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SYMPOSIUM

Mitochondrial Mechanisms Underlying Tolerance to Fluctuating Oxygen Conditions: Lessons from Hypoxia-Tolerant Organisms

Inna M. Sokolova,[1,*,†] Eugene P. Sokolov[‡] and Fouzia Haider[*]

*Department of Marine Biology, University of Rostock, Rostock, Germany; †Department of Maritime Systems, Interdisciplinary Faculty, University of Rostock, Rostock, Germany; ‡Leibniz Institute for Baltic Sea Research, Leibniz ScienceCampus Phosphorus Research Rostock, Warnemünde, Germany

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[1]E-mail: Inna.Sokolova@uni-rostock.de

Synopsis Oxygen (O_2) is essential for most metazoan life due to its central role in mitochondrial oxidative phosphorylation (OXPHOS), which generates >90% of the cellular adenosine triphosphate. O_2 fluctuations are an ultimate mitochondrial stressor resulting in mitochondrial damage, energy deficiency, and cell death. This work provides an overview of the known and putative mechanisms involved in mitochondrial tolerance to fluctuating O_2 conditions in hypoxia-tolerant organisms including aquatic and terrestrial vertebrates and invertebrates. Mechanisms of regulation of the mitochondrial OXPHOS and electron transport system (ETS) (including alternative oxidases), sulphide tolerance, regulation of redox status and mitochondrial quality control, and the potential role of hypoxia-inducible factor (HIF) in mitochondrial tolerance to hypoxia are discussed. Mitochondrial phenotypes of distantly related animal species reveal common features including conservation and/or anticipatory upregulation of ETS capacity, suppression of reactive oxygen species (ROS)-producing electron flux through ubiquinone, reversible suppression of OXPHOS activity, and investment into the mitochondrial quality control mechanisms. Despite the putative importance of oxidative stress in adaptations to hypoxia, establishing the link between hypoxia tolerance and mitochondrial redox mechanisms is complicated by the difficulties of establishing the species-specific concentration thresholds above which the damaging effects of ROS outweigh their potentially adaptive signaling function. The key gaps in our knowledge about the potential mechanisms of mitochondrial tolerance to hypoxia include regulation of mitochondrial biogenesis and fusion/fission dynamics, and HIF-dependent metabolic regulation that require further investigation in hypoxia-tolerant species. Future physiological, molecular and genetic studies of mitochondrial responses to hypoxia, and reoxygenation in phylogenetically diverse hypoxia-tolerant species could reveal novel solutions to the ubiquitous and metabolically severe problem of O_2 deficiency and would have important implications for understanding the evolution of hypoxia tolerance and the potential mitigation of pathological states caused by O_2 fluctuations.

Oxygen (O_2) is essential for metazoan life due to its central role in mitochondrial oxidative phosphorylation (OXPHOS), which generates >90% of the cellular adenosine triphosphate (ATP) in aerobic organisms. Most extant metazoans depend on O_2 at least at some stage of their life cycles, and O_2 fluctuations are intrinsically stressful to aerobic organisms. In terrestrial environments, hypoxia (O_2 deficiency) is uncommon and found at high altitudes where low barometric pressure results in the permanently decreased partial pressure of O_2 (P_{O2}), and in some underground habitats where organism-mediated O_2 consumption exceeds O_2 influx by air exchange. In contrast, aquatic environments often experience O_2 fluctuations (ranging from >400% to 0% of air saturation) reflecting the dynamics of photosynthesis, respiration, and gas exchange with the atmosphere (Burnett 1997; Richards 2011). In estuaries and deep temperate lakes, benthic habitats might become hypoxic in summer when water

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stratification prevents water mixing (and thus replenishment of the O_2 consumed by the organisms) below the thermocline (Diaz and Rosenberg 2008; Fukushima et al. 2017). In the lakes, benthic hypoxia can also occur in the winter when ice formation cuts off the air exchange at the surface. The extent and duration of aquatic hypoxia are increasing due to the increased nutrient pollution that fuels bacterial respiration and leads to formation of the O_2-depleted benthic "dead zones" (Breitburg et al. 2018). Deoxygenation of aquatic habitats is further enhanced by warming of the surface waters (e.g., due to the global climate change) which lowers O_2 solubility, increases stratification, and elevates O_2 consumption rates of the resident biota (Breitburg et al. 2018). These processes may result in the chronic or seasonal (weeks to months) hypoxia in freshwater and marine benthic habitats. In estuaries and tidally influenced coastal zones, the long-term hypoxia combines with the short-term O_2 fluctuations caused by the tidal emersion (∼every 6 h) and the diel rhythms of photosynthesis and respiration causing hypoxia during the night (Truchot and Duhamel-Jouve 1980; Burnett 1997). Together, these factors lead to a complex and often unpredictable O2 dynamics in estuaries, coastal zones, and meromictic lakes posing significant challenge to their inhabitants.

Fluctuations in O_2 concentrations strongly affect mitochondrial respiration as well as the mitochondrial and cellular redox balance. In hypoxia-sensitive species such as most terrestrial mammals, exposure to hypoxia strongly impairs mitochondrial OXPHOS and may result in ATP deficiency, while anoxia leads to cessation of the aerobic ATP production and reliance on the less efficient substrate-level anaerobic phosphorylation (Hochachka and Mommsen 1983; Grieshaber et al. 1994). Furthermore, hypoxia results in the elevated production of reactive oxygen species (ROS) due to the electron slip from the partially reduced upstream electron transport system (ETS) complexes (Solaini et al. 2010; Cadenas 2018) and/or reverse electron flow through mitochondrial Complex I (Chouchani et al. 2014). ROS can oxidize and damage mitochondrial membranes, as well as the ETS and tricarboxylic acid (TCA) enzymes leading to OXPHOS suppression and ROS overproduction (Aragones et al. 2009; Hernansanz-Agustín et al. 2014). The restoration of the O_2 flux to the tissue can bring its own host of associated problems, leading to a burst of ROS production, mitochondrial Ca^{2+} overload and in extreme cases, mitochondrial depolarization, release of the cytochrome c, and apoptosis (Chouchani et al. 2016; Cadenas 2018). This strong sensitivity to O_2 makes mitochondria a central signaling and regulatory hub, as well as a major target for intracellular damage during O_2 fluctuations.

Metabolic adaptations to long-term hypoxia and anoxia have been extensively investigated (review in Hochachka and Lutz 2001; Somero et al. 2016). The adaptive strategies to deal with O_2 deficiency differ between the chronic mild hypoxia (such as, for example, found in high altitude environments), where O_2 levels are sufficient to fuel long-term aerobic metabolism and severe hypoxia and anoxia (such as found in the coastal dead zones), where aerobic metabolism must be put on hold and anaerobic pathways engaged until O_2 returns. Generally, adaptations to moderate chronic hypoxia involve compensatory morphological and physiological adjustments to improve O_2 delivery and mitigate the tissue-level hypoxia (Bavis et al. 2007; Richards 2009; Storz et al. 2010; Richards 2011; Devaux et al. 2019). Common examples of such compensatory adjustments include increased surface area and/or perfusion rate of respiratory organs, elevated hematocrit, increased O_2 affinity of respiratory pigments, changes in the capillary density, and spatial rearrangement of mitochondria bringing them close to the capillary surface (Bavis et al. 2007; Richards 2009; Storz et al. 2010; Richards 2011; Devaux et al. 2019). During severe hypoxia or anoxia, improved survival is associated with metabolic rate depression, a coordinated decrease of ATP production and ATP consumption combined with ion channel arrest (Hochachka et al. 1996; Hochachka and Lutz 2001) and suppressed activity of excitatory tissues (Pamenter et al. 2011; Buck and Pamenter 2018). This serves as an energy-conserving mechanism slowing down the usage of the energy reserves and accumulation of toxic by-products of anaerobic metabolism (Hochachka et al. 1996; Hochachka and Lutz 2001).

The compensatory systemic mechanisms to improve tissue O_2 availability, as well as the mechanisms of metabolic rate depression and anaerobic ATP production have been summarized in several excellent reviews (Hochachka 1993; Hochachka et al. 1996; Hochachka and Lutz 2001; Bickler and Buck 2007; Solaini et al. 2010; Dzal et al. 2015; Murray 2016). However, an important piece of this puzzle is still lacking—namely, understanding of the mechanisms that maintain mitochondrial integrity and function during O_2 deficiency and rapidly restore the aerobic function during reoxygenation. The present review aims to provide an overview of the current state of knowledge and the knowledge gaps concerning the mitochondrial responses to

fluctuating O_2 levels in hypoxia-tolerant organisms and suggest potential directions for future research into the fundamental mechanisms of mitochondrial tolerance to intermittent hypoxia and their role in adaptations to fluctuating O_2 in the environment.

Regulation of the mitochondrial OXPHOS in hypoxia-tolerant organisms

O_2 serves as the main electron acceptor in the mitochondrial ETS, which transfers electrons from energy-rich substrates (NADH and $FADH_2$) to O_2 generating the proton-motive force (Δp) (i.e., the electrochemical and proton concentration gradients) across the inner mitochondrial membrane. The driving force of the ETS activity is the O_2 consumption by cytochrome c oxidase (COX), the main terminal oxidase of aerobic eukaryotes (Blomberg and Siegbahn 2014). Mitochondrial Complexes I, III, and IV act as proton pumps generating Δp, whereas Complex II transfers electrons to the Complex III from $FADH_2$-linked substrates. The energy of Δp generated by the ETS is conserved in the form of ATP by the mitochondrial Complex V (F_O, F_1-ATP synthase) in the process of OXPHOS. O_2-dependence of COX (and therefore, ETS) makes mitochondrial OXPHOS a key target of the hypoxia-reoxygenation (H/R)-induced stress (Paradis et al. 2016).

Generally, hypoxia leads to the substrate (O_2) limitation of COX, slowing down the rate of the electron transfer in the ETS and thereby decreasing the rate of the proton pumping that generates Δp (Kalogeris et al. 2012). This decreases the OXPHOS activity and may result in ATP deficiency in the absence of a concomitant decrease in ATP consumption. Furthermore, a decline in COX activity results in accumulation of partially reduced electron carriers in the mitochondrial ETS, which leads to the electron slip and excessive production of ROS during reoxygenation (Dröse et al. 2016). Succinate accumulation during hypoxia might also contribute to ROS generation by creating the backflow of electrons through the mitochondrial Complex I upon reoxygenation (Chouchani et al. 2014). Elevated ROS production may result in a vicious cascade of ROS release damaging ETS, amplifying ATP deficiency, and eventually resulting in mitochondrial collapse and apoptosis (Zorov et al. 2014). Avoiding these bioenergetic and redox issues is essential for survival of chronic and intermittent hypoxia, making adjustments of OXPHOS and ETS a key candidate mechanism for hypoxic adaptation. Indeed, genetic analysis of 12 species of hypoxia-tolerant mammals (including aquatic, high altitude, and subterranean species) showed evidence of strong positive selection and convergent evolution in the genes involved in ETS, OXPHOS, and TCA cycle (Tian et al. 2017). Interestingly, transition from low to high O_2 environment (such as during evolution of the terrestrial lifestyle in pulmonate snails) was also associated with positive selection in ETS loci encoding Complex I subunits and cytochrome b (Romero et al. 2016). These findings indicate a key role of mitochondrial ETS and OXPHOS adjustments in adaptation to different O_2 conditions in animals.

ETS activity is strongly modulated by H/R, and the mitochondrial responses to H/R differ between the hypoxia-tolerant and hypoxia-sensitive species. In hypoxia-sensitive terrestrial vertebrates, hypoxia results in suppression of the ETS activity that involves both passive mechanisms (i.e., O_2 limitation and oxidative damage to ETS proteins) (Makarov et al. 2002; Ashmore et al. 2014) as well as the active downregulation of ETS activity (Semenza 2007; Zimmer et al. 2016). The key events of this hypoxia-induced ETS suppression involve reversible inactivation of pyruvate dehydrogenase E1 enzyme which interrupts the supply of acetyl-CoA to mitochondria (Semenza 2007; Schönenberger and Kovacs 2015) and suppression of COX activity by reversible phosphorylation and/or by expression of a hypoxia-specific isoform of subunit IV (COX 4-2) (Hüttemann et al. 2012; Aras et al. 2015; Kocha et al. 2015). Similar to mammalian models, hypoxia suppresses COX activity and/or the maximum ETS capacity in hypoxia-sensitive aquatic species, such as the shovelnose ray *Aptychotrema rostrata* (Hickey et al. 2012) and the bay scallop *Argopecten irradians* (Ivanina et al. 2016). In stark contrast, in an extremely hypoxia-tolerant hard shell clam *Mercenaria mercenaria*, hypoxia exposure led to upregulation of the ETS capacity, despite the fact that hypoxic mitochondria were inactive *in vivo* (Ivanina et al. 2016). Protein abundance of several key subunits of mitochondrial Complexes I, II, and IV also increased during anoxia in a hypoxia-tolerant turtle *Trachemys scripta* (Gomez and Richards 2018). In other hypoxia-tolerant species including mollusks (*Crassostrea virginica* and *C. gigas*), fish (killifish *Fundulus heteroclitus*, the epaulette shark *Hemiscyllum ocellatum*, and rainbow trout *Oncorhynchus mykiss*), and the naked mole rats, hypoxia exposure had little effect on the maximum ETS and COX capacity (Kurochkin et al. 2009; Hickey et al. 2012; Ivanina et al. 2012; Sussarellu et al. 2013; Sappal et al. 2016; Du et al. 2016b; Pamenter et al. 2018). Similarly, severe hypoxia (1.6 kPa P_{O2})

suppressed ETS and OXPHOS activity in hypoxia-sensitive lowland locusts (*Locusta migratoria*) but had little or no effect in their hypoxia-tolerant high-altitude counterparts (Zhang et al. 2013). Deer mice (*Peromyscus maniculatus*) from high-altitude populations acclimated to normoxia had higher mitochondrial OXPHOS and ETS capacity in the muscle tissue compared with their lowland counterparts; however, these between-population differences in mitochondrial capacity disappeared after hypoxic acclimation (Mahalingam et al. 2017). Interestingly, in a hypoxia-tolerant turtle *T. scripta*, exposure to anoxia suppressed ETS activity but this suppression appeared to be restricted to Complex I whereas activities of Complexes III and IV remained similar to the normoxic baseline (Pamenter et al. 2016)

Post-hypoxic reoxygenation leads to the collapse of ETS capacity and mitochondrial membrane potential in a hypoxia-sensitive aquatic species, the bay scallop (Ivanina et al. 2016), similar to the typical mitochondrial injury induced by reoxygenation/reperfusion in terrestrial mammals (Kadenbach et al. 2011). In contrast, in hypoxia-tolerant fish, mollusks, and freshwater turtles, ETS capacity is rapidly restored or enhanced during reoxygenation (Kurochkin et al. 2009; Ivanina et al. 2016; Pamenter et al. 2016; Du et al. 2016b; Sokolov et al. 2019). The ability to rapidly restore the ETS flux upon reoxygenation correlates with hypoxia tolerance not only between but also within species as was shown by the comparison of a relatively hypoxia-sensitive brain and less hypoxia-sensitive heart in the epaulette shark (Devaux et al. 2019). Robust mitochondrial ETS capacity may assist in the recovery from the hypoxia-induced energy deficiency and mitigate ROS production during reoxygenation representing a common adaptive mechanism in distantly related hypoxia-tolerant organisms including vertebrates and invertebrates.

It is worth noting that although ETS capacity is conserved or enhanced during H/R stress in hypoxia-tolerant species, OXPHOS rates generally go down during hypoxia in hypoxia-tolerant as well as sensitive species (St-Pierre et al. 2000a; Kurochkin et al. 2009; Hickey et al. 2012; Galli et al. 2013; Onukwufor et al. 2013, 2016; Sussarellu et al. 2013; Sappal et al. 2015). This inhibition of OXPHOS appears to be fully and rapidly reversible in hypoxia-tolerant clams and turtles but not in hypoxia-sensitive scallops (Galli et al. 2013; Ivanina et al. 2016). The hypoxia-induced decrease in OXPHOS reflects (at least in part) inhibition of F_O, F_1-ATPase activity (St-Pierre et al. 2000b; Galli et al. 2013; García-Bermúdez and Cuezva 2016; Ivanina et al. 2016; Pamenter et al. 2016; Gomez and Richards 2018). Inhibition of F_O, F_1-ATPase was proposed as a putative adaptive mechanism to prevent ATP wastage during hypoxia, when this enzyme hydrolyzes ATP in an attempt to preserve the mitochondrial membrane potential (St-Pierre et al. 2000b). In mammalian models, the hypoxia-induced inhibition of F_O, F_1-ATPase involves post-translational modifications (*S*-nitrosylation) of the enzyme and allosteric inhibition by TCA cycle intermediates such as α-ketoglutarate (García-Bermúdez and Cuezva 2016). Furthermore, regulatory proteins such as G0s2 and the ATPase Inhibitory Factor 1 (IF1) can bind to F_O, F_1-ATPase reversibly suppressing its activity during hypoxia (García-Bermúdez and Cuezva 2016). The mechanisms of F_O, F_1-ATPase inhibition are not well studied in hypoxia-tolerant species and require further investigation. In hypoxia-tolerant freshwater turtles *T. scripta*, anoxia-induced inhibition of F_O, F_1-ATPase activity in liver, brain, and heart did not involve *S*-nitrosylation and was associated with a ∼20–30% decreased abundance of ATP synthase subunits B1 and f (Gomez and Richards 2018). In a hypoxia-tolerant Pacific oyster, abundance of F_O, F_1-ATPase subunits did not considerably change in response to H/R stress (Sokolov et al. 2019).

Modulation of the O_2 affinity of mitochondria through evolutionary adaptation or phenotypic plasticity might contribute to the organism's tolerance to low O_2 conditions. O_2 affinity of mitochondrial respiration is commonly measured as a reciprocal of P_{50} ($1/P_{50}$), where P_{50} is the partial O_2 pressure at which mitochondrial O_2 flux is 50% of the maximum. One might expect mitochondria from hypoxia-adapted organisms to have high O_2 affinity (and thus lower P_{50}) thereby ensuring aerobic respiration at lower O_2 tensions. Some studies support this hypothesis. Thus, mitochondria from hypoxia-tolerant intertidal species of sculpins have higher O_2 affinity of COX compared with their hypoxia-sensitive subtidal counterparts (Lau et al. 2017). Similarly, mitochondria from a more hypoxia-tolerant southern population of killifish *F. heteroclitus* had higher O_2 affinity of OXPHOS than those from their hypoxia-sensitive northern conspecifics, albeit this difference is temperature-dependent (Chung et al. 2017). However, analysis of the mitochondrial P_{50} across a broad range of hypoxia-sensitive and tolerant organisms (including endo- and ectotherms) reveals no consistent association with hypoxia tolerance (Table 1) and suggests that other mechanisms might be involved in regulating the mitochondrial O_2 affinity. One possible explanation is that the

Table 1 Oxygen affinity (P_{50}) of mitochondrial OXPHOS and COX in hypoxia-sensitive and tolerant organisms

Species	Tissue	OXPHOS P_{50}, kPa	COX P_{50}, kPa	*T*, in °C	Source
Endotherms					
Rattus norvegicus	Heart	0.035	—	30	Gnaiger et al. (1998b)
Rattus norvegicus	Heart	0.147	—	37	Costa et al. (1997)
Rattus norvegicus	Liver	0.057	—	30	Gnaiger et al. (1998b)
Rattus norvegicus	Liver	0.171	—	37	Costa et al. (1997)
Homo sapiens	Muscle	0.042	—	37	Larsen et al. (2011)
Peromyscus maniculatus	Muscle	0.074	—	37	Mahalingam et al. (2017)
*Peromyscus maniculatus**	Muscle	0.058	—	37	Mahalingam et al. (2017)
Anser anser	Muscle	~0.12	~0.20	41	Scott et al. (2009)
Branta leucopsis	Muscle	~0.16	~0.33	41	Scott et al. (2009)
Anas platyrhynchos	Muscle	~0.15	~0.23	41	Scott et al. (2009)
*Anser indicus**	Muscle	~0.12	~0.23	41	Scott et al. (2009)
Ectotherms					
Rana temporaria	Muscle	0.0521	—	20	St-Pierre et al. (2000c)
Leptocottus armatus	Brain	—	0.047	18	Lau et al. (2017)
Artediuslateralis	Brain	—	0.062	18	Lau et al. (2017)
Blepsias cirrhosus	Brain	—	0.077	18	Lau et al. (2017)
Artedius fenestralis	Brain	—	0.069	18	Lau et al. (2017)
*Oligocottus maculosus**	Brain	—	0.033	18	Lau et al. (2017)
*Fundulus heteroclitus**	Liver	~0.25–0.4	—	15	Chung et al. (2017)
*Fundulus heteroclitus**	Liver	0.070–0.079	—	20	Du et al. (2016a)
*Fundulus heteroclitus**	Liver	~0.09–0.11	—	33	Chung et al. (2017)
*Arctica islandica**	Mantle	0.233	0.199	15	E. P. Sokolov and I. M. Sokolova (unpublished data)
*Mytilus edulis**	Whole body	0.166	0.188	20	E. P. Sokolov and I. M. Sokolova (unpublished data)

Notes: Only data for mitochondria isolated from normoxic organisms are included. Hypoxia-tolerant or hypoxia-adapted organisms are marked with an asterisk. ~ indicates P_{50} data inferred from the graphs where numerical data were not provided. — indicates data not available.

mitochondrial P_{50} has evolved to maintain mitochondrial respiration under the prevailing intracellular O_2 conditions, as was shown in mammals where mitochondria achieve 85–95% of their maximum capacity at intracellular P_{O2} levels (Gnaiger et al. 1998a; Gnaiger 2001). Intracellular O_2 gradients depend on the overall rate of aerobic metabolism, so that cellular O_2 tensions tend to be lower in metabolically active tissues and cells (Gnaiger 2001; Hirakawa et al. 2015). Thus, interspecific differences in mitochondrial P_{50} might reflect differences in the intracellular O_2 tensions determined by the balance of O_2 delivery and tissue O_2 consumption rather than adaptations to the external P_{O2}. Further investigations in a broad comparative framework controlling for the species' phylogeny and overall metabolic rates and taking into account the actual intracellular P_{O2} in different tissues and organisms are needed to test this hypothesis.

Alternative oxidase: a mitochondrial safety valve in hypoxia?

Alternative oxidase (AOX), first discovered in plants, is a ubiquinol oxidase that introduces a branch point in the mitochondrial ETS by accepting electrons from ubiquinol and transferring them onto O_2 (McDonald et al. 2009). This mechanism bypasses mitochondrial Complexes III and IV and uncouples mitochondrial oxidation from ATP synthesis. AOX is found in nine phyla (Porifera, Placozoa, Cnidaria, Mollusca, Annelida, Nematoda, Echinodermata, Hemichordata, and Chordata) and is considered an ancestral trait of animals that was lost in vertebrates (McDonald et al. 2009). One of the postulated adaptive functions of AOX is to prevent excessive ROS formation during COX-inhibited states caused by low O_2 levels, extreme cold, H_2S exposure, or osmotic stress (Camus et al. 2005; Olson and Straub 2016; Hossain and Dietz 2016). Recent studies

indicate that AOX may have a broader role in metabolic regulation acting as a buffering mechanism against stress-induced metabolic fluctuations (Rasmusson et al. 2009). AOX activity thus appears to be a plausible candidate mechanism for mitochondrial adaptation to fluctuating O_2 conditions. Thus, high O_2-regulated AOX activity has been reported in hypoxia-tolerant marine invertebrates including mollusks, annelids, and crustaceans (Tschischka et al. 2000; McDonald et al. 2009; Sussarellu et al. 2013; Tward et al. 2019). Furthermore, AOX mRNA expression and activity was upregulated during anoxia and/or reoxygenation exposure in hypoxia-tolerant bivalves *Diplodon chilensis* (Yusseppone et al. 2018) and *C. gigas*, (Sussarellu et al. 2013). High AOX activity, combined with the suppression of the pathways channeling electrons to ubiquinone (Sokolov et al. 2019) might minimize electron slip from highly reduced electron carriers and thus suppress mitochondrial ROS production during H/R stress. Interestingly, expression of AOX from a tunicate *Ciona intestinalis* mitigated ROS production in transgenic mice (Mills et al. 2016) indicating that the protective mechanism of AOX is highly conserved and can be reconstituted even in vertebrates that lost AOX gene ~500 million years ago (McDonald et al. 2009)

In plants, AOX is constitutively expressed in mitochondria but is mostly inactive in the absence of stress (Rasmusson et al. 2009). Stress-induced mobilization of AOX in plants involves rapid post-translational activation (e.g., by ketoacids such as pyruvate, or by thioredoxin-mediated breakage of a disulphide cross-link between the monomers and AOX dimers) (Rasmusson et al. 2009). The post-transcriptional mechanisms of AOX regulation in animal mitochondria remain unknown, and structural characteristics of animal AOX (such as the lack of the regulatory N-terminal cysteine involved in the disulphide bond formation) indicate that these mechanisms might be different from those found in plants (McDonald et al. 2009). Understanding the contributions of AOX to regulating the mitochondrial function and ROS avoidance during intermittent hypoxia, thus, represents a fruitful avenue for future research facilitated by the broad phylogenetic distribution of AOX in invertebrate taxa with different degrees of hypoxia tolerance.

Mitochondrial responses to hydrogen sulphide

In aquatic habitats, environmental hypoxia and anoxia often coincide with accumulation of hydrogen sulfide (H_2S) in the sediments due to the activities of anaerobic bacteria (Nealson 1997). Sulphide is ubiquitous and serves as an important signaling molecule in biological systems, albeit high sulphide concentrations are toxic to aerobic organisms by inhibiting COX and cellular respiration (Olson 2012; Olson and Straub 2016). H_2S can be enzymatically produced in the tissues of most animals studied to date including marine invertebrates (Julian et al. 2002; Gainey and Greenberg 2005; Huang and Moore 2015; Olson and Straub 2016). In mammals, H_2S is synthesized by three enzymes—cystathionine γ-lyase, cystathionine β-synthetase (CBS), and 3-mercaptopyruvate sulfurtransferase, constitutively expressed in different tissue types (Huang and Moore 2015; Olson and Straub 2016). Biochemical evidence indicates presence of at least two H_2S synthesizing pathways in hypoxia-tolerant marine invertebrates (including the clams *Ruditapes philippinarum* and *M. mercenaria*, and the lugworm *Arenicola marina*): the L-cysteine-dependent CBS pathway and the L-serine sulphydrase pathway(Julian et al. 2002; Gainey and Greenberg 2005). However, the molecular underpinnings and enzymatic mechanisms of H_2S synthesis have not been extensively studied in marine invertebrates, and physiological and signaling roles of intrinsically produced H_2S remain to be established in these organisms.

The foundational studies (Theede et al. 1969; Groenendaal 1980; Shumway et al. 1983; Bestwick et al. 1989) established a strong positive association between tolerance to hypoxia and environmental sulphide in marine invertebrates. Originally this tolerance was ascribed to the presence of symbiotic sulphur-oxidizing bacteria but subsequent research showed that mitochondria of all metazoans possess intrinsic ability to oxidize sulphide in an O_2-dependent fashion (Powell and Somero 1985; Bagarinao and Vetter 1990; Searcy and Lee 1998; Yong and Searcy 2001). Detoxification of sulphide by mitochondrial oxidation decreases O_2 availability for aerobic respiration (Vaquer-Sunyer and Duarte 2010); however, at low H_2S concentrations, mitochondrial sulphide oxidation is coupled with ATP synthesis (Powell and Somero 1986; Bagarinao 1992).

The exact mechanism of H_2S-driven ATP synthesis is debated. In mammalian mitochondria, sulphide donates electrons to a membrane-bound flavoprotein sulphide:quinone oxidoreductase (SQR) similar to bacterial SQR which transfers electrons to Complex III via ubiquinone (Goubern et al. 2007; Lagoutte et al. 2010). A similar mechanism has been described for the sulphide-tolerant lugworm *A. marina* and ribbed mussel *Geukensia demissa* (Parrino et al. 2000; Theissen and Martin 2008). The SQR activity is considered an ancestral trait present in all animal

mitochondria (Lagoutte et al. 2010) and essential for cellular bioenergetics (Módis et al., 2013). Interestingly, in mitochondria of hypoxia- and sulphide-tolerant marine invertebrates, sulphide can donate electrons directly to Complex III and/or cytochrome c bypassing SQR (Bagarinao and Vetter 1990; Oeschger and Vismann 1994; Völkel and Grieshaber 1997; Hildebrandt and Grieshaber 2008a, 2008b). Low sulphide concentrations (1–10 μM, depending on the species) can be utilized for ATP production in mitochondria (Bagarinao and Vetter 1990; Oeschger and Vismann 1994; Völkel and Grieshaber 1997; Yong and Searcy 2001; Hildebrandt and Grieshaber 2008a, 2008b), but as H_2S concentrations increase, ATP yield declines (Bagarinao and Vetter 1990; Hildebrandt and Grieshaber 2008b). Above a certain species-specific threshold (e.g., ~10 μM H_2S in rat, ~100 μM in sulphide-tolerant marine invertebrates such as *A. marina* or *G. demissa*), COX becomes inhibited and H_2S-driven ATP production ceases (Doeller et al. 2001; Hildebrandt and Grieshaber 2008b; Módis et al. 2013). Under these conditions, vertebrate mitochondria stop respiring and may become damaged (Julian et al. 2005). Mitochondria from sulphide-tolerant marine invertebrates continue respiring even at high H_2S levels by channeling the electrons to AOX and bypassing COX; however, no proton gradient is created under these conditions and respiration is fully uncoupled from ATP synthesis (Hildebrandt and Grieshaber 2008a; Yusseppone et al. 2018). Overall, it appears that hypoxia tolerance and sulphide tolerance may have co-evolved in aquatic organisms building on the ancient mechanisms of mitochondrial sulphide metabolism common to all metazoans. The adaptive changes that contribute to high sulphide tolerance include enhancement of the rates of H_2S-metabolizing enzymes, evolution of the additional pathways for electron entry into ETS (e.g., via Complex III or cytochrome c), involvement of AOX in electron transport, and lower sensitivity of mitochondrial COX to H_2S inhibition (Bagarinao and Vetter 1990; Oeschger and Vismann 1994; Völkel and Grieshaber 1997; Doeller et al. 2001; Hildebrandt and Grieshaber 2008a, 2008b; Módis et al. 2013).

Oxidative stress and redox signaling in mitochondrial tolerance to hypoxia

Oxidative damage due to the excessive production of ROS in mitochondria is considered an important mechanism of H/R injury (Zorov et al. 2014). However, ROS also play important regulatory roles in hypoxia stabilizing transcription factors HIF-1α and HIF-2α (Mansfield et al. 2005; Guzy and Schumacker 2006) and activating mitochondrial protein kinases that phosphorylate mitochondrial ETS complexes and modulate their activity (Acin-Perez et al. 2011; Hüttemann et al. 2012; Srinivasan et al. 2013; Acín-Pérez et al. 2014; Lark et al. 2015). This double-faced role of ROS as the damaging and regulatory elements make analysis of their role in hypoxic adaptations challenging, as it is difficult to distinguish between the adaptive and maladaptive aspects of the elevated ROS production in hypoxia. A comparative study in marine bivalves showed similar baseline antioxidant capacity in a hypoxia-tolerant hard shell clam *M. mercenaria* and a hypoxia-sensitive scallop *A. irradians*. Unlike scallops, the hypoxia-tolerant clams upregulated mitochondrial antioxidant capacity during anoxia and avoided oxidative injury to mitochondrial proteins and lipids during reoxygenation (Ivanina et al. 2016; Ivanina and Sokolova 2016). However, in another hypoxia-tolerant bivalve, *C. gigas*, hypoxia and subsequent reoxygenation suppressed expression of mitochondrial antioxidant enzymes peroxiredoxin 5 and 6 (Sokolov et al. 2019). Lower ROS production was found during hypoxia in a hypoxia-tolerant epaulette shark compared with a hypoxia-sensitive shovelnose ray (Hickey et al. 2012) as well as in humans from high altitude populations compared with their lowland counterparts (Storz et al. 2010) and in hypoxia-adapted fruit flies (Ali et al. 2012). In the brain of a hypoxia-tolerant turtle *T. scripta*, ROS generation decreased during anoxia (Pamenter et al. 2007) and returned to the baseline (normoxic) levels during reoxygenation (Milton et al. 2007). In contrast, no correlation was found between the mitochondrial ROS generation and whole-organism hypoxia tolerance in intertidal sculpins (Lau and Richards 2019). Overall, the lack of a consistent pattern linking ROS production, antioxidant levels, and hypoxia tolerance in animals shows that the role of the mitochondrial redox status for hypoxia tolerance may vary across species. Understanding this role would require a species-specific calibration of normal, signaling, and pathological ROS levels in different organisms.

Another important and as yet underexplored aspect of mitochondrial adaptations to fluctuating O_2 concerns the potential role of the hypoxia-induced shifts in the concentrations of metabolic intermediates (particularly, mitochondrial substrates) for post-hypoxic recovery. In particular, in hypoxia-sensitive organisms such as rodents, accumulation of succinate has been proposed as an important contributor to reoxygenation-induced mitochondrial damage due

to the ROS-generating reverse flow of electrons through mitochondrial Complex I (Chouchani et al. 2014). Therefore, one might anticipate lower levels of succinate and/or decreased activity of succinate dehydrogenase in mitochondria of hypoxia-tolerant species, especially during reoxygenation. Some studies in hypoxia-tolerant organisms show results consistent with this hypothesis. Thus, in hypoxia-tolerant Pacific oysters, H/R stress led to upregulation of mitochondrial Complex I and suppression of the pathways channeling electrons to ubiquinone, which might lower ROS production by preventing the backflow of electrons through Complex I (Sokolov et al. 2019). In hypoxia-tolerant carpet sharks (*Hemiscyllium ocellatum* and *Chiloscyllium punctatum*), the affinity of Complex II for succinate decreased during H/R stress (Devaux et al. 2019), albeit this trend was only statistically significant in the more hypoxia-tolerant *H. ocellatum*. In *H. ocellatum* (but not in *C. punctatum)* this change coincided with a decline in the activity and catalytic efficiency of Complex II, consistent with the suppression of the electron flux to ubiquinone (Devaux et al. 2019). In fruit flies *Drosophila melanogaster* adapted to chronic hypoxia, ratio of mitochondrial ETS complexes shifted so that the activity of Complex II decreased and the activities of Complexes I and III increased thereby favoring the forward over the reverse flux of electrons from ubiquinol (Ali et al. 2012).

It is worth noting, however, that recent studies have questioned the role of the succinate-induced ROS generation in mitochondrial injury during H/R stress (Andrienko et al. 2017). This is based on the observations that redox conditions during early reoxygenation are unfavorable for succinate-fueled reverse electron flow through Complex I and that the major increase in ROS production during reoxygenation occurs only after the opening of the mitochondrial permeability transition pore (mPTP) in hypoxia-sensitive mammalian models (Andrienko et al. 2017). Furthermore, earlier studies showed that elevated levels of succinate can be protective rather than damaging to mitochondria. Thus, in rat liver mitochondria, succinate suppressed lipid peroxidation and protected against the loss of the mitochondrial integrity and function (Szabados et al. 1987; Tretter et al. 1987). In the renal proximal tubular cells of humans, elevated levels of succinate ameliorated oxidative stress and prevented inactivation of Complex I (Nowak et al. 2008). Across-species comparisons also show that hypoxia tolerance is not necessarily associated with decreased succinate accumulation. Thus, while some hypoxia-tolerant vertebrates (such as the freshwater turtle T. *scripta*) show lower rates of anoxic succinate accumulation compared with a hypoxia-sensitive rodent (*Mus musculus*), hypoxia-tolerant mollusks and annelids accumulate high levels of succinate during anoxia (de Zwaan 1991; Grieshaber et al. 1994; Sokolova et al. 2000; Sokolova and Pörtner 2001; Kurochkin et al. 2009). Furthermore, ischemic preconditioning that greatly improves mitochondrial tolerance to H/R stress is not associated with attenuated succinate accumulation in mammalian models (Andrienko et al. 2017). Further investigations are needed to resolve the controversy concerning the role of succinate in mitochondrial response and oxidative injury during H/R stress.

Mitochondrial quality control mechanisms in hypoxia

The mitochondrial quality control mechanisms are important for mitochondrial hypoxia tolerance as they maintain mitochondrial integrity, protein homeostasis, and remove damaged proteins or organelles. Accumulation of damaged mitochondria during hypoxia or reoxygenation may result in elevated ROS emission, ATP deficiency, a lower threshold for apoptosis, or release of damage-associated molecular pattern molecules (such as HSP60 or mtDNA) that induce inflammation (Stotland and Gottlieb 2015). Studies in the model organisms showed the critical importance of the quality control mechanisms (including mitochondrial proteases that degrade oxidatively damaged proteins and mitophagy, a selective and controlled degradation of damaged mitochondria) for cell survival during hypoxia and reoxygenation stress (Kuo et al. 2015; Pinti et al. 2015; Zhang et al. 2016, 2017). To date, little is known about the expression and regulation of these mechanisms in hypoxia tolerant organisms. Comparison of a hypoxia-tolerant subterranean blind mole rat *Spalax ehrenbergi* with a common laboratory rat *Rattus norvegicus* showed lower induction of a mitophagic regulator BNIP3 during hypoxia in the mole rat; however, since, BNIP3 is also a pro-apoptotic factor, it remains unclear whether this reflects lower levels of mitophagy or suppression of apoptosis in the hypoxia-tolerant species (Band et al. 2009). In marine bivalves, hypoxia tolerance is associated with the ability to upregulate and/or maintain high levels of ATP-dependent mitochondrial proteases (Sanni et al. 2008; Ivanina et al. 2016; Ivanina and Sokolova 2016) including LON protease which plays a key role in selective degradation of oxidatively damaged proteins (Bota and Davies

2002; Pinti et al. 2015) and regulation of COX activity during hypoxia (Semenza 2007). Hypoxia exposure also led to a strong upregulation of mitochondrial serine/threonine protein phosphatase PGAM5 in oysters (Sokolov et al. 2019), which is a key regulator of mitophagy responsible for degradation of damaged organelles and preventing activation of the apoptotic program during H/R stress (Yang et al. 2017; Hong and Lee 2018). These data indicate that hypoxia-tolerant species such as marine bivalves possess high activity and inducibility of mitochondrial quality control mechanisms, which might help mitigate H/R-induced damage. However, compared with investigations of mitochondrial bioenergetics, the quality control aspect of mitochondrial responses to hypoxia remains understudied in non-model, hypoxia tolerant species. Future comparative studies of the mitophagy and proteostatic pathways in animals with different degrees of hypoxic tolerance represent a fruitful avenue for understanding the mitochondrial adaptations to O_2 deficiency and H/R stress.

Hypoxia-inducible factor in mitochondrial responses to hypoxia

Hypoxia-inducible factor (HIF) is a transcription factor protein complex activated by hypoxia that plays a central role in O_2 sensing and coordinating the cellular response to hypoxia (Semenza 2004). The dimeric HIF complex is composed of an inducible α subunit (of which several isoforms exist, including HIF-1α, 2α and 3α) and a constitutively expressed β subunit. In normoxia, the α subunit is modified in an O_2-dependent manner and rapidly degraded by the proteasome, whereas in hypoxia, this process is hindered and the α subunit accumulates forming an active HIF transcription factor. HIF activates expression of multiple genes involved in erythropoiesis, angiogenesis, energy metabolism, cell survival, and apoptosis thereby orchestrating the adaptive cellular response to O_2 deficiency (Semenza 2004). Recent studies demonstrate a key role of HIF in regulation of the mitochondrial function and dynamics during hypoxia. Thus, HIF regulates the mitochondrial COX activity during hypoxia by activating transcription of the hypoxia-specific COX4-2 subunit and mitochondrial LON protease which degrades the normoxia-specific COX4-1 (Semenza 2007). Similarly, HIF regulates the expression and subunit composition of mitochondrial Complexes I and III with implications for the activity and ROS production (Tello et al. 2011; Fuhrmann and Brüne 2017), expression of the rate-controlling TCA enzymes such as aconitase (Tsui et al. 2013), and PDK1 [PDH (pyruvate dehydrogenase) kinase 1] which inactivates PDH and shunts pyruvate away from the TCA cycle (Semenza 2011; Ali et al. 2012; Fuhrmann and Brüne 2017). The regulatory link between HIF and mitochondrial ETS appears to be a two-way road, as mitochondrial Complex II (specifically, SDHB subunit) and mitochondrial ROS play a role in stabilization of HIF-α subunits (Semenza 2011; Fuhrmann and Brüne 2017). HIF also regulates the mitochondrial dynamics including biogenesis (Ham and Raju 2016; Semenza 2011), fission and fusion (Solaini et al. 2010; Sato et al. 2015), and mitophagy (Schönenberger and Kovacs 2015).

The role of the ancient and conserved HIF transcription factor in hypoxia-tolerant organisms is not well studied. Most data available to date have focused on the DNA structure of HIF-1α or its transcriptional response to hypoxia (Li and Brouwer 2007; Rahman and Thomas 2007; Rytkönen et al. 2007; Soñanez-Organis et al. 2009; Kim et al. 2011; Piontkivska et al. 2011; Kawabe and Yokoyama 2012). Genome sequencing of a hypoxia-tolerant naked mole rat revealed unique mutations in in the regulatory domain of HIF-1α responsible for the binding to von Hippel–Lindau tumor suppressor (VHL) protein as well as a mutation in the VHL protein sequence not found in other mammals (Kim et al. 2011). The functional consequences of these mutations remain unknown, but the predicted structural changes are consistent with the weaker VHL–HIF-1α interactions that might result in relaxation of VHL-mediated degradation of HIF-1α (Kim et al. 2011). In contrast, a comparative study of HIF-1α sequences in vertebrates revealed no association between the primary protein sequence of HIF-1α and hypoxia tolerance (Rytkönen et al. 2007). This indicates that the potential HIF-1α-dependent adaptations to hypoxia in fish might involve differential regulation of its abundance in the cell and/or its interaction with the target genes rather than the change in the protein sequence. Overall, the comparative analysis of the role of HIF in cellular and mitochondrial adaptations to hypoxia remains an open field for future investigations.

Conclusions and perspectives

Comparison of mitochondrial responses to H/R stress in animals with different hypoxia tolerance reveals common (and potentially adaptive) traits of hypoxia-tolerant mitochondrial phenotypes including anticipatory upregulation of ETS capacity, suppression of ROS-producing electron flux through Complexes I and III, reversible suppression of

Table 2 Common features of hypoxia-sensitive and hypoxia-tolerant mitochondrial phenotypes based on the cross-species comparison of hypoxia tolerant and sensitive organisms

Function/trait	Sensitive		Tolerant	
	H	R	H	R
ETS capacity (especially Complex I and IV)	↓	↓↓	~ or ↑	↑↑
OXPHOS capacity (Complex V)	↓	↓↓	↓	↑
Protonmotive force	↓	↓↓	~ or ↑	↑↑
Oxygen affinity of ETS and COX (Complex IV)	~	~	~	~
Substrate transport	↓	↓↓	↓	↑
Electron flux to ubiquinone	?		↓	↓↓
Mitochondrial proteases	↓	↓↓	~ or ↑	~ or ↑
Mitophagic regulators (e.g., PGAM5)	?		↑	↑
Unfolded protein response	↑	~ or ↑	~	~
Mitochondrial biogenesis	↑	↑	?	
Mitochondrial fusion	↓	↓	?	
Mitochondrial fission	↑	↑	?	
HIF-dependent metabolic regulation	↑	~	?	

Note: Question marks indicate potentially important but as yet unexplored pathways.
H, hypoxia; R, reoxygenation.

OXPHOS activity, and investment into the mitochondrial quality control mechanisms (Table 2). Despite the putative importance of oxidative stress in H/R-induced mitochondrial dysfunction (and by extension, in adaptations to intermittent hypoxia), establishing the role of the ROS-dependent mechanisms in hypoxia adaptation is complicated by the difficulties of measuring the relevant ROS levels *in vivo* and establishing the concentration thresholds above which the damaging effects of ROS outweigh their potentially adaptive signaling function. Furthermore, several key aspects of mitochondrial tolerance to hypoxia remain underexplored in hypoxia-tolerant non-model organisms such as the regulation of mitochondrial biogenesis, fusion and fission (disruption of which contributes to mitochondrial H/R injury in sensitive organisms [Li and Liu 2018]), assessment of the apoptosis-inducing mitochondrial damage thresholds, and HIF-dependent regulation of mitochondrial function (Semenza 2007). Future studies of these aspects of mitochondrial responses might reveal a treasure trove of evolutionary solutions to the ubiquitous and metabolically severe problem of O_2 deficiency in hypoxia-tolerant vertebrates and invertebrates, which would have important implications for understanding the evolution of hypoxia tolerance and the potential mitigation of pathological states caused by O_2 fluctuations.

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