the degrees of freedom for the effect and error (in subscripts) are shown. Significant *P* values ( $P<0.05$ ) highlighted in bold.



**Figure 1.** Baseline mitochondrial oxygen consumption (MO<sub>2</sub>) and ROS efflux rates in the permeabilized skeletal muscle and brain cells of *S. scrofa* under the control (normoxic) conditions. (A) oxygen consumption rates, (B) ROS efflux rates, (C) electron leak (the ratio of H<sub>2</sub>O<sub>2</sub> produced to O<sub>2</sub> consumed by the permeabilized cells). Tissues: M—skeletal muscle cells, B—brain cells. Mitochondrial traits were measured in non-phosphorylating mitochondria in the presence of Complex I substrates (LEAK I), the mixture of Complex I and II substrates (LEAK I+II), and in the phosphorylating (ADP-stimulated) mitochondria respiring on the mixture of the Complex I and II substrates (OXPHOS). Asterisks indicate significant differences in the respective traits between different tissues ( $P<0.05$ ). *N*=6–7.

(LEAK I, LEAK I + II, OXPHOS) under control (normoxic) conditions, a two-way ANOVA was conducted followed by Tukey's Honest Significant Difference (HSD) test. Tissue type and respiratory states were designated as between subject factors. The effects of hypoxia and reoxygenation on mitochondrial MO<sub>2</sub> and ROS production were tested using paired two-tailed Student's *t*-test or Wilcoxon test. All statistical analyses were conducted using IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism ver 7.02 (GraphPad Software Inc., La Jolla, CA, USA) software. Differences were considered significant if the probability of Type I error (*P*) was  $<0.05$ .

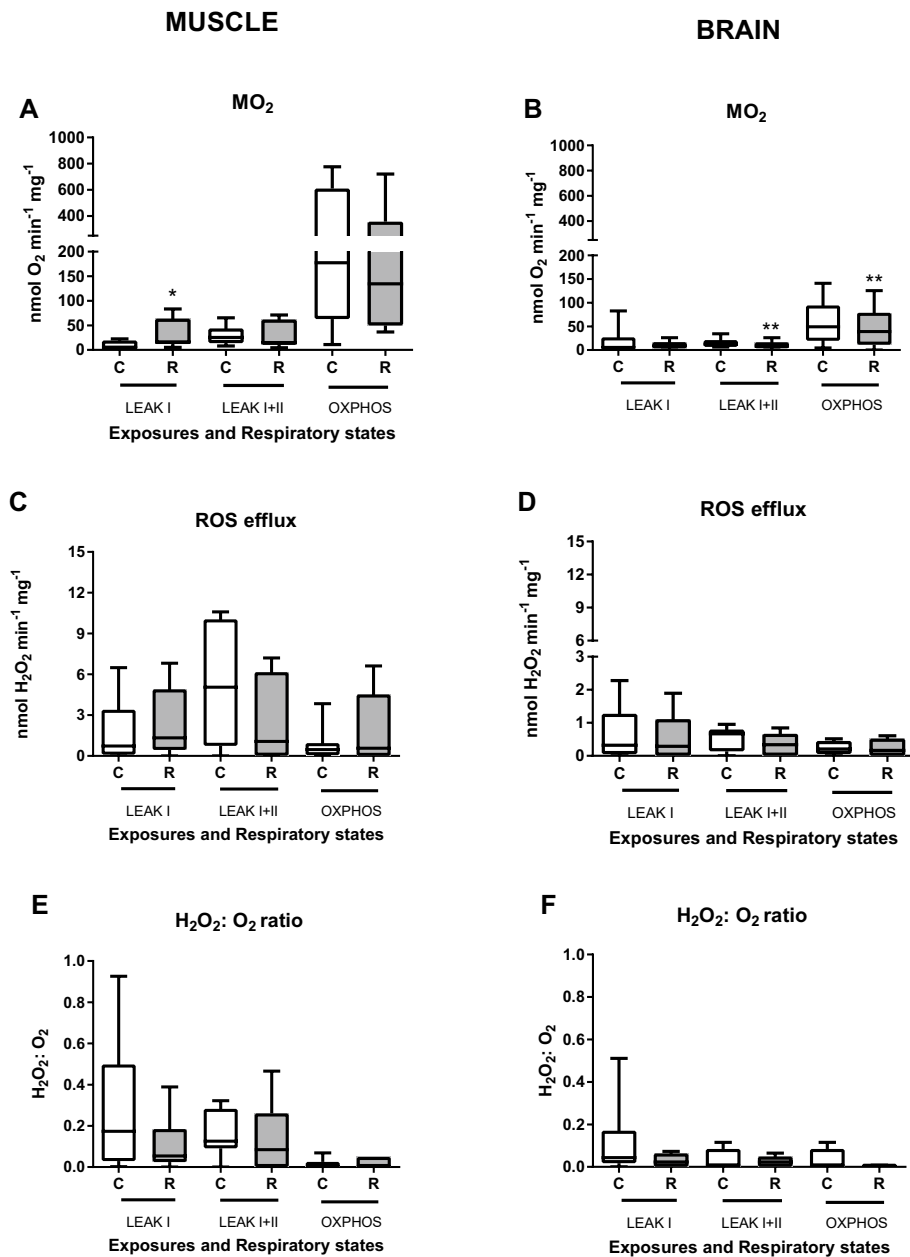
mRNA and miRNA data were analyzed using SAS ver. 9.4. Treatment group (normoxia, hypoxia and reoxygenation) was used as a fixed effect. Samples were used as repeated measures factor using proc mixed procedure. We also compared mRNA and miRNA levels in the tissues immediately after collection (stored at  $-80^{\circ}\text{C}$ ) with the isolated cells from the same tissue samples. The means between the treatment groups were compared using the post hoc Tukey–Kramer test adjusting for multiple comparisons.

We have followed the recommendation of evidence-based language<sup>54</sup> for describing our results. The following thresholds were used:  $P=0.049$ – $0.011$  (moderate evidence),  $P=0.01$ – $0.001$  (strong evidence),  $P<0.001$  (very strong evidence).

## Results

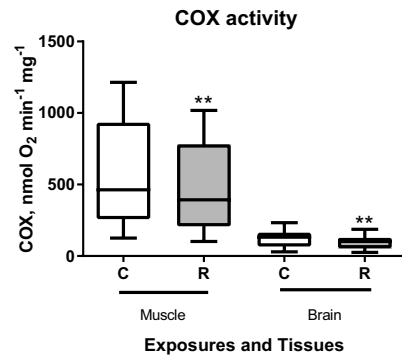
**Tissue-specific mitochondria functional traits and ROS production.** Under normoxic conditions, mitochondria from the isolated porcine cells showed good coupling with the mean RCR of 8.8 and 4.2 respiring on the mixture of Complex I and II substrates. The mean P-L control efficiency with the combination of Complex I and II substrates was 0.88 and 0.76 in the muscle and the brain, respectively. ANOVA showed moderate evidence for the effects of the tissue type on the mitochondrial oxygen consumption and ROS production across three studied mitochondrial states (LEAK I, LEAK I + II and OXPHOS) in normoxia (Table 1). Pairwise comparisons of means showed moderate evidence ( $P<0.05$ ) for higher oxygen consumption rates (MO<sub>2</sub>) and ROS efflux during LEAK I + II respiration in the muscle relative to the brain cells (Fig. 1A,B). Although OXPHOS MO<sub>2</sub> was considerably (~4–9-times) higher than LEAK I + II MO<sub>2</sub>, the ROS efflux rate was lower in the OXPHOS than LEAK state (Fig. 1B, Fig. 2). This was reflected in the lower electron leak (H<sub>2</sub>O<sub>2</sub> to O<sub>2</sub> ratio) in the OXPHOS relative to the LEAK state in both studied tissues (Fig. 1C).

**Effects of H-R stress on mitochondria functional traits.** There was no evidence of difference in the LEAK I + II or OXPHOS MO<sub>2</sub> of the skeletal muscle cells under the control (normoxic) conditions and after



**Figure 2.** Effect of the H-R stress on the mitochondrial oxygen consumption ( $\dot{M}O_2$ ) and ROS efflux rates in the permeabilized skeletal muscle and brain cells of *S. scrofa*. (A, B) oxygen consumption rates, (C, D) ROS efflux rates, (E, F) electron leak (the ratio of  $H_2O_2$  produced to  $O_2$  consumed by the permeabilized cells). Conditions: C—normoxic (control) cells, R—cells after 15 min of severe hypoxia followed by 10 min of reoxygenation. Mitochondrial traits were measured in non-phosphorylating mitochondria in the presence of Complex I substrates (LEAK I), the mixture of Complex I and II substrates (LEAK I+II), and in the phosphorylating (ADP-stimulated) mitochondria respiring on the mixture of the Complex I and II substrates (OXPHOS). Asterisks indicate significant differences in the respective traits between the control and reoxygenation conditions (\* $P < 0.05$ , \*\* $P < 0.01$ ).  $N = 6-7$  for the skeletal muscle and 6 for the brain.

15 min acute exposure to severe hypoxia ( $P > 0.05$ ) (Fig. 2A). However, muscle cell mitochondria showed moderate evidence of increase in LEAK I  $\dot{M}O_2$  (with Complex I substrate) after exposure to H-R stress compared to the normoxic controls ( $P < 0.05$ ). ROS production showed no evidence of change after H-R exposure in the muscle cells regardless of the mitochondrial state ( $P > 0.05$ ) (Fig. 2C). In the brain cell mitochondria, LEAK I+II  $\dot{M}O_2$  and OXPPOS  $\dot{M}O_2$  with Complex I and II substrates showed strong evidence ( $P < 0.01$ ) of suppression after H-R exposure (Fig. 2B). Similar to the muscle cells, there was no evidence for change in ROS production between normoxia and H-R exposure in the brain cells regardless of the mitochondrial state ( $P > 0.05$ ) (Fig. 2D). Exposure to H-R stress had no effect on the RCR or P-L efficiency in the muscle and brain cells ( $P > 0.05$ , data



**Figure 3.** Effect of the H-R stress on the indices of mitochondrial coupling and cytochrome c oxidase (COX) activity in the permeabilized skeletal muscle and brain cells of *S. scrofa*. Conditions: C—normoxic (control) cells, R—cells after 15 min of severe hypoxia followed by 10 min of reoxygenation. Asterisks indicate significant differences in the respective traits between the control and reoxygenation conditions (\*\* $P < 0.01$ ).  $N = 8$  for the skeletal muscle and 7 for the brain.

not shown). COX activity showed strong evidence of decrease ( $P < 0.01$ ) after reoxygenation in both studied tissues (Fig. 3).

**Expression of mRNA transcripts in brain and skeletal muscle tissue.** Cell isolation had a significant effect on the transcript profiles of the muscle and the brain cells. In the masseter, 20 of 41 analyzed mRNA transcripts showed different expressions between the immediately frozen tissue and isolated cells with 14 transcripts showing higher and 6—lower levels in isolated cells than in the fresh tissue (Supplementary Table 3). In the thalamus, 50 of 67 analyzed transcripts showed significantly different expressions between the immediately frozen tissue and isolated cells with most (43) transcripts showing higher levels in isolated cells than the fresh tissue (Supplementary Table 4).

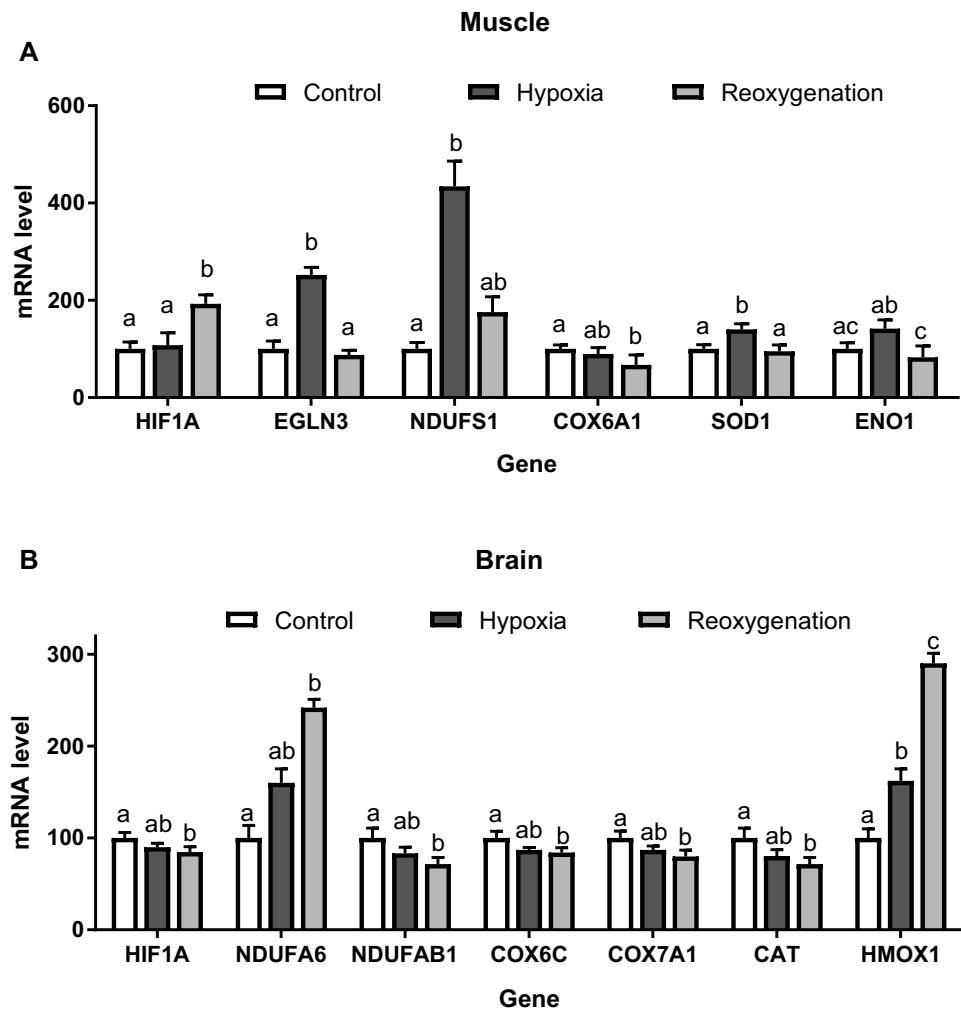
Acute short-term exposure of the isolated muscle cells to hypoxia and reoxygenation significantly altered expression levels of six mRNA transcripts (HIF1A, EGLN3, NDUFS1, COX6A1, SOD1 and ENO1). Three transcripts (EGLN3, NDUFS1 and SOD1) were significantly upregulated in the muscle cells exposed to 15 min of severe hypoxia and returned to the baseline (normoxic) conditions after 10 min of reoxygenation (Fig. 4A). Reoxygenation led to upregulation of HIF1A mRNA levels and a decrease in COX6A1 transcripts relative to the normoxic cells in the muscle cells (Fig. 4A). Transcript levels of ENO1 tended to increase in hypoxia and decrease during reoxygenation relative to the normoxic controls, but the differences in ENO1 mRNA levels were only statistically significant between the muscle cells exposed to hypoxia and those after reoxygenation (Fig. 4A).

Exposure of isolated thalamus cells to acute short-term hypoxia and reoxygenation led to a significant change in the transcript levels of seven mRNA (HIF1A, NDUFA6, NDUFAB1, COX6C, COX7A1, CAT and HMOX1). Hypoxia led to upregulation of HMOX1 transcript levels in the thalamus cells, and this increase was further enhanced by reoxygenation (Fig. 4B). Reoxygenation also led to upregulation of NDUFA6 mRNA. Levels of five transcripts (HIF1A, NDUFAB1, COX6C, COX7A1 and CAT) were not significantly affected by hypoxia, but decreased during post-hypoxic reoxygenation in the thalamus cells (Fig. 4B).

**Expression of miRNA transcripts in brain and skeletal muscle tissue.** In the masseter, 7 of 34 analyzed miRNA showed significantly higher expression in the freshly collected tissue than in isolated cells (Supplementary Table 5). In the thalamus, 33 of 41 analyzed miRNA were differentially expressed in the isolated cells relative to the freshly collected tissue, with higher expression of 31 miRNA and lower expression of 2 miRNA in the isolated cells (Supplementary Table 6).

Of the studied 34 miRNAs in the masseter cells, 9 miRNAs (miR-145-5p, miR-24-3p, miR-140-5p, miR-29-3p, miR-17-3p, miR-107-3p, miR-144, miR-188-3p and miR-199a-3p) showed moderate to very strong evidence of altered expression in response to acute H-R stress (Fig. 5A). The levels of these differentially expressed miRNAs were suppressed in hypoxia and reoxygenation with generally stronger suppression after reoxygenation (Fig. 5A).

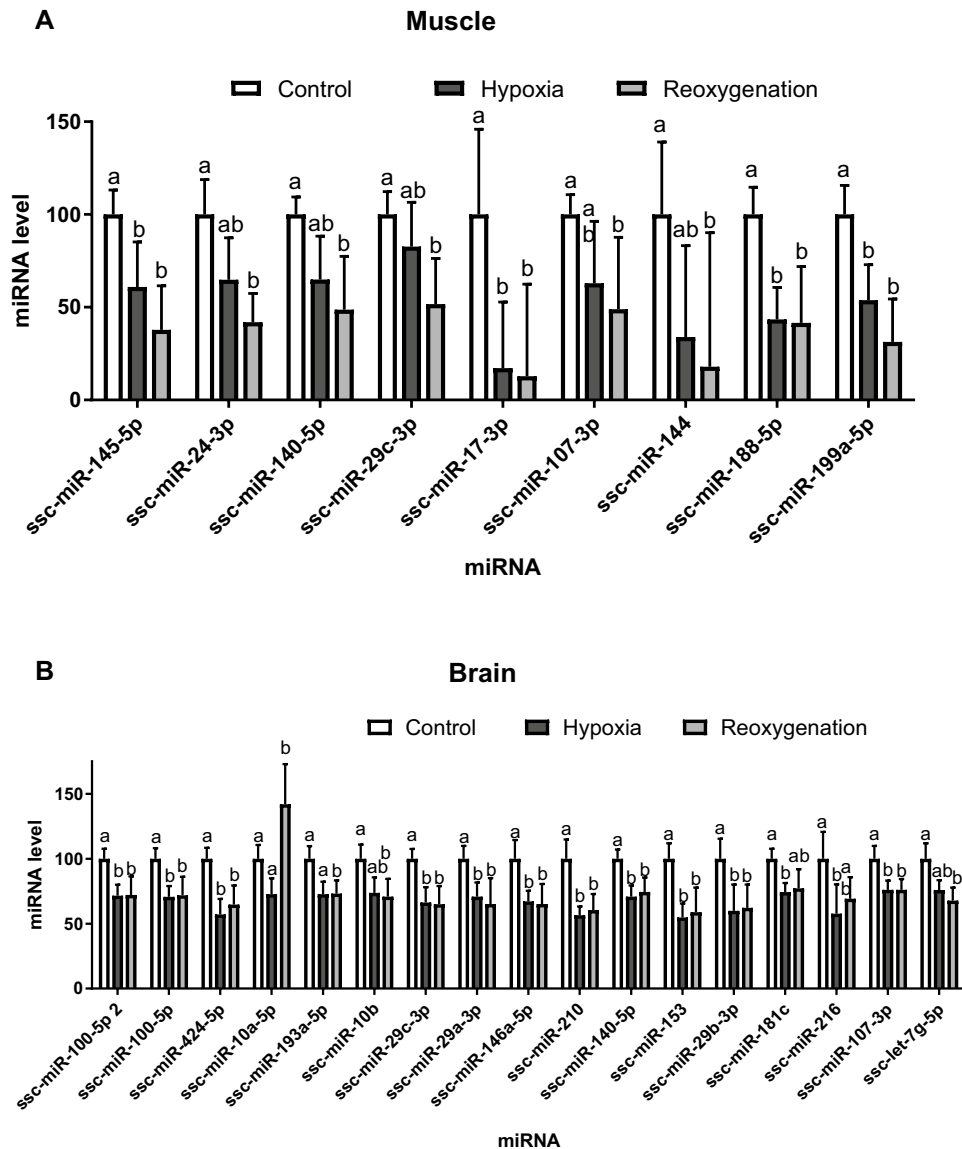
Of the 41 studied miRNAs in the thalamus cells, 17 miRNAs (ssc-miR-100-5p 2, ssc-miR-100-5p, ssc-miR-424-5p, ssc-miR-10a-5p, ssc-miR-193a-5p, ssc-miR-10b, ssc-miR-29c-3p, ssc-miR-29a-3p, ssc-miR-146a-5p, ssc-miR-210, ssc-miR-140-5p, ssc-miR-153, ssc-miR-29b-3p, ssc-miR-181c, ssc-miR-216, ssc-miR-107-3p, ssc-let-7 g-5p) showed moderate to very strong evidence of differential expression after acute H-R stress relative to the normoxic controls (Fig. 5B). Except for miR-10a-5p (significantly upregulated during post-hypoxic recovery), all other differentially expressed miRNAs were suppressed under hypoxia and/or reoxygenation in the brain cells (Fig. 5B).



**Figure 4.** Effect of the H-R stress on mRNA expression of hypoxia-responsive genes in the isolated skeletal muscle and brain cells of *S. scrofa*. HIF1A—Hypoxia Inducible Factor 1 Alpha Subunit, EGLN3—Egl-9 Family Hypoxia Inducible Factor 3, NDUFS1—NADH: Ubiquinone Oxidoreductase Core Subunit S1, COX6A1—Cytochrome C Oxidase Subunit 6A1, SOD1—Superoxide Dismutase 1, HMOX1—Heme Oxygenase 1, ENO1—Enolase 1, NDUFA6—NADH: Ubiquinone Oxidoreductase Subunit A6, SDHC—Succinate Dehydrogenase Complex Subunit C, COX6C—Cytochrome c Oxidase Subunit 6C, COX7A1—Cytochrome C Oxidase Subunit 7A1, and CAT—Catalase. (A) skeletal muscle cells, (B) brain cells. Only the transcripts that showed significant ( $P < 0.05$ ) changes in hypoxia and/or reoxygenation relative to the normoxic controls are shown. Letters indicate differences between the exposure conditions; columns that do not share a letter are significantly different ( $P < 0.05$ ).

## Discussion

**Effects of hypoxia-reoxygenation on mitochondrial activity and gene expression.** Mitochondria of the muscle (masseter) cells of *S. scrofa* were more tolerant to the acute H-R stress than those of the brain (thalamus) cells. Thus, mitochondria of the brain cells showed suppressed mitochondrial respiration in the resting (LEAKI+II) state and a decrease in the OXPHOS (indicative of the maximum ATP synthesis capacity) following acute H-R stress. Furthermore, in three out of eight brain cell isolates, the oxygen consumption was not measurable after H-R stress indicating a complete loss of mitochondrial activity (data not shown). In contrast, mitochondria from the muscle cells showed stable LEAKI+II respiration and OXPHOS capacity following reoxygenation. This indicates that the muscle cells might be able to better restore ATP synthesis after exposure to short-term intermittent hypoxia than the brain cells consistent with the expected higher hypoxia tolerance of the muscle tissue<sup>55</sup>. Interestingly, in both studied cell types exposure to intermittent hypoxia led to the suppression of COX activity by 17–20% and was associated with a decrease in the transcript levels of genes encoding COX subunits (COX6A1 in the muscle and COX6C and COX7A1 in the brain). Possible mechanisms inhibiting COX activity (not tested in our present study) might also include cytochrome c release from the inner mitochondrial membrane<sup>56,57</sup> or phosphorylation of serine or threonine residues on Complex IV subunits<sup>58,59</sup>. In mammals, COX activity can be modulated by the replacement of COX4-1 subunit through the hypoxia-specific COX4-2 subunit during hypoxia<sup>60</sup>. However, this replacement increases COX activity<sup>60,61</sup> and thus cannot explain sup-



**Figure 5.** Effect of the H-R stress on miRNA expression in the isolated skeletal muscle and brain cells of *S. scrofa*. (A) skeletal muscle cells, (B) brain cells. Only the miRNAs that showed significant ( $P < 0.05$ ) changes in expression in hypoxia and/or reoxygenation relative to the normoxic controls are shown. Letters indicate differences between the exposure conditions; columns that do not share a letter are significantly different ( $P < 0.05$ ).

pressed COX activity found in our present study. In porcine cells, COX shows large apparent excess capacity (~2.5–3 fold) relative to the OXPHOS activity, so the observed 17–20% decrease in the maximum COX velocity is unlikely to have a major effect on the ATP synthesis rates under most physiological conditions. However, a modest decrease in COX activity can play an important regulatory role<sup>62</sup> and has been shown to attenuate H-R injury in mammals<sup>63</sup>. Interestingly, the porcine muscle cells showed elevated resting (LEAK I) respiration with pyruvate after H-R stress. Elevated rates of the pyruvate-driven respiration indicate activation of the forward flux of the electrons through mitochondrial Complex I and might represent a mechanism to mitigate ROS production by preventing the reverse electron transport (RET) caused by succinate accumulation in hypoxia<sup>5</sup>. This hypothesis is supported by the observation that succinate addition stimulated ROS efflux in the porcine muscle cells under normoxic conditions, but this increase was attenuated in the cells exposed to H-R stress. An increase in the Complex I activity in the muscle cells after reoxygenation was associated with a strong overexpression of NDUFS1 transcript encoding a key regulatory Complex I subunit<sup>64,65</sup>. NDUFS1 deficiency suppresses Complex I activity and increases ROS production in different types of mammalian cells<sup>64,65</sup>. Overexpression of NDUFS1 mRNA in hypoxia might thus represent an anticipatory response of the porcine muscle cells to support high Complex I activity and mitigate ROS during reoxygenation. In the mitochondria of the brain cells, hypoxia-reoxygenation stress had no effect on pyruvate-driven resting respiration. Interestingly, succinate addition did

not stimulate ROS efflux in the brain mitochondria suggesting that RET might be a less important mechanism of ROS generation in the thalamus cells than in the muscle.

In the porcine brain cells, transcripts of two Complex I subunits, NDUFA6 and NDUFAB1, were differentially expressed in response to H-R stress. The mRNA levels of these subunits showed opposite directions of change during reoxygenation with suppression of NDUFAB1 and upregulation of NDUFA6 mRNA. NDUFAB1 subunit, like NDUFS1, plays an important role in regulating ETS flux and ROS production in mitochondria and its overexpression mitigates ischemia–reperfusion injury in mammalian cells<sup>66</sup>. Therefore, transcriptional downregulation of NDUFAB1 might be maladaptive during H-R and contribute to hypoxia sensitivity of the porcine brain cells. Unlike NDUFS1 and NDUFAB1, the role of NDUFA6 in the regulation of mitochondrial function is less well understood<sup>67,68</sup>. NDUFA6A is an accessory subunit involved in Complex I assembly<sup>69</sup> and contains a site involved in the conversion of Complex I from active to inactive form (A/D conversion) during H-R<sup>70</sup>. Our present data do not permit inferences about the functional implications of the shift in NDUFA6 transcript levels, but the opposite direction of change of NDUFA6 and NDUFAB1 transcripts point out a possible dysregulation of Complex I in the thalamus cells during reoxygenation.

Various studies have reported on ROS production, its detrimental effects and relevance in both skeletal muscle and brain cells during H-R stress<sup>71–73</sup>. However, in our present study, there was no evidence of elevated ROS production during reoxygenation indicating that the observed differences in the mitochondrial response to H-R stress between the muscle and brain cells cannot be attributed to differences in the H-R-induced ROS burst.

**Transcriptional response of HIF-1 pathway and downstream HIF-1 targets.** HIF-1 is a master transcriptional regulator of adaptive response to hypoxia in mammals<sup>19</sup>. HIFs are regulated post-translationally by oxygen-dependent hydroxylation of proline residues by prolyl hydroxylase domain protein (PHD) that targets HIF-1 $\alpha$  for degradation<sup>74</sup>. The PDH activity is inhibited in hypoxia leading to the accumulation of HIF-1 $\alpha$  and transcriptional activation of HIF-1 targets<sup>75,76</sup>. Our present study shows that HIF-1 $\alpha$  and PHD3 (EGLN3) are regulated at the transcriptional level during H-R stress in the porcine cells. In our data, transcripts of three isoforms of PHD (EGLN1/PHD2, EGLN2/PHD1 and EGLN3/PHD3) as well as HIF-1 $\alpha$  and HIF-2 $\alpha$  subunits were found in the muscle and the brain cells of *S. scrofa*. However, only EGLN3 (in the muscle) and HIF-1 $\alpha$  transcripts (in both studied cell types) showed evidence of the impact of H-R stress. EGLN3 transcripts were upregulated during hypoxia in porcine muscle cells, possibly to compensate for lower PHD activity caused by oxygen deprivation. Transcriptional upregulation of PHD3/EGLN3 has been shown to regulate the HIF response under low oxygen conditions ensuring hypoxic cell survival<sup>77,78</sup>. During reoxygenation, a decline in EGLN3 transcript levels was accompanied by upregulation of HIF-1 $\alpha$  transcripts in the porcine muscle cells. An increase in HIF-1 $\alpha$  levels promotes tolerance to H-R in a variety of mammalian systems<sup>79–81</sup> and transcriptional upregulation of HIF-1 $\alpha$  mRNA contributes to such increase<sup>82</sup>. Interestingly, a strong increase in HIF-1 $\alpha$  expression was found during reoxygenation in an extremely hypoxia-tolerant marine invertebrate, the hard shell clam *Mercenaria mercenaria* suggesting the adaptive role of HIF-1 in post-hypoxic survival<sup>83</sup>. In porcine brain cells, no transcriptional upregulation of EGLN3 or HIF-1 $\alpha$  was found during hypoxia and reoxygenation. Furthermore, HIF-1 $\alpha$  mRNA levels were suppressed during reoxygenation indicating that muted HIF-1 response might contribute to lower H-R tolerance of this cell type.

Upregulation of HIF-1 induces an adaptive switch to glycolysis in mammalian cells<sup>40</sup>. HIF-1 upregulates the transcription of multiple glycolytic enzymes such as enolase 1, aldolase, hexokinase and glyceraldehyde-3-phosphate dehydrogenase to enhance anaerobic ATP production under oxygen deficiency<sup>74,84</sup>. In the isolated porcine cells, all studied genes encoding glycolytic or glycogenolytic enzymes (including ENO1, PYGM, GPI, PGK1 and LDHB) showed stable transcript levels during H-R stress. This lack of glycolytic activation might be due to the short (15 min) hypoxia exposure that was insufficient to trigger anaerobic metabolism in the porcine cells in the present study.

Hypoxia has been reported to modulate antioxidant defense<sup>85–87</sup>. In our present study, out of the six studied antioxidant enzymes (heme oxygenase (HMOX1), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, thioredoxin and copper chaperone for superoxide dismutase), three (CAT, SOD1 and HMOX1) were transcriptionally modulated by H-R exposure. In the muscle cells, SOD1 transcripts encoding cytosolic Cu, Zn-SOD were modestly and transiently upregulated during hypoxia returning to the baseline levels during reoxygenation. No other studied antioxidant transcripts responded to H-R stress in the isolated muscle cells. In the brain cells, HMOX1 was significantly overexpressed in both hypoxia and recovery, whereas CAT transcripts were suppressed during the post-hypoxic recovery in the brain cells. HMOX1 is associated with the degradation of heme and is involved in alleviating ischemic injury<sup>86</sup>. While the upregulation of HMOX1 transcripts might be considered protective, transcriptional suppression of CAT indicates dysregulation of antioxidant response during the post-hypoxic recovery of the brain cells. Taken together, these findings show that acute short-term H-R exposure did not induce strong oxidative stress consistent with the observation of the stable ROS emission during H-R stress in the isolated muscle and brain cells.

**Effects of hypoxia-reoxygenation on cellular miRNA profiles.** MicroRNAs are important post-transcriptional regulators involved in the adaptive and maladaptive responses to hypoxia (ischemia) and reoxygenation<sup>88–90</sup>. In the porcine muscle cells, 4 of 34 studied miRNA were suppressed in hypoxia and 9 were suppressed in reoxygenation relative to the normoxic controls. This suppression implies transcriptional derepression of mRNA targets of respective miRNAs. Three hypoxamiRs suppressed during H-R in the muscle cells (ssc-miR-107-3p and ssc-miR-29b-3p regulating apoptosis and ssc-miR-140-5p regulating angiogenesis) also showed a decrease during reoxygenation in the brain cells indicating that they are a part of a general cellular response to H-R stress. In human pulmonary artery smooth muscle cells, hypoxia-mediated downregulation of

miR-140-5p inhibits proliferation and promotes apoptosis by targeting and regulating DNA methyltransferase 1 (DNMT1) and SOD2 expression<sup>91</sup>. The miR-29 family also includes many important apoptosis regulators. Thus, downregulation of miR-29a-3p induces both pro- and anti-apoptotic functions in cells<sup>92</sup> whereas upregulation of miR-29b-3p induces apoptosis and activates caspase 3 proteins<sup>93</sup>. Hence, decreased expression of these miRNAs in our study might indicate stimulation of apoptosis-related pathways in the muscle and brain cells exposed to H-R stress.

