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RESEARCH ARTICLE

Intermittent hypoxia leads to functional reorganization of mitochondria and affects cellular bioenergetics in marine molluscs

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ABSTRACT

Fluctuations in oxygen (O_2) concentrations represent a major challenge to aerobic organisms and can be extremely damaging to their mitochondria. Marine intertidal molluscs are well-adapted to frequent O_2 fluctuations, yet it remains unknown how their mitochondrial functions are regulated to sustain energy metabolism and prevent cellular damage during hypoxia and reoxygenation (H/R). We used metabolic control analysis to investigate the mechanisms of mitochondrial responses to H/R stress (18 h at <0.1% O_2 followed by 1 h of reoxygenation) using hypoxia-tolerant intertidal clams *Mercenaria mercenaria* and hypoxia-sensitive subtidal scallops *Argopecten irradians* as models. We also assessed H/R-induced changes in cellular energy balance, oxidative damage and unfolded protein response to determine the potential links between mitochondrial dysfunction and cellular injury. Mitochondrial responses to H/R in scallops strongly resembled those in other hypoxia-sensitive organisms. Exposure to hypoxia followed by reoxygenation led to a strong decrease in the substrate oxidation (SOX) and phosphorylation (PHOS) capacities as well as partial depolarization of mitochondria of scallops. Elevated mRNA expression of a reactive oxygen species-sensitive enzyme aconitase and Lon protease (responsible for degradation of oxidized mitochondrial proteins) during H/R stress was consistent with elevated levels of oxidative stress in mitochondria of scallops. In hypoxia-tolerant clams, mitochondrial SOX capacity was enhanced during hypoxia and continued rising during the first hour of reoxygenation. In both species, the mitochondrial PHOS capacity was suppressed during hypoxia, likely to prevent ATP wastage by the reverse action of F_O,F_1-ATPase. The PHOS capacity recovered after 1 h of reoxygenation in clams but not in scallops. Compared with scallops, clams showed a greater suppression of energy-consuming processes (such as protein turnover and ion transport) during hypoxia, indicated by inactivation of the translation initiation factor EIF-2α, suppression of 26S proteasome activity and a dramatic decrease in the activity of Na^+/K^+-ATPase. The steady-state levels of adenylates were preserved during H/R exposure and AMP-dependent protein kinase was not activated in either species, indicating that the H/R exposure did not lead to severe energy deficiency. Taken together, our findings suggest that mitochondrial reorganizations sustaining high oxidative phosphorylation flux during recovery, combined with the ability to suppress ATP-demanding cellular functions during hypoxia, may contribute to high resilience of clams to H/R stress and help maintain energy homeostasis during frequent H/R cycles in the intertidal zone.

KEY WORDS: Hypoxia, Metabolic control analysis, Mitochondria, Phosphorylation, Proton leak, Substrate oxidation

INTRODUCTION

Fluctuations in oxygen (O_2) availability represent a major challenge to aerobic organisms. The degree of hypoxia tolerance varies among animals, from terrestrial mammals that survive only minutes to hours without O_2 to hypoxia-tolerant fish, reptiles and invertebrates capable of withstanding anoxia for weeks to months (Grieshaber et al., 1994; Bickler and Buck, 2007). Marine intertidal molluscs are among the champions of hypoxia tolerance, experiencing frequent and extreme fluctuations of O_2 concentrations. Diurnal cycles of respiration and photosynthesis in the coastal zones commonly lead to O_2 swings from near-anoxia during the night to hyperoxia during the day (Burnett, 1997; Ringwood and Keppler, 2002). Intertidal rhythms also result in O_2 deprivation during low tide, when intertidal animals close the shells to prevent water loss (McMahon, 1988). Many estuaries and coastal zones also experience long-term hypoxia lasting weeks to months because of the formation of O_2-depleted dead zones (Vaquer-Sunyer and Duarte, 2008). These conditions put intertidal organisms under a strong selective pressure requiring prolonged survival without O_2 and fast recovery of aerobic metabolism when O_2 returns.

Adaptations for prolonged survival in anoxia have been extensively studied in animals (Grieshaber et al., 1994; Hochachka et al., 1996; Storey, 2002) including molluscs (de Zwaan et al., 1991; Sokolova et al., 2000a,b; Babarro and De Zwaan, 2008). These adaptations involve metabolic rate depression, use of alternative glycolytic pathways that produce more ATP and fewer metabolic protons per unit substrate, generation of volatile end products easily released from the body, maintenance of high glycogen levels, and increased proton buffering capacities of the tissues (Willmer et al., 2000). Although the role of these mechanisms in anoxic survival is well understood, an important piece of the puzzle is still missing – namely, knowledge about the mechanisms that allow hypoxia-tolerant animals to preserve mitochondrial functions during hypoxia and quickly restore aerobic metabolism upon reoxygenation. This question is especially important because in hypoxia-sensitive organisms, mitochondrial dysfunction plays a key role in hypoxia–reoxygenation (H/R)-induced injury (Honda et al., 2005; Kadenbach et al., 2011; Hüttemann et al., 2012). However, many hypoxia-tolerant invertebrates, such as intertidal molluscs, endure frequent H/R cycles without any apparent ill effects, and restore mitochondrial respiration within minutes of recovery (Ellington, 1983; Vismann and Hagerman, 1996; Kurochkin et al., 2009). Currently, the physiological mechanisms responsible for such mitochondrial resilience to O_2 fluctuations remain unknown.

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List of symbols and abbreviations

AMPKα	AMP-activated protein kinase α
ASW	artificial seawater
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
ETS	electron transport system
GLM	generalized linear model
H/R	hypoxia–reoxygenation
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIF1-α	hypoxia-inducible factor 1α
HNE	4-hydroxynonenal
LEAK	proton leak
MCA	metabolic control analysis
MDA	malondialdehyde
ME	malic enzyme
NKA	Na^+/K^+-ATPase
OXPHOS	oxidative phosphorylation
PEPCK	phosphoenolpyruvate carboxykinase
PHOS	phosphorylation
PK	pyruvate kinase
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time PCR
RCR	respiratory control ratio
ROS	reactive oxygen species
SOX	substrate oxidation
TCA	tricarboxylic acid
TPP^+	tetraphenyl phosphonium
Δp	proton-motive force
$\Delta\psi$	mitrochondrial membrane potential

This study aimed to elucidate mitochondrial and cellular responses to severe H/R stress in marine bivalves and identify the potential mechanisms involved in mitochondrial resilience to H/R stress. We determined the effects of H/R stress on mitochondrial bioenergetics and control over respiratory flux in two species of marine bivalves – the hard clam *Mercenaria mercenaria* and the bay scallop *Argopecten irradians* – and assessed the potential links between mitochondrial dysfunction and cellular energy homeostasis and injury. Hard clams are an intertidal species that can survive several weeks in anoxia (~14 days at 20°C) whereas subtidal scallops can tolerate the lack of O_2 for only a few hours (<24 h at 20°C) (I.M.S. and A.V.I., unpublished data; Savage, 1976; Vaquer-Sunyer and Duarte, 2011). To identify the mitochondrial subsystems affected by H/R stress, we applied top-down metabolic control analysis (MCA), a powerful approach to analyze regulation and homeostasis in complex metabolic systems such as mitochondria (Brand, 1998). In top-down MCA, the mitochondrial reactions are partitioned into three interconnected blocks [phosphorylation (PHOS), proton leak (LEAK) and substrate oxidation (SOX) subsystems] linked by a common intermediate, the proton-motive force Δp (Brand, 1997, 1998; Suarez, 2004). The SOX subsystem creates Δp through the activity of the electron transport system (ETS) supported by the tricarboxylic acid (TCA) cycle and substrate transporters. The PHOS and LEAK subsystems dissipate Δp. The PHOS subsystem (including F_O,F_1-ATPase, adenylate and inorganic phosphate transporters) uses Δp to synthesize ATP, and the LEAK subsystem dissipates Δp without ATP production because of the activities of futile cation cycles. Excessive LEAK can reduce efficiency of mitochondria, while mild LEAK serves as a 'safety valve' to curb reactive oxygen species (ROS) production (Brand, 2000; Miwa et al., 2003). This mechanistic comparison of mitochondrial responses using top-down MCA allows determination of the critical mitochondrial functions modulated by H/R stress and serves as a foundation for further comparative studies to address the role of these functions in evolutionary adaptation to intermittent hypoxia.

Because mitochondrial dysfunction has implications for cellular energy and redox homeostasis, we also assessed effects of H/R stress on cellular energy balance, oxidative damage and unfolded protein response in scallops and clams. We anticipated that more hypoxia-tolerant clams will demonstrate stronger metabolic rate depression, enhanced energy homeostasis and better protection against oxidative damage compared with the hypoxia-sensitive clams. Anaerobic capacity was assessed by determining accumulation of anaerobic end products (L-alanine, succinate and acetate) (de Zwaan, 1991) and by measuring the enzyme activities at aerobic/anaerobic branchpoint – pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK) and malic enzyme (ME). In facultative anaerobes including molluscs, PK and PEPCK act as a metabolic switch, channeling glycolytic substrates to aerobic or anaerobic ATP production, respectively, and ME acts in concert with PEPCK diverting glycolytic substrates to anaerobic pathways (Zammit and Newsholme, 1978; van Hellemond et al., 2003). Therefore, a lower PK/PEPCK ratio and higher ME activity indicate activation of anaerobic pathways in molluscs (de Vooys, 1980; Sokolova and Pörtner, 2001; van Hellemond et al., 2003). Energy status was assessed by tissue levels of adenylates and energy stores, as well as expression of a key cellular energy sensor, AMP-activated protein kinase (AMPKα) (Hardie, 2014). Effects of H/R stress on major ATP-consuming processes were determined by assessing the cellular markers of protein synthesis (expression of the eukaryotic initiation factor eIF-2α; Larade and Storey, 2002, 2007), protein breakdown (activity of the 26S proteasome; Coux et al., 1996; Götze et al., 2014) and ion transport activity [Na^+/K^+-ATPase (NKA)] (Hochachka, 1985). Cellular damage was assessed by measuring levels of end products of lipid peroxidation, as well as expression of molecular chaperones (HSP60 and HSP70 family) and mRNA expression of ROS-sensitive enzymes mitochondrial aconitase and Lon protease (Bota and Davies, 2002; Bota et al., 2002; Bulteau et al., 2003). This comprehensive assessment of mitochondrial and cellular bioenergetics and stress response provides insights into the potential mechanisms involved in the mitochondrial resilience to H/R and furthers our understanding of the metabolic regulation during intermittent hypoxia in molluscs. Such multivariate analysis can also strengthen inferences about the potential links of the studied physiological traits with hypoxia tolerance within the limited framework of a two-species comparison (Garland and Adolph, 1994) and inform future comparative research to uncover the role of natural selection in shaping adaptations of mitochondrial and cellular bioenergetics to hypoxia.

MATERIALS AND METHODS

Animal maintenance

Clams and scallops were obtained from local suppliers (Inland Seafood, Charlotte, NC, USA, and UNC Wilmington's Shellfish Research Hatchery, Wilmington, NC, USA). Molluscs were kept in tanks with aerated artificial seawater (ASW) (Instant Ocean, Kent Marine, Acworth, GA, USA) at 20±1°C and salinity 30±1 (practical salinity units). Molluscs were fed *ad libitum* (3 ml per 20–25 animals every day for scallops and every other day for clams) with a commercial algal blend (DT's Live Marine Phytoplankton, Sycamore, IL, USA).

To induce hypoxia, molluscs were placed in covered plastic trays (four animals in 5 litres ASW) and hypoxic conditions were created by bubbling ASW with nitrogen (Robert Oxygen, Charlotte, NC, USA) to achieve 0.04–0.1% O_2. Exposure to 18 h of hypoxia led to

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28% and 0% mortality in scallops and clams, respectively. After 18 h of hypoxia, animals were returned into well-aerated tanks and allowed to recover for 1 h. Tissues were collected at the end of the hypoxic exposure and after 18 h of hypoxia followed by 1 h normoxic recovery (i.e. reoxygenation). Control animals were maintained in normoxia.

Because of the limited tissue amount, all traits could not be measured in the same tissues. