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RESEARCH ARTICLE

Effects of intermittent hypoxia on oxidative stress and protein degradation in molluscan mitochondria

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ABSTRACT

Oxygen fluctuations represent a common stressor in estuarine and intertidal environments and can compromise the mitochondrial integrity and function in marine organisms. We assessed the role of mitochondrial protection mechanisms (ATP-dependent and -independent mitochondrial proteases, and antioxidants) in tolerance to intermittent hypoxia or anoxia in three species of marine bivalves: hypoxia-tolerant hard clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*), and a hypoxia-sensitive subtidal scallop (*Argopecten irradians*). In clams and oysters, mitochondrial tolerance to hypoxia (18 h at 5% O_2), anoxia (18 h at 0.1% O_2) and subsequent reoxygenation was associated with the ability to maintain the steady-state activity of ATP-dependent and -independent mitochondrial proteases and an anticipatory upregulation of the total antioxidant capacity under the low oxygen conditions. No accumulation of end-products of lipid or protein peroxidation was found during intermittent hypoxia or anoxia in clams and oysters (except for an increase in protein carbonyl concentration after hypoxia–reoxygenation in oysters). In contrast, hypoxia/anoxia and reoxygenation strongly suppressed activity of the ATP-dependent mitochondrial proteases in hypoxia-sensitive scallops. This suppression was associated with accumulation of oxidatively damaged mitochondrial proteins (including carbonylated proteins and proteins conjugated with a lipid peroxidation product malondialdehyde) despite high total antioxidant capacity levels in scallop mitochondria. These findings highlight a key role of mitochondrial proteases in protection against hypoxia–reoxygenation stress and adaptations to frequent oxygen fluctuations in intertidal mollusks.

KEY WORDS: ATP-dependent proteases, Antioxidants, Bivalves, Hypoxia–reoxygenation, Mitoproteases, Oxidative lesions

INTRODUCTION

Oxygen deficiency (hypoxia) represents a major challenge for most metazoans as it negatively affects the rates and efficiency of ATP synthesis. Aquatic habitats often experience extreme fluctuations in O_2 content ranging from near anoxia (<1% O_2) to hyperoxia (300–500% O_2), reflecting the local dynamics of photosynthesis, respiration and atmospheric gas exchange (Diaz and Rosenberg, 2008; McMahon, 1988). These fluctuations expose aquatic organisms to the tidal, diurnal and seasonal cycles of hypoxia–reoxygenation (H/R), with hypoxia periods lasting from several hours to several weeks (Diaz and Rosenberg, 2008; McMahon, 1988). Long-term hypoxia can lead to mass mortalities (Rabalais et al., 2009), but even short-term H/R cycles are potentially damaging owing to the effects on the energy levels and oxidative stress (Piper et al., 2003; Sadek et al., 2003).

Mitochondria generate over 90% of cellular ATP and are involved in cell signaling, Ca^{2+} homeostasis and life–death decisions. They are also the main site of generation of reactive oxygen species (ROS) in the cell and a key target of the H/R-induced injury. Exposure to hypoxia leads to accumulation of highly reduced metabolic intermediates in mitochondria, which, upon reoxygenation, can result in a surge of ROS production and damage mitochondrial proteins, lipids and DNA (Piper et al., 2003; Sadek et al., 2003; Zenebe et al., 2007). The ROS-induced mitochondrial damage is critically involved in ischemia–reperfusion injury during stroke, infarction, and tissue and organ transplantation (Levraut et al., 2003; Piper et al., 2003; Sadek et al., 2002, 2003; Tirapelli et al., 2008). However, some animals such as diving birds and mammals or intertidal invertebrates can undergo frequent H/R cycles without any apparent tissue damage. Recent studies showed that hypoxia-tolerant organisms (such as fish and mollusks) maintain mitochondrial integrity during H/R via rapid functional reorganization of mitochondria that upregulates activity of the electron transport system (ETS) and suppresses ATP wastage in hypoxia (Ivanina et al., 2016, 2012; Kurochkin et al., 2009). Mitochondrial tolerance to H/R stress in hypoxia-tolerant organisms also requires that the oxidative damage is prevented and/or repaired during the H/R cycles. However, to date, our knowledge of the mitochondrial protective mechanisms that mitigate damage during H/R stress in hypoxia-tolerant species is limited (Hermes-Lima et al., 1998; Hermes-Lima and Zenteno-Savín, 2002; Hickey et al., 2012; Rivera-Ingraham et al., 2013).

Mitochondrial proteases (mitoproteases) are involved in the protein quality control in mitochondria and implicated in both adaptive and pathological responses to cellular stress (Martinelli and Rugarli, 2010; Quiros et al., 2015). ATP-dependent mitoproteases (such as Lon and ClpP proteases) play a pivotal role in maintaining the integrity of mitochondrial proteome by selectively degrading misfolded and oxidatively damaged proteins (Baker et al., 2011; Bota and Davies, 2002; Quiros et al., 2015; Smakowska et al., 2014). Suppression of the activity of ATP-dependent mitoproteases leads to decreased mitochondrial respiration, elevated ROS production and upregulated expression of mitochondrial chaperones reflecting proteotoxicity in diverse organisms including mammals, plants and fungi (Pinti et al., 2015; Quirós et al., 2014, 2015). Studies in rodents showed that ATP-dependent mitoproteases play a key role in mitigating oxidative damage and cellular injury during H/R stress (Bezawork-Geleta et al., 2015; Ngo et al., 2013; Teng et al., 2013). However, the involvement of mitoproteases in the response to intermittent hypoxia has not been extensively studied in hypoxia-tolerant organisms including marine mollusks.

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List of symbols and abbreviations

A/R	anoxia–reoxygenation
ASW	artificial seawater
DNPH	2,4-dinitrophenyl hydrazine
ETS	electron transport system
FITC	fluorescein isothiocyanate
HNE	4-hydroxynonenal
H/R	hypoxia–reoxygenation
HSP60	heat shock protein 60
MDA	malondialdehyde
PBS	phosphate-buffered saline
ROS	reactive oxygen species
TAOC	total antioxidant capacity

The goal of the present study was to determine the effects of intermittent hypoxia on mitochondrial oxidative stress and activity of mitoproteases in marine bivalves with different degree of hypoxia tolerance. Three common Western Atlantic species were used: the bay scallop, *Argopecten irradians* (Lamarck 1819), the eastern oyster, *Crassostrea virginica* Gmelin 1791, and the hard clam, *Mercenaria mercenaria* Linnaeus 1758. *Argopecten irradians* is a subtidal species whose distribution is confined to well-oxygenated seagrass beds (Hernández Cordero et al., 2012). It cannot tolerate prolonged hypoxia and experiences mass mortalities after only hours of oxygen deprivation (Ivanina et al., 2016). In contrast, oysters and clams are intertidal species that are regularly exposed to intermitted hypoxia because of the tidal cycles and can survive days to weeks without oxygen, depending on the environmental temperature (Kennedy et al., 1996; Kraeuter and Castagna, 2001). We hypothesized that activities of mitoproteases (especially the ATP-dependent proteases involved in the degradation of oxidatively damaged proteins) and antioxidants will be more strongly induced during H/R stress in hypoxia-tolerant intertidal species (oysters and clams) compared with scallops, rendering protection from ROS-induced injury. To test this, we measured activities of ATP-dependent and -independent mitoproteases and total antioxidant capacity (TAOC) in mitochondria of scallops, oysters and clams exposed to hypoxia or near-anoxia (18 h at ~5% or <0.1% O_2, respectively) followed by 1 h of recovery. We also tested a hypothesis that higher induction of mitoproteases and/or TAOC will be associated with the reduced levels of oxidative lesions to mitochondrial proteins and lipids, measured by the levels of carbonylated proteins, end-products of lipid peroxidation [malondialdehyde (MDA) and 4-hydroxynonenal (HNE)], and the degree of inactivation of a ROS-sensitive mitochondrial enzyme aconitase. Levels of a mitochondrial chaperone, HSP60, were measured to assess the unfolded protein response during H/R stress. This study provides insights into the involvement of mitochondrial proteolysis and antioxidant defense in the maintenance of mitochondrial integrity during H/R stress and the potential role of these mechanisms in exceptional hypoxia tolerance of animal extremophiles such as the intertidal mollusks.

MATERIALS AND METHODS

Animal maintenance

Scallops, oysters and clams were obtained from local suppliers (Inland Seafood, Charlotte, NC, USA, for clams and oysters and UNC Wilmington's Shellfish Research Hatchery, Wilmington, NC, USA, for scallops). Mollusks were kept in tanks with artificial seawater (ASW) (Instant Ocean, Kent Marine, Acworth, GA, USA) at 20±1°C and a salinity of 30±1 PSU. Mollusks were fed *ad libitum* with a commercial algal blend containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* spp. (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Because of the differences in the metabolic rates and food demand between the species, oysters and clams were fed every other day and scallops every day. At each feeding, 2–3 ml of the algal blend were added per tank containing 20–25 animals.

Two levels of hypoxic exposures were used: near-anoxia (0.04–0.1% O_2, later referred to as anoxia) and hypoxia (5% O_2). Mollusks were placed in plastic trays with lids (five animals per 5 liters of ASW) containing water pre-equilibrated either with nitrogen or with a gas mixture containing 5% O_2, 0.04% CO_2 and balance of nitrogen (Robert Oxygen, Charlotte, NC, USA) to achieve anoxia and hypoxia, respectively. Mollusks were maintained under the anoxic or hypoxic conditions for 18 h with continuous bubbling of the respective gases to maintain target O_2 levels. All exposures were conducted at 20±1°C. After exposures, animals were placed into well-aerated ASW to recover for 1 h. Control animals were maintained under normoxic conditions (21% O_2) for the duration of the experiment. Exposure to anoxia (18 h) led to 47% mortality in scallops but caused no mortality in clams or oysters. No mortality was observed during hypoxic exposure in any of the studied species. Mitochondrial isolations were conducted in animals maintained in normoxia (controls), at the end of the 18 h exposure to anoxia or hypoxia, or after 1 h of recovery.

Mitochondrial isolation

Mitochondria were isolated from gills of clams, oysters and scallops using a method modified from Sokolova (2004). Gills are the main organ of gas exchange in bivalves and are exposed to H/R cycles. For each mitochondrial isolation, gill tissues from two bivalves were pooled. Pooled gills (2–4 g) were placed in ice-cold buffer containing 300 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 50 mmol l^{-1} NaCl, 8 mmol l^{-1} EGTA and 30 mmol l^{-1} Hepes, pH 7.5, 5 mmol l^{-1} sodium citrate, 0.5 mmol l^{-1} DTT and 0.1% β-mercaptoethanol. Tissues were homogenized with several passes of a Potter–Elvenhjem homogenizer and a loosely fitting Teflon pestle at 200 rpm. The homogenate was centrifuged at 2000 ***g*** for 8 min to remove cell debris, and the supernatant was centrifuged at 8500 ***g*** for 8 min to obtain a mitochondrial pellet. The mitochondrial pellet was resuspended in a buffer containing 30 mmol l^{-1} Hepes, pH 7.4, 10 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $MgCl_2$, 150 mmol l^{-1} NaCl, 150 mmol l^{-1} KCl, 50 mmol l^{-1} sucrose, 100 mmol l^{-1} mannitol, 5 mmol l^{-1} sodium citrate, 0.5 mmol l^{-1} DTT and 0.1% β-mercaptoethanol, centrifuged again at 8500 ***g*** for 8 min and resuspended in 1 ml of assay buffer containing 30 mmol l^{-1} Hepes, pH 7.4, 10 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $MgCl_2$, 150 mmol l^{-1} NaCl, 150 mmol l^{-1} KCl, 200 mmol l^{-1} sucrose and 250 mmol l^{-1} mannitol. Protein concentrations in isolated mitochondria (treated with 0.1% Triton X-100 to solubilize mitochondrial membranes) were measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of the proteolytic activity

Proteolytic activity was determined as a rate of degradation of fluorescein isothiocyanate (FITC)-conjugated casein (Life Technology Corporation, Grand Island, NY, USA). To distinguish between ATP-dependent and -independent mitoproteases, proteolytic activity of mitochondrial extract was assessed in the presence and absence of ATP as described elsewhere (Bulteau et al., 2005). It is worth noting that this approach measures the total

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activity of ATP-dependent and -independent mitoproteases and does not distinguish between individual proteases within each of these broad functional groups. Freshly isolated mitochondria were diluted to 0.5 mg ml^{-1} in an assay buffer containing 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} DTT, 0.05% Triton X-100 and 50 mmol l^{-1} Tris, pH 7.9. Proteolytic activity was measured at 20°C in the presence or absence of 8 mmol l^{-1} ATP to distinguish between the activities of ATP-dependent and -independent proteases (Bulteau et al., 2005). After 30 min incubation, a 20 µl aliquot was taken, and proteins (including the undigested FITC-casein substrate) were precipitated with 10% (w/v) trichloracetic acid. Samples were centrifuged at 15,000 ***g*** for 30 min at +4°C. The supernatant containing the peptide fragments was neutralized by addition of 100 µl of 2 mol l^{-1} potassium borate (pH 10). Amount of the fluorescent peptide fragments was determined by a fluorescent plate reader (CytoFluor Series 4000, Framingham, MA, USA) at excitation and emission wavelengths of 495 and 515 nm, respectively.

Total antioxidant capacity

Isolated mitochondria were diluted with ice-cold phosphate-buffered saline (PBS) to a final concentration of 1 mg l^{-1} of protein. TAOC was measured using a colorimetric microplate assay for total antioxidant power (Oxford Biomedical Research, Oxford, MI, USA) following the manufacturer's protocol. TAOC was expressed as Cu^{2+}-reducing equivalents calibrated against a Trolox standard and normalized for the protein content of the sample.

Aconitase activity

Aconitase activity was measured spectrophotometrically with a UV-Vis spectrophotometer (VARIAN Cary 50Bio, Cary, NC, USA) using an aconitase-specific NADPH-coupled assay (Talbot and Brand, 2005). Isolated mitochondria were added to the assay medium containing 30 mmol l^{-1} Hepes, pH 7.4, 10 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $MgCl_2$, 150 mmol l^{-1} NaCl, 150 mmol l^{-1} KCl, 200 mmol l^{-1} sucrose, 250 mmol l^{-1} mannitol, 38.5 mmol l^{-1} succinate, 3.25 mmol l^{-1} citrate, 0.52 mmol l^{-1} NADP and 0.3 U ml^{-1} isocitrate dehydrogenase (IDH) (Sigma-Aldrich, St Louis, MO, USA). Aconitase activity was determined as the rate of increase in the absorbance of NADPH at 340 nm. After recording the background absorbance, mitochondria were solubilized with 2.5% of Triton X-100 to release matrix enzymes. Following the Triton addition, aconitase activity was recorded for 10–15 min. After this, freshly prepared Fe-Cys buffer (154 mmol l^{-1} Tris, pH 7.4, 10 mmol l^{-1} cysteine, 10 mmol l^{-1} Fe^{2+} as $FeSO_4$) was added to the assay in order to reactivate aconitase through substitution of Fe^{2+} in the enzyme's active center. Activity of reactivated aconitase was measured for an additional 5–10 min. The reactions were linear for the complete span of the measurements. Background-corrected activities of active and total aconitase were calculated from the reaction slopes after the addition of Triton-X and Fe-Cys buffer, respectively, and used to calculate the specific enzyme activities (U g^{-1} protein) and percent of the active enzyme in the total pool of mitochondrial aconitase.

Oxidative lesions

Isolated mitochondria were diluted with ice-cold PBS to a final concentration of 1 mg l^{-1} of protein. Proteins conjugated to MDA and HNE were measured as biomarkers of lipid peroxidation using enzyme-linked immunosorbent assays (MDA OxiSelect MDA adduct ELISA Kit and HNE OxiSelect HNE-His adduct ELISA Kit, respectively) according to the manufacturer's protocol (Cell Biolabs, San Diego, CA, USA).

For carbonyl content determination, 100 µl of isolated mitochondria were incubated with 1% (v:v) of streptomycin sulfate for 15 min at room temperature and centrifuged at 6000 ***g*** for 10 min to remove nucleic acid contamination. Carbonyl content of extracted proteins was determined using the 2,4-dinitrophenyl hydrazine (DNPH) assay as described elsewhere (Levine et al., 2000). For each sample, a blank was prepared using 2 mol l^{-1} HCl instead of DNPH. Proteins in the DNPH-stained samples and HCl-treated blanks were precipitated with trichloracetic acid, collected by centrifugation 11,000 ***g*** for 3 min, and washed three times in ethanol:ethyl acetate mixture (1:1 v:v). The pellets were dried, dissolved with 6 mol l^{-1} guanidine HCl in 20 mmol l^{-1} KH_2PO_4, pH 2.4, centrifuged at 11,000 ***g*** for 5 min, and absorbance of the supernatant was determined at 360 nm. Carbonyl concentrations were calculated using an extinction coefficient of ε=22,000 $(mol\ l^{-1})^{-1}$ cm^{-1} for DNPH. Protein content of the samples was determined using the Bradford assay (Bio-Rad Laboratories) with BSA standard prepared in 6 mol l^{-1} guanidine HCL and 20 mmol l^{-1} KH_2PO_4 (pH 2.4). Carbonyl content was normalized to the protein concentration of the mitochondria.

Expression of heat shock protein 60

Expression of heat shock protein 60 (HSP60) was determined in isolated mitochondria by standard immunoblotting. Proteins (20 µg per lane) were loaded onto 10% polyacrylamide gels and run at 72 mA for 2 h at room temperature. The resolved proteins were transferred onto nitrocellulose membrane in 96 mmol l^{-1} glycine, 12 mmol l^{-1} Tris and 20% methanol (v/v) using a Trans-Blot SD (Bio-Rad Laboratories). Equal loading was verified with Amido Black staining (McDonough et al., 2015). The membranes were blocked for 1 h with 3% non-fat milk in Tris-buffered saline, pH 7.6, containing 0.1% Tween-20 (TBST). Membrane was probed overnight with a primary monoclonal antibody against HSP60 (SPA-805, Stressgen Bioreagent, Ann Arbor, MI, USA) in TBST with 5% bovine serum albumin at +4°C. After washing off the primary antibody, membranes were probed with the polyclonal secondary antibody conjugated with horseradish peroxidase (Jackson Immunoresearch, West Grove, PA, USA) and proteins were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Densiometric analysis of the signal was performed by Gel Doc EZ imager and Image Lab 4.1 software (Bio-Rad Laboratories). Each blot included a control sample (the same on all blots) as an internal standard.

Statistics

Because of significant deviations of the data from the normal distribution and/or heteroscedasticity of variances, the effects of oxygen conditions on the mitochondrial traits were analyzed separately for each species using Kruskal–Wallis non-parametric ANOVA and *post hoc* comparisons as implemented in JMP10 software (SAS Institute, Cary, NC, USA). To determine the species-specific differences in the baseline values of the studied mitochondrial traits (except HSP60 expression), a Kruskal–Wallis test was conducted on data for the control (normoxic) animals. The effects of the factor 'species' were not tested for the expression of HSP60 because of the possible species-specific differences in the antibody's affinity to HSP60, which could affect the level of signal. For each experimental group, a total of five to six biological replicates were obtained, each representing a mitochondrial isolate

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from the pooled tissues of two animals. Effects were considered significant if the probability of Type I error (P) was less than 0.05. The data are presented as means+s.e.m. unless indicated otherwise.

RESULTS

Mitochondrial proteolysis

Baseline activities of ATP-dependent and -independent mitoproteases were similar in mitochondria of oysters and scallops, but significantly higher in the mitochondria of clams (Table 1, Fig. 1). In scallops, anoxia and hypoxia caused no changes in the activity of ATP-dependent proteases, while post-anoxic and post-hypoxic recovery led to a strong suppression of ATP-dependent mitoproteases (Fig. 1A). In oyster mitochondria, ATP-dependent protease activity remained stable in anoxia and post-anoxic reoxygenation (Fig. 1B). Hypoxia suppressed activity of ATP-dependent mitoproteases in oysters, but this activity was restored during post-hypoxic recovery (Fig. 1B). In clams, the activity of ATP-dependent mitoproteases was significantly downregulated in anoxia but remained high (similar to the control levels) during hypoxia as well as during the post-anoxic and post-hypoxic recovery (Fig. 1C).

Activity of ATP-independent mitochondrial proteases was significantly inhibited during anoxia in scallops, returning to control levels after 1 h of post-anoxic recovery (Fig. 1D). Hypoxia and H/R had no effect on the activity of ATP-independent mitochondrial proteases in scallops. In oysters, activity of ATP-independent mitochondrial proteases increased during anoxia and during post-hypoxic recovery (Fig. 1E). In clam mitochondria, ATP-independent protease activity was not affected by experimental oxygen levels (Fig. 1F).

Total antioxidant capacity

The baseline TAOC was higher in the mitochondria of scallops compared with those of clams or oysters (Table 1, Fig. 2). Exposure to anoxia significantly suppressed TAOC levels in scallop mitochondria, which returned to control levels during post-anoxic recovery. Hypoxia and post-hypoxic recovery had no effect on TAOC level in scallop mitochondria (Fig. 2A). In oysters, TAOC level significantly increased during hypoxia (but not anoxia), whereas in clams, hypoxic and anoxic exposures stimulated the mitochondrial TAOC activity (Fig. 2B,C). TAOC returned to control levels during the first hour of post-anoxic or post-hypoxic recovery in oysters and clams (Fig. 2B,C).

Oxidative lesions and HSP60 expression

The baseline (control) MDA levels were highest in oysters, intermediate in clams and lowest in scallops, while the species-specific differences in HNE levels showed an opposite trend (i.e. highest levels in scallops followed by clams and oysters) (Table 1, Fig. 3A–F). The baseline levels of carbonyls were similar in the mitochondria of scallops and clams and lower than those in oysters (Table 1, Fig. 3G–I). In scallop mitochondria, anoxia and post-anoxic recovery resulted in a significant increase in the levels of MDA, while the levels of carbonylated proteins increased during anoxia–reoxygenation (A/R) and H/R stress (Fig. 3A,G). Anoxia (but not hypoxia) also led to an increase in MDA levels in scallop mitochondria (Fig. 3A). Mitochondrial HNE levels were not significantly affected by the experimental oxygen conditions in scallops (Fig. 3D). In oysters, H/R stress resulted in a significant increase in the mitochondrial levels of protein carbonyls (but not MDA or HNE); all other experimental conditions had no effect on the levels of oxidative lesions in oyster mitochondria (Fig. 3B,E,H). In clams, the experimental oxygen conditions had no effect on the levels of the oxidative lesions in mitochondria except a small but significant decrease in the levels of the protein carbonyls after 18 h of hypoxia (Fig. 3C,F,I).

Expression of a mitochondrial chaperone HSP60 was not affected by the experimental oxygen conditions in mitochondria of oysters or clams (Fig. 3K,L) and significantly increased after 1 h of post-hypoxic recovery in scallops (Table 1, Fig. 3J).

Table 1. Effects of species and oxygen exposure on the studied mitochondrial traits in marine bivalves

	K–W: effect of species on baseline trait levels	K–W: effects of experimental oxygen conditions		
		Scallops	Oysters	Clams
Total antioxidant capacity	**χ^2_2=8.38, P=0.015**	χ^2_4 =8.89	**χ^2_4=9.76**	**χ^2_4=12.34**
	[O=C]<S	P=0.064	**P=0.045**	**P=0.015**
ATP-dependent protease activity	χ^2_2 =5.45, P=0.066	**χ^2_4=10.96**	χ^2_4=7.19	χ^2_4=4.53
	S<C, S=O, O=C	**P=0.027**	P=0.126	P=0.339
ATP-independent protease activity	**χ^2_2 =8.35, P=0.015**	χ^2_4=8.22	**χ^2_4=10.25**	χ^2_4=1.25
	C=O, S<O, S<C	P=0.084	**P=0.036**	P=0.870
MDA	**χ^2_2=10.26, P=0.006**	**χ^2_4=19.55**	χ^2_4=3.71	χ^2_4=3.18
	O=C, S<C, S<O	**P=0.0006**	P=0.446	P=0.528
HNE	**χ^2_2=15.16, P=0.0005**	**χ^2_4=9.79**	χ^2_4=5.15	χ^2_4=3.11
	S<O<C	**P=0.044**	P=0.273	P=0.540
Carbonyls	**χ^2_2=8.56, P=0.014**	**χ^2_4=1.58**	χ^2_4=6.65	χ^2_4=8.03
	O<C, S=O, S=C	**P=0.032**	P=0.156	P=0.090
Aconitase (active)	χ^2_2=3.75, P=0.153	χ^2_4=6.94	χ^2_4=7.76	**χ^2_4=10.55**
	C=S=O	P=0.139	P=0.101	**P=0.032**
Aconitase (total)	χ^2_2=4.43, P=0.109	**χ^2_4=10.76**	**χ^2_4=15.15**	χ^2_4=2.80
	S=O=C	**P=0.029**	**P=0.004**	P=0.592
% of active aconitase	**χ^2_2=12.49, P=0.002**	χ^2_4=4.19	χ^2_4=5.96	**χ^2_4=16.34**
	O<S<C	P=0.381	P=0.203	**P=0.003**
HSP60 expression	ND	χ^2_4=3.52	χ^2_4=1.75	χ^2_4=2.26
		P=0.476	P=0.781	P=0.688

Chi-square values with the degrees of freedom for the effect and P-values are given for Kruskal–Wallis (K–W) analysis. Species abbreviations: O, oysters; C, clams; S, scallops. For *post hoc* comparisons, the equals (=) sign indicates that the respective means are not statistically different (P>0.05), while > or < signs indicate significant differences between the species (P<0.05). Effects of the experimental conditions (normoxia, anoxia, post-anoxic recovery, hypoxia and post-hypoxic recovery) were tested separately for each species. HNE, 4-hydroxynonenol; MDA, malondialdehyde; ND, not determined.

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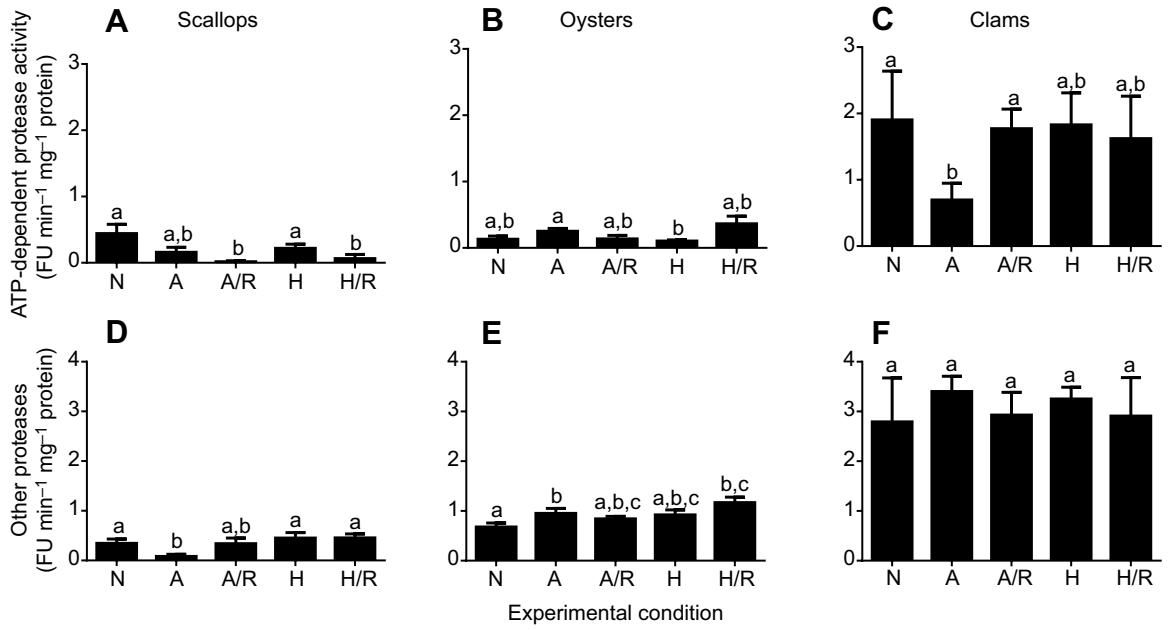

Fig. 1. Effect of anoxia–reoxygenation (A/R) or hypoxia–reoxygenation (H/R) on ATP-dependent and -independent mitoproteases. (A–C) Activity of ATP-dependent proteases. (D–F) Activity of ATP-independent proteases. Data are for scallops (A,D), oysters (B,E) and clams (C,F). *N*=5–6. Different letters above the columns indicate values that are significantly different among the treatments (P<0.05). N, normoxia; A, anoxia; H, hypoxia.

Aconitase activity

Baseline activity of the total (Fe-Cys-reactivated) aconitase, reflective of the overall aconitase content of the mitochondria, was similar in the control groups of all three studies species, while levels of the enzymatically active aconitase were higher in the mitochondria of scallops and clams compared to oysters (Fig. 4A–F). As a result, the fraction of total aconitase that was enzymatically active was lowest in the mitochondria of oysters and highest in those of clams (Table 1, Fig. 4D–F). The native aconitase activity and the fraction of the active enzyme decreased during anoxia and post-anoxic reoxygenation in clams but did not change in response to the experimental oxygen conditions in scallops or oysters (Fig. 4A–C). The total amount of aconitase in mitochondria was suppressed during hypoxia in scallops and during anoxia and A/R stress in oysters, but did not change in clams (Fig. 4D–F). In scallops, the fraction of active aconitase in the total aconitase pool was not affected by H/R stress. In oysters, the highest percentage of active aconitase was found after 1 h of post-anoxic recovery, whereas in clams, anoxia and post-anoxic recovery resulted in a decrease in the fraction of the active enzyme (Fig. 4G–I).

DISCUSSION

Exposure to H/R results in functional reorganization of the mitochondria in bivalves, which markedly differs in two hypoxia-tolerant species (oysters or clams) and a hypoxia-sensitive species (e.g. scallops) (Ivanina et al., 2016, 2012; Kurochkin et al., 2008). Clams show a notably greater mitochondrial tolerance to H/R stress compared with oysters and especially scallops, as indicated by their ability to upregulate the activity of the ETS and oxidative phosphorylation capacity during hypoxia and subsequent reoxygenation (Ivanina et al., 2016, 2012; Kurochkin et al., 2008). Oysters also showed an upregulation of ETS activity during H/R stress (albeit to a lesser degree than clams), whereas in scallops, H/R stress led to a suppression of the ETS activity, collapse of the oxidative phosphorylation capacity, and a decrease in the mitochondrial membrane potential (Ivanina et al., 2016). Mitochondrial injury during H/R stress is driven by the increased ROS production during oxygen influx combined with the high concentrations of reduced metabolic intermediates that accumulate in hypoxia (Clanton, 2007; Korge et al., 2015). Therefore, mitochondrial tolerance to H/R stress will depend on the ability to mitigate and counteract the oxidative injury during reoxygenation. The results of our present study are consistent with this hypothesis, showing elevated expression of mitochondrial protective mechanisms (such as mitoproteases and antioxidants) during H/R or A/R stress in hypoxia-tolerant bivalve species.

Hard clams, which are the most tolerant to H/R stress among the three studied species, had the highest activity of ATP-dependent and

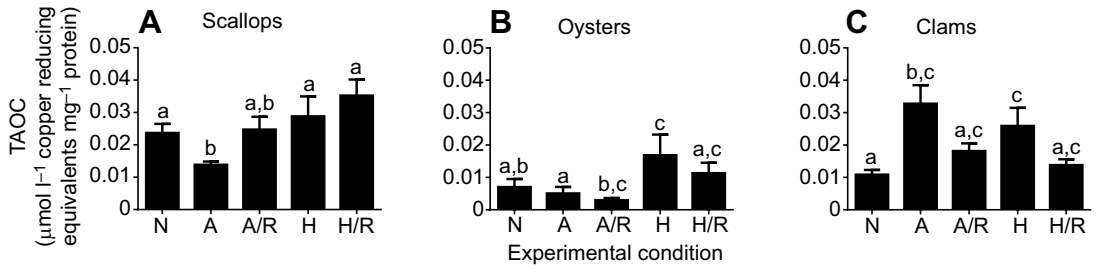

Fig. 2. Effect of A/R or H/R on total antioxidant capacity (TAOC) in mitochondria. (A) Scallops (*N*=3–4); (B) oysters (*N*=6); (C) clams (*N*=4). Different letters above the columns indicate values that are significantly different among the treatments (P<0.05).

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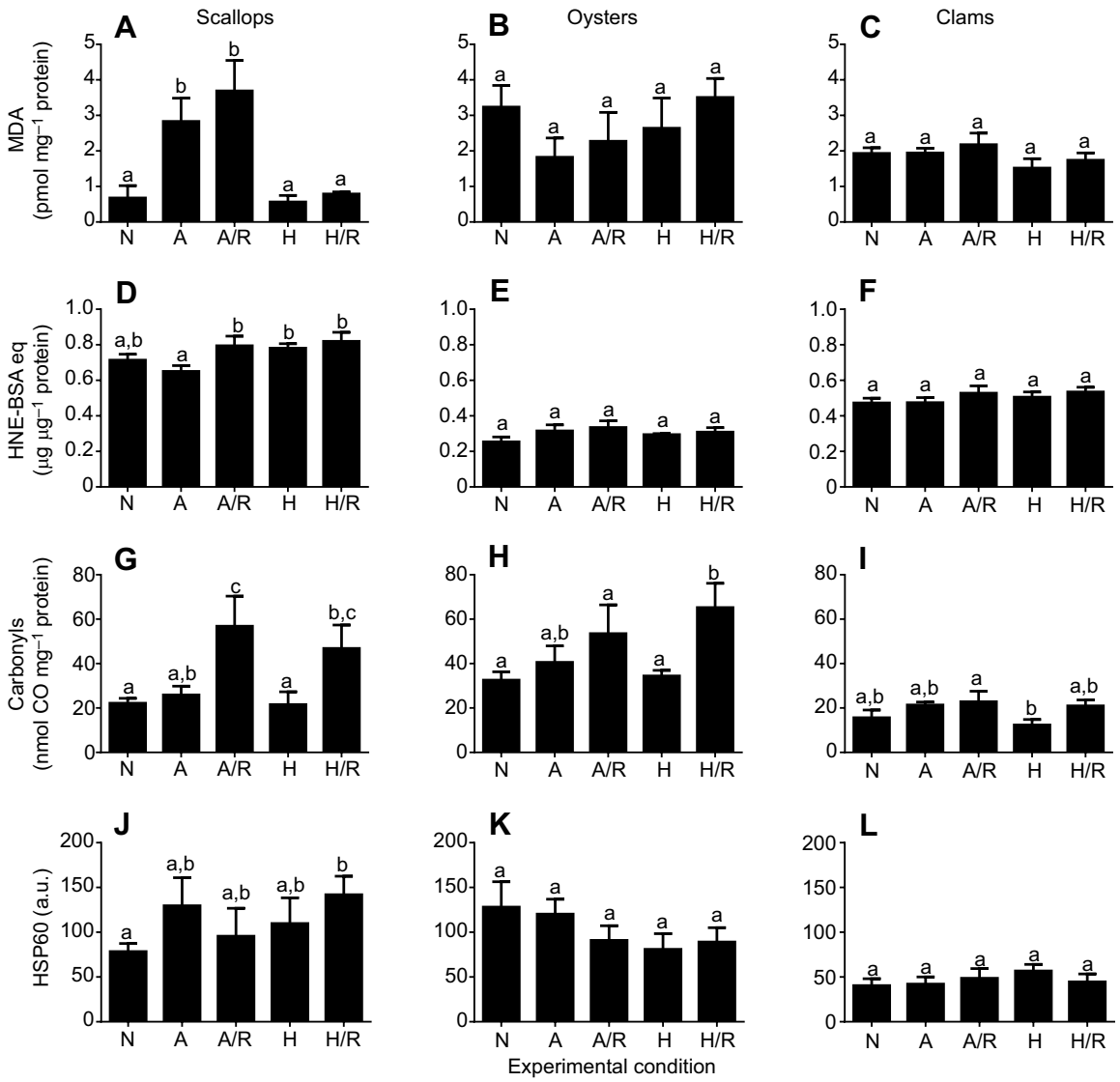

Fig. 3. Effect of A/R or H/R on oxidative lesions in mitochondria. (A–C) Malondialdehyde (MDA)–protein conjugates; (D–F) 4-Hydroxynonenol (HNE)–protein conjugates; (G–I) protein carbonyls; (J–L) expression of mitochondrial HSP60. Data are for scallops (A,D,G,J), oysters (B,E,H,K) and clams (C,F,I,L). *N*=5–6. Different letters above the columns indicate values that are significantly different among the treatments ($P<0.05$).

-independent mitoproteases (4- to 14-fold higher than in scallops or oysters) under control conditions as well as during H/R and A/R stress. ATP-dependent mitochondrial proteases (including Lon and ClpXP proteases) are essential components of the mitochondrial protective system against oxidative stress. They degrade damaged mitochondrial proteins, preventing their accumulation and aggregation, and often demonstrate selectivity for oxidatively damaged proteins (Ngo et al., 2013). High steady-state activity of ATP-dependent mitoproteases in clam mitochondria may therefore protect these organelles during oxygen fluctuations by maintaining integrity of the mitochondrial proteome. In oysters, the activity of ATP-dependent proteases remained at steady-state levels during oxygen fluctuations even though the overall activity was lower than in clams. In contrast, in hypoxia-sensitive scallops, the activity of ATP-dependent mitoproteases was suppressed by twofold to threefold during exposure to anoxia and hypoxia, and by up to sevenfold to 28-fold during reoxygenation. During anoxia, a decline in ATP-independent protease activity was accompanied by an approximately fourfold decrease in the activity of ATP-independent proteases, indicating a global suppression of mitochondrial proteolysis in scallops. Suppression of ATP-dependent and -independent mitoproteases during oxygen deficiency and reoxygenation can negatively affect the mitochondrial integrity and function of hypoxia-sensitive scallops by slowing down degradation of irreparably damaged mitochondrial proteins (Teng et al., 2013), and may explain the earlier findings of rapid deterioration of mitochondrial function during reoxygenation in this species (Ivanina et al., 2016).

Suppression of ATP-dependent proteases during H/R and A/R exposure in scallops goes hand in hand with accumulation of oxidatively damaged proteins, including carbonylated proteins and proteins conjugated with the end products of lipid peroxidation (MDA). In scallops, MDA–protein adducts mostly increased in response to anoxia (<0.1% O_2) and post-anoxic reoxygenation, while carbonylated proteins showed the strongest accumulation after 1 h of post-anoxic and post-hypoxic recovery. This indicates that different oxygen regimes may cause injury to different mitochondrial components in susceptible organisms. Our data also indicate that direct oxidative damage to the proteins (indicated by carbonylation) mostly occurs during the recovery following

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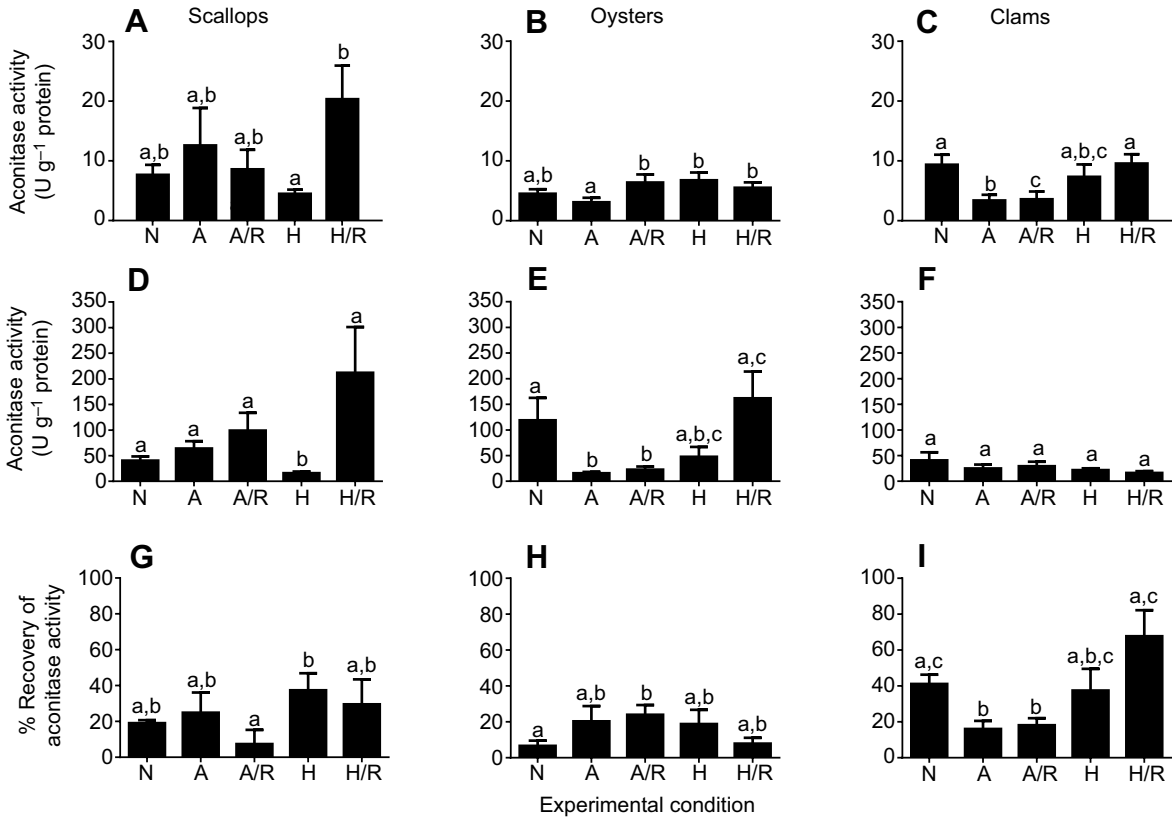

Fig. 4. Effect of A/R or H/R on activity of mitochondrial aconitase. (A–C) Specific aconitase activity; (D–F) Fe-cysteine reactivated aconitase activity; (G–I) percentage of aconitase activity recovery. Data are for scallops (A,D,G), oysters (B,E,H) and clams (C,F,I). *N*=5–6. Different letters above the columns indicate values that are significantly different among the treatments ($P<0.05$).

oxygen-deficient periods. In the two hypoxia-tolerant species, there was no accumulation of the oxidative lesions except for an increase in the carbonylated protein levels during post-hypoxic recovery in oysters. Notably, baseline TAOC was considerably higher in scallops compared with oysters or clams, indicating higher steady-state production of ROS in scallops. However, these high levels of antioxidants did not prevent formation of the oxidative lesions in scallop mitochondria during A/R and H/R stress. In contrast to scallops, the baseline mitochondrial TAOC of oysters and clams was low but significantly increased during hypoxia and/or anoxia. Such anticipatory increase in antioxidants during oxygen-deficient conditions has been previously described in a variety of hypoxia-tolerant species and appears to be a common adaptation to tolerate rapid changes in oxygen availability (Hermes-Lima and Zenteno-Savín, 2002).

Aconitase is a ROS-sensitive enzyme involved in the mitochondrial tricarboxylic acid cycle. It is reversibly inactivated by superoxide because of the release of iron from the active center, which results in the loss of enzymatic activity; this activity can be restored by iron donors (such as Fe^{2+}-Cys) under the reducing conditions (Gardner et al., 1997). Aconitase inactivation thus serves as a marker of the mitochondrial ROS production in many species including mollusks (Cherkasov et al., 2007; Sanni et al., 2008). Moderate levels of aconitase inactivation may act as a 'safety valve' limiting supply of the reducing equivalents from the tricarboxylic acid cycle and thereby curbing the ETS activity and ROS production (Andersson et al., 1998; Bota et al., 2002; James et al., 2002). However, aconitase inactivation can aggravate oxidative stress by increasing the iron release in the mitochondria (Andersson et al., 1998; Bota et al., 2002; Bulteau et al., 2003; James et al., 2002). In the three studied species of bivalves, only clams showed partial inactivation of aconitase during anoxia and post-anoxic recovery, indicated by a decline in the percentage of active aconitase from ∼40 to ∼20%. In oysters or scallops, the fraction of active aconitase in the total aconitase pool did not change during intermittent hypoxia or anoxia. These findings are consistent with the putative protective role of moderate aconitase inactivation to limit the electron supply to the ETS in the most hypoxia-tolerant species, the hard clam.

As a corollary, mitochondrial tolerance to fluctuating oxygen levels in the studied bivalve species correlates with the differences in expression of the mitochondrial protection mechanisms translating into the different susceptibility of the mitochondrial membranes and proteins to oxidative damage. Highly tolerant hard clams that can survive for weeks in anoxic and sulfidic sediments (Altieri, 2008; Kraeuter and Castagna, 2001; Savage, 1976) show the highest levels of mitochondrial protection, indicated by the constitutively high activity of mitoproteases, strong anticipatory induction of antioxidants during anoxia and hypoxia and lack of accumulation of the oxidative lesions in mitochondria during intermittent hypoxia. Similarly, hypoxia-tolerant intertidal oysters that can survive several days to weeks without oxygen (Ivanina et al., 2010, 2012; Vaquer-Sunyer and Duarte, 2008) achieve mitochondrial protection by upregulating mitochondrial antioxidants and/or mitoproteases during H/R or A/R stress. In scallops, suppression of ATP-dependent protease activity during intermittent anoxia or hypoxia is associated with accumulation of oxidatively damaged proteins and is not alleviated by the high baseline activity of antioxidants. These findings suggest that molluscan mitoproteases play a crucial

role in mitochondrial protection against oxygen fluctuations common in the intertidal zone, likely by maintaining mitochondrial protein homeostasis during redox stress.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization, A.V.I. and I.M.S.; Methodology, A.V.I. and I.M.S.; Investigation A.V.I.; Writing – Original Draft, I.M.S. and A.V.I.; Writing – Review & Editing, I.M.S. and A.V.I., Funding Acquisition, I.M.S.; Formal Analysis, A.V.I. and I.M.S.

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