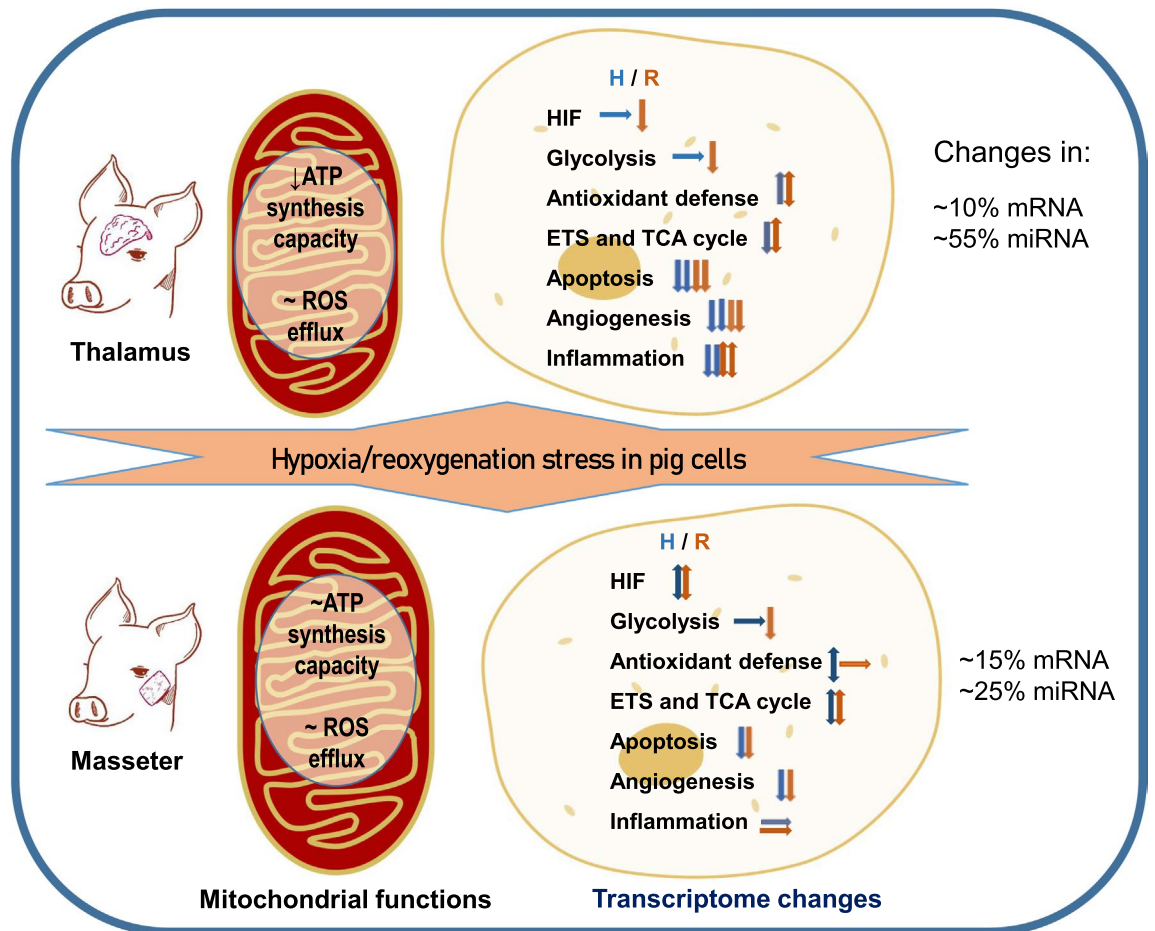
In the muscle cells, hypoxamiRs downregulated by hypoxia involved ssc-miR-199a-5p controlling HIF1A expression<sup>94</sup>, a regulator of angiogenesis and mitochondrial antioxidants (ssc-miR-17-3p)<sup>95</sup> and two miRNAs regulating autophagy and apoptosis (ssc-miR-145-5p and ssc-miR-188-5p)<sup>96–99</sup>. Suppression of miR-199a-5p protects against H-R stress in mammalian heart and brain cells<sup>100–103</sup>, attenuates apoptosis<sup>104,105</sup>, and contributes to higher aerobic capacity and tolerance to high-altitude hypoxia in humans<sup>106</sup>. Previous studies have associated lower levels of ssc-miR-17-3p with elevated mitochondrial enzyme activities in human colorectal cancer cells<sup>95,107</sup>. If similar mechanisms exist in the porcine masseter cells, suppression of miR-199a-5p and ssc-miR-17-3p might partially explain the ability of these cells to maintain high aerobic capacity during H-R stress found in our present study. Furthermore, downregulation of miR-145 and ssc-miR-188-5p suppresses autophagy and cell death<sup>96,108</sup> and might enhance post-hypoxic survival of the muscle cells.

In the porcine brain cells, substantially more (17 of 41 studied) miRNAs were suppressed after H-R stress. This indicates that the hypoxia-induced metabolic reorganization involving hypoxamiRs affects a larger swath of the cellular pathways in the brain than in the muscle cells. Unlike the muscle cells, H-R stress suppressed the expression of hypoxamiRs regulating key energy-conserving metabolic pathways such as mitochondrial ETS, TCA cycle (3 miRNAs) and glycolysis (3 miRNAs). These findings indicate that the brain cells engage compensatory mechanisms to increase ATP synthesis in response to H-R stress. Other suppressed hypoxamiRs in the brain involved those regulating apoptosis (5 miRNAs), angiogenesis (3 miRNAs) and inflammation (3 miRNAs). Upregulation of inflammatory pathways in the H-R exposed brain cells is consistent with the damage reflecting lower hypoxia tolerance of this cell type compared with the muscle cells. Overexpression of ssc-miR-424 and ssc-miR-210 stabilizes HIF1A and attenuates hypoxia-induced apoptosis, angiogenesis and stress response in mammalian cells<sup>109,110</sup>. Downregulation of these miRNAs found in both hypoxic and post-hypoxic brain cells might therefore negatively affect survival of the brain cells.

Interestingly, ssc-miR-10a-5p showed a different pattern of H-R-induced expression compared with other studied miRNAs increasing during the post-hypoxic recovery in the brain cells. miR10a-5p has been reported as a key regulator of tissue inflammation<sup>111</sup>. In stem and kidney cells downregulation of miR-10a-5p during hypoxia inhibits inflammatory responses, suppresses inflammatory gene expression and increases the proliferative ability of cells<sup>112,113</sup>. In contrast, in rat liver, miR-10a-5p was upregulated under hypoxic exposure and suggested to be responsible for regulating cell survival<sup>111,114</sup>. In primary murine adipocytes, elevated miR-10a5p facilitated cell cycle and interfered with fat deposition<sup>115</sup>. Given this controversy, the implications of the observed increase of ssc-miR-10a-5p during reoxygenation in the brain cells remain unclear and require further investigation.

**Conclusions, limitations and outlook.** The present study using isolated primary cells demonstrated that differences in mitochondrial tolerance and transcriptional regulation underlie the greater sensitivity of the brain cells to H-R stress compared with the skeletal muscle cells (Fig. 6). At the mitochondrial level, H-R stress led to a loss of the ATP synthesis capacity in the brain cells whereas in the skeletal muscle the ATP synthesis capacity was preserved and the forward flux through Complex I enhanced after short-term H-R exposure. The impaired mitochondrial aerobic capacity in the brain cells went hand-in-hand with the transcriptional upregulation of anaerobic glycolysis, likely as a compensatory mechanism to cover ATP deficiency. Notably, no elevated ROS efflux (above the respective tissue-specific baseline) was recorded in either cell type during the H-R stress showing that existing cellular antioxidants were sufficient to maintain the normal ROS levels during acute short-term H-R stress in isolated pig cells. Amplex UltraRed (used in the present study) is a highly sensitive probe for ROS and can detect low levels of H<sub>2</sub>O<sub>2</sub> and (in the presence of extraneous SOD) superoxide. Superoxide and H<sub>2</sub>O<sub>2</sub> are considered the main mitochondrial ROS in mammals<sup>116</sup>, but we cannot exclude production of other types of reactive oxygen species (such as singlet oxygen) during reoxygenation that cannot be detected by Amplex UltraRed. It is also worth noting that the present study used short-term (15 min) exposure of the cells to hypoxia followed by 10 min of reoxygenation, and longer exposures might induce stronger mitochondrial response and result in greater damage in the studied cell types.

The key elements of oxygen sensing (including HIF-1 and/or PHD) were transcriptionally modulated by H-R stress in both studied cell types, but the downstream effects differed between the brain and the skeletal muscle cells. In the muscle cells, mitochondrial ETS as well as protective mechanisms (including antioxidants and mitochondrial quality control) were transcriptionally unregulated, whereas in the brain cells apoptosis and inflammation pathways were the most responsive indicating cell damage. Although our results cannot be generalized for other tissues due to the tissue-specific differences in hypoxia response, we speculate that the transcriptional regulation of the various pathways observed in our study might be applicable to other tissues with similar levels of hypoxia tolerance including sensitive (e.g. heart) or tolerant (e.g. vascular muscle, liver and kidney) tissues. These pathways can be further explored to fully understand the mechanisms associated with hypoxia tolerance across functionally different tissues. Furthermore, identification of the pathways modulated in the tolerant but not in the sensitive tissues might open avenues for future clinical interventions that mimic the tolerant molecular phenotype and desensitize tissues to H-R stress. Unexpectedly, our study showed transcriptional upregulation of multiple pathways related to metabolism, antioxidant defense and stress survival caused by the isolation of the muscle and brain cells from the respective tissues. This might be due to the improved nutrient and oxygen delivery to isolated cells suspended in the substrate-enriched media *ex situ*. These findings demonstrate that



**Figure 6.** Summary of the observed mitochondrial and transcriptomic changes in response to acute H-R stress in the brain and the muscle cells of the pigs. Mitochondrial functions were assessed only after reoxygenation. mRNA and miRNA profiles were measured after hypoxia (blue arrows) and reoxygenation (red arrows). Downward arrows show a decrease, upwards arrows—increase, horizontal arrows—no change. Single arrows—moderate change, double arrows—strong change.

assessment of the transcriptional shifts between the tissues and isolated cells might serve as a sensitive marker for the deviation of the phenotype of isolated primary cells from the native *in situ* state and can be used in future studies as a measure of the preservation of the native metabolic phenotype during isolation of the primary cells.

### Data availability

The data that supports the findings of this study are available in the supplementary material of this article. Additional data such as metadata can be obtained from the corresponding authors (mRNA and miRNA data: Dr. Siriluck Ponsuksili, ponsuksili@fhn-dummerstorf.de; mitochondrial data: Dr. Inna Sokolova inna.sokolova@uni-rostock.de) upon request.

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## Author contributions

L.A.—Investigation, Data Curation, Validation, Formal analysis, Visualization, Writing—Original Draft. E.P.S.—Methodology, Investigation, Data Curation, Validation, Writing—Review & Editing. K.W.—Methodology, Resources, Writing—Review & Editing, I.M.S and S.P.—Conceptualization, Methodology, Validation, Resources, Data Curation, Writing—Review & Editing, Supervision, Project administration, Funding acquisition.

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# Intrinsic Mechanisms Underlying Hypoxia-Tolerant Mitochondrial Phenotype During Hypoxia-Reoxygenation Stress in a Marine Facultative Anaerobe, the Blue Mussel *Mytilus edulis*

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Hypoxia is common in marine environments and a major stressor for marine organisms inhabiting benthic and intertidal zones. Several studies have explored the responses of these organisms to hypoxic stress at the whole organism level with a focus on energy metabolism and mitochondrial response, but the intrinsic mitochondrial responses that support the organelle's function under hypoxia and reoxygenation (H/R) stress are not well understood. We studied the effects of acute H/R stress (10 min anoxia followed by 15 min reoxygenation) on mitochondrial respiration, production of reactive oxygen species (ROS) and posttranslational modifications (PTM) of the proteome in a marine facultative anaerobe, the blue mussel *Mytilus edulis*. The mussels' mitochondria showed increased OXPHOS respiration and suppressed proton leak resulting in a higher coupling efficiency after H/R stress. ROS production decreased in both the resting (LEAK) and phosphorylating (OXPHOS) state indicating that *M. edulis* was able to prevent oxidative stress and mitochondrial damage during reoxygenation. Hypoxia did not lead to rearrangement of the mitochondrial supercomplexes but impacted the mitochondrial phosphoproteome including the proteins involved in OXPHOS, amino acid- and fatty acid catabolism, and protein quality control. This study indicates that mussels' mitochondria possess intrinsic mechanisms (including regulation via reversible protein phosphorylation) that ensure high respiratory flux and mitigate oxidative damage during H/R stress and contribute to the hypoxia-tolerant mitochondrial phenotype of this metabolically plastic species.

**Keywords:** bioenergetics, mitochondria, respiration, oxidative stress, proteomics, posttranslational modification (PTM), bivalve, supercomplexes

## INTRODUCTION

Oxygen deficiency (hypoxia) is a major stressor for aerobic eukaryotes that depend on oxygen to drive mitochondrial ATP synthesis. For these organisms, oxygen fluctuations are stressful due to the limitations of energy supply during hypoxia, as well as the oxidative stress and high costs of recovery during reoxygenation (Garbarino et al., 2015; Lesnefsky et al., 2017; Sokolova et al., 2019). Oxygen is rarely limiting in terrestrial habitats (except underground burrows and high altitudes) but in aquatic environments oxygen fluctuations are common. Many benthic marine organisms – particularly sessile species from the habitats where oxygen availability varies regularly and predictably such as the intertidal zone – have adapted to survive under the fluctuating oxygen conditions. The ecological success of these species is often linked with their ability to transition to anaerobiosis and enter the metabolically depressed state during hypoxia (Hochachka and Mustafa, 1972; Guppy et al., 1994; Hochachka and Somero, 2002). The metabolic rate depression in facultative anaerobes involves complex regulatory mechanisms that ensure a concomitant decrease in ATP consumption and production, onset of anaerobic glycolysis, and changes in membrane properties to maintain ion homeostasis with minimum energy input (Hochachka and Mustafa, 1972; Hochachka and Mommsen, 1983; Hochachka and Guppy, 1987; Buck and Hochachka, 1993; Guppy et al., 1994; Storey and Storey, 2004; Storey, 2015). However, the metabolically arrested state allows only time-limited survival, and to continue the life cycle, the organisms depend on the return of oxygen. Therefore, tolerance to frequent oxygen fluctuations requires maintenance of the mitochondrial integrity and rapid restoration of the energetic and redox homeostasis during reoxygenation (Honda et al., 2005; Pamerter, 2014; Pell et al., 2016; Sokolova et al., 2019).

Recent studies indicate that mitochondria of hypoxia-tolerant organisms are capable of preserving the mitochondrial integrity and function during hypoxia-reoxygenation (H/R) stress via rapid suppression and restoration of oxidative phosphorylation (OXPHOS) subject to oxygen availability, mitochondrial proteome reorganization to limit production of reactive oxygen species (ROS), and upregulation of mitochondrial quality control pathways (Buck and Pamerter, 2006; Pamerter, 2014; Pamerter et al., 2016; Bundgaard et al., 2019; Sokolov et al., 2019; Steffen et al., 2020). These changes are observed during long-term (hours to days) exposures of the organisms to hypoxia and subsequent reoxygenation *in vivo* (Kurochkin et al., 2008; Ivanina et al., 2010, 2011, 2012, 2016; Pamerter et al., 2016; Steffen et al., 2020; Ouillon et al., 2021) and likely reflect a complex integrated response encompassing the direct impact of the oxygen fluctuations on the mitochondria, indirect effects of the changes in intracellular milieu, responses driven by retrograde signaling to the nucleus, as well as endocrine and/or neural regulation (Lee et al., 2020; Du et al., 2021). This complexity, while physiologically relevant and important, makes it difficult to disentangle the contributions of different mechanisms to the functional changes of the mitochondria during H/R stress. Therefore, a reductionist approach using a simplified and thus more tractable system (such as the isolated

mitochondria) could provide useful insights. Mitochondria are semi-autonomous organelles that possess their own DNA and protein translation machinery, as well as a dedicated system of quality control and post-translational regulatory mechanisms (McBride et al., 2006) making them capable of direct responses to H/R transitions independently of the rest of the cell. The functional changes during acute H/R exposures of isolated mitochondria provide evidence for such intrinsic mitochondrial regulation (Onukwufor et al., 2014, 2016, 2017; Sappal et al., 2015), but the molecular mechanisms of this regulation have not yet been investigated.

The intrinsic mitochondrial responses to H/R transitions must rely on post-translational regulatory mechanisms such as the changing levels of the metabolic intermediates, shifts in the state of the electron carriers of the ETS, and post-translational modifications (PTM) of mitochondrial proteins. Among the PTM mechanisms, assembly of the respiratory supercomplexes and reversible protein phosphorylation (catalyzed by mitochondrial phosphatases and kinases) are proposed to play a key role in the modulation of mitochondrial metabolism (Pagliarini and Dixon, 2006; Lapuente-Brun et al., 2013; Chaban et al., 2014; Mathers and Staples, 2019). Mitochondrial supercomplexes (SC) involve a close association between complexes CI, CIII, and CIV as a potential mechanism to channel electron flux, increase the efficiency of OXPHOS and mitigate ROS generation (Guerra-Castellano et al., 2018). The functional consequences of SC rearrangement are debated, but recent studies showed links between SC stability and plasticity and metabolic responses to stress in animals (Bundgaard et al., 2018, 2020a; Falfushynska et al., 2020). Furthermore, reversible phosphorylation of the proteins of the mitochondrial complexes CI and CIV can contribute to the regulation of the mitochondrial function during H/R stress by modulating ETS activity, protein-protein and protein-lipid interactions, and ROS production (Helling et al., 2012; Gowthami et al., 2019; Kalpage et al., 2019; Mathers and Staples, 2019). Stress-induced shifts in phosphoproteome and modulation of the activity of protein phosphatases and kinases have been shown in marine mollusks exposed to H/R stress (Sokolov et al., 2019; Falfushynska et al., 2020); however, these studies have been conducted during the whole-organism exposures to H/R stress, which does not allow discerning the intrinsic mitochondrial mechanisms of the observed changes. Overall, the potential contribution of the supercomplex rearrangement and reversible protein phosphorylation to hypoxia-tolerant mitochondrial phenotype has not been well studied in facultative anaerobes and warrants further investigation.

The mussels of the genus *Mytilus* are widespread facultative anaerobes with ecological and economical importance (Astorga, 2014). Their habitats (including the intertidal zone, eutrophicated estuaries, and coastal habitats) are characterized by recurrent oxygen fluctuations, hence they are highly adapted to these conditions (Gosling, 1992). The energy metabolism pathways of *Mytilus* spp. are well characterized (Gosling, 1992; Babarro et al., 2007; Connor and Gracey, 2011), and the genomes and transcriptomes of several *Mytilus* species have been recently published (Martino et al., 2019; Paterno et al., 2019;

Li et al., 2020) making these organisms an excellent model to study the molecular and physiological mechanisms of mitochondrial responses to H/R stress. Our present study aimed to elucidate the potential autonomous regulatory mechanisms (i.e., those independent of the cellular metabolic context and regulatory mechanisms) of mitochondrial responses to H/R stress in the blue mussels *Mytilus edulis*. We hypothesized that isolated mitochondria of a hypoxia-tolerant facultative anaerobe, *M. edulis*, possess intrinsic mechanisms that permit maintaining the respiratory flux and ATP synthesis and minimize ROS production during acute H/R stress. We also hypothesized that posttranslational mechanisms such as the rearrangement of supercomplexes and reversible protein phosphorylation would be involved in the intrinsic mitochondrial response to H/R transitions. To test these hypotheses, we investigated the direct effects of hypoxia and reoxygenation on the respiration rate under phosphorylating conditions (measured as the OXPHOS activity), proton leak and ROS generation in the isolated mitochondria from the digestive gland of *M. edulis*. Furthermore, we determined the abundance of the supercomplexes (formed by associations between CI, CIII, and CIV) and shifts in the mitochondrial phosphoproteome in the isolated mitochondria of *M. edulis* exposed to H/R stress *in vitro*. The results of this study shed light on the metabolic plasticity of mitochondrial function and demonstrate autonomous mitochondrial mechanisms involved in adaptive metabolic responses to oxygen fluctuations in the hypoxia-tolerant facultative anaerobe *M. edulis*.

## MATERIALS AND METHODS

### Animal Collection and Maintenance

Experimental blue mussels *Mytilus edulis* were collected in Warnemünde harbor in the Baltic Sea near Rostock, Germany. The animals were acclimated at salinity 15 and 15°C for a week before the experiment. The acclimation temperature and salinity were close to the habitat conditions at the time of collection. During the acclimation, the mussels were continuously fed *ad libitum* with Premium Reef Blend (CoralSands, Wiesbaden, Germany).

### Mitochondrial $\dot{M}O_2$ and ROS Production

Mitochondria were isolated from the mussels' digestive gland as described elsewhere (Haider et al., 2018; Sokolov et al., 2019). The digestive gland was chosen as one of the most metabolically active organs and a main site of energy storage in mussels (Gosling, 1992). The osmolarity of isolation and assay buffers were optimized in pilot experiments to achieve high quality of mitochondrial isolations (assessed by the respiratory control ratio and cytochrome c test of mitochondrial integrity) for the studied *M. edulis* population (data not shown). Briefly, 0.5–1.2 g tissue was homogenized on ice in a glass-Teflon homogenizer containing 10 ml of isolation buffer (~630 mOsm) containing 100 mM sucrose, 200 mM KCl, 50 mM NaCl, 30 mM HEPES pH 7.5, and 8 mM EGTA supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and 2  $\mu$ g/ml

aprotinin). Homogenate was centrifuged at 4°C and 2000  $\times$  g for 5 min, the supernatant was collected and centrifuged at 8000  $\times$  g for 8 min. The resulting mitochondrial pellet was resuspended in isolation buffer and kept on ice. All assays commenced within 5 min of mitochondrial isolation.

Mitochondrial respiration rate ( $\dot{M}O_2$ ) and ROS production were measured simultaneously in the high-resolution respirometer Oxygraph 2 k (Oroboros, Innsbruck, Austria) at 15 and 25°C using the DatLab6 software. The assay temperatures were chosen to reflect the acclimation temperature (15°C) typical for early summer at the study site (June average monthly surface water temperature ~14.8°C), and the elevated temperature close to the summer maximum (25°C). Mitochondrial suspension was added to the instrument's chamber filled with 2 ml of assay buffer (~525 mOsm) containing 30 mM HEPES pH 7.2, 185 mM sucrose, 130 mM KCl, 10 mM NaCl, buffer, 10 mM glucose, 1 mM  $MgCl_2$ , 10 mM  $KH_2PO_4$ , and 1% fatty acid-free bovine serum albumin (BSA). The final mitochondrial protein concentrations in the assay chamber were ~1–1.5 mg ml<sup>-1</sup>. Because earlier studies in bivalves (Sokolov and Sokolova, 2019; Haider et al., 2020) and mammals (Hansen et al., 2010; Wang et al., 2016) showed that a common intracellular osmolyte, taurine, might improve mitochondrial function, we tested the potential mitoprotective effect of taurine in the mussel mitochondria. For this, assay buffer was supplemented either with 50 mM taurine or 50 mM glycine. Glycine is the most abundant free amino acid in oyster tissues (Haider et al., 2020) and was used to distinguish taurine-specific effects on mitochondria (if any) from non-specific effects of amino acid addition. After addition of mitochondrial suspension and stabilization of the polarographic oxygen sensor, the following substrate–uncoupler–inhibitor titration (SUIT) protocol was used: 1) 5 mM pyruvate, 0.5 mM malate to spark Complex I-driven respiration (LEAK I); 2) 10 mM succinate to stimulate Complex II (LEAK I + II); 3) 2.5 mM ADP to stimulate OXPHOS; 4) 2.5  $\mu$ M oligomycin to inhibit mitochondrial  $F_0F_1$ -ATPase followed by titration (in 0.5  $\mu$ M steps) with the uncoupler carbonyl cyanide-chlorophenyl hydrazine (CCCP) to measure maximum ETS activity. ROS production was measured in parallel with  $\dot{M}O_2$  using the Oxygraph-2K LED2 module with Fluorescent Sensor Green (525 nm) and Amplex Red (Thermo Fisher Scientific, Waltham, MA, United States) as described elsewhere (Ouillon et al., 2021). Addition of cytochrome C (0.8  $\mu$ M) in the OXPHOS state (Gnaiger, 2012) increased mitochondrial respiration by <10% indicating integrity of isolated mitochondria (data not shown). The LEAK state  $\dot{M}O_2$  indicates the proton leak rate of resting (non-phosphorylating) mitochondria,  $\dot{M}O_2$  of ADP-stimulated mitochondria corresponds to the OXPHOS capacity, and CCCP-uncoupled  $\dot{M}O_2$  reflects the maximum ETS capacity. Respiratory control ratio was calculated by dividing OXPHOS by LEAK I + II respiration (Estabrook, 1967).

To test for the effects of H/R stress on mitochondrial respiration and ROS production, steps 1–3 (up to and including ADP addition) of the above-described SUIT protocol were carried out to measure the pre-hypoxic (baseline) LEAK I + II and OXPHOS oxygen consumption and ROS production rates. The OXPHOS respiration continued until all oxygen in the

chamber was consumed, and mitochondria were held in anoxia for 10 min. The chamber was then opened for reoxygenation (2.5–5 min to achieve >80% of air saturation), closed and the post-reoxygenation OXPHOS oxygen consumption rate was measured. Next, 2.5  $\mu\text{M}$  of oligomycin was added to record oxygen consumption in the post-reoxygenation LEAK state (LEAK I + II). At the end of the assay, 0.5  $\mu\text{M}$  of rotenone and 2.5  $\mu\text{M}$  of antimycin A were added to assess non-mitochondrial respiration (<10% of the total oxygen consumption rate, data not shown). The effects of H/R stress on the oxygen consumption rates were tested at 15 and 25°C, and ROS production at 25°C due to low ROS generation rates at 15°C.

The impact of H/R stress on mitochondrial ETS activity was measured in CCCP-uncoupled mitochondria at 15°C using pyruvate (5 mM), succinate (10 mM), or the mixture of pyruvate and succinate as substrates. Malate (0.5 mM) was added to spark the respiration. Mitochondria were allowed to respire until all oxygen was consumed in the chamber, kept in anoxia for 10 min, and re-oxygenated to measure the post-hypoxic ETS activity.

After completion of the assays, an aliquot of each mitochondrial suspension was lysed in hypoosmotic solution using three freezing-thawing cycles followed by sonication, and mitochondrial protein content was measured according to Bradford using a commercial kit (Merck KGaA, Darmstadt, Germany) with BSA as a standard. Respiration rates were expressed in  $\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$  protein and ROS production in  $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}$  protein.

## Isolation of Phosphoproteins for LC/MS Analysis

Isolation of mitochondrial phosphoproteins was done using a chromatography approach with the Pro-Q® Diamond Phosphoprotein Enrichment Kit (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's recommendations. Briefly, 40–60 mg of mitochondrial suspension was lysed with a proprietary Invitrogen's buffer, diluted to 0.1–0.2 mg  $\text{ml}^{-1}$  protein and applied to a prewashed chromatographic column. After the lysate passed through the column, the column was intensively washed to remove unbound proteins, followed by elution of an enriched fraction of phosphoproteins. The phosphoproteins were concentrated using several rounds of centrifugation with Vivaspin concentrators (Sartorius, Göttingen, Germany). Samples were stored at  $-80^\circ\text{C}$  in 25 mM Tris, pH 7.5, 0.25% CHAPS until further analyses.

## LC/MS Analysis

Sample preparation for mass spectrometric analysis of enriched *M. edulis* mitochondrial phosphoproteins was performed as described in detail previously (Sokolov et al., 2019). Five biological replicates per condition including normoxic controls (i.e., isolated mitochondria prior to H/R stress) and mitochondria exposed to reoxygenation following 10 min of experimental anoxia *in vitro* were prepared for MS analysis. In short, protein extracts were concentrated to 15  $\mu\text{l}$ , mixed with sample buffer [200 mM Tris-HCl pH 6.8, 50 mM EDTA pH 8.0, 40% glycerol, 8% SDS, 0.08% bromophenol blue, 1 mM dithiothreitol

(DTT)], denatured at 90°C for 10 min, and briefly separated in 10% SDS mini gels. After Coomassie staining, the protein-containing gel areas (upper 10 mm of each gel band) were excised, dried in a vacuum centrifuge, reduced (20 mM DTT, 30 min, 55°C, 800 rpm), alkylated (40 mM iodoacetamide, 30 min, room temperature in the dark), and dehydrated (100% acetonitrile HPLC grade, 2 min, room temperature). Proteins were then digested (4  $\mu\text{g}/\mu\text{l}$  trypsin solution, 37°C, 16 h), and peptides were purified by Stage Tipping, adjusted to 2  $\mu\text{g}/\mu\text{l}$  and frozen at  $-80^\circ\text{C}$  until analysis. Purified peptides were analyzed by reversed phase liquid chromatography (LC) electrospray ionization (ESI) MS/MS. Thus, an EASY nLC 1000 (Thermo Fischer Scientific, Bremen, Germany) was coupled to a QExactive mass spectrometer (Thermo Fischer Scientific, Bremen, Germany). Peptides were loaded onto in-house self-packed fused silica columns (100  $\mu\text{m} \times 20 \text{ cm}$ ) containing reverse-phase C18 material (ReproSil-Pur 120-AQ 3.0  $\mu\text{m}$ , Dr. Maisch GmbH, Ammerbuch, Germany). Peptides were eluted using a non-linear binary gradient of 166 min from 2 to 99% solvent B [0.1% (v/v) acetic acid in acetonitrile] in solvent A [0.1% (v/v) acetic acid] at a constant flow rate of 300 nl/min. The full scan was recorded with a mass range from 300 - 1650 m/z and a resolution of 70,000 at 200 m/z. The 15 most abundant ions were isolated with an isolation width of 2 Th and fragmented by higher-energy collisional dissociation (HCD) at a normalized collision energy (NCE) of 27.5. Fragment ion spectra were recorded with a resolution of 17,500 at 200 m/z. Ions with unassigned charge states as well as charge 1 and higher than 6 were excluded from fragmentation. Fragmented ions were excluded from fragmentation for 30 s. Lock mass correction was enabled.

## MS Data Analysis

MS/MS spectra were extracted and analyzed using the Sorcerer Sequest software (v5.1.1, Sage-N Research) and a combined target-decoy database. As a dedicated complete *M. edulis* genome sequence was not yet available at the time, we constructed the database from all available related *Mytilus* species. To this end, all NCBI protein entries listed for the taxon “Mytilus” (77,824 proteins on 09/08/2020; **Supplementary Table 1**) were retrieved and clustered at 97% identity with Cd-Hit (Li and Godzik, 2006) to remove redundant proteins. A set of common laboratory contaminants was added and all sequence entries were reversed and appended to the database as decoy sequences (to allow for false discovery rate (FDR) calculation), resulting in a total of 127,890 sequences in the database. MS/MS-based peptide and protein identifications of all 10 samples were merged and evaluated with Scaffold version 4.8.4<sup>1</sup>, with the clustering feature enabled. Protein level FDR and peptide level FDR were set to 1%. A minimum of 2 peptides was required for protein identification. For semi-quantitative analysis and comparison of relative protein abundances between samples, normalized spectral abundance factors (NSAF) were calculated using total spectrum counts (Zybailov et al., 2006). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the

<sup>1</sup><http://www.proteomesoftware.com>

PRIDE partner repository (Vizcaíno et al., 2016) with the dataset identifier PXD027955 and 10.6019/PXD027955.

## Blue Native PAGE

Mitochondrial membrane proteins from isolated *M. edulis* mitochondria were separated by Blue Native PAGE (BN-PAGE) as previously described (Bundgaard et al., 2018, 2020a). Membrane proteins were extracted with 1% dodecyl maltoside (DDM) or 3 g g<sup>-1</sup> protein digitonin on ice for 15 or 5 min, respectively. Mouse mitochondrial membrane proteins extracted with 1% DDM were used as a size marker to identify the *M. edulis* bands. In-gel complex I activity was assessed as a purple stain after incubation for 30 min at room temperature with 1 mg/mL nitroblue tetrazolium with 0.1 μM NADH (Greggio et al., 2017; Bundgaard et al., 2020a). The gels were scanned using a Bio-Rad ChemiDoc MP imager and analyzed using the Bio-Rad Image Lab band detection software (Bio-Rad, Hercules, CA, United States). The intensity of the supercomplex band was expressed relative to the intensity of the strong complex V (ATP synthase) band to control for loading differences.

## Statistics

The significance of the effects of amino acid addition or H/R stress on the studied mitochondrial traits was tested using paired Student's *t*-test. The effects of H/R stress on the abundance of mitochondrial supercomplexes were assessed using unpaired *t*-test. Statistical analyses were conducted using GraphPad Prism v. 7.02 (GraphPad Software Inc., La Jolla, CA, United States) software. All differences were considered significant if the probability of Type II error was <0.05. Testing for statistically significant differences in protein abundance between hypoxia and control samples in the phosphoproteome NSAF data set was performed with Perseus (Tyanova and Cox, 2018) using a *p*-value-based Welch's *t*-test (*p*-value threshold 0.05) after imputation of missing values.

## RESULTS

### Effects of Amino Acids and Temperature on Mitochondrial Functions

Addition of 50 mM taurine or glycine had no significant effect on mitochondrial oxygen consumption or ROS production regardless of the temperature (Table 1). Therefore, amino acid addition has not been considered in the further analyses of the effects of temperature and H/R stress on the studied mitochondrial traits.

Elevated temperature (25°C) led to an increase in mitochondrial respiration and ROS emission in the LEAK and OXPHOS states (Figure 1). Likewise, ETS activity was higher at 25°C than at 15°C (Figure 1). Q<sub>10</sub> for the mitochondrial oxygen consumption was 2.2–2.5 regardless of the mitochondrial activity state. Q<sub>10</sub> for ROS emission was 2.4–2.5 in the LEAK state and 1.6 in the OXPHOS state.

The rate of the ROS emission was higher in the LEAK state compared with the OXPHOS state regardless of the temperature

(Table 1 and Figure 1). At both 15 and 25°C, mitochondria in the LEAK state converted ~5.4–5.9% of consumed oxygen into H<sub>2</sub>O<sub>2</sub> when respiring on pyruvate. When a mixture of pyruvate and succinate was used, the fraction of consumed O<sub>2</sub> converted to H<sub>2</sub>O<sub>2</sub> in the LEAK state decreased to ~3.6–3.8%. Mitochondria in the OXPHOS state respiring on pyruvate and succinate converted ~1.3–1.9% of consumed O<sub>2</sub> into H<sub>2</sub>O<sub>2</sub>.

### Effects of H/R Stress on Mitochondria Functional Traits and Supercomplex Formation

Effects of H/R stress on mitochondrial oxygen consumption were similar at 15 and 25°C showing suppression of LEAK respiration, elevated OXPHOS activity and increased RCR after H/R stress (Figure 2). ETS activity was elevated after H/R stress in mitochondria respiring on succinate but not in those using pyruvate or pyruvate-succinate mixture (Figure 3).

To improve the assay sensitivity and decrease the time for aerobic-anaerobic transition, all subsequent analyses (including ROS emission, supercomplex formation and phosphoproteomics) were conducted in mitochondria respiring at 25°C where the mitochondrial activity was higher. At 25°C, ROS emission in the LEAK state was strongly (by 56%) suppressed after the H/R exposure, whereas ROS emission in the OXPHOS state was not significantly affected by the H/R stress (Figure 4). The rate of the electron leak (measured as a ratio of H<sub>2</sub>O<sub>2</sub> production to O<sub>2</sub> consumption) decreased by 49 and 28% in the LEAK and OXPHOS state mitochondria, respectively, after the H/R stress (Figure 4C). The LEAK state mitochondria converted ~3.6% of consumed O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub> under the control conditions, but only ~1.9% after the H/R stress (Figure 2C). In the OXPHOS state, the mussels' mitochondria converted ~1.3% and <1% of consumed O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub> under before and after the H/R stress, respectively (Figure 4C).

Supercomplex (CI-CIII) formation was detected in mussels but it was not affected by exposure of isolated mussel mitochondria to H/R stress (Figure 5). The SC abundance relative to that of complex V was instead dependent on the use of detergent, as found previously in several species (Bundgaard et al., 2020a,b).

### Mitochondrial Phosphoproteome Shifts During the H/R Stress

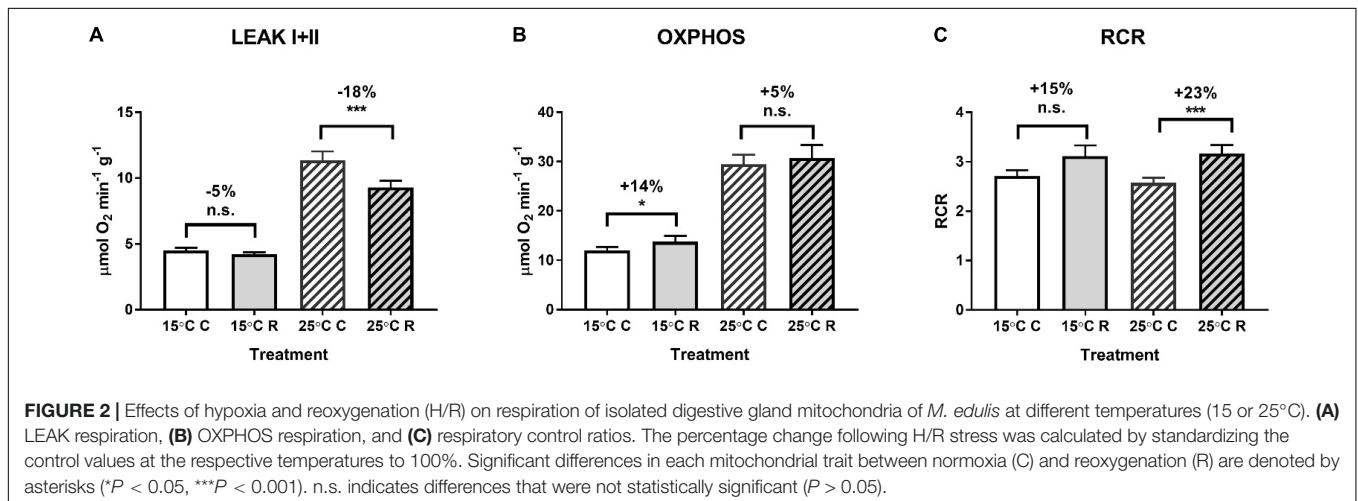
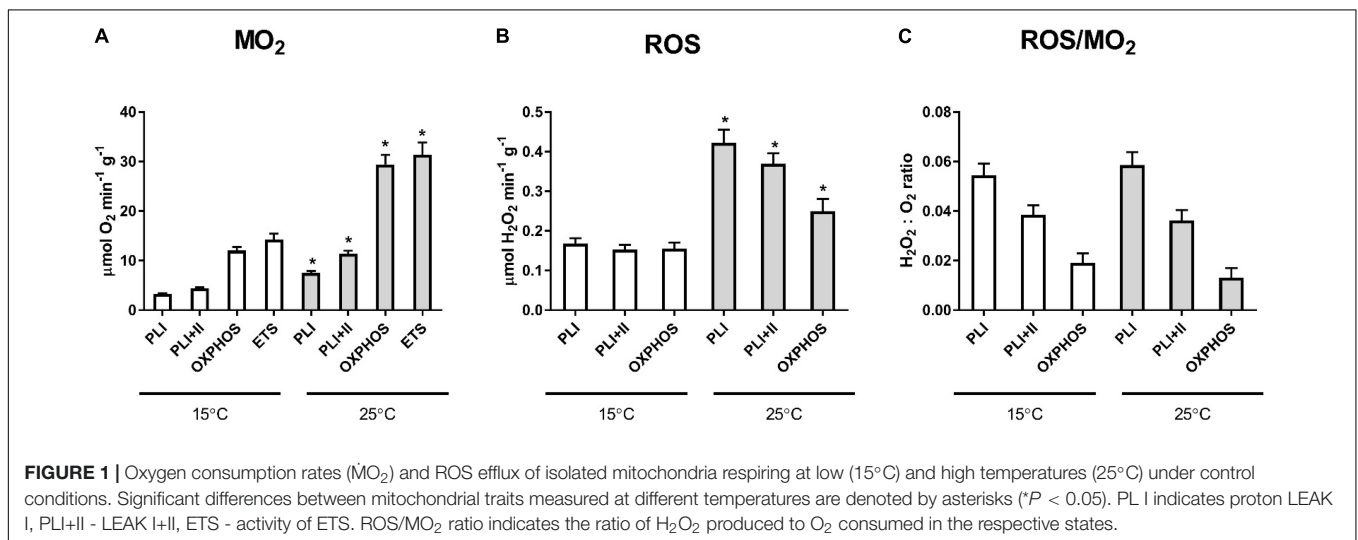
Proteomic analysis identified 169 mitochondrial phosphorylated proteins in the isolated mitochondria of *M. edulis* (Supplementary Table 2) out of which 25 (15%) significantly changed in abundance in response to the H/R stress (Welch's *t*-test, *P* < 0.05; Table 2; Supplementary Table 2). Of these, 16 phosphorylated proteins showed an increase in abundance, whereas abundance of 9 phosphorylated proteins decreased.

Among the phosphoproteins upregulated during the H/R stress, two (the NDUFA1 subunit of the mitochondrial Complex I and A-kinase anchoring protein 1) showed an “off-on” response and were found exclusively in the samples exposed to the H/R stress but not in normoxia (Table 2). Furthermore, eight more phosphorylated proteins showed a large increase in abundance

**TABLE 1** | Effect of amino acid addition (50 mM taurine or glycine) on oxygen consumption ( $\dot{M}_{O_2}$ ) and ROS efflux of isolated mitochondria of *M. edulis*.

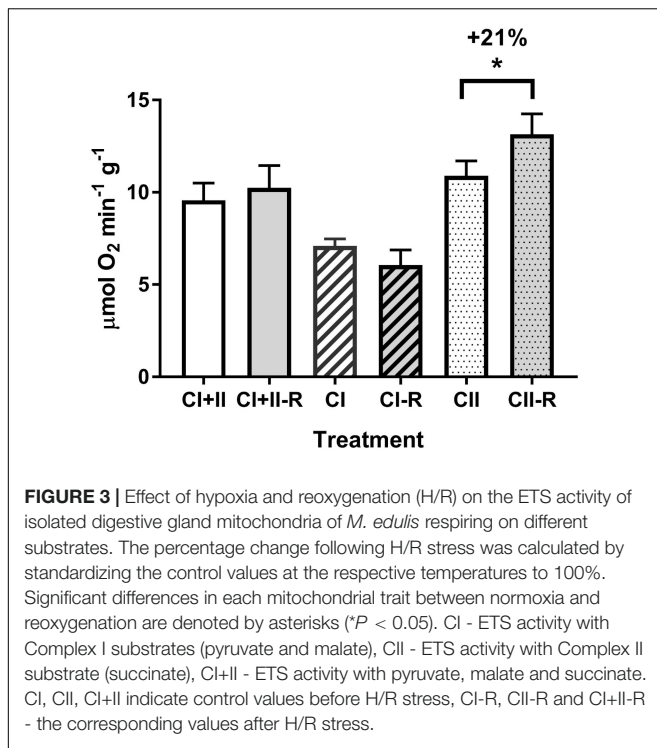
Temperature		$\dot{M}_{O_2}$ LEAK I		$\dot{M}_{O_2}$ LEAK I + II		$\dot{M}_{O_2}$ OXPHOS		$\dot{M}_{O_2}$ ETS		ROS LEAK I		ROS LEAK I + II		ROS OXPHOS	
		15°C	25°C	15°C	25°C	15°C	25°C	15°C	25°C	15°C	25°C	15°C	25°C	15°C	25°C
Control	Mean	3.26	7.59	4.42	11.38	11.73	28.64	14.06	30.40	0.18	0.43	0.17	0.36	0.17	0.24
	SEM	0.20	0.51	0.31	0.85	0.93	2.57	1.54	3.60	0.02	0.04	0.02	0.03	0.02	0.03
	N	17	13	17	13	17	13	12	13	15	13	15	13	15	13
Taurine	Mean	3.29	7.78	4.48	11.79	12.58	32.08	15.21	34.65	0.17	0.38	0.16	0.33	0.15	0.17
	SEM	0.23	0.73	0.33	1.12	1.04	3.00	2.01	4.03	0.02	0.06	0.02	0.05	0.02	0.03
	N	13	8	13	8	13	8	8	8	12	8	12	8	12	8
Glycine	Mean	3.47	7.11	4.68	10.66	11.92	27.38	13.06	28.67	0.10	0.49	0.10	0.44	0.12	0.40
	SEM	0.41	1.08	0.71	1.77	2.51	5.19	3.03	4.88	0.01	0.08	0.01	0.09	0.02	0.10
	N	4	5	4	5	4	5	4	5	4	5	4	5	4	5

Paired *t*-test with Bonferroni correction showed no significant differences between the control and amino acid-treated mitochondria ( $P > 0.05$ ). Respiration rates were expressed in  $\mu\text{mol O}_2 \text{ min}^{-1} \text{ g}^{-1}$  protein, and  $\text{H}_2\text{O}_2$  production in  $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}$  protein.



after the H/R stress including kynurenine/ $\alpha$ -aminoadipate aminotransferase (7.1-fold increase), propionyl-CoA carboxylase  $\beta$  chain (4.9-fold), cytochrome c oxidase assembly protein (3.8-fold), Golgi phosphoprotein 3 (2.9-fold), Bax inhibitor

1 (2.7-fold), the catalytic subunit of mtDNA polymerase  $\gamma$  (2.4-fold), phosphoenolpyruvate carboxykinase (PEPCK) (2.2-fold) and arginine kinase (1.9-fold) (Table 2). Other upregulated mitochondrial phosphoproteins (involved in the



ETS, TCA cycle, amino acid metabolism, and protein processing) showed a 10–70% increase in abundance in response to H/R exposure (Table 2).

Among the phosphorylated mitochondrial proteins that were significantly less abundant after the H/R stress, two proteins (mitochondrial metalloendopeptidase OMA1 and NADH:ubiquinone oxidoreductase subunit AB1) showed an “on-off” response (i.e. were not detected in the phosphorylated fraction after the H/R stress), and two (glutaryl-CoA-dehydrogenase and hydroxyacid-oxoacid transhydrogenase) showed a 2.5-fold decrease in the abundance after the H/R stress (Table 2). Six phosphorylated proteins including the NDUFB11 subunit of the mitochondrial Complex I, F-type H<sup>+</sup>-transporting ATPase subunits  $\gamma$  and  $f$ , mitochondrial ribosomal protein S22, and two enzymes involved in the intermediary metabolism [ $\Delta(3,5)$ - $\Delta(2,4)$ -dienoyl-CoA isomerase, and 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase] showed a modest but statistically significant decrease (by 30–40%) in abundance after the H/R stress (Table 2). Two more proteins showed large but statistically non-significant changes in the abundance of the phosphorylated form after H/R stress, including translocase of outer membrane 20 (TOM20) that was not found in the mitochondrial phosphoproteome after H/R stress, and reticulon-4-interacting protein that showed a >5-fold decrease in phosphorylated form abundance (Supplementary Table 2).

## DISCUSSION

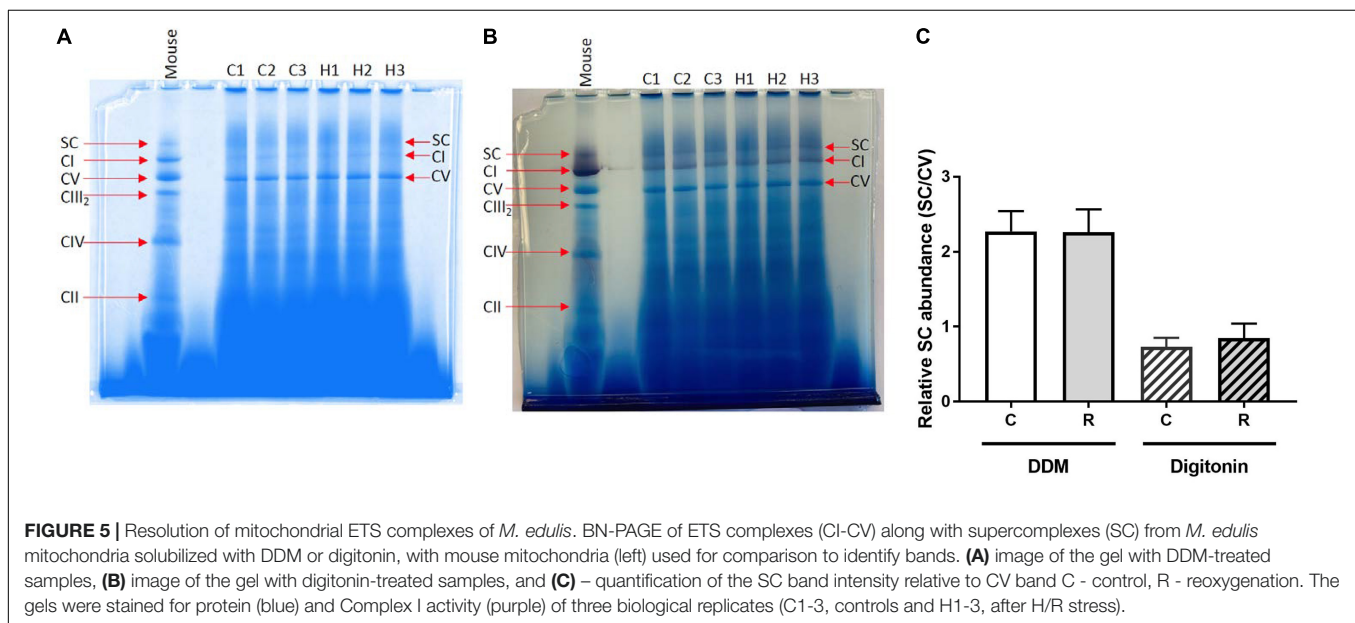
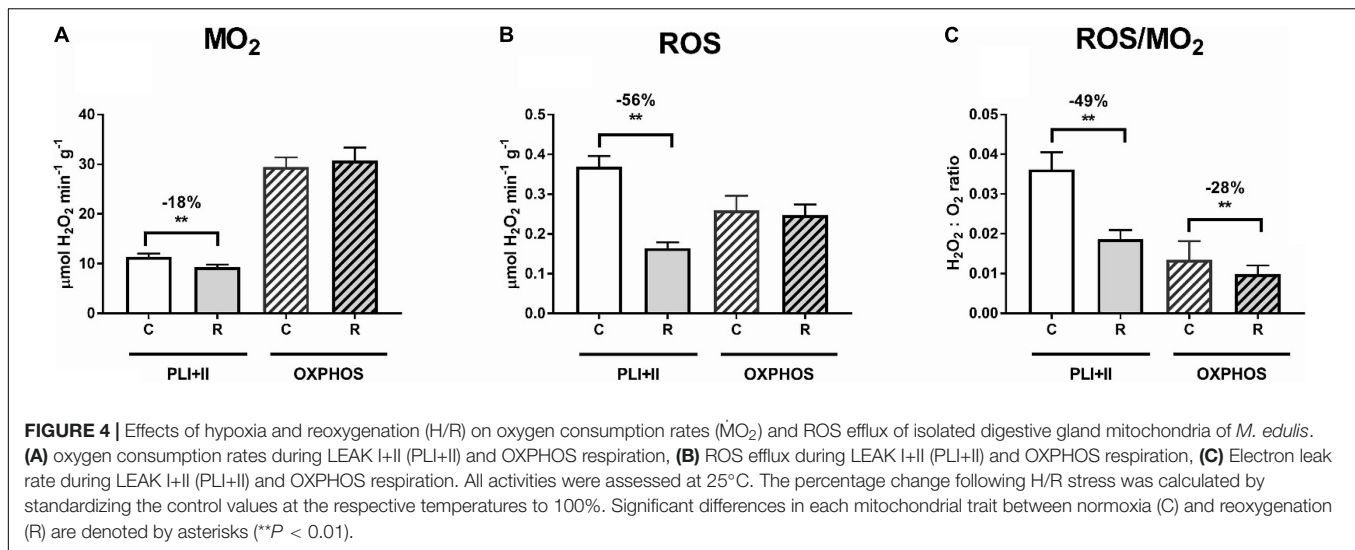
Mussels’ mitochondria show robust coupling and 2.2–2.5-fold increase in both LEAK and OXPHOS respiration during a

temperature increase from 15 to 25°C. Elevated temperature led to higher ROS efflux in the mussels mitochondria proportional to the increase in MO<sub>2</sub> so that electron leak rate (i.e., the fraction of consumed oxygen converted to ROS) did not change during warming. Interestingly, the ROS emission in the OXPHOS state was less temperature sensitive ( $Q_{10} = 1.6$ ) than ROS emission of the resting (LEAK state) mitochondria ( $Q_{10} = 2.4$ – $2.5$ ). Such thermally robust mitochondrial function might reflect adaptations to fluctuating temperature regimes in this typical intertidal species that colonized the subtidal zone of the Baltic Sea about 10,000 years ago (Śmietanka et al., 2014).

## H/R Stress Improves Mitochondrial Efficiency and Suppresses ROS Formation

Mitochondrial oxygen consumption is a driving force of the electron flux in the ETS and ATP production by OXPHOS, as well as a protective mechanism against oxygen toxicity (Sung et al., 2010). As the major oxygen consumers and ROS producers, mitochondria are key targets of H/R stress. Mitochondrial tolerance to intermittent hypoxia requires the ability to maintain high respiration flux while avoiding excessive ROS production during oxygen fluctuations (Sokolova et al., 2019). Our present study indicates that the mitochondria of the blue mussels *M. edulis* show features consistent with the hypoxia-tolerant phenotype. Thus, isolated mitochondria of *M. edulis* exposed to H/R stress *in vitro* showed a 5–18% decrease in proton leak rate and a 5–14% increase in OXPHOS activity resulting in higher coupling efficiency of mitochondria. Mitochondrial proton leak causes uncoupling of oxygen consumption from ATP synthesis and must be compensated by consumption of additional oxygen and substrates to maintain the activity of ETS proton pumps and prevent depolarization of the mitochondrial membrane. Therefore, lower proton leak during reoxygenation in the mussel mitochondria can contribute to improved ATP synthesis efficiency and decrease energy costs of mitochondrial maintenance. Furthermore, an increase in the OXPHOS rate after reoxygenation indicates improved ATP synthesis capacity in the mussel mitochondria. This increase appears largely driven by improved capacity of the mitochondrial ETS to utilize succinate whereas the ability to oxidize NADH-linked substrates such as pyruvate is not affected. Succinate is a common anaerobic end product in marine bivalves that accumulates in high concentrations during hypoxia (Hochachka and Mustafa, 1972; Bayne, 2017). High succinate oxidation capacity might be metabolically beneficial during post-hypoxic recovery in mussels helping to generate ATP with a highly abundant substrate (succinate) and restore normal levels of metabolic intermediates. Elevated OXPHOS and ETS capacity after H/R stress might thus assist in restoring of energy homeostasis after hypoxia and contribute to the increase in oxygen consumption during post-hypoxic recovery (so called “oxygen debt”) commonly found in hypoxia-tolerant invertebrates and fish (Ellington, 1983; Lewis et al., 2007; Vismann and Hagerman, 2008).

Exposure of isolated mussels’ mitochondria to H/R stress strongly modulated mitochondrial ROS emission, which is



considered a main driver of H/R-induced mitochondrial stress and injury (Honda et al., 2005; Cadenas, 2018). In hypoxia-sensitive organisms such as terrestrial mammals, post-hypoxic reoxygenation commonly leads to a burst of ROS production causing damage to mitochondrial membrane and proteins and suppression the ETS activity and ATP production (Honda et al., 2005; Venditti et al., 2013; Cadenas, 2018). The damage to ETS further enhances electron leak and ROS production resulting in a vicious cycle of ROS-induced ROS release (Zorov et al., 2006). Unlike this detrimental pattern commonly observed in mammals, mitochondria of *M. edulis* suppressed electron leak in response to H/R stress. The overall rate of ROS emission and the electron leak rate were strongly (by ~56 and ~49%, respectively) suppressed in the LEAK state mitochondria of mussels after H/R stress. The ROS emission rate in the OXPPOS state did not significantly decrease after

H/R stress, but the opposing effects of H/R stress on the oxygen consumption (i.e., a slight increase) and ROS emission (a slight decrease) combined to produce a significant (by ~28%) suppression of the electron leak rate in the OXPPOS state mitochondria. These changes in the ROS emission and electron leak rates induced by the H/R stress can prevent oxidative stress and mitochondrial damage during reoxygenation and might reflect adaptation of the mussels to frequent fluctuation in the oxygen conditions experienced in intertidal and estuarine habitats. From the perspective of oxidative stress mitigation, suppression of the ROS emission in the LEAK state, where electron leak rates and ROS generation rates are high due to the high mitochondrial membrane potential (Miwa and Brand, 2003; Lambert and Brand, 2004), might play an especially important protective role during H/R stress in mussel mitochondria.

**TABLE 2** | List of the mitochondrial phosphoproteins that significantly changed in abundance in response to the exposure of the isolated mitochondria of *M. edulis* to H/R stress *in vitro* according to a *p*-value-based Welch's *t*-test (*p*-value threshold 0.05) performed in Perseus.

Accession number	Protein	Function	Molecular weight (kDa)	Fold change in H/R
<b>Phosphorylation increased during the H/R stress</b>				
CAC5370403.1	NADH:ubiquinone oxidoreductase subunit AB1	Electron transport, ETS Complex I	22	INF
CAC5410092.1	A-kinase anchoring protein 1	Reversible protein phosphorylation, anchoring of the PKA regulatory subunits	78	INF
CAC5372201.1	Kynurenine/alpha-aminoadipate aminotransferase	Amino acid metabolism	47	7.1
CAC5394308.1	Propionyl-CoA carboxylase beta chain	Fatty acid and amino acid catabolism	29	4.9
CAC5385545.1	Cytochrome c oxidase assembly protein	ETS Complex IV assembly	29	3.8
CAC5412268.1	Golgi phosphoprotein 3	Control of Golgi localization, activated by mtDNA depletion	33	2.9
AGK88248.1	Bax inhibitor-1	Apoptosis	27	2.7
CAC5369661.1	mtDNA polymerase $\gamma$ , catalytic subunit	mtDNA synthesis	121	2.4
OPL21205.1	PEPCK	Anaerobic glycolysis	22	2.2
CAC5356853.1	Arginine kinase	Phosphagen metabolism	61	1.9
CAC5415815.1	Mitochondrial inner membrane protease ATP23	Assembly of the mitochondria $F_0F_1$ -ATPase	31	1.7
ALX27209.1	NADH:Ubiquinone oxidoreductase beta subcomplex subunit 6	Electron transport, ETS Complex I	25	1.6
CAC5371863.1	Malate dehydrogenase	TCA cycle	34	1.4
CAC5400267.1	NADH:Ubiquinone Oxidoreductase Core Subunit S2	Electron transport, ETS Complex I	54	1.3
CAC5389134.1	Peptidase, Mitochondrial Processing Subunit Beta (PMPCB)	Mitochondrial protein import and maturation	57	1.3
CAC5380024.1	3-methylcrotonyl-CoA carboxylase subunit $\alpha$	Amino acid (L-Leu) and fatty (isovaleric) acid catabolism	79	1.2
<b>Phosphorylation decreased in response to H/R stress</b>				
CAC5393689.1	OMA1 metalloendopeptidase	Protein quality control, mitochondria stress response	55	INF
CAC5377560.1	Glutaryl-CoA dehydrogenase	Amino acid (L-Lys, L-hydroxylysine and L-Try) catabolism	70	2.5
CAC5388599.1	Hydroxyacid-oxoacid transhydrogenase	Alcohol dehydrogenase, glutamate catabolism	32	2.5
CAC5410310.1	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11	Electron transport, ETS Complex I	20	1.4
CAC5403955.1	Mitochondrial ribosomal protein S22	Mitochondrial protein synthesis	22	1.4
CAC5419341.1	2-methoxy-6-polyprenyl-1,4-benzoquinol methylase	Ubiquinone biosynthesis	37	1.4
CAC5396165.1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	Fatty acid $\beta$ -oxidation	34	1.3
CAC5379931.1	F-type $H^+$ -transporting ATPase subunit $\gamma$	ATP synthesis	32	1.3
CAC5392483.1	F-type $H^+$ -transporting ATPase subunit f	ATP synthesis	19	1.3

Fold changes were calculated from NSAF values in Scaffold. INF – indicates an “on-off” response where a phosphorylated protein was exclusively found in the normoxic samples but not in those exposed to H/R stress, or vice versa. Accession numbers refer to the sequences from the NCBI reference genomes (*M. coruscus* or *M. edulis*) used for protein identification.

Earlier studies using exposures to H/R *in vivo* (e.g., in whole organisms) or *in situ* (e.g., during ischemia-reperfusion of organs, tissues, or intact cells) also show robust mitochondrial ETS and OXPHOS function in hypoxia-tolerant species contrasting the loss of OXPHOS capacity and suppressed ETS activity

commonly observed in hypoxia-tolerant species (Pamenter, 2014; Sokolova, 2018; Cheng et al., 2021). Thus, OXPHOS respiration and ETS activity were greatly impaired after H/R exposure in hypoxia-sensitive organisms like rainbow trout (*Oncorhynchus mykiss*) (Onukwufor et al., 2014, 2016, 2017), bay scallops

(*Argopecten irradians*) (Ivanina et al., 2016) and mammals (Paradis et al., 2016; Lesnefsky et al., 2017). A decrease in the mitochondrial OXPHOS rates was also found in isolated rainbow trout mitochondria (Onukwufor et al., 2014) and in permeabilized heart fibers of a hypoxia-sensitive shovelnose ray (*Aptychotrema rostrata*) (Hickey et al., 2012) after exposure to H/R stress *in vitro*. Mitochondrial ETS and OXPHOS rates of hypoxia-tolerant organisms such as freshwater turtles, fish, and benthic and intertidal marine bivalves (clams, oysters, mussels) remained constant or increased after H/R exposure *in vivo* (Hickey et al., 2012; Cook et al., 2013; Galli and Richards, 2014; Du et al., 2016; Ivanina et al., 2016; Gerber et al., 2019; Napolitano et al., 2019; Ouillon et al., 2021) but not after anoxic exposure in freshwater turtles, where mitochondrial respiration was suppressed (Galli et al., 2013; Bundgaard et al., 2018, 2020a). Similarly, an increase in OXPHOS activity was found in isolated mitochondria of hypoxia-tolerant zebrafish (*Danio rerio*) after H/R exposure *in vitro* (Napolitano et al., 2019) but a decrease in OXPHOS was found after *in vivo* prolonged hypoxia of zebrafish in permeabilized skeletal muscle fibers (Cadiz et al., 2019). Taken together, our present findings and earlier published research indicate that, although different responses to hypoxia may take place in some vertebrate species (Pamenter, 2014; Pamenter et al., 2016, 2018; Bundgaard et al., 2020b), in bivalves the mitochondrial phenotype associated with hypoxia tolerance involves the ability to maintain or enhance OXPHOS capacity, mitigate ROS emission and prevent collapse of the ETS during reoxygenation, and that these mitochondrial traits are partially independent of cellular or organismal regulatory mechanisms.

### Mitochondrial Proteome Rearrangements During H/R Stress: Dynamics of Supercomplexes

Supercomplexes are dynamic structures consisting of different combinations of complexes involved in the ETS system that can undergo rearrangement in response to varying physiological or environmental conditions including hypoxia (Ramírez-Aguilar et al., 2011; Timón-Gómez and Barrientos, 2020). In hypoxia-exposed mammalian mitochondria, CI was reversibly deactivated (Galkin et al., 2009) likely due to dissociation of the large supercomplexes containing CI, CIII, and CIV, although the capacity of the individual respiratory complexes was intact (Rosca et al., 2008). In plants, SCs were observed to dissociate and reassemble during H/R stress as a result of cellular acidification (Ramírez-Aguilar et al., 2011). However, our present study in mussels as well as recent research in hypoxia-tolerant freshwater turtles (Bundgaard et al., 2018) shows that the dynamic rearrangement of SCs is not involved in response to hypoxia in these species. In *M. edulis*, H/R stress did not influence the SCs' abundance (Figure 5), and stability of CIIICIII<sub>2</sub> SC during H/R stress was reported in freshwater turtles (Bundgaard et al., 2018). The functional significance of SC rearrangement remains debatable (Signes and Fernandez-Vizcarra, 2018; Timón-Gómez and Barrientos, 2020), and the role of SC stability in maintaining robust ETS activity or in limiting ROS during oxygen fluctuations in hypoxia-tolerant organisms requires further investigation. It is

worth noting that SC of ectotherms appear generally more stable than those of mammals, regardless of the species-specific hypoxia tolerance (Bundgaard et al., 2020a,b). Thus, it is possible that the stability of SCs in the mitochondria of mussels observed in our present study is a shared ectotherm trait rather than adaptation to hypoxic stress.

### Phosphoproteome Shifts

Reversible phosphorylation is emerging as a pivotal posttranslational modification (PTM) mechanism involved in the regulation of mitochondrial functions (Pagliarini and Dixon, 2006; Helling et al., 2012). The steadily increasing numbers of reported mitochondrial kinases, phosphatases, and phosphoproteins imply an important role of protein phosphorylation in different mitochondrial processes, including responses to H/R stress (Goldenthal and Marín-García, 2004; Horbinski and Chu, 2005). Mitochondrial proteome phosphorylation patterns and their role in the responses to H/R stress are less well studied in hypoxia-tolerant organisms including mollusks. Differential phosphorylation of proteins associated with OXPHOS, Krebs cycle, and intermediary metabolism has been reported during H/R exposure *in vivo* in the hypoxia-tolerant Pacific oyster, *C. gigas* (Sokolov et al., 2019). Furthermore, H/R exposure *in vivo* modulated activities of protein kinases A and C in the mitochondria of *M. edulis*, *C. gigas*, and *Arctica islandica* (Falfushynska et al., 2020). Our present study demonstrates that reversible protein phosphorylation is also involved in the intrinsic mitochondrial responses to H/R stress that act independently of the cellular context during *in vitro* exposures to fluctuating oxygen conditions. The pathways regulated by reversible phosphorylation during H/R stress in isolated mussel mitochondria include ETS and OXPHOS (10 out of 25 differentially phosphorylated proteins), amino acid and fatty acid metabolism (6 proteins), mitochondrial protein and mtDNA homeostasis (5 proteins), and substrate-level phosphorylation (2 proteins). It is worth noting that in our present study, the change in the protein abundance in the phosphoprotein fraction after H/R stress was interpreted as a change in the phosphorylation state of the respective protein due to PTM. In principle, changes in the abundance of a certain phosphoprotein might also reflect changes in the total protein abundance in the mitochondria due to *de novo* synthesis and/or protein degradation. However, these alternative explanations appear less likely given the cell-free system and the short exposure times (10 min in anoxia followed by immediate sample collection after reoxygenation) used in the present study.

Of note, H/R exposure of isolated mussels' mitochondria led to a major increase in abundance of phosphorylated A-kinase anchoring protein 1 (AKAP1). AKAP1 is a scaffold protein that recruits protein kinase A (PKA) and other signaling proteins, as well as RNA, to the outer mitochondrial membrane (Liu et al., 2020). AKAP1 acts as a signaling hub regulating metabolic homeostasis and mitochondrial quality control and is involved in response to H/R stress in mitochondria (Merrill and Strack, 2014; Liu et al., 2020). In mammalian models, AKAP1 becomes phosphorylated in response to energy stress via AMP-activated protein kinase (AMPK)-dependent mechanism, and

this phosphorylation is essential for activation of mitochondrial respiration (Liu et al., 2020). Although the mechanisms of AKAP1-dependent mitochondrial regulation are not yet fully unraveled, multiple studies indicate that functional AKAP1 is essential for cell survival during H/R stress and is involved in regulation of PKA activity in mitochondria [review in Merrill and Strack (2014); Liu et al. (2020)]. If similar mechanisms exist in mussel mitochondria, phosphorylation of AKAP1 might indicate an adaptive response to a decrease in ATP levels during H/R stress and positively modulate mitochondrial respiration and PKA recruitment. The latter hypothesis is also consistent with an important role of PKA in regulating the mitochondrial ETS activity in mussels (Falfushynska et al., 2020).

Mitochondrial Complex I is an important target for PTM-dependent regulation during H/R stress in animals including mammals (Gowthami et al., 2019) and mollusks (Falfushynska et al., 2020). Complex I activity is often down-regulated in response to H/R stress as a mechanism to suppress mitochondrial ROS generation by reversible electron transport (RET) (Pell et al., 2016); however, the role of reversible protein phosphorylation in this regulation is not known. In marine bivalves including mussels, Complex I activity is responsive to experimental manipulation of the phosphorylation status (Falfushynska et al., 2020). In *M. edulis*, PKA-dependent phosphorylation led to an increase in Complex I activity whereas PKC-dependent phosphorylation had no effect (Falfushynska et al., 2020). Unspecific dephosphorylation (by cerium oxide) suppressed Complex I activity (Falfushynska et al., 2020). Our present study strongly indicates that H/R stress affects phosphorylation status of several Complex I subunits in *M. edulis*. Thus, the subunit NDUFB1 of Complex I was exclusively phosphorylated in the mitochondrial samples exposed to H/R, and the abundance of the phosphorylated forms of NDUFS2 and NDUFB6 increased by 30–60% after the H/R stress. In contrast, the abundance of the phosphorylated NDUFB11 decreased by 40% after the H/R stress. Functional implications of PTM of these subunits are not known in *M. edulis*. Interestingly, the subunit NDUFB1 that is exclusively phosphorylated in the mussels after H/R stress is known to play a protective role during H/R stress in mammalian mitochondria supporting high respiratory flux, stabilizing supercomplexes and mitigating ROS generation (Hou et al., 2019). It is tempting to speculate that a similar protective mechanism mediated by the PTM of NDUFB1 subunit might be in play in the mussels' mitochondria during H/R stress, but this hypothesis requires further investigation.

It is worth noting that mitochondrial Complex IV was not affected by PTM during H/R stress in the mussels' mitochondria. In mammals, Complex IV is considered a key regulatory target during hypoxia whose activity and oxygen sensitivity is modulated by PTM (including reversible phosphorylation) (Prabu et al., 2006; Kadenbach, 2021) and differential expression of hypoxia-specific subunits (Kocha et al., 2015). In *M. edulis*, Complex IV can also be modulated by experimental phosphorylation/dephosphorylation, albeit to a lesser extent than Complex I (Falfushynska et al., 2020). In marine mollusks including *M. edulis*, cytochrome c oxidase is not considered rate-limiting due to the high apparent excess capacity

of this enzyme relative to the maximum ETS activity (Sokolov et al., 2019; Sokolov and Sokolova, 2019; Ouillon et al., 2021). Furthermore, oxygen affinity of cytochrome c oxidase does not appear modulated by H/R stress in marine bivalves (Ouillon et al., 2021). This indicates that cytochrome c oxidase might be less important as a regulatory target of ETS during H/R stress in mollusks. Consistent with this notion, none of the Complex IV subunits were identified as differentially phosphorylated during H/R stress in our present analysis. Interestingly, H/R stress led to a major increase (by ~3.8-fold) of the phosphorylated form of cytochrome c oxidase assembly protein COX11. This protein acts as copper chaperone delivering  $\text{Cu}^{2+}$  to the active center of cytochrome c oxidase and might be involved in mitigation of ROS generation in mitochondria (Radin et al., 2015). It is possible that elevated phosphorylation levels of this protein might affect redox properties or  $\text{Cu}^{2+}$  binding affinity of this protein; however, at present the functional implications of COX11 PTM remain speculative.

H/R stress resulted in a modest (by ~30%) but statistically significant decrease in the abundance of phosphorylated subunits  $\gamma$  and f of the mitochondrial  $F_0$ ,  $F_1$ -ATPase in the mussels and a ~70% increase in abundance of phosphorylated mitochondrial protease ATP23 responsible for  $F_0$ ,  $F_1$ -ATPase assembly. These findings indicate that  $F_0$ ,  $F_1$ -ATPase is another target of regulatory PTM during H/R stress in bivalve mitochondria. Phosphorylation of ATPase subunits (particularly subunit  $\gamma$ ) plays an important role in  $F_0$ ,  $F_1$ -ATPase assembly (Reinders et al., 2007). In yeast, dephosphorylation of subunit  $\gamma$  facilitates oligomerization of  $F_0$ ,  $F_1$ -ATPase enhancing its activity (Reinders et al., 2007). If a similar mechanism exists in mussels, dephosphorylation of subunit  $\gamma$  of  $F_0$ ,  $F_1$ -ATPase would activate  $F_0$ ,  $F_1$ -ATPase through enhanced oligomerization in the inner mitochondrial membrane and might contribute to the observed increase in the OXPHOS activity after the H/R stress in mussels.

Amino acid and fatty acid catabolism in mitochondria represents another key target for regulation through PTM in bivalve mitochondria as shown by differential phosphorylation levels induced by the H/R stress in multiple enzymes involved in the respective pathways. Overall, six enzymes involved in amino acid and fatty acid catabolism appeared differentially phosphorylated in the control and H/R exposed mitochondria of mussels (Table 2). Most of these phosphorylated enzymes showed a modest change in abundance (typically, ~20–40% change compared with the controls), except glutaryl-CoA dehydrogenase (showed a 2.5 decrease in the phosphorylation level) and phosphorylated kynurenine/alpha-aminoacidate aminotransferase and propionyl-CoA carboxylase beta chain that showed a ~–5–7-fold higher abundance after H/R stress. In oysters (*C. gigas*), phosphorylated forms of enoyl-CoA isomerase and acyl-CoA dehydrogenase family enzymes (involved in amino acid and fatty acid catabolism) decreased in abundance after H/R stress *in vivo* (Sokolov et al., 2019). Presently, the functional consequences of reversible phosphorylation of these enzymes are not known but their high representation in differentially expressed mitochondrial phosphoproteome of bivalves after H/R stress indicates the importance of modulation of amino acid and fatty acid metabolism, possibly to facilitate the use of

diverse mitochondrial substrates for ATP production during post-hypoxic recovery.

Mitochondrial protein quality control mechanisms play a key role in maintaining the integrity of the mitochondrial proteome and are commonly regulated during H/R stress at the transcriptional, translational and post-translational levels (Sokolov et al., 2019; Falfushynska et al., 2020; Steffen et al., 2020). Among these proteins, mitochondrial metalloendopeptidase OMA1 appears an important target for PTM. In *M. edulis*, the abundance of phosphorylated OMA1 form was strongly down-regulated in H/R stress, so that no phosphorylated form of OMA1 was found in the mitochondrial proteome after H/R stress. Similarly, in *C. gigas* exposed to hypoxia *in vivo* the abundance of phosphorylated OMA1 decreased ~6-fold in hypoxia, and no phosphorylated OMA1 was found after 1 h of reoxygenation (Sokolov et al., 2019). These findings indicate that dephosphorylation of OMA1 plays an important role in response to H/R stress, particularly the post-hypoxic recovery, in facultative anaerobes such as marine bivalves, and can occur via intrinsic mitochondrial mechanisms. Dephosphorylation of OMA1 in response to low mitochondrial ATP levels might activate this enzyme, thereby fostering mitochondrial fragmentation and suppressing mitochondrial fusion in stress (Baker et al., 2014; Bohovych et al., 2015; Sokolov et al., 2019). Other important proteins involved in the maintenance of mitochondrial proteome showed an apparent change in phosphorylation status after the H/R stress (including the mitochondrial ribosomal protein S22, TOM20 and mitochondrial processing peptidase subunit beta), consistent with the key role of protein homeostasis in mitochondrial stress resistance (Sokolova, 2018). However, the functional implications of PTM of these proteins are not yet known even in model organisms and therefore, no conclusions can presently be drawn with regard to their possible effects on mussel mitochondria.

In conclusion, our study shows that isolated digestive gland mitochondria of the blue mussel *M. edulis* possess intrinsic regulatory mechanisms that improve mitochondrial efficiency and limit ROS generation during hypoxia-reoxygenation transition. These adaptive functional changes are triggered by oxygen fluctuations in isolated mitochondria within minutes and thus reflect autonomous mitochondrial mechanisms that do not require an intact cellular environment or retrograde nuclear signaling. The intrinsic mitochondrial responses to H/R stress are at least in part dependent on the shifts in the mitochondria phosphoproteome including such key pathways as OXPHOS, amino acid and fatty acid catabolism, and protein quality control, but do not appear to involve a change in SC formation. The findings of our present study complement earlier research that showed an important role of PTM in regulating glycolytic metabolic fluxes and substrate-level phosphorylation

(Brooks and Storey, 1995, 1997; Russell and Storey, 1995; Dawson et al., 2013; Lama et al., 2013; Storey, 2015) and highlight the importance of reversible protein phosphorylation as a global metabolic regulatory mechanism during oxygen fluctuations in facultative anaerobes.

## DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaíno et al., 2016) with the dataset identifier PXD027955 and doi: 10.6019/PXD027955.

## AUTHOR CONTRIBUTIONS

ES and AB: methodology, investigation, data curation, validation, formal analysis, and writing - review and editing. LA: data curation, visualization, writing - original draft, and writing - review and editing. SM: data curation, validation, formal analysis, resources, and writing - review and editing. AF: methodology, resources, supervision, and writing - review and editing. DB: methodology, resources, supervision, validation, and writing - review and editing. CH: methodology, investigation, data curation, validation, and writing - review and editing. IS: conceptualization, methodology, validation, formal analysis, resources, supervision, project administration, funding acquisition, writing - original draft, and writing - review and editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.773734/full#supplementary-material>

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## RESEARCH ARTICLE

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

Linda Adzibli<sup>1,2</sup>, Eugene P. Sokolov<sup>3</sup>, Siriluck Ponsuksili<sup>1</sup> and Inna M. Sokolova<sup>2,4,\*</sup>**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<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> to O<sub>2</sub> 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

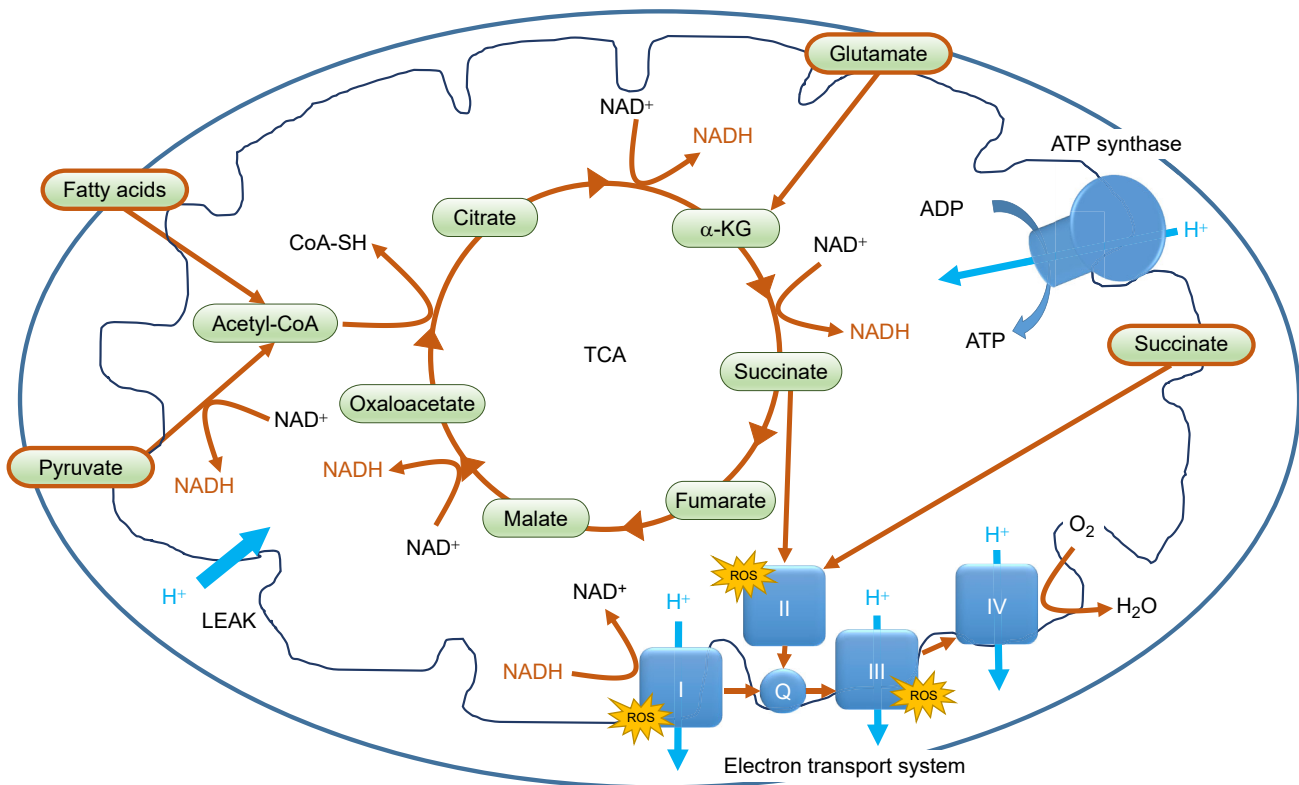
ATP synthesis, whereas reoxygenation can cause a surge in reactive oxygen species (ROS), resulting in oxidative damage to DNA, lipids and proteins (Andrienko et al., 2017). In aerobic organisms, mitochondria represent key targets of hypoxia–reoxygenation (H/R) stress, with mitochondrial damage implicated in the disruption of energy and redox homeostasis, and subsequent cellular injury and death (Honda et al., 2005; Klöner, 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; Pamerter 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; Pamerter 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 Pamerter, 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

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<sub>2</sub> 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<sup>-1</sup> 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.5, 100 mmol l<sup>-1</sup> sucrose, 100 mmol l<sup>-1</sup> NaCl, 200 mmol l<sup>-1</sup> KCl, 8 mmol l<sup>-1</sup> ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 50 µg l<sup>-1</sup> aprotinin, 1 mmol l<sup>-1</sup> phenylmethylsulfonyl fluoride (PMSF), using several passes of a Potter–Elvehjem 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<sup>-1</sup> HEPES pH 7.2, 185 mmol l<sup>-1</sup> sucrose, 10 mmol l<sup>-1</sup> glucose, 130 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> NaCl, 1 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 10 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> 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<sub>2</sub> s<sup>-1</sup> ml<sup>-1</sup>), 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<sup>-1</sup> ADP to achieve ADP-stimulated oxidative phosphorylation (OXPHOS) state; (3) 5 µmol l<sup>-1</sup> 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<sub>2</sub> 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<sup>-1</sup> oligomycin to inhibit mitochondrial F<sub>0</sub>, F<sub>1</sub>-ATPase and measure post-hypoxic LEAK respiration (state 4); (2) 1 µmol l<sup>-1</sup> rotenone to inhibit electron flux through complex I; (3) 2.5 µmol l<sup>-1</sup> antimycin A to inhibit electron flux through complex III; (4) 40 mmol l<sup>-1</sup> 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<sup>-1</sup> palmitoyl-DL-carnitine (PALM), (2) 5 mmol l<sup>-1</sup> pyruvate with 2 mmol l<sup>-1</sup> malate to spark respiration (PM), (3) 10 mmol l<sup>-1</sup> glutamate with 2 mmol l<sup>-1</sup> malate (G); (4) 10 mmol l<sup>-1</sup> succinate (S), (5) 10 mmol l<sup>-1</sup> glutamate, 2 mmol l<sup>-1</sup> malate and 10 mmol l<sup>-1</sup> succinate (GS); (6) 10 mmol l<sup>-1</sup> glutamate, 2.5 mmol l<sup>-1</sup> pyruvate, 2 mmol l<sup>-1</sup> malate and 10 mmol l<sup>-1</sup> 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<sub>2</sub>O<sub>2</sub> 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<sup>-1</sup> Amplex Red, 1 U ml<sup>-1</sup> horseradish peroxidase stock solution, and 5 U ml<sup>-1</sup> superoxide dismutase (SOD) (Makrecka-Kuka et al., 2015). A two-step calibration was conducted with addition of 0.1 µmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> before and after the addition of the mitochondrial suspension. ROS production was measured as the rate of H<sub>2</sub>O<sub>2</sub> 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) ( $\Delta$ <5%,  $P$ >0.05). Therefore, both LEAK state 2 and LEAK state 4

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

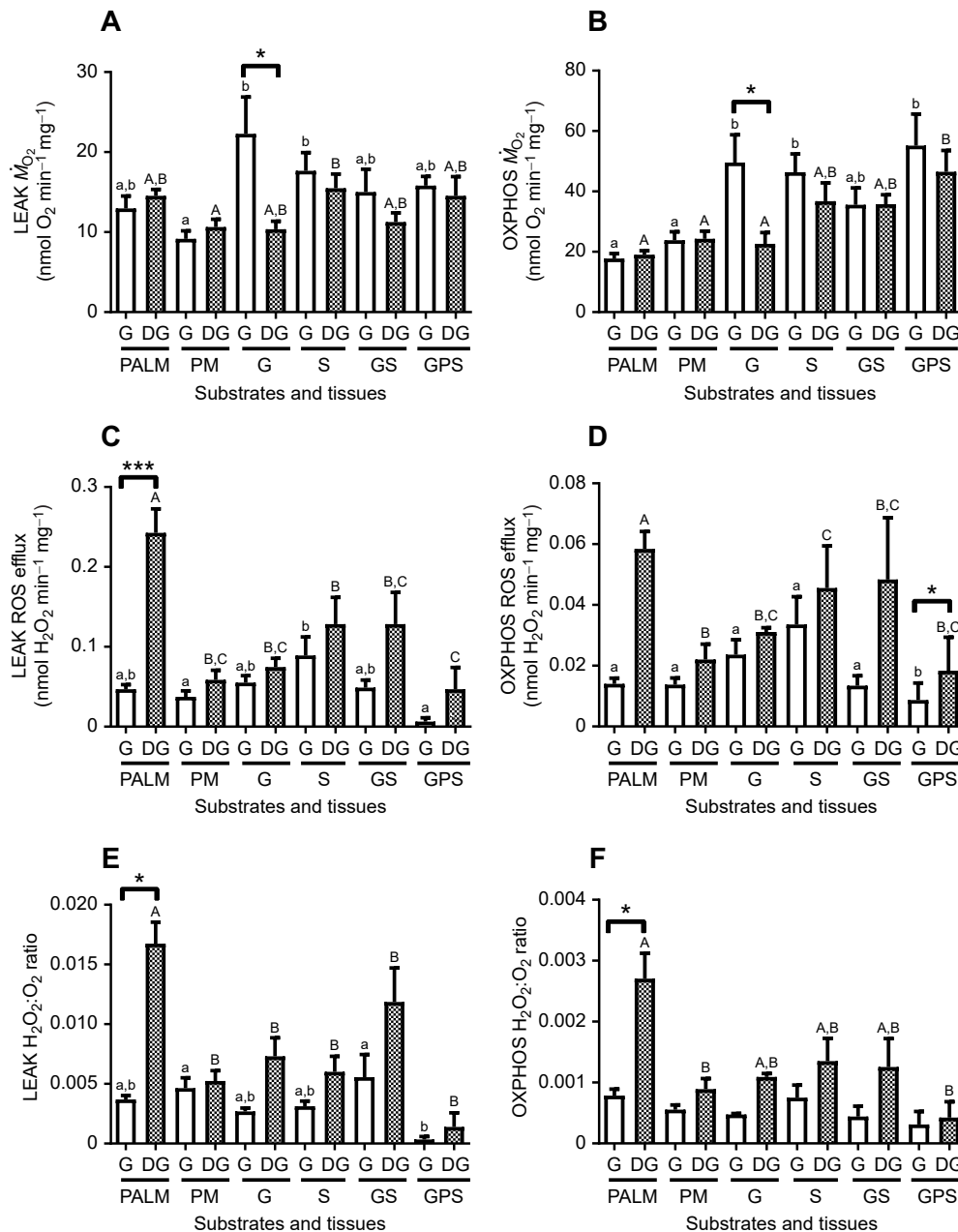
**Table 1. ANOVA: Effects of the tissue, substrate and their interaction on mitochondrial traits of *Crassostrea gigas***

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

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.

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).

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



**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.**

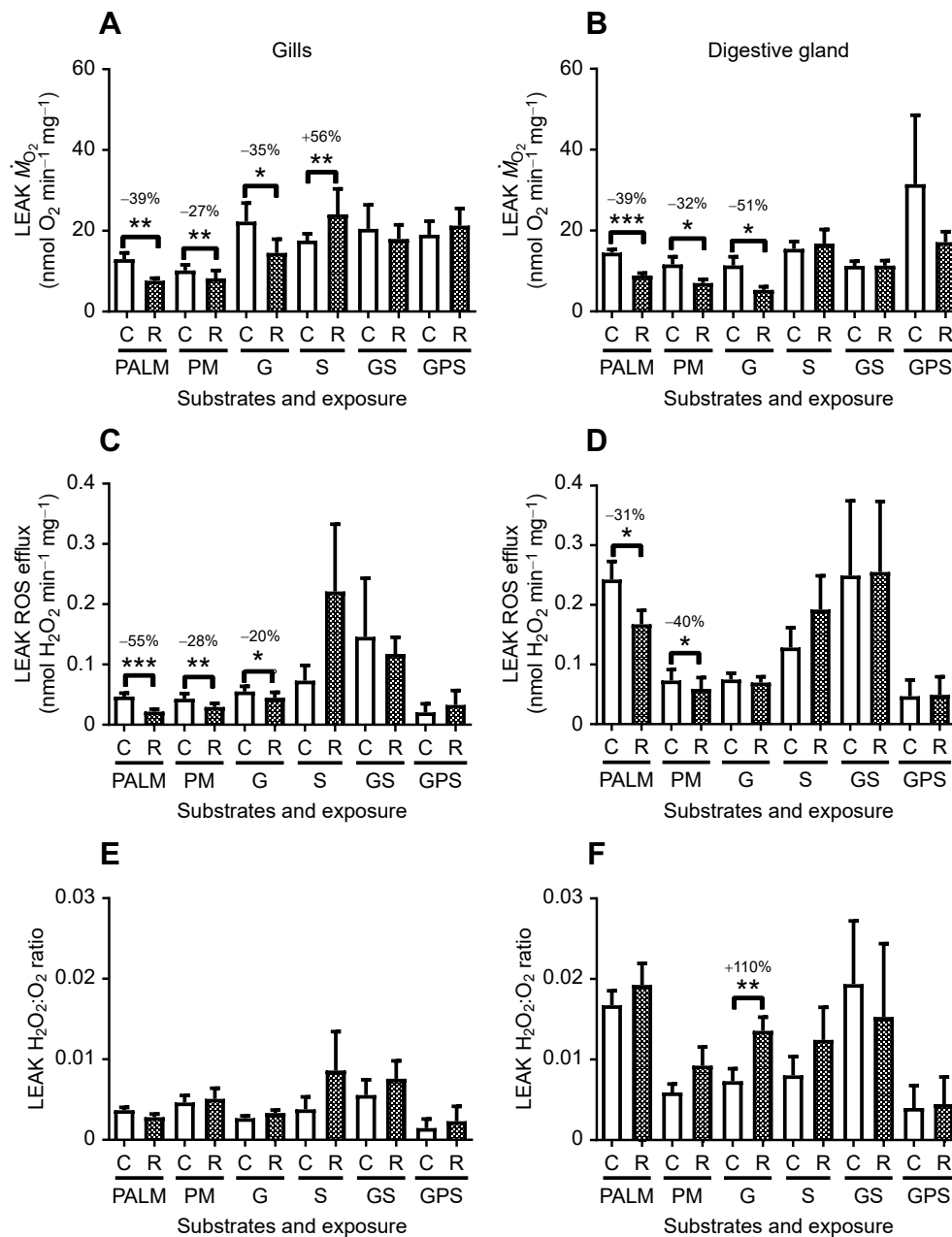
Mitochondria were isolated from the gill (G) or the digestive gland (DG) of *C. gigas* and supplied with palmitate (PALM), pyruvate (PM), glutamate (G), succinate (S), glutamate+succinate (GS) or glutamate, pyruvate and succinate mixture (GPS). (A,C,E) LEAK  $\dot{M}_{O_2}$ , ROS efflux and  $H_2O_2:O_2$  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 to 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 than 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 to 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



**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).

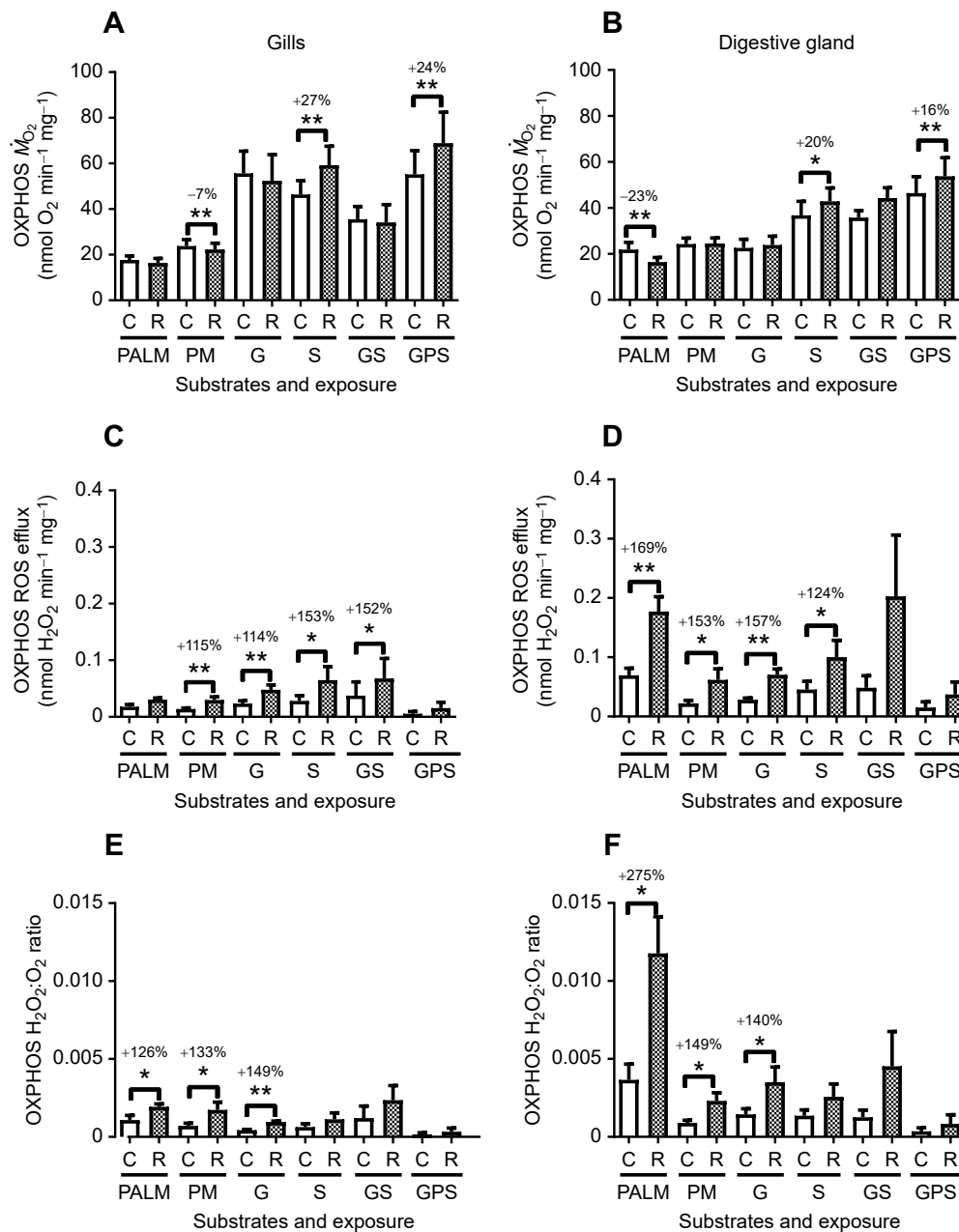
Exposure to H/R stress suppressed OXPHOS rate in the gill mitochondria respiring on pyruvate (by 7%) and in the digestive gland mitochondria respiring on palmitate (by 23%) (Fig. 4A,B). In both studied tissues, H/R stress stimulated mitochondrial OXPHOS respiration driven by succinate (by 20–27%) or 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



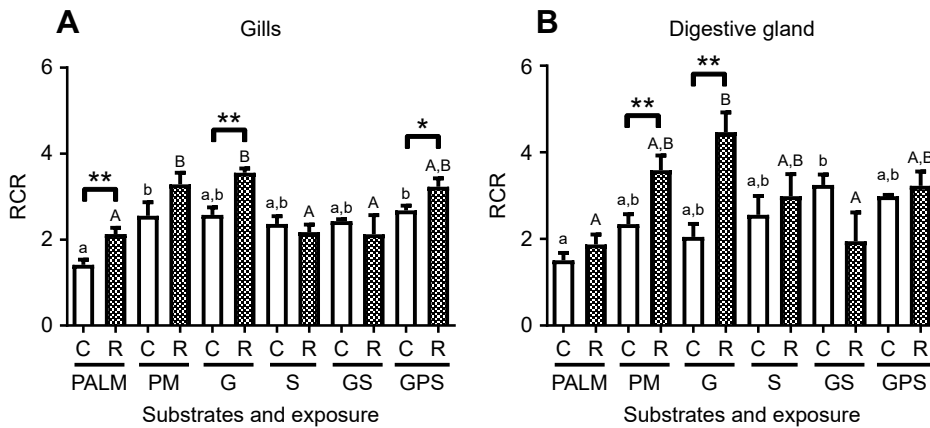
**Fig. 4. Effects of hypoxia/reoxygenation (H/R) on OXPPOS respiration of mitochondria isolated from *C. gigas* respiring on different substrates.** Mitochondria were isolated from the gills (A,C,E) or the digestive glands (B,D,F) of oysters and treated with palmitate (PALM), pyruvate (PM), glutamate (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 OXPPOS state (Figs 6 and 7). After H/R stress, the succinate-driven LEAK and OXPPOS  $\dot{M}O_2$  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 OXPPOS 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 OXPPOS state (Fig. 7E). A similar trend for rotenone-induced suppression of electron leak was found in the gill mitochondria in the OXPPOS 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,



**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  $N = 12$ .

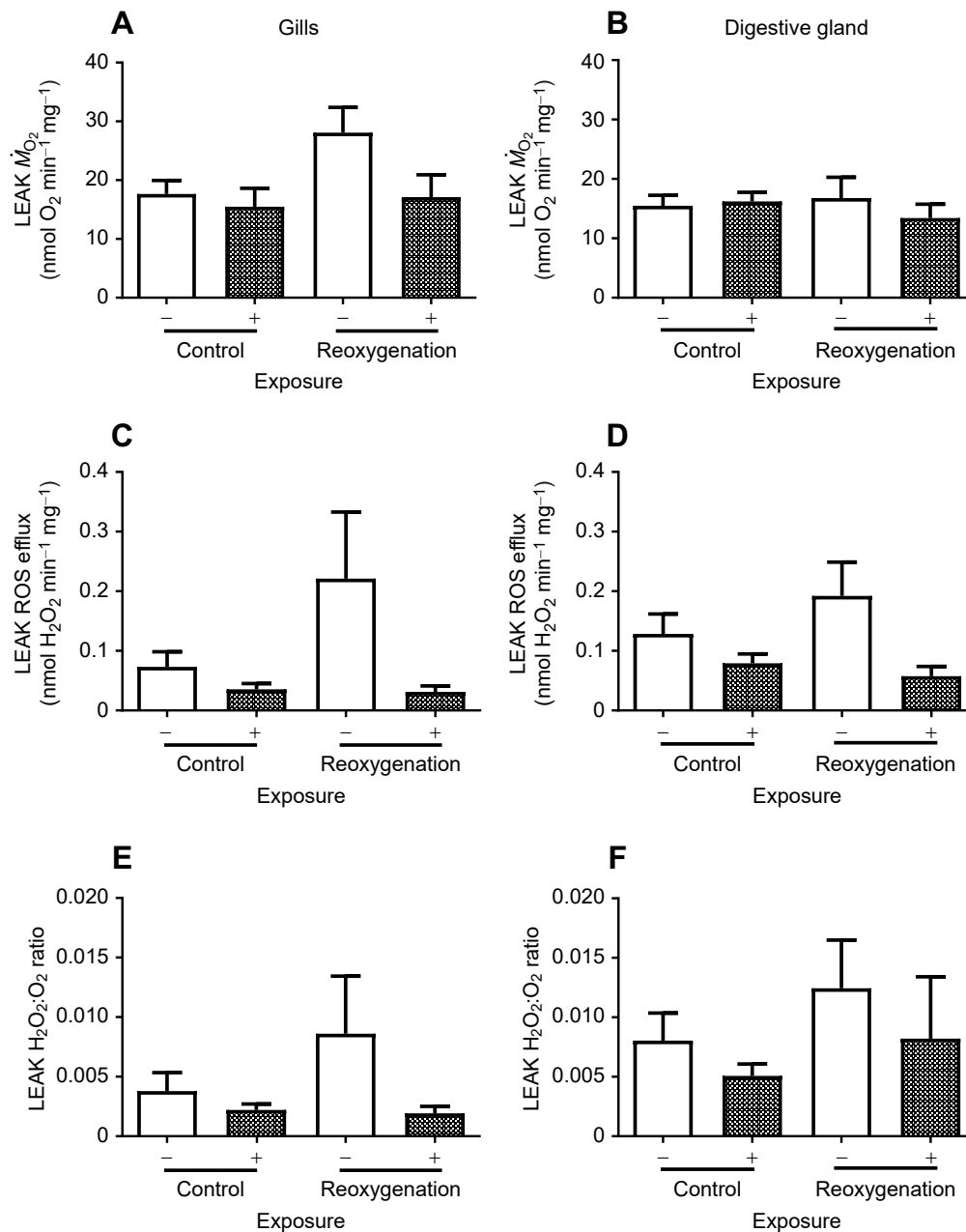
mitochondrial substrate preference has been studied in insects and varies depending on the aerobic endurance, with carbohydrates predominantly used by the short-range (flies and beetles) fliers and fatty acids utilized in the long-range (butterflies and locusts) fliers (Bailey, 1975; Krogh and Weis-Fogh, 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  $\alpha$ -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; Pamerter

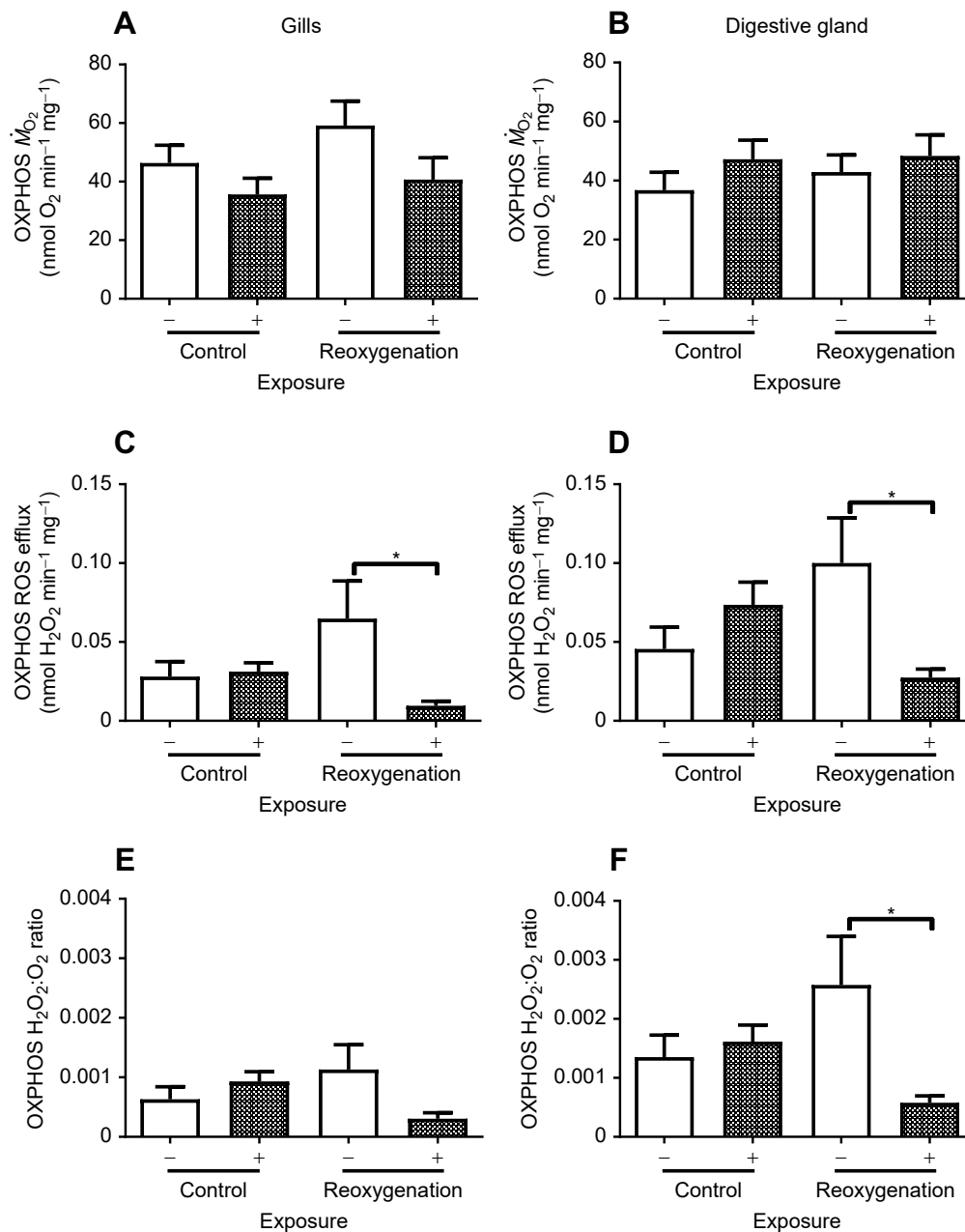


**Fig. 6. Effect of complex I inhibitor (rotenone) on succinate-driven respiration and ROS efflux in the LEAK state of *C. gigas* mitochondria.** Mitochondria were isolated from the gills (A,C,E) or the digestive glands (B, D,F) of oysters. Mitochondrial traits were assessed immediately after isolation (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



**Fig. 7. Effect of complex I inhibitor (rotenone) on succinate-driven respiration and ROS efflux in the OXPPOS 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 between 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<sub>2</sub>O<sub>2</sub> in the gill and ~1.4% in the digestive gland. In contrast, in the ADP-stimulated (OXPPOS 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<sub>2</sub> converted to H<sub>2</sub>O<sub>2</sub> 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 OXPPOS 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 OXPPOS 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<sub>2</sub>-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<sub>2</sub>O<sub>2</sub> to O<sub>2</sub> 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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## Mitochondrial responses to constant and cyclic hypoxia depend on the oxidized fuel in a hypoxia-tolerant marine bivalve *Crassostrea gigas*

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Sessile benthic organisms like oysters inhabit the intertidal zone, subject to alternating hypoxia and reoxygenation (H/R) episodes during tidal movements, impacting respiratory chain activities and metabolome compositions. We investigated the effects of constant severe hypoxia (90 min at ~ 0% O<sub>2</sub>) followed by 10 min reoxygenation, and cyclic hypoxia (5 cycles of 15 min at ~ 0% O<sub>2</sub> and 10 min reoxygenation) on isolated mitochondria from the gill and the digestive gland of *Crassostrea gigas* respiring on pyruvate, palmitate, or succinate. Constant hypoxia suppressed oxidative phosphorylation (OXPHOS), particularly during Complex I-linked substrates oxidation. It had no effect on mitochondrial reactive oxygen species (ROS) efflux but increased fractional electron leak (FEL). In mitochondria oxidizing Complex I substrates, exposure to cyclic hypoxia prompted a significant drop after the first H/R cycle. In contrast, succinate-driven respiration only showed significant decline after the third to fifth H/R cycle. ROS efflux saw little change during cyclic hypoxia regardless of the oxidized substrate, but Complex I-driven FEL tended to increase with each subsequent H/R cycle. These observations suggest that succinate may serve as a beneficial stress fuel under H/R conditions, aiding in the post-hypoxic recovery of oysters by reducing oxidative stress and facilitating rapid ATP re-synthesis. The impacts of constant and cyclic hypoxia of similar duration on mitochondrial respiration and oxidative lesions in the proteins were comparable indicating that the mitochondrial damage is mostly determined by the lack of oxygen and mitochondrial depolarization. The ROS efflux in the mitochondria of oysters was minimally affected by oxygen fluctuations indicating that tight regulation of ROS production may contribute to robust mitochondrial phenotype of oysters and protect against H/R induced stress.

**Keywords** Mitochondrial substrate preference, Succinate, Electron transport system, Hypoxia-reoxygenation, Oxidative stress, Bioenergetics

Aquatic ecosystems worldwide are experiencing a significant reduction in dissolved oxygen levels, a phenomenon known as hypoxia<sup>1,2</sup>. Coastal ecosystems are particularly vulnerable to hypoxia due to the combination of natural features (such as enrichment with organic matter, stratification and shallow depth) and anthropogenic nutrient pollution that stimulate bacterial respiration outstripping the oxygen input through photosynthesis, mixing and diffusion<sup>1,2</sup>. Depending on the local conditions, hypoxic episodes can last from several hours (during diurnal cycles of photosynthesis and respiration) to days and weeks in coastal dead zones<sup>1-3</sup>. Oxygen is essential for survival and development of most metazoans, making permanent dead zones incompatible with animal life and leading to a major loss of benthic biodiversity<sup>4</sup>. However, areas with periodic oxygen fluctuations (such as the intertidal zone or margins of the oxygen minimum zones) can support high diversity and biomass of benthic organisms adapted to hypoxia and reoxygenation stress<sup>5-9</sup>. Survival strategies during prolonged hypoxia center on energy conserving strategies such as the metabolic rate suppression and use of alternative anaerobic pathways

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with higher ATP yields<sup>10–12</sup>. Recovery from hypoxia presents additional challenges requiring rapid restoration of the homeostasis and avoidance of oxidative damage during reoxygenation<sup>13–15</sup>.

Mitochondria are a major target of hypoxia due to their key role in the oxygen-dependent ATP production and generation of reactive oxygen species (ROS). Under normal conditions, oxygen consumed by the mitochondria through the electron transfer system (ETS) generates the proton motive force driving synthesis of ATP in the process called oxidative phosphorylation (OXPHOS). Hypoxia disrupts ETS activity leading to lower rates of ATP production and elevated generation of ROS<sup>16</sup>. During reoxygenation, ROS efflux is further enhanced leading to mitochondrial damage<sup>17</sup>. The response of mitochondria to hypoxia-reoxygenation (H/R) stress differs between hypoxia-tolerant and intolerant species<sup>15,18</sup>. In hypoxia-intolerant species like terrestrial mammals and highly aerobic species of aquatic invertebrates, a suppression of ETS activity, mitochondrial depolarization and oxidative injury is observed after a single H/R cycle<sup>19–21</sup>. In contrast, in hypoxia-tolerant species like intertidal bivalves and some fishes, the ETS activity is stabilized or enhanced following H/R exposure<sup>21–25</sup>. Several adaptive mechanisms that might contribute to this metabolic resilience have been proposed including upregulation of ETS and antioxidant activity, enhanced protein quality control and suppression of ATP wastage<sup>26–28</sup>. Additionally, research findings have also revealed that changes in the intracellular milieu (such as shifts in the levels of metabolic intermediates) might contribute to the mitochondrial stress responses due to the use of alternative fuels for mitochondrial respiration<sup>29,30</sup>. Despite these advancements, our understanding of the mitochondrial mechanisms that contribute to the tolerance of animals to H/R stress remains limited. Critical questions remain unanswered, including the influence of the frequency and duration of hypoxic stress on mitochondrial damage and resilience, as well as the implications of alternative mitochondrial substrates on stress-induced changes in mitochondrial bioenergetics and ROS generation.

To fill these gaps in our knowledge, we conducted a study on the mitochondrial responses to varying H/R regimes in the Pacific oyster *Crassostrea (Magallana) gigas* Thunberg 1793, a marine bivalve known for its exceptional tolerance to hypoxia. *C. gigas* is a common intertidal species native to the Pacific coast of Asia and a successful invader in the intertidal zones worldwide, partially due to its high tolerance to abiotic stressors including oxygen fluctuations<sup>31</sup>. This makes the Pacific oyster an excellent model species to study mitochondrial flexibility under H/R stress. We focused on the intrinsic mechanisms of mitochondrial responses to different H/R regimes (constant and fluctuating hypoxia) using mitochondria isolated from two metabolically important organs of oysters—the gill and the digestive gland. Both organs are involved in energy metabolism with the gill responsible for oxygen uptake and filter-feeding<sup>26,32</sup>, and the digestive gland—for digestion and energy storage<sup>33,34</sup>. Here we studied how the mitochondria from the gill and the digestive gland of oysters responded to constant and intermittent hypoxia of similar duration (~90–125 min) and examined the dependence of the mitochondrial responses to H/R stress on the type of substrate used to fuel the respiration. While the duration of hypoxic exposures in our experiment was shorter than typical coastal hypoxia events<sup>2,3</sup>, it's important to recognize that experiments with isolated mitochondria are limited by their viability window, necessitating shorter exposures. Nonetheless, the oxygen regime used in our in vitro studies remains relevant for situations involving short-term oxygen fluctuations, such as those induced by valve closure and gaping behavior commonly observed in bivalves<sup>35–37</sup>.

We hypothesized that the constant (~90 min) severe hypoxia followed by reoxygenation will be more damaging to the oyster mitochondria than the cyclic H/R stress of similar total duration. Drawing on earlier research demonstrating the potential of succinate as a recovery fuel in hypoxia-tolerant marine bivalves<sup>14,27,38</sup>, we hypothesized that the capacity for mitochondrial succinate oxidation will increase in response to H/R stress without generating excess ROS, thereby mitigating the negative effects associated with H/R stress. To test our hypotheses, we conducted mitochondrial assays measuring basal and ADP-stimulated oxygen consumption rates and ROS efflux in isolated mitochondria from the gills and digestive gland of *C. gigas* under normoxic conditions and two H/R regimes: constant severe hypoxia (90 min at ~0% O<sub>2</sub>) followed by 10 min of reoxygenation, and cyclic H/R stress comprising five cycles of 15 min severe hypoxia and 10 min of reoxygenation each. We used a fatty acid (palmitate) and a carboxylic acid (pyruvate) as Complex I substrates, and succinate as a Complex II substrate. To assess the extent of ROS-induced damage to the mitochondria, we measured protein carbonyl accumulation in isolated gill mitochondria respiring on different substrates after constant and cyclic hypoxia exposures. Our findings offer insight into the mitochondrial mechanisms by which oysters adapt to H/R stress, potentially informing the development of new strategies for mitigating the negative effects of such stress.

## Materials and methods

### Chemicals

All chemicals were purchased from Fisher Scientific (Schwerte, Germany), Sigma Aldrich (Munich, Germany), or Carl Roth (Karlsruhe, Germany) and were of analytical grade or higher.

### Animals

Adult Pacific oysters *C. gigas* collected from the island of Sylt in the German Wadden Sea were transported within 24 h of collection to the University of Rostock. On arrival, oysters were acclimated for 3–6 weeks at 15 ± 1 °C temperature and 32 ± 1 (practical salinity units) salinity in aerated natural Baltic Sea water adjusted to a salinity of 32 with Instant Ocean sea salt (Aquarium Systems, Sarrebourg, France). The specified salinity and temperature conditions were representative of the habitat conditions during the time of collection. The 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

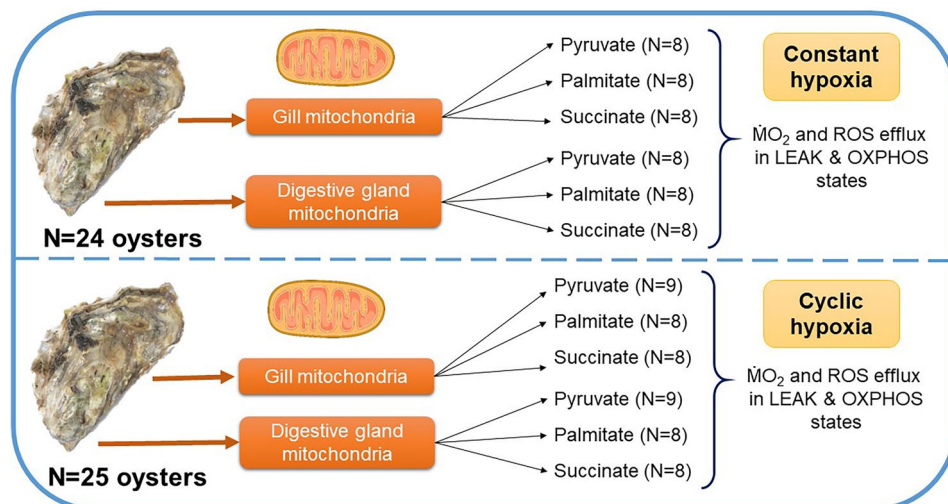
Mitochondria isolates were obtained from the gill and digestive gland tissues of oysters. For each substrate and oxygen regime, mitochondria were isolated from the gill and the digestive gland of 8–9 oysters (Fig. 1). Overall, 24 and 25 oysters were used in the constant and cyclic hypoxia experiments, respectively, yielding a total of 98 mitochondrial isolates. Mitochondria from individual oysters were isolated as described elsewhere<sup>38</sup> in an isolation medium (30 mmol l<sup>-1</sup> 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.5, 100 mmol l<sup>-1</sup> sucrose, 100 mmol l<sup>-1</sup> NaCl, 200 mmol l<sup>-1</sup> KCl, 8 mmol l<sup>-1</sup> ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 1 mmol l<sup>-1</sup> phenylmethylsulfonyl fluoride, 50 µg l<sup>-1</sup> aprotinin). Mitochondrial pellets were resuspended in an ice-cold assay medium (30 mmol l<sup>-1</sup> HEPES pH 7.2, 390 mmol l<sup>-1</sup> sucrose, 10 mmol l<sup>-1</sup> glucose, 130 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> NaCl, 1 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 10 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> and 1% fatty acid free bovine serum albumin, BSA).

## Mitochondrial respiration indices and ROS measurements

Oxygen consumption rate ( $\dot{M}O_2$ ) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) efflux rate were measured in isolated mitochondria at 15 °C using a high resolution Oxygraph 2-k respirometer (Oroboros, Innsbruck, Austria) with integrated DatLab 6 software. The H<sub>2</sub>O<sub>2</sub> efflux rate is determined by the balance between the mitochondrial H<sub>2</sub>O<sub>2</sub> production and consumption<sup>39</sup> and referred to as ROS efflux.  $\dot{M}O_2$  was measured using a Clark-type electrode and H<sub>2</sub>O<sub>2</sub> with a Fluorescence-Sensor Green, both integrated with the Oxygraph-2k. Detailed procedures for the Oxygraph-2k calibration and substrate–uncoupler–inhibitor titration for measuring mitochondrial respiration and ROS efflux are outlined in<sup>38</sup>. Saturating concentrations of the respective substrates were used: (1) 10 µmol l<sup>-1</sup> palmitoyl-DL-carnitine, (2) 5 mmol l<sup>-1</sup> pyruvate with 2 mmol l<sup>-1</sup> malate to spark respiration, (3) 10 mmol l<sup>-1</sup> succinate. Baseline (LEAK) respiration (indicative of the rate of proton leak) was determined as the respiration of non-phosphorylating mitochondria in the presence of saturating concentrations of substrates. LEAK respiration was achieved either by addition of substrate without the addition of ADP (State II, before H/R) or by addition of an F<sub>0</sub>, F<sub>1</sub> ATPase inhibitor oligomycin (State IV, after H/R). Pilot studies showed that the difference between the LEAK rate of the oyster mitochondria in State II and State IV is below 5% (data not shown). OXPHOS rate (reflective of ATP synthesis capacity) was determined as the rate of ADP-stimulated mitochondrial respiration.

## Hypoxia and reoxygenation exposures

Both constant and cyclic hypoxia exposures were conducted as separate experiments on isolated mitochondria in the respirometer chamber. After the addition of substrates and ADP to stimulate OXPHOS (State III respiration), the mitochondria were allowed to respire until all oxygen in the chamber was exhausted achieving severe hypoxia near anoxia (~0% O<sub>2</sub>). For constant hypoxia, the period of severe hypoxia was maintained for 90 min, after which the oxygen tension was raised to ~80% of air saturation and the mitochondria were allowed to recover for 10 min. For cyclic hypoxia, severe hypoxia was maintained for 15 min, after which the oxygen tension was raised to ~50–80% of air saturation for 10 min. The mitochondria were then again allowed to exhaust all oxygen in the chamber and underwent another 15 min of severe hypoxia. The hypoxia and reoxygenation process was conducted for five consecutive cycles, and the last reoxygenation period was maintained for 10 min. Pilot experiments showed that there was no ADP limitation throughout the entire exposure period to the constant or cyclic hypoxia (data not shown). After the last reoxygenation cycle, 2.5 µmol l<sup>-1</sup> oligomycin was added to the chamber to inhibit mitochondrial F<sub>0</sub>, F<sub>1</sub>-ATPase and measure LEAK respiration. This experimental design resulted in two values of respiration and ROS efflux measured in the LEAK state before the first and after the last H/R cycle. For the OXPHOS respiration and ROS efflux, two values (before and after H/R) were obtained in the constant hypoxia exposure, and six values (prior to hypoxia exposure and after each of the five cycles of



**Figure 1.** Schematic representation of the experimental design of the study.

H/R) were measured in the cyclic hypoxia exposures. Additionally, to ensure that the effects observed from the experiments were due to H/R stress and not the loss of mitochondria viability over time, parallel measurements were conducted in the mitochondria maintained for the same duration of time under normoxic conditions. The result revealed no difference in mitochondrial functional parameters of mitochondria maintained for ~125 min under normoxia (data not shown) eliminating the loss of mitochondrial viability over time under normoxic conditions.

Protein concentration was measured in the mitochondrial suspensions using the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as a standard and corrected for the BSA content of the resuspension media. Respiration rates were expressed in  $\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$  protein, and  $\text{H}_2\text{O}_2$  efflux in  $\text{nmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1}$  protein. The respiratory control ratio (RCR) was calculated as a ratio of OXPHOS to LEAK respiration rates<sup>40</sup>. The fractional electron leak (FEL) rate was determined by dividing the  $\text{H}_2\text{O}_2$  efflux rate by the oxygen consumption rate in the same mitochondrial isolate.

### Measurement of protein carbonyls

Concentrations of carbonyls (as a proxy for oxidative damage of proteins) were determined by enzyme-linked immunosorbent assay (ELISA) in gill mitochondria only. After the mitochondrial assays were completed, mitochondria suspensions were collected from the control (normoxic) conditions and after both constant and cyclic hypoxia stress and stored at  $-80^\circ\text{C}$ . For cyclic hypoxia, the suspensions were collected after the third H/R cycle to ensure the overall duration similar to the constant hypoxia exposures. Samples were lysed and diluted to  $10 \mu\text{g ml}^{-1}$  protein with phosphate buffered saline (PBS) solution. ELISA protocol was adapted from<sup>41</sup> with modifications. Carbonyl standards were prepared by mixing varying amounts of oxidized BSA solution (IgG-free BSA dissolved in  $\text{H}_2\text{O}_2$ ) and reduced BSA solution (IgG-free BSA dissolved in PBS) to a protein content of  $10 \mu\text{g ml}^{-1}$ . Carbonyl concentrations in the oxidized BSA were determined spectrophotometrically. Aliquots ( $100 \mu\text{l}$ ) of samples or standards were placed into ELISA microwell plates and incubated overnight at  $4^\circ\text{C}$ . Plates were washed three times with  $250 \mu\text{l}$  of PBS.  $100 \mu\text{l}$  solution of  $5 \text{ mM}$  2, 4-dinitrophenylhydrazine was added to each well and incubated in the dark for 45 min at room temperature. After incubation, the plate was washed five times with PBS: ethanol (1:1 v:v) mixture and twice with PBS. The plate was blocked with  $200 \mu\text{l}$  of  $1 \text{ mg ml}^{-1}$  BSA solution for 2 h at room temperature and washed thrice with 0.05% Tween solution. The primary anti-DNP antibody (mouse monoclonal, Sigma Aldrich MAB2223) was incubated for 1 h. After three washes with 0.05% Tween, the plates were incubated with a secondary anti-mouse antibody (Abcam) for 1 h at room temperature. The plate was washed five times with 0.05% Tween solution.  $100 \mu\text{l}$  TMB/E ultra-sensitive blue Horseradish Peroxidase substrate was added to each well and upon color development (10 min),  $100 \mu\text{l}$  2 M sulfuric acid was added to stop the reaction. The absorbance was read at 450 nm. For protein carbonyls, sample size was 4–6 per substrate and hypoxia exposure group, with each biological replicate representing a separate mitochondrial isolate.

### Statistics

We conducted a normality test on the raw data using the Shapiro–Wilk Test and detected outliers with the Box and Whiskers plot using IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA). Data points were considered outliers if they fell outside 1.5 times the interquartile range and removed from the final analysis. Negative values in ROS efflux measurements, typically occurring during late cycles of cyclic H/R due to low mitochondrial activity, were removed as physiologically unfeasible. Final sample sizes are noted in the figure legends. To evaluate the interactive effect of the hypoxic state and respiratory substrate on mitochondrial oxygen consumption, ROS efflux, FEL and protein carbonyl accumulation, we used a repeated measures two-way general linearized ANOVA model with hypoxic state as a within-subject factor and respiratory substrate as a between-subject factor. The hypoxic state was treated as a within-subject factor since measurements of the mitochondrial traits under various oxygen treatments (normoxia and reoxygenation) were conducted in the same mitochondrial isolate. For constant hypoxia, the assumption of sphericity (equal variance) in the data was confirmed, leading to the recording of degrees of freedom and p-values for the within-subject effect based on the assumption of sphericity. However, for cyclic hypoxia stress, the assumption of sphericity was violated for some data sets. Therefore, degrees of freedom and p-values for the within-subject effect were calculated using the Greenhouse–Geisser correction. We used the Least Significant Difference (LSD) and Tukey's honest significant differences (HSD) tests for planned comparisons of the group means. 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  $P$  was  $< 0.05$ . We have followed the recommendation of evidence-based language<sup>42</sup> for describing our results. The following thresholds were used:  $P \geq 0.05$  (no evidence of effect),  $P = 0.049–0.011$  (moderate evidence),  $P = 0.01–0.001$  (strong evidence),  $P < 0.001$  (very strong evidence of effect).

## Results

### Effects of constant hypoxia on mitochondrial functions

#### LEAK state

Our data showed strong evidence of the interactive effects of the substrate and constant (~90 min) hypoxia on the LEAK respiration in the gill mitochondria (Table 1). LEAK respiration was significantly suppressed in the gill mitochondria oxidizing palmitate and succinate but not affected in those oxidizing pyruvate (Fig. 2A). In the mitochondria from the digestive gland, there was moderate evidence for the effect of substrate on the LEAK respiration but no evidence for the effects of constant hypoxia or factor interactions (Table 1). LEAK respiration

Mitochondrial state	Factors		
	Oxygen regime	Substrate	Interactive effect
MO <sub>2</sub>			
LEAK <sub>GILL</sub>	<b>F<sub>1,20</sub> = 83.31, P &lt; 0.001</b>	F <sub>2,19</sub> = 3.02, P = 0.071	<b>F<sub>2,20</sub> = 8.29, P = 0.002</b>
LEAK <sub>DG</sub>	F <sub>1,20</sub> = 2.95, P = 0.101	F <sub>2,20</sub> = 5.05, P = <b>0.017</b>	F <sub>2,20</sub> = 0.18, P = 0.833
OXPHOS <sub>GILL</sub>	<b>F<sub>1,20</sub> = 81.73, P &lt; 0.001</b>	F <sub>2,20</sub> = 11.91, P = <b>0.001</b>	<b>F<sub>2,20</sub> = 5.45, P = 0.013</b>
OXPHOS <sub>DG</sub>	<b>F<sub>1,20</sub> = 20.38, P &lt; 0.001</b>	<b>F<sub>2,20</sub> = 4.99, P = 0.017</b>	F <sub>2,20</sub> = 1.45, P = 0.257
ROS efflux			
LEAK <sub>GILL</sub>	<b>F<sub>1,19</sub> = 5.09, P = 0.036</b>	F <sub>2,19</sub> = 1.30, P = 0.296	<b>F<sub>2,19</sub> = 3.63, P = 0.046</b>
LEAK <sub>DG</sub>	<b>F<sub>1,19</sub> = 6.96, P = 0.016</b>	F <sub>2,19</sub> = 5.50, P = <b>0.013</b>	F <sub>2,19</sub> = 2.54, P = 0.106
OXPHOS <sub>GILL</sub>	F <sub>1,19</sub> = 0.43, P = 0.519	<b>F<sub>2,19</sub> = 6.04, P = 0.009</b>	F <sub>2,19</sub> = 0.88, P = 0.430
OXPHOS <sub>DG</sub>	F <sub>1,19</sub> = 0.000, P = 0.999	<b>F<sub>2,19</sub> = 6.54, P = 0.007</b>	F <sub>2,19</sub> = 1.90, P = 0.177
FEL			
LEAK <sub>GILL</sub>	F <sub>1,19</sub> = 1.86, P = 0.189	F <sub>2,19</sub> = 2.93, P = 0.078	F <sub>2,19</sub> = 2.26, P = 0.131
LEAK <sub>DG</sub>	F <sub>1,19</sub> = 0.37, P = 0.552	<b>F<sub>2,19</sub> = 5.24, P = 0.015</b>	F <sub>2,19</sub> = 0.10, P = 0.903
OXPHOS <sub>GILL</sub>	<b>F<sub>1,20</sub> = 9.95, P = 0.005</b>	<b>F<sub>2,20</sub> = 5.07, P = 0.017</b>	<b>F<sub>2,20</sub> = 4.97, P = 0.018</b>
OXPHOS <sub>DG</sub>	<b>F<sub>1,20</sub> = 17.67, P &lt; 0.001</b>	<b>F<sub>2,20</sub> = 8.35, P = 0.002</b>	<b>F<sub>2,20</sub> = 4.08, P = 0.033</b>

**Table 1.** Repeated measures ANOVA: Effects of constant hypoxia/reoxygenation and substrates on the respiration, ROS efflux and FEL in the mitochondria from the gill and the digestive gland (DG) of *C. gigas*. Mitochondrial parameters were measured in the resting (LEAK) and actively phosphorylating (OXPHOS) states. F-values with the degrees of freedom for the effect and the error (in subscript) and P-values are given. Significant effects ( $P < 0.05$ ) are highlighted in bold.

was suppressed after 90 min of hypoxia in the digestive gland mitochondria respiring on palmitate but not in those oxidizing pyruvate or succinate (Fig. 2B).

ROS efflux in the gill mitochondria in the LEAK state showed moderate interactive effect of constant hypoxia and substrate (Table 1). In the gill mitochondria respiring on pyruvate, ROS efflux in the LEAK state was suppressed after constant hypoxia (Fig. 2C). No evidence for change in ROS flux was found in the LEAK mitochondria respiring on palmitate or succinate (Fig. 2C). Moderate evidence was found for the effects of constant hypoxia and substrate on ROS efflux in the LEAK state mitochondria from the digestive gland (Table 1). ROS efflux rate and FEL tended to be lower in the digestive gland mitochondria respiring on succinate compared with those oxidizing pyruvate or palmitate (Fig. 2D). No evidence for the impact of prolonged hypoxia and reoxygenation was found for the ROS efflux or FEL, regardless of the substrate (Fig. 2D,F). The FEL in the gill mitochondria in the LEAK state remained unchanged after hypoxia exposure regardless of the substrate (Fig. 2E).

#### OXPHOS state

Moderate evidence for the interactive effects of the substrate and constant (~90 min) hypoxia was found for OXPHOS respiration of the gill mitochondria (Table 1). In the digestive gland mitochondria, OXPHOS respiration was significantly affected by constant hypoxia and substrate, but not by their interactions (Table 1). Generally, OXPHOS respiration was suppressed after prolonged hypoxia exposure in oyster mitochondria. The suppression was greater in the mitochondria respiring on Complex I substrates (pyruvate and palmitate) than in those oxidizing succinate (Fig. 3A,B).

ROS efflux in the actively phosphorylating (OXPHOS state) mitochondria from the gills and the digestive gland showed strong evidence of the effect of oxidized substrate but no effect of hypoxia exposure (Table 1, Fig. 3C,D). ROS efflux rates in the succinate oxidizing mitochondria were lower than in those respiring on pyruvate and palmitate (Fig. 3C,D). The FEL in the OXPHOS state mitochondria from the gill and the digestive gland showed moderate evidence of the interactive effects of hypoxia and substrate (Table 1). This reflected a major increase in the FEL after reoxygenation in the gill and the digestive gland mitochondria energized by pyruvate and (to a lesser degree) palmitate, which was not observed in the mitochondria oxidizing succinate (Fig. 3E,F).

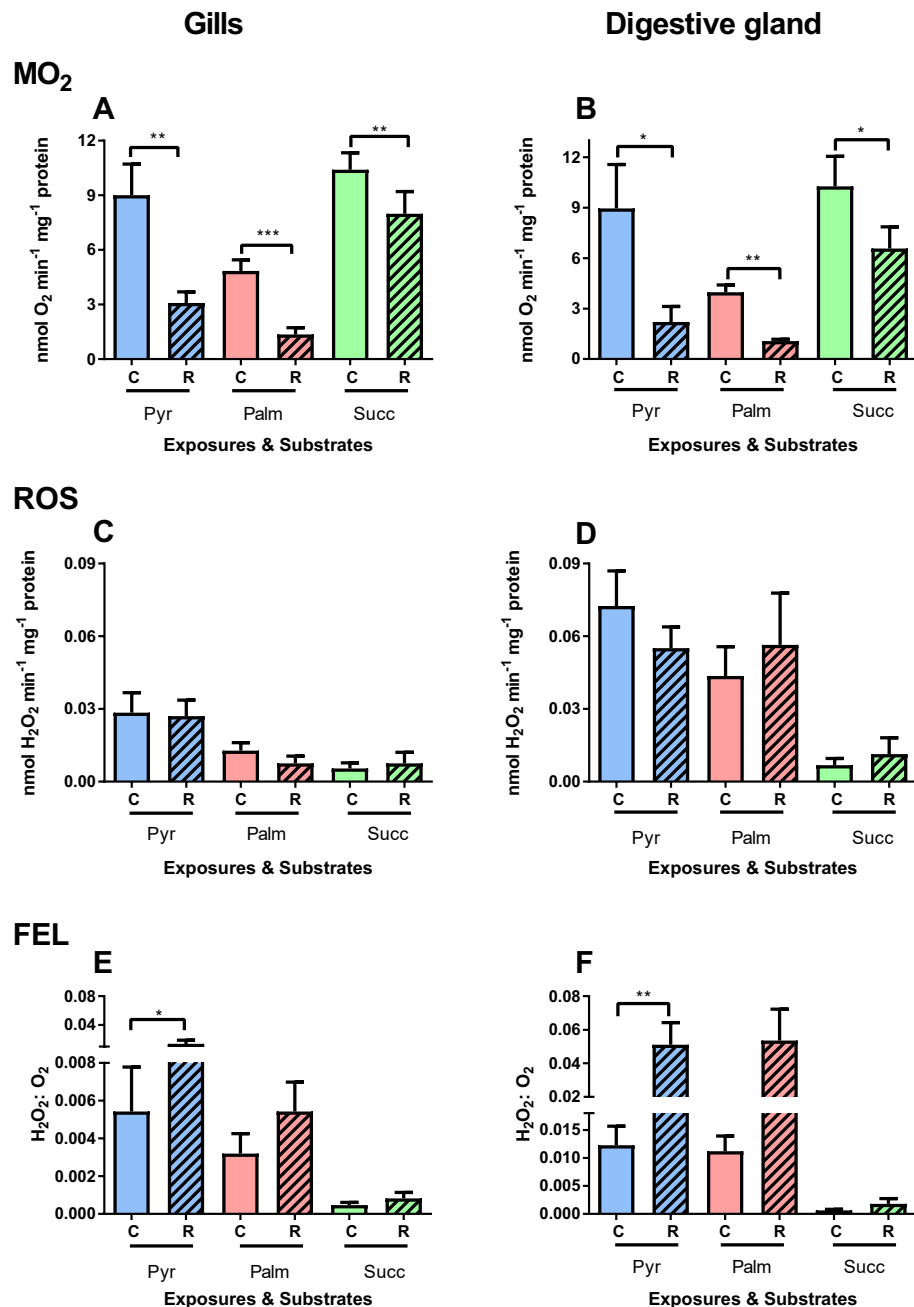
### Effects of cyclic hypoxia on mitochondrial functions

#### LEAK state

In the gill mitochondria, there was no evidence for the effects of substrates or factor interactions on LEAK respiration but very strong evidence of the effect of cyclic hypoxia (Table 2). This reflects a strong suppression in LEAK respiration observed after five H/R cycles with all studied substrates (Fig. 4A). In the mitochondria from the digestive gland, there was moderate evidence for the interactive effect of cyclic hypoxia and substrate (Table 2). In the digestive gland mitochondria, palmitate-driven LEAK respiration was significantly suppressed after H/R exposures whereas the pyruvate- and succinate-driven LEAK respiration did not change (Fig. 4B).

There was no evidence for the effect of the cyclic hypoxia, substrate, or factor interactions on ROS efflux or FEL in the LEAK state mitochondria from the gill or the digestive gland (Table 2). In the gill mitochondria, no change in the ROS efflux or FEL was found before and after the cyclic H/R stress (Fig. 4C,E). In the digestive gland





**Figure 3.** Effects of constant (~90 min) hypoxia and reoxygenation on OXPHOS respiration and ROS efflux of mitochondria isolated from the gills (A,C,E) or the digestive glands (B,D,F) of *C. gigas*. Substrates: Pyr—pyruvate, Palm—palmitate, and Succ—succinate. (A,B) Oxygen consumption rate, (C,D) ROS efflux rates, (E,F) FEL. Significant differences in a specific mitochondrial trait between normoxia (C, solid bars) and reoxygenation (R, striped bars) are denoted by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).  $N = 7$  for pyruvate, and 8 for palmitate and succinate.

gland mitochondria, there was strong evidence for the interactive effect of substrate and cyclic hypoxia on OXPHOS respiration (Table 2). Similar to the gills, a decline in the OXPHOS respiration rate with NADH-linked substrate was observed after the first H/R cycle in the digestive gland mitochondria (Fig. 5B). A significant decline in succinate-driven OXPHOS respiration was observed after the third H/R cycle in the digestive gland mitochondria (Fig. 5B).

During OXPHOS respiration, there was no evidence for the effect of oxygen regime, substrate, or factor interaction on ROS efflux and FEL in the gill mitochondria (Table 2). In the gill mitochondria respiring on pyruvate, there was a significant decline in ROS efflux after the second H/R cycle and increase in FEL after the fifth cycle (Fig. 5C,E). ROS efflux and FEL of the gill mitochondria respiring on palmitate or succinate showed no evidence of change during cyclic hypoxia (Fig. 5C,E). There was no evidence of the effects of oxygen

Mitochondrial state	Factors		
	Oxygen regime	Substrate	Interactive effect
MO <sub>2</sub>			
LEAK <sub>GILL</sub>	<b>F<sub>1,17</sub> = 40.71, P &lt; 0.001</b>	F <sub>2,17</sub> = 0.28, P = 0.757	F <sub>2,17</sub> = 1.29, P = 0.301
LEAK <sub>DG</sub>	<b>F<sub>1,20</sub> = 24.66, P &lt; 0.001</b>	<b>F<sub>2,20</sub> = 5.42, P = 0.013</b>	<b>F<sub>2,20</sub> = 5.16, P = 0.016</b>
OXPPOS <sub>GILL</sub>	<b>F<sub>1,5,25</sub> = 20.85, P &lt; 0.001</b>	<b>F<sub>2,17</sub> = 4.60, P = 0.025</b>	F <sub>2,9,35</sub> = 1.17, P = 0.339
OXPPOS <sub>DG</sub>	<b>F<sub>1,3,26</sub> = 60.84, P &lt; 0.001</b>	<b>F<sub>2,20</sub> = 8.07, P = 0.003</b>	<b>F<sub>2,6,26</sub> = 6.76, P = 0.002</b>
ROS efflux			
LEAK <sub>GILL</sub>	F <sub>1,16</sub> = 2.29, P = 0.149	F <sub>2,16</sub> = 1.47, P = 0.260	F <sub>2,16</sub> = 0.42, P = 0.663
LEAK <sub>DG</sub>	F <sub>1,20</sub> = 3.14, P = 0.092	F <sub>2,20</sub> = 0.02, P = 0.979	F <sub>2,20</sub> = 1.52, P = 0.243
OXPPOS <sub>GILL</sub>	F <sub>1,5,24</sub> = 1.14, P = 0.323	F <sub>2,16</sub> = 1.60, P = 0.232	F <sub>3,24</sub> = 0.62, P = 0.610
OXPPOS <sub>DG</sub>	F <sub>1,1,20,7</sub> = 1.24, P = 0.284	F <sub>2,19</sub> = 0.11, P = 0.894	F <sub>2,2,20,7</sub> = 0.41, P = 0.684
FEL			
LEAK <sub>GILL</sub>	F <sub>1,16</sub> = 0.17, P = 0.690	F <sub>3,16</sub> = 0.55, P = 0.655	F <sub>3,16</sub> = 0.33, P = 0.806
LEAK <sub>DG</sub>	F <sub>1,18</sub> = 0.23, P = 0.635	F <sub>2,18</sub> = 0.91, P = 0.420	F <sub>2,18</sub> = 0.31, P = 0.740
OXPPOS <sub>GILL</sub>	F <sub>1,2,19,6</sub> = 1.34, P = 0.270	F <sub>2,16</sub> = 2.66, P = 0.101	F <sub>2,5,19,6</sub> = 1.18, P = 0.336
OXPPOS <sub>DG</sub>	F <sub>1,1,21,4</sub> = 3.56, P = 0.069	F <sub>2,19</sub> = 0.88, P = 0.431	F <sub>2,2,21,4</sub> = 0.49, P = 0.639

**Table 2.** Repeated measures ANOVA: Effects of cyclic hypoxia (5 cycles of 15 min anoxia followed by reoxygenation) and substrates on the respiration, ROS efflux and FEL in the mitochondria from the gill and the digestive gland (DG) of *C. gigas*. Mitochondrial parameters were measured in the resting (LEAK) and actively phosphorylating (OXPHOS) states. F-values with the degrees of freedom for the effect and the error (in subscript) and P-values are given. Significant effects ( $P < 0.05$ ) are highlighted in bold.

regime, substrate, or factor interaction on ROS efflux in the digestive gland mitochondria (Table 2; Fig. 5D), whereas FEL was significantly affected by cyclic hypoxia showing increasing trend with each consequent H/R cycle (Table 2; Fig. 4F).

#### Mitochondrial coupling

In the gill mitochondria, exposure to constant hypoxia suppressed mitochondrial RCR during pyruvate-driven oxidation and increased it under succinate-driven oxidation (Fig. 6A). In the digestive gland, constant hypoxia led to a decline in RCR of the mitochondria respiring on pyruvate and palmitate, but no change was found in those oxidizing succinate (Fig. 6B). A similar pattern was found in the mitochondria exposed to the cyclic hypoxia (Fig. 6C,D).

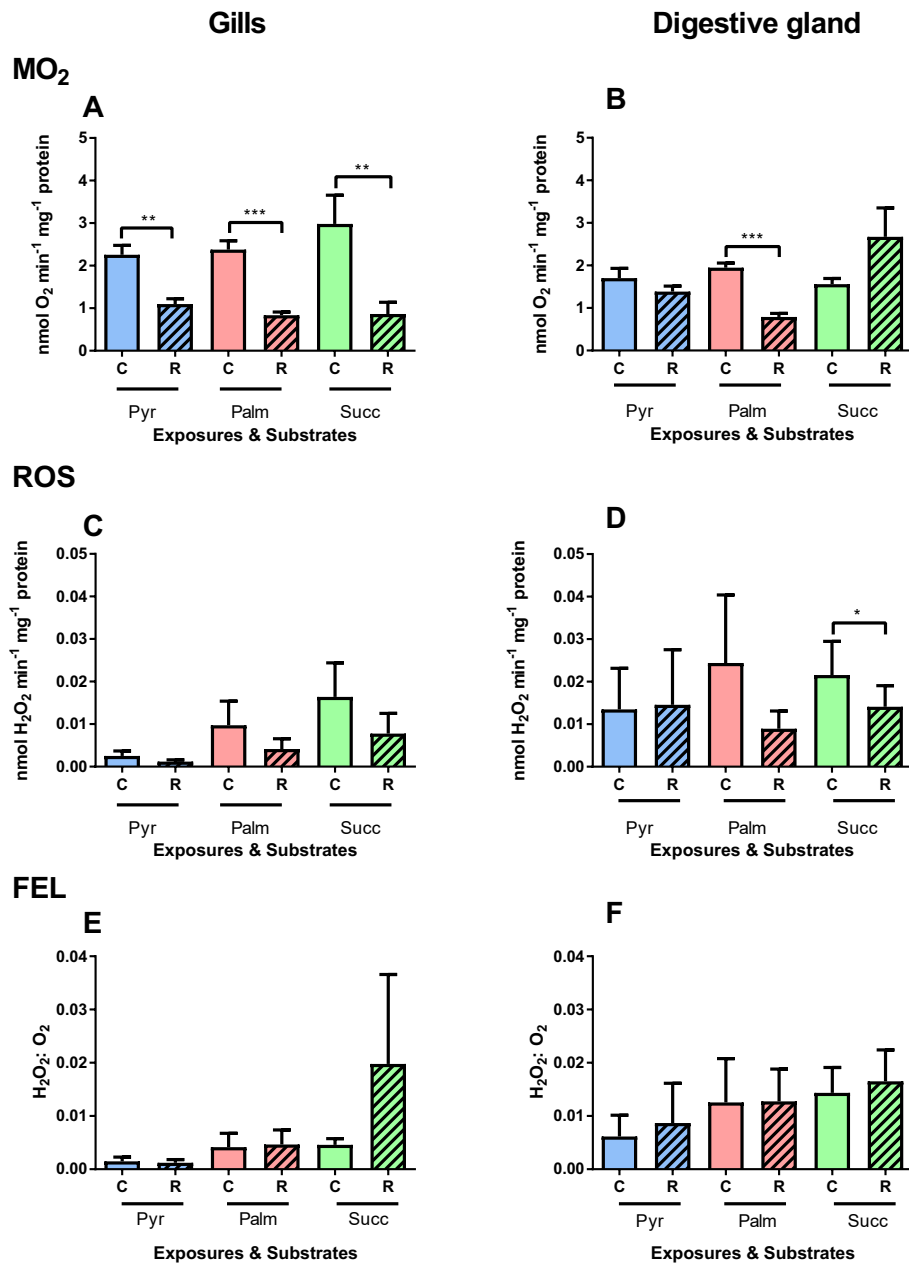
#### Oxidative damage

Constant and cyclic hypoxia led to an increase in the carbonyl content of proteins in isolated gill mitochondria under most experimental conditions (Table 3, Fig. 7). This increase was significant in palmitate-oxidizing mitochondria after constant hypoxia and in succinate-oxidizing mitochondria after cyclic H/R stress.

## Discussion

Pacific oysters are exceptionally stress-tolerant bivalves able to survive exposures to extreme temperature, salinity and oxygen fluctuations<sup>43–45</sup>. In oysters, exposure to severe hypoxia leads to metabolic rate suppression with simultaneous activation of anaerobic pathways for ATP generation and antioxidant activities<sup>46</sup>. One of the major consequences of anaerobic transition is alteration of the metabolome composition including changes in the concentrations of mitochondrial substrates<sup>32,46</sup>. During hypoxia, reduced intermediates like NADH and succinate accumulate and can drive mitochondrial ROS production during reoxygenation<sup>30,47,48</sup>. Our findings demonstrate that mitochondrial responses to H/R stress in a hypoxia-tolerant marine bivalve are modulated by the substrate oxidized by mitochondria. This is indicated by significant interactive effects of substrate and oxygen regime on mitochondrial oxygen consumption in both LEAK and OXPPOS states during constant hypoxia in the gills (Table 1) and during cyclic hypoxia in the digestive gland (Table 2). Notably, no evidence of the interactive effects of substrate and oxygen regime was found on the mitochondrial ROS efflux indicating that net ROS production during H/R stress is similar with Complex I (pyruvate and palmitate) and Complex II (succinate) substrates (Table 2; Figs. 4C,D, 5C,D). This finding is consistent with the earlier studies showing negligible ROS production due to the reverse electron flux in oysters<sup>38</sup> in contrast to hypoxia-intolerant species such as some terrestrial mammals where succinate oxidation leads to a major increase of ROS production due to the reverse electron transport (RET)<sup>30,49</sup>.

Constant and cyclic hypoxia suppressed oxygen consumption in the mitochondria of *C. gigas*. The oyster mitochondria were considerably more susceptible to H/R-induced loss of respiration capacity during oxidation of Complex I substrates compared with the Complex II substrate. Thus, OXPPOS rate in the oyster mitochondria oxidizing pyruvate or palmitate declined by 63–70%, whereas those oxidizing succinate decreased by 24–31% after 90 min of constant hypoxia. Similarly, three H/R cycles (of comparable duration to the constant hypoxia in our present study) led to a 45–52% decrease in OXPPOS respiration fueled by pyruvate, 61–62% decline in

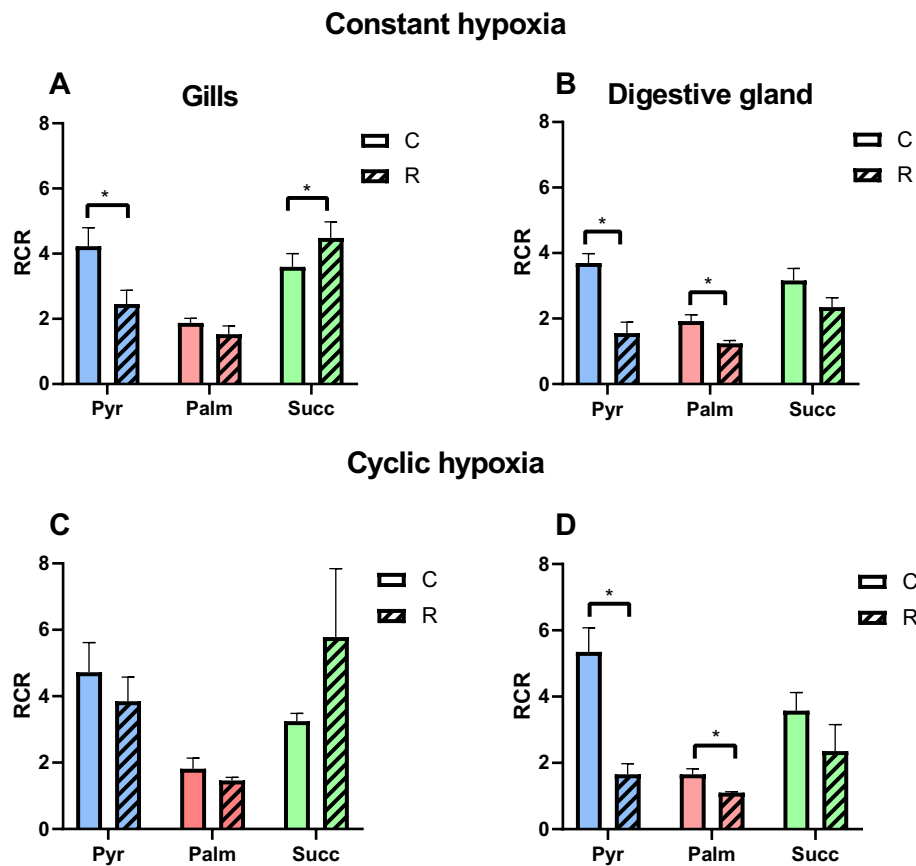


**Figure 4.** Effects of cyclic hypoxia on LEAK respiration and ROS efflux of mitochondria isolated from the gills (A,C,E) or the digestive glands (B,D,F) of *C. gigas*. Substrates: Pyr—pyruvate, Palm—palmitate, and Succ—succinate. Significant differences in a specific mitochondrial trait between normoxia (C, solid bars) and reoxygenation (R, striped bars) are denoted by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).  $N = 7$ –9 for pyruvate, 4–6 for palmitate, and 7–8 for succinate.

palmitate-driven OXPHOS and only 25–30% decrease in the succinate-driven OXPHOS respiration. The decline in OXPHOS rate continued during cyclic hypoxia with the loss of 55–73%, 71–73%, and 51–52% of OXPHOS activity with pyruvate, palmitate and succinate, respectively, after five H/R cycles. Generally, palmitate oxidation generated the lowest respiratory flux under normoxia and the highest decline both under constant and cyclic hypoxia in oyster mitochondria. A similar observation was made in *C. gigas* mitochondria exposed to acute short-term (15 min) hypoxia<sup>38</sup> suggesting the inability of oyster mitochondria to effectively utilize fatty acids especially during intermittent hypoxia. Studies of other marine mollusks also reveal their limited capacity in oxidizing fatty acids<sup>50–52</sup>.

Interestingly, the resting (LEAK) respiration with Complex I substrates was generally less susceptible to cyclic hypoxia than OXPHOS rate (Fig. 3C,D). As a result of this discrepancy, the mitochondrial coupling efficiency (RCR) decreased after cyclic hypoxia in the oyster mitochondria respiring on Complex I substrates. This was not observed during the succinate oxidation where the mitochondrial coupling efficiency increased or remained



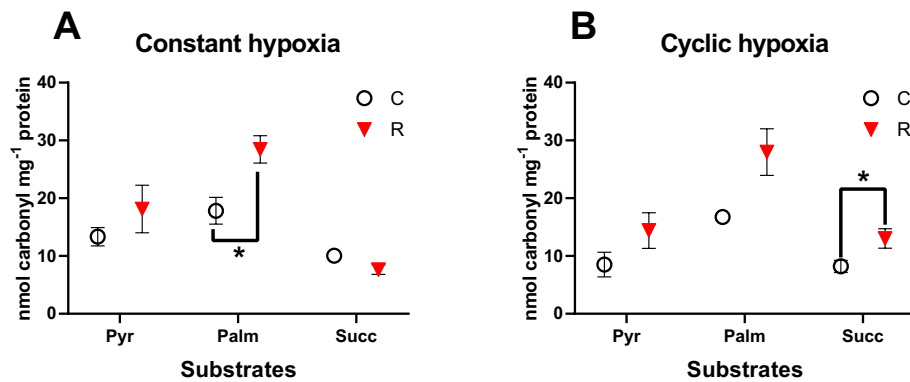


**Figure 6.** Effects of constant (A,B) and cyclic (C,D) hypoxia on respiratory control ratio (RCR) of mitochondria isolated from the gills (A,C) or the digestive glands (B,D) of *C. gigas*. Substrates: Pyr—pyruvate, Palm—palmitate, and Succ—succinate. Significant differences in a specific mitochondrial trait between normoxia (C, solid bars) and reoxygenation (R, striped bars) are denoted by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).  $N = 7$ – $9$  for pyruvate,  $5$ – $8$  for palmitate, and  $7$ – $8$  for succinate.

	Oxygen regime	Substrates	Interactive effect
Constant hypoxia	$F_{1,12} = 6.94$ , $P = 0.022$	$F_{1,12} = 10.74$ , $P = 0.002$	$F_{1,12} = 5.62$ , $P = 0.019$
Cyclic hypoxia	$F_{1,20} = 83.31$ , $P < 0.001$	$F_{1,20} = 3.02$ , $P = 0.071$	$F_{2,20} = 8.29$ , $P = 0.002$

**Table 3.** Repeated measures ANOVA: Effects of the constant and cyclic hypoxia and reoxygenation and substrates on the protein carbonyl content of the mitochondria isolated from the gills of *C. gigas*. Significant values are in bold.

caused by H/R stress<sup>55,56</sup>. Suppression of Complex I activity can impair OXPHOS flux capacity that is predominantly controlled by ETS activity in bivalves<sup>59–61</sup> and can explain the observed suppression in OXPHOS fueled by pyruvate and palmitate in oysters. However, suppression of Complex I activity might also serve as a protective mechanism preventing excessive ROS production during reoxygenation, since Complex I is one of the main sites of ROS generation<sup>54,62</sup>. Here, we observed that the impact of H/R on ROS efflux via Complex I-linked substrate oxidation was dependent on the mitochondrial activity state. Thus, in the resting (LEAK state) mitochondria respiring on Complex I-linked substrate, constant (90 min) hypoxia followed by reoxygenation suppressed ROS efflux by 34–47%. Under these conditions, the decrease in ROS generation was roughly proportional to the decline in LEAK respiration, hence the FEL rate did not change. Similarly, after five H/R cycles the change in ROS efflux rate of the LEAK state mitochondria respiring with pyruvate or palmitate were proportional to the changes in the oxygen consumption resulting in a relatively stable FEL. In contrast, pyruvate- and palmitate-driven ROS efflux remained unchanged or slightly increased in OXPHOS state mitochondria after constant hypoxia leading to an increased FEL. A similar increasing trend of FEL was found during sequential H/R cycles in OXPHOS state mitochondria energized by pyruvate or palmitate. Elevated FEL was associated with higher levels of the oxidative damage (indicated by accumulation of protein carbonyls) in oyster mitochondria respiring on Complex I substrates, particularly during the oxidation of palmitate.



**Figure 7.** Effects of constant (A) and cyclic (C) hypoxia on protein carbonyl content of isolated gill mitochondria of *C. gigas* respiring on different substrates. Substrates: Pyr—pyruvate, Palm—palmitate, and Succ—succinate. Significant differences in a specific mitochondrial trait between normoxia (C, circles) and reoxygenation (R, triangles) are denoted by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).  $N = 4-6$ .

Unlike Complex I substrates, oxidation of Complex II substrate (succinate) was more robust to hypoxia and reoxygenation in oyster mitochondria. Thus, a decline in succinate-driven respiration was 2–3 times lower than that observed during oxidation of Complex I substrates. Previous studies in oysters exposed to H/R stress *in vivo* revealed stimulation of succinate-driven LEAK and OXPHOS respiration in the gill mitochondria<sup>27,63</sup>. *In vitro* exposure of mussel and oyster mitochondria to short-term (15 min) hypoxia and subsequent reoxygenation also stimulated rather than suppressed Complex II-driven respiration<sup>14,38</sup>. In *Drosophila*, exposure to high temperatures switched the mitochondrial substrate preference from Complex I substrates to succinate thus maintaining respiration despite the heat-induced decrease in Complex I activity<sup>64,65</sup>. Taken together, these findings indicate that succinate might be a preferred mitochondrial fuel under stress conditions like H/R exposures or heat stress<sup>66</sup>. Furthermore, succinate oxidation acts as a regulatory and compensatory mechanism for maintaining of the mitochondrial membrane potential, ATP synthesis and adenylate pool<sup>167-69</sup>. In marine bivalves including oysters, succinate is the major anaerobic end product that accumulates in high concentrations during hypoxia<sup>25,32,46,70,71</sup>. Thus, high capacity for succinate oxidation might be adaptive during post-hypoxic recovery in oysters helping to rapidly restore ATP levels and remove excess succinate from tissues.

Succinate has been reported to strongly stimulate ROS generation due to the RET through mitochondrial Complex I<sup>49</sup>. This mechanism has been observed in mitochondria of mammals<sup>30,48</sup> and reptiles<sup>47,72</sup>. However, in our previous study on *C. gigas*, we observed that RET does not contribute to the ROS efflux both under normal and short-term (15 min) H/R conditions<sup>38</sup>. Consistent with the notion of lack of RET, our present study found no increase in ROS efflux or FEL rate in succinate-energized oyster mitochondria after constant or cyclic H/R stress. There was also no accumulation of oxidative damage to proteins (indicated by protein carbonyls) after constant hypoxia in succinate-energized mitochondria. These findings indicate that a mild decrease in Complex II activity combined with a more significant suppression of Complex I effectively prevented RET and oxidative damage in oyster mitochondria under these conditions. However, after five H/R cycles a modest but statistically significant increase in the protein carbonylation was detected, indicating that the mitochondrial antioxidant systems might become overwhelmed under prolonged and frequent oxygen fluctuations.

## Conclusions and outlook

Mitochondrial bioenergetics of oysters is modulated by the constant hypoxia and cyclic oxygen fluctuations such as might occur during the shell closure and periodical valve gaping during the low tide. Mitochondrial Complex I appears to be the main target of H/R stress showing a gradual loss of activity with increasing duration of hypoxic exposure. There appears to be no major difference between the impact of constant and cyclic hypoxia of similar duration indicating that the mitochondrial damage is mostly determined by the lack of oxygen that leads to mitochondrial depolarization<sup>73</sup>. The damage to Complex I by H/R stress leads to a decrease in mitochondrial coupling efficiency and ATP synthesis capacity in oysters. In contrast to Complex I, Complex II-dependent succinate oxidation is considerably more robust to H/R stress showing only modest decrease after prolonged hypoxia (90–125 min, this study) and an increase after a short-term (15 min) hypoxia<sup>27,74</sup>. Combined with the lack of succinate-driven RET, highly robust succinate oxidation by oyster mitochondria might be considered an adaptive mechanism that permits flexible use of metabolic fuels and circumvents the limitations of Complex I during oxygen fluctuations.

Notably, the ROS efflux in the mitochondria of oysters was minimally affected by the H/R stress with no consistent evidence of the oxidative damage to mitochondria. This suggests that unlike the mitochondria of mammals where reoxygenation is associated with ROS burst<sup>19,53,75,76</sup>, mitochondria of stress-tolerant intertidal bivalves like oysters tightly control ROS efflux during environmental stress like oxygen (this study) and salinity<sup>77</sup> fluctuations. In oysters, the robust forward electron flux with succinate combined with suppression of Complex I activity can minimize RET and stabilize ROS production in mitochondria during H/R stress. This aspect is particularly important in the intertidal species that often experience oxygen fluctuations from near anoxia to

normoxia or even hyperoxia. Nevertheless, oxidative damage to proteins accumulates early if the respiration is fueled by NADH-linked substrates, and later after repeated H/R cycles with succinate. Further studies are needed to determine the possible functional consequences of mitochondrial protein carbonylation and determine the mechanisms responsible for the selective inactivation of Complex I by H/R stress in mitochondria of oysters and other stress-tolerant marine invertebrates.

### Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

LA—Investigation, methodology, validation, data curation, formal analysis, visualization, writing—original draft preparation, writing—review and editing; SP—funding acquisition, writing—review and editing; IMS—conceptualization, funding acquisition, project administration, resources, supervision, writing—review and editing.

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1 **Effects of hypoxia-reoxygenation on the bioenergetics and**  
2 **oxidative stress in the mitochondria of the king scallop, *Pecten***  
3 ***maximus* (Linnaeus, 1758)**  
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23 **Keywords:** Short-term hypoxia, Long-term hypoxia, Mitochondria, Oxidative stress,  
24 Bioenergetics  
25  
26

27 **Abstract**

28 Subtidal species like the king scallop inhabit the subtidal zones with infrequent and  
29 unpredictable hypoxic events. Due to the high energy consuming muscle activity of  
30 scallops, they require an oxygenated environment for recovery from hypoxia, thus  
31 rendering them sensitive to low oxygen conditions. Hypoxia reoxygenation (H-R)  
32 conditions influence the metabolic activities, especially those of mitochondrial functions  
33 and metabolome composition. This study explored the effects of different hypoxic  
34 episodes on mitochondrial functional indices of the king scallop *P. maximus*. We exposed  
35 isolated mitochondria from two scallop tissues, gill and digestive gland, respiring on  
36 different substrates (pyruvate, palmitate, and succinate) to short-term H-R stress (15 min  
37 at ~0% O<sub>2</sub>, and 10 min reoxygenation) and long-term H-R stress (90 min at ~0% O<sub>2</sub>, and  
38 10 min reoxygenation). Our findings revealed that the scallop mitochondria have  
39 preference for carboxylic acid (pyruvate) oxidation under normoxia. After H-R exposure,  
40 mitochondria of both tissues had a better post-hypoxic recovery oxidizing succinate as  
41 opposed to mitochondria oxidizing Complex I-linked substrates although coupling  
42 efficiency decreased during long-term exposure in the digestive gland mitochondria.  
43 Scallop mitochondria utilizing both glycolytic products and fatty acids saw a significant  
44 decline in ATP synthesis capacity (due to mitochondrial uncoupling) during post-hypoxic  
45 recovery in both studied tissues. Our findings also revealed the ability of the gill  
46 mitochondria of *P. maximus* to effectively regulate ROS efflux and electron leak as  
47 compared to the digestive gland mitochondria during H-R stress. Overall, our findings  
48 revealed the lack of H-R induced oxidative stress irrespective of the H-R induced changes  
49 in respiration presenting an opportunity to investigate the mitochondrial antioxidant  
50 activity during H-R stress in scallop mitochondria.

## 51 **Introduction**

52 Mitochondria play a central role in cellular metabolism, homeostasis and responses to  
53 stress<sup>1</sup>. They host the machinery for oxidative phosphorylation, producing over 90% of  
54 the cellular ATP, alongside housing multiple metabolic pathways such as fatty acid  $\beta$ -  
55 oxidation, the tricarboxylic acid cycle, and the urea cycle <sup>2</sup>. Furthermore, mitochondria  
56 are involved in vital biosynthetic activities, regulate intracellular calcium metabolism and  
57 signaling, generate the majority of cellular reactive oxygen species (ROS), and act as the  
58 cell's gatekeeper for programmed cell death (apoptosis) <sup>2</sup>. Mitochondrial bioenergetics is  
59 a complex process that entails the oxidation of diverse organic molecules—such as  
60 carbohydrates, fatty acids, and amino acids—yielding hydrogen reducing equivalents.  
61 These equivalents are then channeled into the electron transport system (ETS), where  
62 they contribute to the creation of a proton motive force, driving ATP synthesis via  
63 oxidative phosphorylation (OXPHOS)<sup>3</sup>. A portion of the proton motive force is dissipated  
64 without ATP synthesis through the phenomenon known as mitochondrial proton leak.  
65 While contributing to mitochondrial inefficiency, this process also plays a crucial role in  
66 regulating mitochondrial membrane potential and ROS <sup>4</sup>. The efficiency of mitochondria,  
67 the yield of ATP, and the rates of ROS generation all rely on the mitochondrial state,  
68 cellular energy demand, and the type of fuel substrate being oxidized by the mitochondria.  
69 Given their pivotal role in cellular physiology, mitochondria are among the frontline  
70 responders to various stressors that challenge cellular and organismal homeostasis <sup>2</sup>.  
71 Mitochondrial flexibility and robustness are crucial for adapting to stressors, ensuring  
72 cellular survival and maintaining energy homeostasis<sup>5-7</sup>.

73 Hypoxia, prevalent in aquatic environments due to factors like organic matter overload,  
74 nutrient pollution and stratification, poses significant challenges, particularly in coastal  
75 areas heavily impacted by anthropogenic activities<sup>8,9</sup>. Permanent hypoxia, as observed in  
76 coastal dead zones, is incompatible with metazoan life; however, even shorter periods of  
77 hypoxia, resulting from tidal, diurnal, or seasonal fluctuations in dissolved oxygen levels  
78 in coastal areas, present significant stress to marine organisms <sup>10</sup>. The ability to withstand  
79 oxygen fluctuations and hypoxia varies among organisms adapted to different habitats,  
80 with tolerance increasing from the subtidal zone, where infrequent and unpredictable  
81 hypoxic events occur, to the intertidal zone, home to organisms adapted to survive  
82 periodic hypoxia induced by low-tide emersion <sup>11-13</sup>. Strategies enhancing the ability of  
83 these hypoxia-tolerant organisms, including intertidal benthic invertebrates, revolve

84 around energy-conserving mechanisms like metabolic rate suppression, supported by  
85 high glycogen stores and reliance on alternative pathways for anaerobic ATP generation,  
86 which produce more ATP and less metabolic waste per unit substrate <sup>14</sup>.

87 Mitochondria emerge as critical targets of hypoxia and reoxygenation (H-R) stress due to  
88 their central role in aerobic energetics and signaling pathways. While mitochondria  
89 normally generate ATP via ETS activity and OXPHOS in the presence of oxygen, hypoxic  
90 conditions disrupt the ETS, slowing ATP generation and increasing reactive oxygen  
91 species (ROS) production<sup>15</sup>. Upon recovery from hypoxia, ROS levels surge, leading to  
92 mitochondrial damage, and a momentary collapse of ETS and OXPHOS, and oxidative  
93 stress <sup>16,17</sup>. Despite these challenges, some intertidal and benthic organisms have adapted  
94 their aerobic machinery to enhance their survival, with varying degrees of tolerance  
95 observed <sup>18-20</sup>. Previous studies have identified intertidal bivalves such as mussels and  
96 oysters, as well as some intertidal fish species as particularly hypoxia-tolerant compared  
97 to subtidal species, such as scallops. Hypoxia-tolerant species maintain or even enhance  
98 mitochondrial functional traits like OXPHOS and ETS activity <sup>19,21-23</sup>, whereas intolerant  
99 species experience loss of ETS activity, mitochondrial membrane depolarization, and  
100 oxidative stress <sup>16,24,25</sup>.

101 Recent evidence suggests that changes in intracellular milieu, such as shifts in metabolic  
102 intermediates, may contribute to mitochondrial responses to stress by altering fuel  
103 utilization for mitochondrial respiration <sup>26,27</sup>. Mitochondria exhibit metabolic flexibility in  
104 selecting substrate fuels for energy production during pathological and stressful  
105 conditions. This adaptability is observed in mammals under conditions like nutrient  
106 scarcity, type 2 diabetes, and hypoxia<sup>3-5</sup>, and in invertebrates and some rodents during  
107 thermal stress and oxygen limitation <sup>20,28-30</sup>. Notably, in hypoxia-tolerant oysters,  
108 succinate has been identified as a major stress response fuel, highlighting the importance  
109 of assessing mitochondrial flexibility in substrate utilization as a response mechanism to  
110 H-R stress<sup>19</sup>. Nevertheless, the contribution of mitochondrial fuels to metabolic responses  
111 to stressors, including H-R stress, remains poorly understood in non-model species such  
112 as marine invertebrates, with a literature bias toward hypoxia-tolerant species <sup>5,31,32</sup>. This  
113 knowledge gap has sparked interest in evaluating the mitochondrial flexibility of hypoxia-  
114 sensitive organisms in utilizing substrate fuels as a mechanism of response to H-R stress,  
115 thereby providing valuable insights into the differing metabolic response strategies  
116 between hypoxia-tolerant and hypoxia-intolerant species.

117 To investigate this, we examined the mitochondrial responses of the hypoxia-sensitive  
118 scallop *Pecten maximus* (Linnaeus, 1758), a subtidal bivalve species found in the Eastern  
119 Atlantic, the eastern North Sea coast, and the Mediterranean Sea <sup>33,34</sup>. Most scallops stand  
120 out among bivalves due to their mobility as adults, exhibiting swimming and jumping  
121 behavior as predator escape reactions <sup>35,36</sup>. Their energy-demanding vigorous muscle  
122 activity during valve clapping for swimming and jumping as well as their inability to fully  
123 close their shells renders scallops reliant on oxygenated environments during recovery,  
124 making them highly sensitive to H-R stress <sup>37</sup>. The intense activity frequently leads to  
125 functional anaerobiosis in scallops, as the demand for oxygen exceeds its supply during  
126 vigorous exertion <sup>38</sup>. The reliance on mitochondria during activity bouts and subsequent  
127 recovery in scallops highlights the importance of understanding their mitochondrial  
128 responses to H-R stress, a topic that warrants further investigation. In this study, we  
129 exposed mitochondria from two tissues of *P. maximus*, the gill and digestive gland, to  
130 short-term H-R (15 min hypoxia, 10 minutes reoxygenation) and long-term H-R (90 min  
131 hypoxia, 10 min reoxygenation), and evaluated the substrate and tissue-specific  
132 mitochondrial responses to these varying periods of H-R stress. Both gill and digestive  
133 gland tissues are metabolically important organs: gills facilitate oxygen uptake, sensitive  
134 to oxygen fluctuations, while the digestive gland serves digestion and energy storage  
135 functions<sup>39</sup>. We hypothesized that gill tissue would exhibit heightened sensitivity to H-R  
136 stress compared to the digestive gland, with long-term hypoxia expected to induce more  
137 significant damage to mitochondrial functional traits and elevate oxidative stress levels  
138 in the gills. Drawing on prior research indicating succinate as a recovery fuel in hypoxia-  
139 tolerant marine bivalves <sup>19,20,22</sup>, we speculated that the capacity for mitochondrial  
140 succinate oxidation would be compromised under long-term H-R stress in scallops,  
141 potentially to a greater extent than observed in hypoxia-tolerant bivalves like mussels and  
142 oysters. To test our hypotheses, we measured basal and ADP-stimulated oxygen  
143 consumption rates and ROS efflux in isolated mitochondria from *P. maximus* gills and  
144 digestive gland under normoxic conditions and two H-R regimes: short-term (15 min)  
145 severe hypoxia (~0% O<sub>2</sub>) followed by 10 min of reoxygenation, and long-term (~90 min)  
146 followed by 10 min of reoxygenation. We used a fatty acid (palmitate) and a carboxylic  
147 acid (pyruvate) as Complex I substrates, and succinate as a Complex II substrate.  
148 Additionally, to assess ROS-induced damage, we measured protein carbonyl accumulation  
149 and lipid peroxidation (levels of 4-hydroxynonenal, HNE) in isolated gill mitochondria of  
150 scallops respiring on different substrates during short-term and long-term H-R

151 exposures. Our findings aim to elucidate mechanisms associated with hypoxia sensitivity  
152 and shed light on the distinctions in mitochondrial resilience between hypoxia-tolerant  
153 and hypoxia-intolerant species of marine bivalves<sup>18</sup>.

## 154 **Materials and methods**

155 **Chemicals.** All chemicals were purchased from Sigma Aldrich (Munich, Germany), Fisher  
156 Scientific (Schwerte, Germany), or Carl Roth (Karlsruhe, Germany) and were of analytical  
157 grade or higher.

158 **Animals.** Adult king scallops *P. maximus* were collected in the estuary of Vigo, Spain  
159 (42°14'46.6"N 8°44'18.5"W) at 10 m depth and transported by car, submerged under  
160 aerated water at 10°C to the Alfred Wegener Institute (AWI), Helmholtz Centre for Polar  
161 and Marine Research in Bremerhaven, Germany. Upon arrival, scallops were transferred  
162 to the AWI aquarium system filled with North Sea water (10 °C, salinity 32 practical  
163 salinity units) and acclimated for at least 4 weeks. Scallops were fed 3 times a week by a  
164 mixture of commercial algal blend (Nyos, PhytoMaxx) and a self-cultivated algal mixture  
165 including *Rhodomonas* sp., *Phaeodactylum tricornutum*, *Chaetocerus* sp., and *Isochrysis*  
166 *galbana* (minimum 3000 cells ml<sup>-1</sup> SW).

167 **Mitochondrial isolation.** Mitochondria isolates were obtained from gill and digestive  
168 glands tissues of scallops. Briefly, 1–2 g of gill or digestive gland tissue were homogenized  
169 in ice-cold isolation buffer (50 mmol l<sup>-1</sup> NaCl, 300 mmol l<sup>-1</sup> sucrose, 130 mmol l<sup>-1</sup> KCl, 30  
170 mmol l<sup>-1</sup> 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES), 8 mmol l<sup>-1</sup>  
171 ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1% fatty acid  
172 free bovine serum albumin (BSA), 50 µg l<sup>-1</sup> aprotinin, 1 mmol l<sup>-1</sup> phenylmethylsulfonyl  
173 fluoride (PMSF) at pH 7.5) using several passes of a Potter–Elvehjem homogenizer at  
174 200 r.p.m. The homogenate was centrifuged for 4 min at 2000×g to remove cell debris.  
175 The supernatant was collected and centrifuged for 8 min at 8500×g to acquire the  
176 mitochondrial pellet. The pellet was washed twice with the isolation media and  
177 recollected by centrifugation (5 min at 8500×g). All centrifugation steps were conducted  
178 at 4 °C. The mitochondrial pellet was resuspended in ice-cold assay media (30 mmol l<sup>-1</sup>  
179 HEPES, 550 mmol l<sup>-1</sup> sucrose, 10 mmol l<sup>-1</sup> glucose, 130 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> NaCl, 1  
180 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 10 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 7.2, and 1% fatty acid free bovine serum albumin  
181 (BSA). Both gill and digestive gland mitochondria were isolated from the same scallop.

182 **Mitochondrial oxygen consumption ( $\dot{M}O_2$ ) and ROS efflux rates.**  $\dot{M}O_2$  and ROS efflux  
183 were measured simultaneously at 15 °C using an Oxygraph 2k high-resolution  
184 respirometer (Oroboros, Innsbruck, Austria) and integrated DatLab 7.4 software. Oxygen  
185 consumption rate of isolated mitochondria was measured using a Clark-type electrode  
186 calibrated with 100% (air-saturated assay buffer) and 0% (saturated solution of sodium  
187 dithionite). The background flux for the oxygen measurements was  $\pm 1$  pmol  $O_2$   $s^{-1}$   $ml^{-1}$ .  
188 The ROS efflux was measured as the release of  $H_2O_2$  reflecting the net balance between  
189 the mitochondrial  $H_2O_2$  production and consumption <sup>40</sup>. Measurements were conducted  
190 with a fluorometer integrated into the Oxygraph 2k (Fluorescence-Sensor Green, 525 nm)  
191 in an assay buffer containing 10  $\mu$ mol  $l^{-1}$  Amplex Red, 1 U  $ml^{-1}$  horseradish peroxidase  
192 stock solution, and 5 U  $ml^{-1}$  superoxide dismutase (SOD) <sup>41</sup>. Calibration was conducted  
193 with addition of 0.1  $\mu$ mol  $l^{-1}$   $H_2O_2$  before and after the addition of the mitochondrial  
194 suspension to the chamber containing 2 ml of temperature-equilibrated assay buffer. A  
195 substrate-uncoupler-inhibitor titration (SUIT protocol) was conducted by sequential  
196 additions of substrates and inhibitors as follows: 1) 5 mmol  $l^{-1}$  pyruvate with 2 mmol  $l^{-1}$   
197 malate, 2) 10 mmol  $l^{-1}$  palmitoyl-DL-carnitine, 3) 10 mmol  $l^{-1}$  succinate; 4) 2.5 mmol  $l^{-1}$   
198 ADP to achieve ADP-stimulated OXPHOS state.

199 After the ADP addition, mitochondria were permitted to respire until all oxygen in the  
200 chamber was consumed and near-anoxia ( $\sim 0\%$   $O_2$ ) was achieved. Depending on the  
201 mitochondrial activity, this took 10-20 min. The mitochondria were then maintained  
202 under near-anoxia for 15 min (short-term hypoxia) or 90 min (long-term hypoxia).  
203 Oxygen tension was then raised to  $\sim 80$ -100% of air saturation, and mitochondria were  
204 allowed to recover for 10 min (reoxygenation). Following 10 min reoxygenation, post-  
205 hypoxic OXPHOS respiration rate was recorded, and the SUIT continued as follows: 5) 2.5  
206  $\mu$ mol  $l^{-1}$  oligomycin to inhibit mitochondrial  $F_1F_0$ -ATPase and measure post-hypoxic  
207 LEAK respiration (state 4); 6) 40 mmol  $l^{-1}$  KCN to measure non-mitochondrial respiration  
208 ( $< 10\%$  of the total oxygen consumption rate, data not shown). This experiment resulted  
209 in four values of respiration and ROS efflux measured for each mitochondrial isolate as  
210 follows: 1) baseline LEAK respiration and ROS efflux prior to H-R after addition of the  
211 substrates before the addition of ADP (State II; step 3 of the SUIT); 2) OXPHOS respiration  
212 and ROS efflux prior to H-R (state III, step 4 of the SUIT); 3) OXPHOS respiration and ROS  
213 efflux after H-R (15 or 90 min of hypoxia followed by 10 min of recovery); 4) LEAK  
214 respiration and ROS efflux after the H-R measured following addition of the  $F_1F_0$ -ATPase

215 inhibitor oligomycin (State IV; step 5 of the SUIT protocol). Pilot studies indicated a less  
216 than 5% difference in LEAK rate between scallops mitochondria in State II and State IV  
217 (data not shown). To confirm that observed effects were due to H-R stress rather than loss  
218 of mitochondrial viability over time, parallel measurements were done under normoxic  
219 conditions during prolonged exposures (~100-120 min). Results showed no variance in  
220 mitochondrial functional parameters under normoxia, indicating no loss of mitochondrial  
221 viability over time in normoxic conditions (data not shown).

222 Protein concentrations in the mitochondrial suspensions were measured using a Bio-Rad  
223 Bradford protein assay (Bio-Rad, Hercules, CA, USA) <sup>42</sup> using BSA as a standard. Protein  
224 concentrations of the mitochondrial suspensions were corrected for the BSA content of  
225 the assay media. Mitochondrial respiration and ROS efflux rates were expressed as nmol  
226 O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> protein and nmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> protein, respectively. The respiratory  
227 control ratio (RCR) was calculated as a ratio of OXPHOS to LEAK respiration <sup>43,44</sup>. The  
228 fractional electron leak rate (FEL) was determined by dividing the H<sub>2</sub>O<sub>2</sub> efflux rate by the  
229 oxygen consumption rate in the same mitochondrial isolate. For each experimental group  
230 (pyruvate, succinate, and palmitate), eight scallops were used with gill and the digestive  
231 gland mitochondria isolated from each scallop.

232 ***Measurement of oxidative stress indices.*** Concentrations of carbonyls (as a proxy for  
233 oxidative damage of proteins) and HNE (proxy of lipid peroxidation) were determined by  
234 Enzyme-linked Immunosorbent Assay (ELISA). After the mitochondrial functional assays  
235 were completed, aliquots of gill mitochondria suspensions were collected from the  
236 control (normoxic) conditions and after short-term H-R stress and stored at -80 °C.  
237 Samples were lysed and diluted to 10 µg ml<sup>-1</sup> and 5 mg ml<sup>-1</sup> protein with phosphate  
238 buffered saline (PBS) solution for carbonyl and HNE measurements, respectively.

239 The protein carbonyl ELISA protocol was adapted from Buss and Winterbourn <sup>45</sup> with  
240 modifications. Carbonyl standards were prepared by mixing varying amounts of oxidized  
241 BSA solution (IgG-free BSA dissolved in H<sub>2</sub>O<sub>2</sub>) and reduced BSA solution (IgG-free BSA  
242 dissolved in PBS) to a protein content of 10 µg ml<sup>-1</sup>. Carbonyl concentrations in the  
243 oxidized BSA were determined spectrophotometrically. Aliquots (100 µl) of samples or  
244 standards were placed into ELISA microwell plates and incubated overnight at 4 °C. Plates  
245 were washed three times with 250 µl of PBS. 100 µl solution of 5 mM 2, 4-  
246 dinitrophenylhydrazine (DNPH) was added to each well and incubated in the dark for 45

247 min at room temperature. After incubation, the plate was washed five times with PBS:  
248 ethanol (1:1 v:v) mixture and twice with PBS. The plate was blocked with 200  $\mu$ l of 1 mg  
249  $\text{ml}^{-1}$  BSA solution for 2 h at room temperature and washed three times with 0.05% Tween  
250 solution. The primary anti-DNP antibody (monoclonal, host mouse from Sigma Aldrich  
251 (MAB2223)) was added and incubated for 1 h. After three washes with 0.05% Tween, the  
252 plates were incubated with a secondary anti-mouse antibody (Abcam) for 1 h at room  
253 temperature. The plate was washed five times with 0.05% Tween solution. 100  $\mu$ l TMB/E  
254 ultra-sensitive blue Horseradish Peroxidase substrate was added to each well and upon  
255 color development (10 min), 100  $\mu$ l 2 M sulfuric acid was added to stop the reaction. The  
256 absorbance was read at 450 nm.

257 The HNE ELISA protocol was modified from Weber et al.,<sup>46</sup> and Borovic et al.,<sup>47</sup>. 4-HNE-  
258 dimethylacetal solution (Sigma Aldrich) was dried under constant nitrogen flow and  
259 dissolved in 1 mM HCl at room temperature. The concentration of HNE solution was  
260 determined by measuring absorbance at 450 nm. HNE standards were prepared by  
261 mixing varying amount of HNE solution with 5 mg/ml fatty acid free BSA dissolved in PBS  
262 to desired concentration and aliquots stored at  $-20^{\circ}\text{C}$  until analysis. ELISA plates (Nunc  
263 Immuno Maxisorp, Thermo Scientific) were coated with 200  $\mu$ l of 0.05 M carbonate  
264 binding buffer (pH 9.6; sodium carbonate) and 20  $\mu$ l of standard or samples. All samples  
265 were measured in triplicates. Plates were incubated overnight at  $4^{\circ}\text{C}$ , washed with 300  $\mu$ l  
266 PBS, and blocked with 5% fat free dry milk in carbonate binding buffer for 2.5 h at room  
267 temperature. The plate was then washed five times with 0.1% Tween 20 in PBS. 200  $\mu$ l of  
268 the primary antibody (mouse monoclonal anti-4-Hydroxynonenal antibody from Abcam,  
269 (AB48506)) was added and incubated for 2 h at  $37^{\circ}\text{C}$ . The plate was washed five times  
270 and incubated with peroxidase solution (3%  $\text{H}_2\text{O}_2$  in PBS) for 30 min. The washing was  
271 repeated prior to the addition of 100  $\mu$ l goat anti-mouse secondary antibody solution. The  
272 plates were incubated for 1 h at room temperature, washed once, incubated for 10 min  
273 with 200  $\mu$ l TMB substrate solution, and 50  $\mu$ l of 2 M sulfuric acid was added to stop the  
274 reaction. The absorbance of samples and standards was measured at 450 nm. The amount  
275 of HNE measured was expressed as nmol HNE/mg protein.

276 **Statistics.** We conducted a normality test to detect outliers in the mitochondrial  
277 functional traits ( $\dot{\text{M}}\text{O}_2$  and ROS efflux) and oxidative stress markers. To evaluate the  
278 interactive effect of the exposure (control or H-R) and respiratory substrate on  
279 mitochondrial oxygen consumption, ROS efflux, FEL, and protein carbonyl and HNE

280 accumulation, we used a repeated measures two-way ANOVA model. H-R exposures were  
281 denoted as a within-subject factor and respiratory substrate as a between-subject factor.  
282 The exposure was treated as a within-subject factor since measurements of the  
283 mitochondrial traits under various oxygen treatments (control and H-R) were conducted  
284 in the same mitochondrial isolate. For the tissue-specific substrate utilization, we used  
285 Tukey's honest significant differences (HSD) tests for planned comparisons of the group  
286 means. The effects of hypoxia and reoxygenation on mitochondrial  $\dot{M}O_2$  and ROS efflux  
287 were tested using the paired Student's t-test with the paired values measured in the same  
288 mitochondrial isolate before and after H-R exposure. All statistical analyses were  
289 conducted using IBM® SPSS® Statistics ver. 22.0.0.0 (IBM Corp., Armonk, NY, USA) and  
290 GraphPad Prism v. 7.02 (GraphPad Software Inc., La Jolla, CA, USA) software. Differences  
291 were considered significant if the probability of Type II error (P) was <0.05. For  
292 mitochondrial  $\dot{M}O_2$  and ROS efflux, sample size (N) was 8 for all groups. In some groups,  
293 N was lower owing to the removal of 1–2 statistically significant outliers (P<0.05) as  
294 indicated in figure legends. For oxidative stress markers, sample size (N) was 5 for all  
295 measurements.

296 We have followed the recommendation of evidence-based language <sup>48</sup> for describing our  
297 results. The following thresholds were used: P=0.049-0.011 (moderate evidence), P=0.01-  
298 0.001 (strong evidence), P<0.001 (very strong evidence).

## 299 **Results**

300 ***Tissue-specific mitochondrial  $\dot{M}O_2$  and ROS efflux in normoxia.*** We found strong to  
301 very strong evidence for the effect of the oxidized substrate on LEAK and OXPHOS  
302 respiration rates of scallop mitochondria (Table 1). For the OXPHOS state respiration,  
303 there was also strong evidence for the interactive effects of substrate and tissue (Table 1).  
304 Generally, the LEAK respiration rate was highest during succinate oxidation and the  
305 OXPHOS rate was highest with pyruvate as a substrate in mitochondria from both studied  
306 tissues (Fig. 1A). The ROS efflux rate and FEL in both the LEAK and OXPHOS state  
307 mitochondria exhibited moderate to strong evidence of substrate effects and very strong  
308 evidence of tissue-specific differences, with no significant interactive effects (Table 1). In  
309 the gill mitochondria, the ROS efflux rate in the LEAK state increased in the order pyruvate  
310 < palmitate < succinate (Fig. 1C). In the gill mitochondria in the OXPHOS state, the ROS  
311 efflux was higher during palmitate oxidation compared with the oxidation of pyruvate or

312 succinate (Fig. 1C). Similar but statistically not significant substrate-dependent trends of  
313 ROS efflux were found in the digestive gland mitochondria (Fig. 1C, D). In the gill  
314 mitochondria in both LEAK and OXPHOS states, the lowest FEL was found during  
315 oxidation of pyruvate compared with palmitate or succinate (Fig. 1E, F). In the digestive  
316 gland mitochondria, no significant substrate-specific differences in ROS efflux or FEL were  
317 found (Fig. 1E, F). Notably, substrate-specific ROS efflux rates and FEL were higher in the  
318 digestive gland mitochondria compared to those from the gills, despite similar or lower  
319 mitochondrial respiration rates in the digestive gland compared to the gill (cf. Fig. 1 C-F  
320 and 1A, B).

321 ***Effect of H-R stress on scallop mitochondria: LEAK state.*** During short-term hypoxia,  
322 there was very strong evidence for the interactive effect of hypoxia exposure and  
323 substrate on LEAK  $\text{MO}_2$  in the gill and digestive gland mitochondria (Table 2). Likewise,  
324 the interaction of hypoxia exposure and substrate showed very strong evidence of effect  
325 on the ROS efflux in the gills but not in the digestive gland mitochondria (Table 2). In the  
326 gill mitochondria, we found no evidence of effect of exposure, substrate or their  
327 interactions on FEL. In the digestive gland mitochondria, ROS efflux and FEL showed very  
328 strong evidence of hypoxia exposure only (Table 2).

329 Under long-term hypoxia, there was very strong evidence for the interactive effect of H-R  
330 exposure and substrate on LEAK  $\text{MO}_2$  in the gill mitochondria, whereas in the digestive  
331 gland, only a moderate effect of the substrate on LEAK  $\text{MO}_2$  was found (Table 3). No  
332 evidence of the interactive effects of substrate and H-R on ROS efflux was found in the gill  
333 or digestive gland mitochondria after long-term H-R exposures (Table 3). In the gill,  
334 substrate had an effect on the ROS efflux in the LEAK state ( $P=0.002$ ), whereas in the  
335 digestive gland, only the H-R exposure effect was significant ( $P<0.001$ ). For LEAK state  
336 FEL, we found very strong interactive effect of H-R and substrate in the gill mitochondria,  
337 and no evidence of effects of studied factors or their interactions in the digestive gland  
338 mitochondria (Table 3).

339 Both short- and long-term hypoxia increased LEAK state oxygen consumption in gill  
340 mitochondria oxidizing pyruvate, without a simultaneous increase in ROS efflux or FEL  
341 (Fig. 2A, C). Short- and long-term hypoxia suppressed the palmitate-driven LEAK  
342 respiration. However, LEAK state ROS efflux did not change but FEL increased after H-R  
343 in mitochondria oxidizing palmitate (Fig. 2C, E). There was no evidence for the H-R effect

344 on the succinate-driven LEAK  $\dot{M}O_2$  of the gill mitochondria (Fig. 2A). The ROS efflux in the  
345 LEAK state decreased after H-R and FEL did not change in response to H-R in the LEAK  
346 state gill mitochondria respiring on succinate (Fig. 2C, E).

347 Similar to the gill mitochondria, H-R resulted in increased LEAK  $\dot{M}O_2$  in the digestive gland  
348 mitochondria oxidizing pyruvate, although this effect was only statistically significant  
349 after short-term H-R (Fig. 3A). However, during palmitate and succinate oxidation, LEAK  
350  $\dot{M}O_2$  in the digestive gland mitochondria decreased after long-term (with palmitate) and  
351 short-term (with succinate) H-R (Fig. 3A). The ROS efflux in the LEAK state decreased  
352 after H-R exposure of the digestive gland mitochondria irrespective of the oxidized  
353 substrate (Fig. 3C). FEL also decreased in the digestive gland mitochondria oxidizing  
354 NADH-linked substrate (pyruvate and palmitate) after the short-term H-R whereas for  
355 the long-term H-R, FEL decreased only during succinate driven LEAK state (Fig. 3E).

356 ***Effect of H-R stress on scallop mitochondria: OXPHOS state.*** After short-term H-R  
357 exposure, no evidence of interactive effects of H-R and substrate on OXPHOS  $\dot{M}O_2$ , ROS  
358 efflux and FEL was found in the gill mitochondria, and only a moderate evidence of the  
359 interactive effects on ROS efflux was found in the digestive gland mitochondria (Table 2).  
360 After short-term H-R, OXPHOS  $\dot{M}O_2$  showed moderate to very strong effects of substrate  
361 and H-R in the gill and the digestive gland mitochondria. For OXPHOS ROS efflux,  
362 moderate evidence of the substrate effect was found in the gill, and strong evidence of H-  
363 R effect – in the digestive gland mitochondria (Table 2). In the digestive gland  
364 mitochondria, no evidence of the effects of the studied factors or their interaction was  
365 found for FEL (Table 2).

366 Under long-term H-R exposure, we found moderate to very strong evidence for the  
367 interactive effects of H-R exposure and oxidized substrate on OXPHOS  $\dot{M}O_2$  and FEL in  
368 both studied tissue types (Table 3). No evidence of the interactive effects on OXPHOS ROS  
369 efflux was found. The OXPHOS ROS efflux after long-term H-R There was very strong  
370 evidence for the effect of oxidized substrate on ROS efflux in the phosphorylating gill  
371 mitochondria and no effect of long-term H-R showed very strong evidence of substrate  
372 effects in the gills, and of the H-R-exposure in the digestive gland mitochondria (Table 3).

373 The OXPHOS respiration in the gill mitochondria of scallops was strongly suppressed by  
374 H-R, regardless of the oxidized substrate or hypoxia duration (Fig. 2B). The ROS efflux and

375 FEL in the OXPHOS state generally remained at baseline level after H-R exposure, except  
376 for the increase in FEL in the palmitate-respiring mitochondria in the OXPHOS state (Fig.  
377 2D, F).

378 Similar to the gills, the OXPHOS respiration in the digestive mitochondria of scallops was  
379 consistently suppressed by H-R, regardless of the oxidized substrate or the duration of  
380 hypoxia (Fig. 3B). Additionally, there was also a tendency for the H-R induced decrease in  
381 ROS efflux in the digestive gland mitochondria in the OXPHOS state, albeit the pattern was  
382 less consistent than for  $\dot{M}O_2$  (Fig. 3D). Short-term H-R had no significant effect on OXPHOS  
383 FEL, whereas the effects of long-term H-R on OXPHOS FEL were substrate-dependent,  
384 showing a decrease for pyruvate and succinate and an increase for palmitate oxidation  
385 (Fig. 3F).

386 ***Mitochondrial coupling.*** In gill and digestive gland mitochondria, exposure to both short-  
387 and long-term hypoxia suppressed mitochondrial coupling during Complex I-linked  
388 (pyruvate and palmitate) substrate oxidation (Fig. 4A, B). However, mitochondrial  
389 coupling remained at baseline levels during succinate-driven respiration in the gill  
390 mitochondria under both short- and long-term hypoxia conditions (Fig. 4A). In the  
391 digestive gland mitochondria, respiratory coupling with succinate decreased after long-  
392 term H-R but remained stable after short-term H-R (Fig. 4B).

393 ***Oxidative damage after short-term hypoxia.*** The analyzed oxidative stress markers  
394 showed no evidence of interactive or individual effects of substrates and H-R exposure  
395 (Table 4). Mitochondrial concentrations of protein carbonyls and HNE remained at  
396 baseline levels after H-R exposure (Fig. 5A, B).

## 397 **Discussion**

398 The mitochondria's ability to utilize different substrates is vital under different  
399 physiological and metabolic states <sup>49</sup>. In animals, the substrate preference of different  
400 tissue varies. Thus, in mammals, mitochondria from the brain have preference for glucose  
401 <sup>50</sup>, heart mitochondria oxidize fatty acids, amino acids, and lactate <sup>51 52</sup>, liver mitochondria  
402 show preference for succinate <sup>53</sup>, whereas kidney and skeletal muscle mitochondria can  
403 effectively oxidize fatty acids and glucose <sup>54,55</sup>. In our study of the mitochondria of the king  
404 scallop *P. maximus*, we found preference for carbohydrate (pyruvate) oxidation in both  
405 the gill and the digestive gland tissues as shown by high OXPHOS rate and coupling

406 efficiency (indicated by high RCR). Furthermore, the ROS efflux rate and FEL in the scallop  
407 mitochondria respiring on pyruvate tended to be lower than with succinate and palmitate.  
408 Contrarily, in the Pacific oyster *C. gigas*, we found preference for glutamate and succinate  
409 oxidation especially in the digestive gland as the gill mitochondria had broader substrate  
410 preference from carbohydrate, amino acids and fatty acids<sup>19</sup>. Just like the scallop  
411 mitochondria, palmitate and succinate oxidation recorded higher ROS efflux than  
412 pyruvate in the oyster mitochondria. Increased ROS efflux during palmitate oxidation  
413 have also been recorded in different mammalian cells and tissues and associated with  
414 endoplasmic reticulum stress and Ca<sup>2+</sup> overload leading to oxidative stress and eventually  
415 cellular damage and death (Ly et al., 2017). Mitochondrial succinate oxidation has been  
416 reported to strongly stimulate ROS generation via reverse electron transport (RET)  
417 through mitochondrial Complex I<sup>56</sup>. Pyruvate oxidation on the other hand produces NADH  
418 by pyruvate dehydrogenase and NADH is known to directly act as an antioxidant by  
419 scavenging ROS in the mitochondria and an indirect antioxidant via the glutathione  
420 reaction thereby suppressing the ROS efflux<sup>57</sup>. The antioxidant activity of pyruvate might  
421 explain our observation of low ROS efflux recorded in the scallop mitochondria oxidizing  
422 pyruvate. However, the digestive gland mitochondria of scallops generally showed higher  
423 ROS efflux and elevated electron leak rates during respiration with all three tested  
424 substrates than the gill mitochondria. This indicates that the digestive gland tissues might  
425 be more prone to oxidative stress than the gill in scallops. Additionally, the higher ROS  
426 efflux might also be an isolation artifact (higher levels of digestive enzymes released  
427 during the homogenization of the digestive gland tissue). The mechanisms underlying this  
428 difference – such as potential differences in the levels of mitochondria antioxidants or the  
429 efficiency of the electron transfer in the ETS in different tissues - require further  
430 investigation.

431 Marine bivalves of the family Pectinidae including the king scallop *P. maximus*  
432 demonstrate notable sensitivity to hypoxia compared to other marine bivalves<sup>25</sup>. Our  
433 study reveals that even relatively short durations of hypoxia (15-90 min) can cause injury  
434 to scallop mitochondria, consistent with their low tolerance to hypoxia at the organism  
435 level<sup>58,59</sup>. Both short-term (15 min) and long-term (90 min) hypoxia-reoxygenation (H-R)  
436 stress led to a reduction in respiratory capacity in scallop mitochondria. However, this  
437 mitochondrial response to H-R stress in *P. maximus* varied depending on the tissue and  
438 substrate. In scallop gill and digestive gland mitochondria, the most significant decline in

439 ATP synthesis capacity during H-R occurred during palmitate oxidation (by ~45-85%),  
440 followed by pyruvate oxidation (by ~15-55%). Additionally, the LEAK respiration, which  
441 indicates the futile proton leak not associated with ATP synthesis, increased in scallop  
442 mitochondria respiring on pyruvate (by ~40-165% depending on the tissue and duration  
443 of hypoxia). Consequently, exposure to H-R resulted in a substantial decrease in  
444 mitochondrial coupling efficiency during pyruvate oxidation, as evidenced by the loss of  
445 the RCR. A less pronounced degree of H-R-induced uncoupling was observed in the scallop  
446 mitochondria respiring on palmitate. Unlike pyruvate oxidation, this loss of coupling with  
447 palmitate reflected the stronger suppression of OXPHOS respiration by H-R compared to  
448 LEAK respiration. Taken together, these findings suggest that mitochondrial Complex I in  
449 *P. maximus* is highly susceptible to H-R induced stress, which is likely to strongly diminish  
450 ATP synthesis capacity utilizing both glycolytic products and fatty acids in scallop  
451 mitochondria during post-hypoxic recovery. The susceptibility of mitochondrial Complex  
452 I have also been reported in organisms like turtles<sup>60</sup>, and other bivalves like quahogs<sup>61</sup>,  
453 Pacific oyster and blue mussels<sup>19,22</sup>. In the Pacific oyster *C. gigas*, H-R stress led to a  
454 decrease in LEAK respiration with no effect on OXPHOS during Complex I substrate  
455 oxidation<sup>19</sup>. This decline might be due to the modulation of proton conductance or a  
456 decrease in the resting mitochondrial membrane potential. Contrary to that observed in  
457 the oyster, the scallop mitochondria showed a significant decline in OXPHOS respiration  
458 during H-R stress. This decline might be due to the inactivation and damage to Complex I  
459 as well as the activation of uncoupling proteins hence lower Complex I enzymatic  
460 activity<sup>60,61</sup>.

461 In the scallop gill and digestive gland mitochondria, Complex II-linked substrate oxidation  
462 also declined after H-R stress. However, this decline in respiratory capacity was only ~20  
463 - 40% of that observed during Complex I-linked substrate oxidation. The decline was also  
464 similar between both gill and digestive gland mitochondria except for LEAK respiration  
465 (~2 - 20%) in gill mitochondria. Additionally, mitochondrial coupling efficiency (RCR)  
466 was maintained in both tissue mitochondria oxidizing succinate after H-R stress except  
467 for a slight decline after long-term H-R stress in the digestive gland mitochondria.  
468 Previous studies by Ivanina et al.,<sup>25</sup> reported that the exposure of scallops to intermittent  
469 hypoxia induced a strong decline in the succinate substrate oxidation and  
470 phosphorylation capacities in scallop mitochondria. Contrary to the scallop mitochondria,  
471 the mitochondria of hypoxia tolerant bivalves oxidizing succinate maintain or enhance

472 OXPHOS respiration capacity after H-R stress<sup>19,22</sup>. Succinate has been reported as a key  
473 metabolic intermediate that accumulates (anaerobiosis) in aerobic organisms including  
474 bivalves during hypoxia<sup>62</sup>. On reoxygenation, succinate oxidation capacity was enhanced  
475 which might facilitate post-hypoxic recovery by rapidly restoring ATP levels and  
476 removing excess succinate. Although our present study did not observe enhanced  
477 succinate oxidation after H-R stress, the decline after succinate-driven respiration was  
478 lower than pyruvate- and palmitate-driven respiration. Hence, we can suggest that scallop  
479 mitochondria respond better to H-R stress when oxidizing succinate as opposed to  
480 Complex I-linked substrates. However, the scallop mitochondria from gills and digestive  
481 gland are apparently not as well adapted for succinate oxidation after H-R stress as  
482 mitochondria of hypoxia-tolerant species.

483 Transitioning from hypoxia to high oxygen conditions commonly leads to increased ROS  
484 production, primarily attributed to the accumulation of reduced intermediates such as  
485 reduced coenzyme Q and succinate in the mammalian mitochondria<sup>63</sup>. Succinate,  
486 particularly in vertebrates, is recognized as a significant contributor to elevated ROS  
487 production because it drives reverse electron transport (RET) through mitochondrial  
488 Complex I, resulting in a high rate of superoxide generation<sup>64-66</sup>. The adaptive  
489 mechanisms that limit the dangers posed by RET during H-R in vertebrates are commonly  
490 associated with suppression of Complex I activity and/or succinate accumulation<sup>67-69</sup>. In  
491 bivalves, succinate is a major end product of anaerobic metabolism that accumulates to  
492 high levels during hypoxia<sup>62,70,71</sup>. In scallops, amino acid derivatives (opines) also  
493 accumulate in hypoxia and during functional anaerobiosis caused by physical activity<sup>72,73</sup>  
494 . Our study found that the gill mitochondria of *P. maximus* are more effective at regulating  
495 ROS efflux and electron leak during H-R stress compared to mitochondria from the  
496 digestive gland. During active phosphorylation (OXPHOS state), the substrate-specific  
497 rate of ROS release in gill mitochondria remained unaffected by the tested H-R conditions.  
498 In the resting state (LEAK), a decrease in ROS efflux was observed during succinate  
499 oxidation in the gill mitochondria, while ROS efflux during pyruvate and palmitate  
500 oxidation showed no change in response to H-R stress. These findings suggest a lack of  
501 significant succinate-driven RET in *P. maximus* gill mitochondria. Contrarily, in the gill  
502 mitochondria of the hypoxia-tolerant Pacific oyster, H-R stress induced higher succinate-  
503 driven ROS efflux than that with NADH-linked substrates especially in the phosphorylated  
504 mitochondria as opposed to the resting mitochondria<sup>19</sup>. The heightened ROS efflux was

505 partially alleviated by rotenone addition suggesting the contribution of RET to H-R-  
506 induced ROS efflux. Similar observations were also made in the digestive gland  
507 mitochondria of the hypoxia-tolerant subtidal *Arctica islandica* where succinate-induced  
508 ROS efflux increased during both LEAK and OXPHOS respiration after H-R stress<sup>74</sup>.  
509 However, this increase was suppressed with rotenone confirming the contribution of RET.  
510 Although there was lack of RET contribution to ROS in *P. maximus*, there was an increase  
511 in fatty acid oxidation-induced electron leak in gill mitochondria, indicating a potential  
512 association between fatty acid oxidation and oxidative stress during post-hypoxia  
513 recovery. This suggests that under H-R stress, fatty acid oxidation is associated with both  
514 oxidative stress and impaired ATP synthesis capacity in the scallops' gills. However,  
515 investigating levels of oxidative stress markers (protein carbonyls and lipid peroxidation  
516 products) revealed the lack of oxidative stress irrespective of the oxidized substrate.  
517 Hence our earlier suggestion that fatty acid oxidation is associated with oxidative stress  
518 is invalid.

519 Unlike the gill mitochondria, H-R stress decreased ROS release from scallop digestive  
520 gland mitochondria in both LEAK and OXPHOS states of mitochondria across all studied  
521 substrates. Following H-R exposure, ROS efflux from *P. maximus* digestive gland  
522 mitochondria decreased by ~15-70% during NADH-linked substrate oxidation and by  
523 ~35-75% during succinate oxidation. This decline wasn't entirely proportional to the  
524 decrease in oxygen consumption rates, as indicated by significant changes in the  
525 fractional electron leak (FEL) in many cases post H-R. During palmitate oxidation, FEL  
526 was suppressed after short-term H-R and significantly elevated after long-term H-R,  
527 suggesting a decrease in electron transfer efficiency in the latter case. These findings  
528 support the hypothesis that fatty acid oxidation is notably dysregulated during post-  
529 hypoxic recovery in both studied tissue types of scallop mitochondria. The dysregulation  
530 of fatty acid oxidation during post-hypoxic recovery have also been reported in the Pacific  
531 oyster especially the digestive gland mitochondria<sup>19</sup>.

## 532 **Conclusions**

533 Taken together, our findings reveal the robustness of scallop gills and digestive gland  
534 mitochondria to oxidize succinate during intermittent hypoxia and the potential of  
535 succinate as a stress fuel. Additionally, hypoxia revealed similar effects like the damage to  
536 protein complexes, loss of ETS function, and loss of mitochondrial quality control on the

537 hypoxia-intolerant scallop mitochondria as reported in other intolerant species like the  
538 highly researched mammalian models<sup>60,75-77</sup>. However, effects like oxidative stress and  
539 damage were not associated with H-R induced changes in the scallop mitochondria. The  
540 lack of oxidative stress and damage associated with intermittent hypoxia in the scallop  
541 mitochondrial suggests an active functioning of the scallop antioxidant system during H-  
542 R stress.

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### 549 **Conflict of interest statement**

550 Authors have no conflict of interest.

### 551 **Data sharing and availability**

552 The data that supports the findings of this study are available in the manuscript.  
553 Additional data supporting this manuscript will be available on request.

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- 778

779 **Table 1.** Repeated measures ANOVA: Effects of tissue type (gills and digestive gland) and  
 780 substrates on the respiration, ROS efflux and FEL in the mitochondria of *P. maximus* under  
 781 normal oxygen condition.

782 Mitochondrial parameters were measured in the resting (LEAK) and actively  
 783 phosphorylating (OXPHOS) states. F-values with the degrees of freedom for the effect and  
 784 the error (in subscript) and P-values are given. Significant effects ( $P < 0.05$ ) are highlighted  
 785 in bold.

786

	Tissue	Substrate	Interactive effect
$\dot{M}O_2$ LEAK	$F_{1,45}=1.181, P=0.283$	$F_{2,45}=\mathbf{11.037}, P<\mathbf{0.001}$	$F_{2,45}=0.069, P=0.933$
$\dot{M}O_2$ OXPHOS	$F_{1,45}=3.544, P=0.066$	$F_{2,45}=\mathbf{20.950}, P<\mathbf{0.001}$	$F_{2,45}=\mathbf{5.193}, P=\mathbf{0.009}$
ROS LEAK	$F_{1,42}=\mathbf{24.742}, P<\mathbf{0.001}$	$F_{2,42}=\mathbf{4.480}, P=\mathbf{0.017}$	$F_{2,16}=1.071, P=0.352$
ROS OXPHOS	$F_{1,42}=\mathbf{32.789}, P<\mathbf{0.001}$	$F_{1,42}=\mathbf{3.612}, P=\mathbf{0.036}$	$F_{2,42}=0.300, P=0.742$
FEL LEAK	$F_{1,41}=\mathbf{22.173}, P<\mathbf{0.001}$	$F_{2,41}=\mathbf{4.081}, P=\mathbf{0.024}$	$F_{2,41}=1.992, P=0.149$
FEL OXPHOS	$F_{1,42}=\mathbf{21.189}, P<\mathbf{0.001}$	$F_{2,42}=\mathbf{7.592}, P=\mathbf{0.002}$	$F_{2,42}=0.767, P=0.471$

787

788 **Table 2.** Repeated measures ANOVA: Effects of short-term H-R and substrates on the  
 789 respiration, ROS efflux and FEL in the mitochondria from the gill and the digestive gland  
 790 (DG) of *P. maximus*.

791 Mitochondrial parameters were measured in the resting (LEAK) and actively  
 792 phosphorylating (OXPHOS) states. F-values with the degrees of freedom for the effect and  
 793 the error (in subscript) and P-values are given. Significant effects (P<0.05) are highlighted  
 794 in bold.

795

	Exposure	Substrate	Interactive effect
<b><i>Mo<sub>2</sub></i></b>			
LEAK <sub>GILL</sub>	<b><math>F_{1,21}=5.980, P=0.023</math></b>	<b><math>F_{2,21}=16.323, P&lt;0.001</math></b>	<b><math>F_{2,21}=23.169, P&lt;0.001</math></b>
LEAK <sub>DG</sub>	<b><math>F_{1,22}=5.235, P=0.032</math></b>	<b><math>F_{1,22}=8.302, P=0.002</math></b>	<b><math>F_{2,22}=20.551, P&lt;0.001</math></b>
OXPHOS <sub>GILL</sub>	<b><math>F_{1,21}=113.114, P&lt;0.001</math></b>	<b><math>F_{2,21}=19.827, P&lt;0.001</math></b>	$F_{2,21}=0.016, P=0.984$
OXPHOS <sub>DG</sub>	<b><math>F_{1,22}=50.012, P&lt;0.001</math></b>	<b><math>F_{2,22}=4.982, P=0.016</math></b>	$F_{2,22}=1.951, P=0.166$
<b><i>ROS</i></b>			
LEAK <sub>GILL</sub>	<b><math>F_{1,20}=11.148, P=0.003</math></b>	<b><math>F_{2,19}=4.617, P=0.022</math></b>	<b><math>F_{2,20}=15.287, P&lt;0.001</math></b>
LEAK <sub>DG</sub>	<b><math>F_{1,22}=42.340, P&lt;0.001</math></b>	$F_{2,22}=0.053, P=0.949$	$F_{2,22}=3.411, P=0.051$
OXPHOS <sub>GILL</sub>	$F_{1,20}=3.686, P=0.069$	<b><math>F_{2,20}=4.453, P=0.025</math></b>	$F_{2,20}=0.577, P=0.571$
OXPHOS <sub>DG</sub>	<b><math>F_{1,22}=14.929, P=0.001</math></b>	$F_{2,22}=0.053, P=0.948$	$F_{2,22}=1.201, P=0.320$
<b><i>FEL</i></b>			
LEAK <sub>GILL</sub>	$F_{1,21}=0.704, P=0.411$	$F_{2,21}=1.914, P=0.172$	$F_{2,21}=1.774, P=0.194$
LEAK <sub>DG</sub>	<b><math>F_{1,22}=35.650, P&lt;0.001</math></b>	$F_{2,22}=2.467, P=0.108$	$F_{2,22}=0.996, P=0.385$
OXPHOS <sub>GILL</sub>	<b><math>F_{1,20}=6.440, P=0.020</math></b>	<b><math>F_{2,20}=5.271, P=0.015</math></b>	<b><math>F_{2,20}=4.813, P=0.020</math></b>
OXPHOS <sub>DG</sub>	$F_{1,22}=0.338, P=0.567$	$F_{2,22}=1.299, P=0.293$	$F_{2,22}=1.076, P=0.365$

796

797 **Table 3.** Repeated measures ANOVA: Effects of long-term H-R and substrates on the  
 798 respiration, ROS efflux and FEL in the mitochondria from the gill and the digestive gland  
 799 (DG) of *P. maximus*.

800 Mitochondrial parameters were measured in the resting (LEAK) and actively  
 801 phosphorylating (OXPHOS) states. F-values with the degrees of freedom for the effect and  
 802 the error (in subscript) and P-values are given. Significant effects ( $P < 0.05$ ) are highlighted  
 803 in bold.  
 804

	Exposure	Substrate	Interactive effect
<b><i>MO<sub>2</sub></i></b>			
LEAK <sub>GILL</sub>	$F_{1,21}=0.310, P=0.584$	<b><math>F_{2,19}=7.955, P=0.003</math></b>	<b><math>F_{2,21}=26.189, P&lt;0.001</math></b>
LEAK <sub>DG</sub>	$F_{1,20}=0.504, P=0.486$	<b><math>F_{2,20}=3.928, P=0.036</math></b>	$F_{2,20}=2.576, P=0.101$
OXPHOS <sub>GILL</sub>	<b><math>F_{1,21}=188.101, P&lt;0.001</math></b>	<b><math>F_{2,21}=30.773, P&lt;0.001</math></b>	<b><math>F_{2,21}=25.472, P&lt;0.001</math></b>
OXPHOS <sub>DG</sub>	<b><math>F_{1,20}=56.209, P&lt;0.001</math></b>	$F_{2,20}=1.489, P=0.250$	<b><math>F_{2,20}=3.529, P=0.049</math></b>
<b><i>ROS</i></b>			
LEAK <sub>GILL</sub>	$F_{1,19}=3.366, P=0.082$	<b><math>F_{2,19}=8.878, P=0.002</math></b>	$F_{2,19}=2.697, P=0.093$
LEAK <sub>DG</sub>	<b><math>F_{1,20}=32.385, P&lt;0.001</math></b>	$F_{2,20}=1.460, P=0.256$	$F_{2,20}=1.822, P=0.188$
OXPHOS <sub>GILL</sub>	$F_{1,20}=0.021, P=0.885$	<b><math>F_{2,20}=17.011, P&lt;0.001</math></b>	$F_{2,20}=0.709, P=0.504$
OXPHOS <sub>DG</sub>	<b><math>F_{1,20}=36.652, P&lt;0.001</math></b>	$F_{2,20}=2.017, P=0.159$	$F_{2,20}=0.516, P=0.604$
<b><i>FEL</i></b>			
LEAK <sub>GILL</sub>	<b><math>F_{1,19}=10.815, P=0.004</math></b>	<b><math>F_{2,19}=18.403, P&lt;0.001</math></b>	<b><math>F_{2,19}=13.407, P&lt;0.001</math></b>
LEAK <sub>DG</sub>	$F_{1,21}=1.252, P=0.276$	$F_{2,21}=0.949, P=0.403$	$F_{2,21}=2.081, P=0.150$
OXPHOS <sub>GILL</sub>	<b><math>F_{1,20}=77.255, P&lt;0.001</math></b>	<b><math>F_{2,20}=69.665, P&lt;0.001</math></b>	<b><math>F_{2,20}=66.748, P&lt;0.001</math></b>
OXPHOS <sub>DG</sub>	<b><math>F_{1,20}=5.472, P=0.030</math></b>	<b><math>F_{2,20}=6.973, P=0.005</math></b>	<b><math>F_{2,20}=9.871, P=0.001</math></b>

805

806

807 **Table 4.** Repeated measures ANOVA: Effects of short-term H-R and substrates on  
 808 oxidative stress indices of gill mitochondria of *P. maximus*

	Exposure	Substrate	Interactive effect
Carbonyls	$F_{1,11}=0.303, P=0.593$	$F_{1,11}=2.076, P=0.172$	$F_{2,11}=0.717, P=0.509$
HNE	$F_{1,12}=0.288, P=0.601$	$F_{1,12}=0.714, P=0.510$	$F_{2,12}=1.125, P=0.357$

809

810 **FIGURE CAPTIONS.**

811 **Figure 1.** Oxygen consumption rates ( $\dot{M}O_2$ ) and ROS efflux of isolated mitochondria from  
812 the gill and digestive gland (DG) tissue of *P. maximus* respiring on different mitochondrial  
813 substrates under normoxic conditions. Substrates: pyruvate, palmitate, and succinate.  
814 Significant differences in a specific mitochondrial trait between normoxia (Control) and  
815 reoxygenation (H-R) are denoted by asterisks ( $*P < 0.05$ ). N=16 for all substrates except  
816 in the gill mitochondria oxidizing pyruvate and palmitate (N=7 for each).

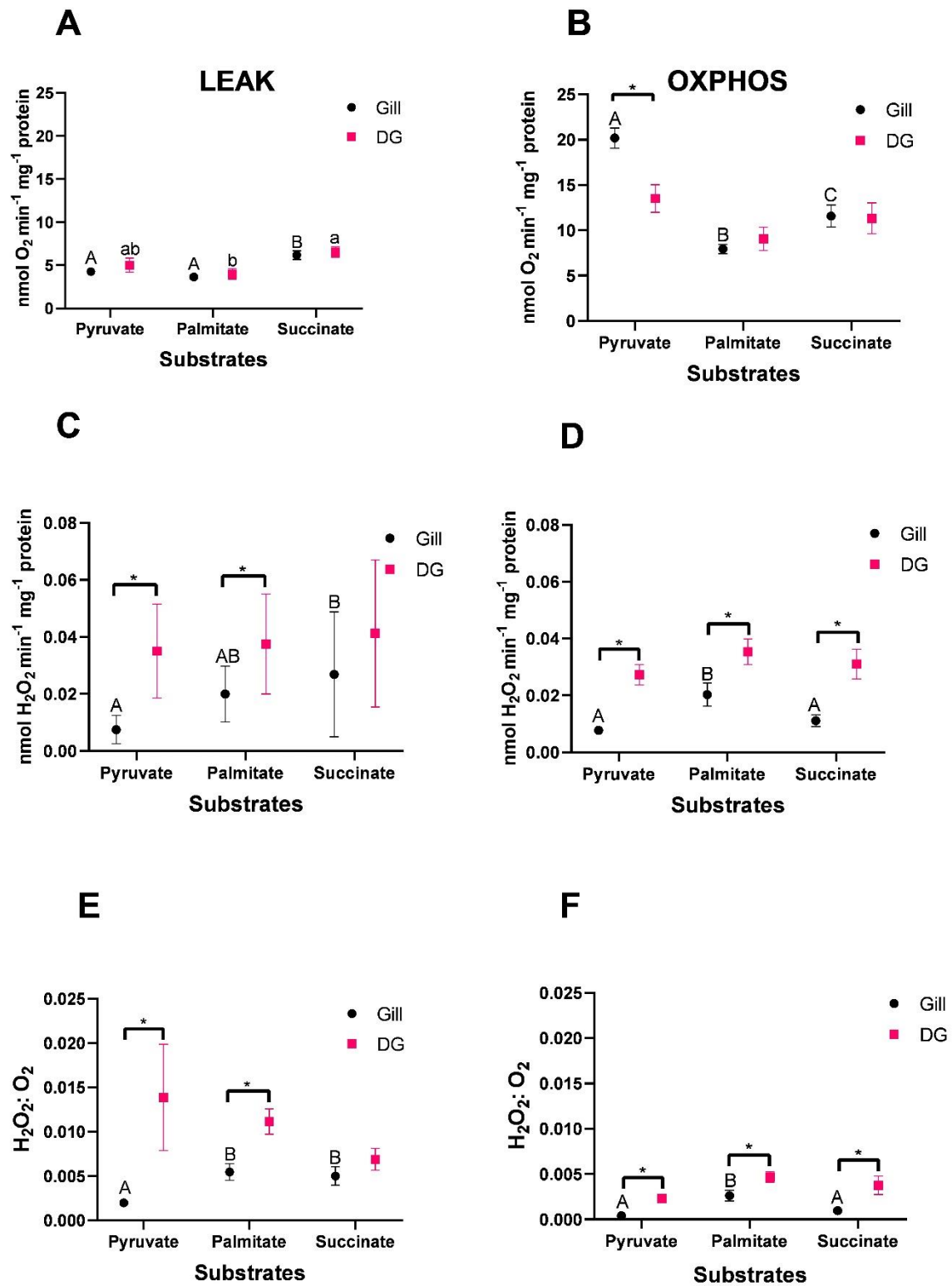
817 **Figure 2.** Effects of short-term (ST) and long-term (LT) hypoxia and reoxygenation (H-R)  
818 on LEAK (A, C, E) and OXPHOS (B, D, F) respiration of mitochondria isolated from the gill  
819 tissue of *P. maximus* respiring on different substrates. Substrates: pyruvate, palmitate, and  
820 succinate. Significant differences in a specific mitochondrial trait between normoxia  
821 (Control) and reoxygenation (H-R) are denoted by asterisks ( $*P < 0.05$ ). N=8 for all  
822 substrates except palmitate (N=7 for palmitate-driven ROS and FEL in long-term  
823 hypoxia).

824 **Figure 3.** Effects of short-term (ST) and long-term (LT) hypoxia and reoxygenation (H-R)  
825 on LEAK (A, C, E) and OXPHOS (B, D, F) respiration of mitochondria isolated from the  
826 digestive gland (DG) tissue of *P. maximus* respiring on different substrates. Substrates:  
827 Pyruvate, Palmitate, and Succinate. Significant differences in a specific mitochondrial trait  
828 between normoxia (Control) and reoxygenation (H-R) are denoted by asterisks ( $*P <$   
829  $0.05$ ). N=8 for all substrates except palmitate (N=7 for palmitate-driven ROS and FEL in  
830 long-term hypoxia).

831 **Figure 4.** Effects of short-term (ST) and long-term (LT) hypoxia and reoxygenation (H-R)  
832 on respiratory control ratio (RCR) of mitochondria isolated from the gill and digestive  
833 gland (DG) tissue of *P. maximus* respiring on different substrates. Substrates: Pyruvate,  
834 Palmitate, and Succinate. Significant differences in a specific mitochondrial trait between  
835 normoxia (Control) and reoxygenation (H-R) are denoted by asterisks ( $*P < 0.05$ ). N=8 for  
836 all substrates.

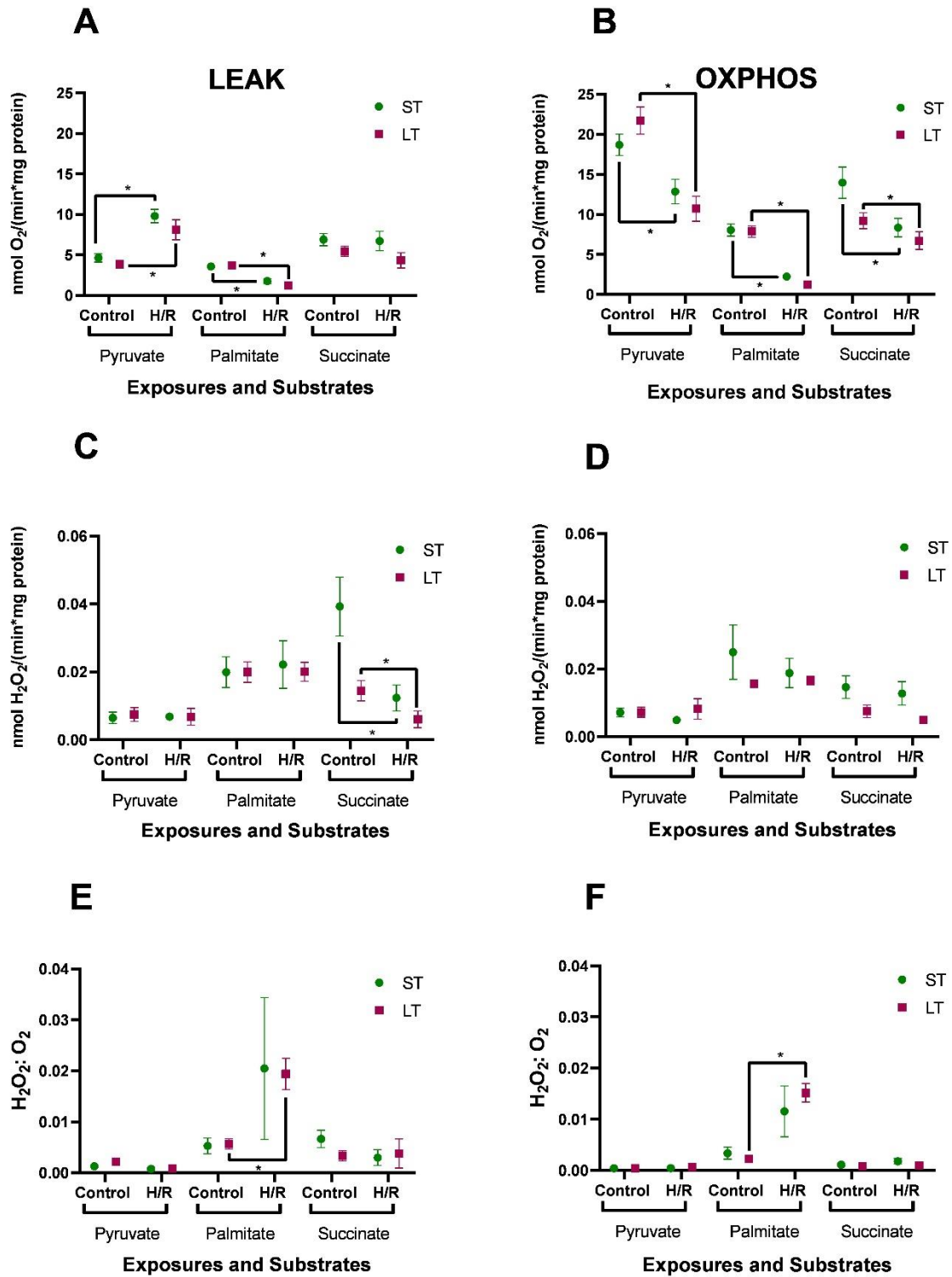
837 **Figure 5.** Effects of short-term (ST) hypoxia and reoxygenation (H-R) on carbonyl (A) and  
838 HNE (B) content of mitochondria isolated from the gill tissue of *P. maximus* respiring on  
839 different substrates. Substrates: Pyruvate, Palmitate, and Succinate. Significant  
840 differences in a specific mitochondrial trait between normoxia (C) and reoxygenation (H-  
841 R) are denoted by asterisks ( $*P < 0.05$ ). N=5 for all substrates.

842



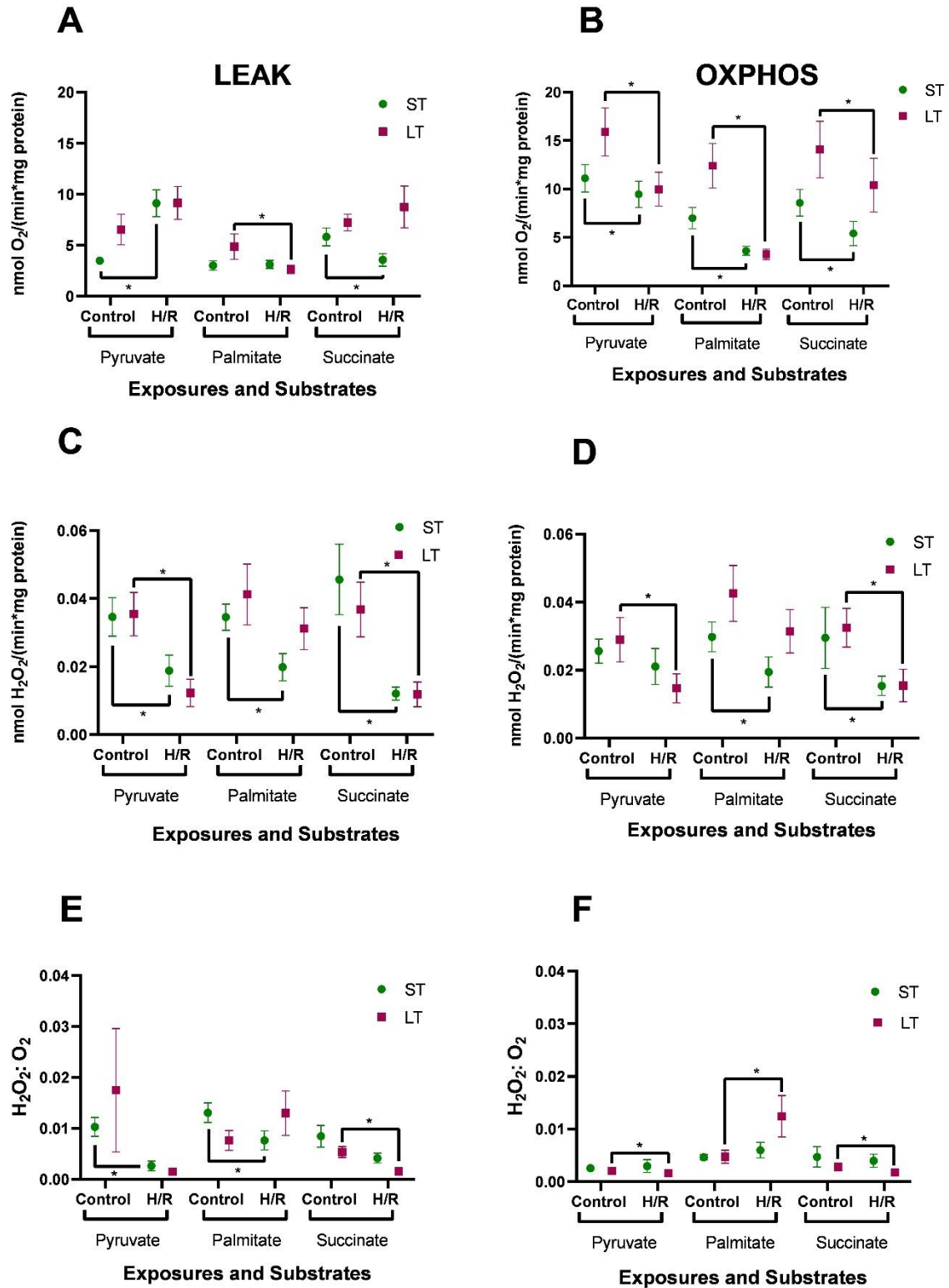
843

844 Figure 1.



845

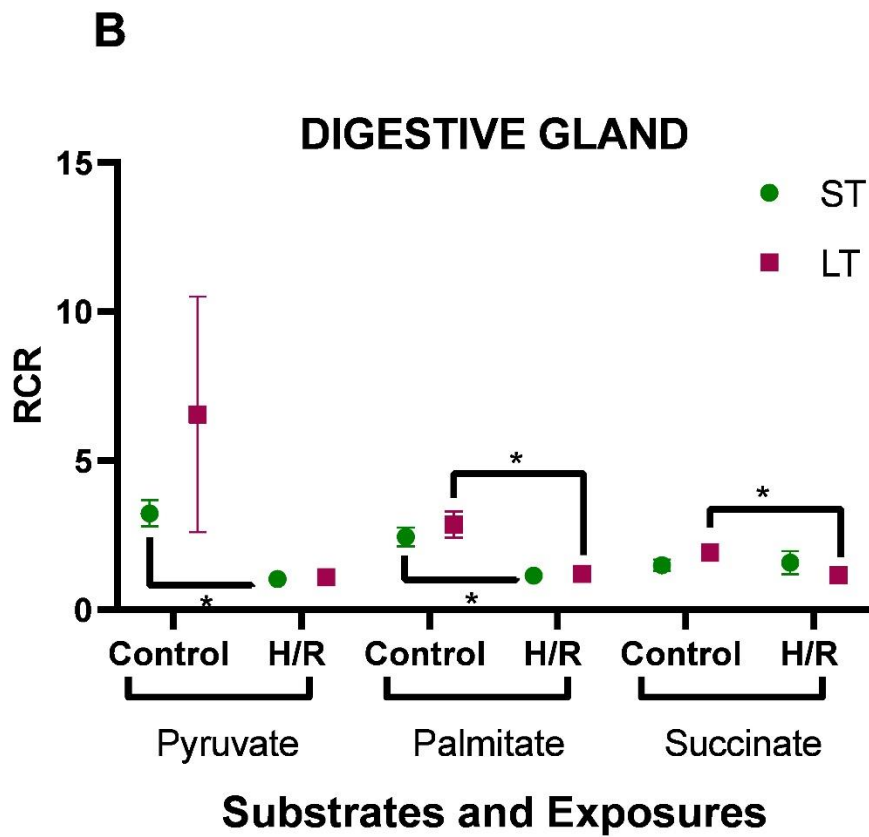
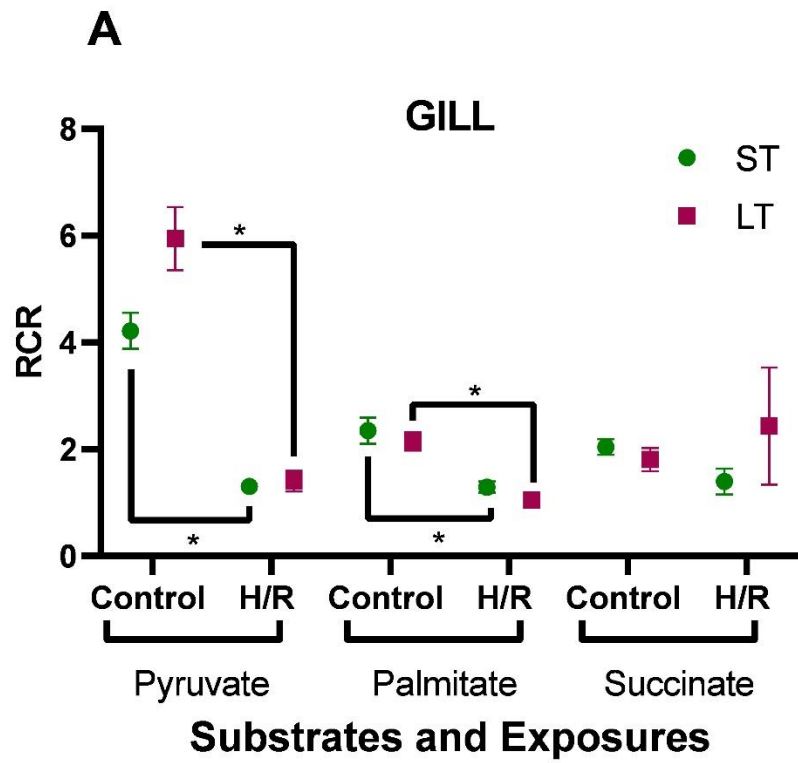
846 Figure 2.



847

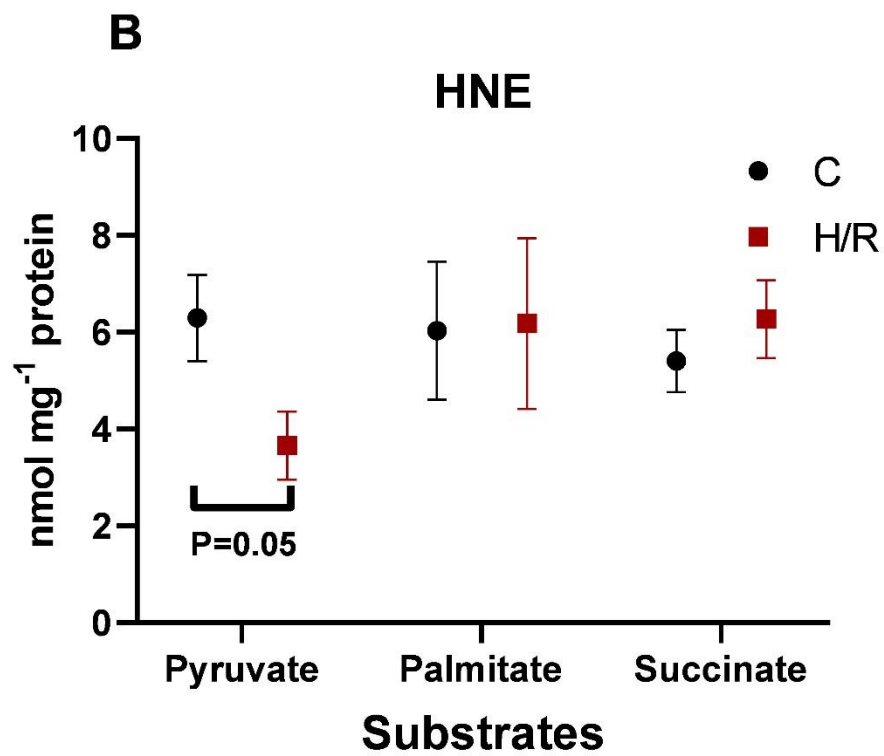
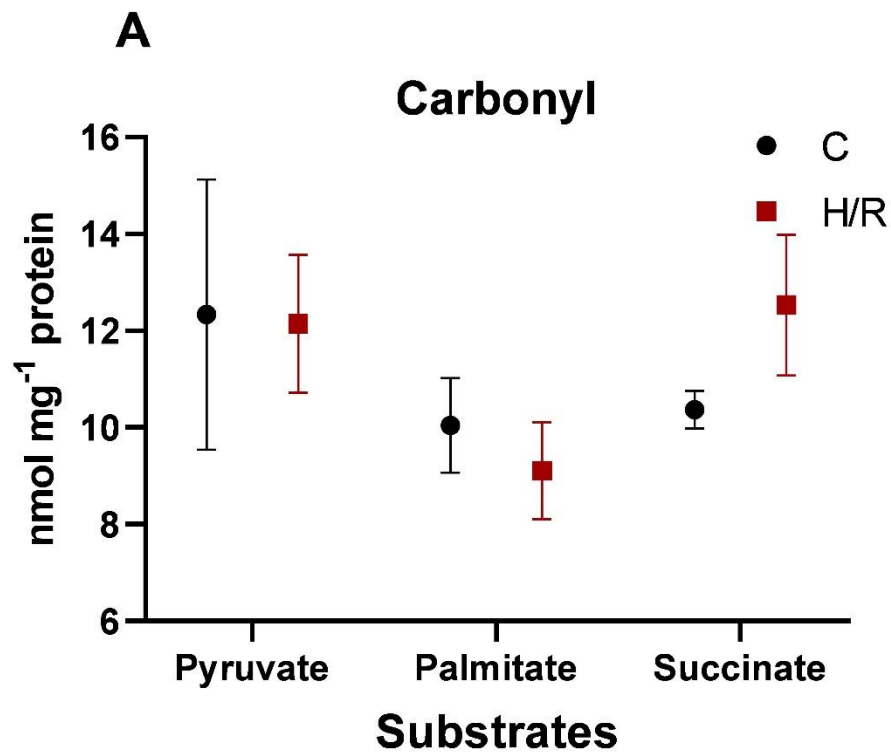
848 Figure 3.

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850

851 Figure 4.



852

853 Figure 5.

## PUBLICATION LIST

1. **Adzibbli, L.**, Ponsuksili, S. & Sokolova, I (2024). Mitochondrial responses to constant and cyclic hypoxia depend on the oxidized fuel in a hypoxia-tolerant marine bivalve *Crassostrea gigas*. *Sci Rep* **14**, 9658. <https://doi.org/10.1038/s41598-024-60261-w>
2. **Adzibbli L.**, Sokolov P.E., Wimmers K., Sokolova M. I., Ponsuksili S. (2022). Effects of hypoxia and reoxygenation on mitochondrial functions and transcriptional profiles of isolated brain and muscle porcine cells. *Scientific Reports*, 12, 19881. [10.1038/s41598-022-24386-0](https://doi.org/10.1038/s41598-022-24386-0)
3. **Adzibbli L.**, Sokolov P.E., Ponsuksili S., Sokolova M. I (2021). Tissue- and substrate-dependent mitochondrial responses to acute hypoxia-reoxygenation stress in a marine bivalve *Crassostrea gigas* (Thunberg, 1793). *Journal of Experimental Biology*, 225(1) [10.1242/jeb.243304](https://doi.org/10.1242/jeb.243304)
4. Sokolov P.E., **Adzibbli L.**, Markert S., Bundgaard A., Fago A., Becher D., Hirschfeld C., Sokolova M. I (2021). Intrinsic Mechanisms Underlying Hypoxia-Tolerant Mitochondrial Phenotype during Hypoxia-Reoxygenation Stress in a Marine Facultative Anaerobe, the Blue Mussel *Mytilus edulis*. *Frontiers in Marine Science*, 8:773734. [10.3389/fmars.2021.773734](https://doi.org/10.3389/fmars.2021.773734)
5. Zheng Z., Liao Y., Ye J., Yang C., **Adzibbli L.**, Wang Q., Du X., Deng Y. (2021) Microbiota Diversity in Pearl Oyster *Pinctada fucata martensii* Intestine and Its Aquaculture Environment. *Frontiers in Marine Science*, 8:655698. [10.3389/fmars.2021.655698](https://doi.org/10.3389/fmars.2021.655698)
6. Zhouxin L., **Adzibbli L.**, Chen Q., Hao R., Liao Y., Deng Y., Wang Q. (2021). Identification and Allelic Variants Associated with Cold Tolerance of *PmPIAS* in *Pinctada fucata martensii*. *Frontiers in Physiology*, 12:634838. [10.3389/fphys.2021.634838](https://doi.org/10.3389/fphys.2021.634838)
7. Hao R., Mo C., **Adzibbli L.**, Yang C., Deng Y., Wang Q. (2020). Molecular Cloning and Polymorphism Analysis of *PmFGF18* from *Pinctada fucata martensii*. *Journal of Marine Science and Engineering* 8(11):896. [10.3390/jmse8110896](https://doi.org/10.3390/jmse8110896)
8. Wang Z., **Adzibbli, L.**, Zheng Z., Yang C., Deng, Y. (2020). How cultured pearls acquire their colour. *Aquaculture Research*, 51: 3925– 3934. <https://doi.org/10.1111/are.14765>
9. **Adzibbli L.**, Hao R., Jiao Y., Deng Y., Du, X., Wang Q., Huang R. (2020). Immune response of pearl oysters to stress and diseases. *Reviews in Aquaculture*, 12(2), 513-523. [10.1111/raq.12329](https://doi.org/10.1111/raq.12329)
10. **Adzibbli L.**, Zhao Z., Wang Z., Yang C., Hao R., Deng Y. (2020). Characterization of cyclin dependent kinase-7 (CDK-7) and its differential response to grafting challenge in the black colored selected line and control stocks of *Pinctada fucata martensii*. *Fish & Shellfish Immunology*, 101:277-283. [10.1016/j.fsi.2020.04.008](https://doi.org/10.1016/j.fsi.2020.04.008).

11. **Adzibli L.**, Zheng Z., Liao Y., Deng Y., Du X., Yang C. (2020). Characterization of thioredoxin-like PROTEIN-5 (TRXLP-5) and its differential response to grafting challenge in the black colored selected line and control stocks of *Pinctada fucata martensii*. *Developmental & Comparative Immunology* 106, 103635. [10.1016/j.dci.2020.103635](https://doi.org/10.1016/j.dci.2020.103635)
12. **Adzibli L.**, Wang Z., Li, J., Deng, Y. (2020), Survival, retention rate and immunity of the black shell colored stocks of pearl oyster *Pinctada fucata martensii* after grafting operation. *Fish & Shellfish Immunology*, 98, 691-698. [10.1016/j.fsi.2019.11.003](https://doi.org/10.1016/j.fsi.2019.11.003)
13. **Adzibli, L.**, Wang Z., Zhao Z., Yang C., Li, J., Deng, Y. (2019), Growth in pearl oysters: A review of genetic and environmental influences. *Aquaculture Research*, 15, 100214. [10.1111/are.14365](https://doi.org/10.1111/are.14365)
14. **Adzibli, L.**, Wang Z., Lai, Z., Li, J., Deng, Y. (2019), Sex determination in pearl oyster: A mini review. *Aquaculture Reports* 15, 100214 [10.1016/j.aqrep.2019.100214](https://doi.org/10.1016/j.aqrep.2019.100214)
15. **Adzibli, L.**, Yu, W., Li, J., Yang, C., Deng, Y. (2019), A comparison of pearl productions performance, enzymatic activity and immune related gene expression of different aged pearl oyster *Pinctada fucata martensii*. *North Am J Aquaculture* 81, 430-437. [10.1002/naaq.10111](https://doi.org/10.1002/naaq.10111)
16. Deng, Y., **Adzibli, L.**, (2018). Assessing the impact of oil spills on marine organisms. *Journal of Oceanography and Marine Research* 06, 1-7. [10.4172/2572-3103.1000179](https://doi.org/10.4172/2572-3103.1000179)
17. Adzibli, L., Bacosa P.H., Deng, Y., (2018). Response of microbial communities to oil spill in the Gulf of Mexico: A review. *African Journal of Microbiology Research*. 12(23), 536-545, [10.5897/AJMR2018.8843](https://doi.org/10.5897/AJMR2018.8843)

## CONFERENCES TALKS AND POSTERS

1. Leibniz science campus Phosphorus research Rostock symposium 2023, Rostock Germany (Oral presentation – “Mitochondrial responses of organisms to hypoxia-reoxygenation stress”)
2. Society of Experimental Biology (SEB) Centenary conference 2023, Edinburgh, UK (Oral presentation - “Metabolic fuel alters responses to hypoxia-reoxygenation stress in marine bivalves”, Poster - “Effects of hypoxia and reoxygenation (H/R) on mitochondrial functions and transcriptional profiles of isolated brain and muscle porcine cells”.
3. Leibniz science campus Phosphorus research Rostock symposium 2022, Warnemunde, Germany (Oral presentation - “Mitochondrial responses of marine bivalves to hypoxia-reoxygenation stress”)
4. Society of Experimental Biology (SEB) annual conference 2022, Montpellier, France (Oral presentation - “Tissue- and substrate-dependent mitochondrial responses to hypoxia-reoxygenation stress in oysters *Crassostrea gigas* (Thunberg, 1793)”).
5. Canadian Society of Zoologists (CSZ) annual conference 2022 (Poster –

“Tissue and substrate dependent mitochondrial responses to hypoxia reoxygenation (H/R) stress in oysters *Crassostrea gigas* (Thunberg, 1793)”.

6. Leibniz science campus Phosphorus research Rostock symposium 2021, Rostock Germany (Poster - “Tissue- and substrate-dependent mitochondrial responses to acute hypoxia-reoxygenation stress in a marine bivalve *Crassostrea gigas* (Thunberg, 1793)”)

## **AWARDS**

- Guangdong ocean university outstanding student, 2017
- Guangdong Provincial scholarship, 2018
- Guangdong ocean university outstanding student, 2019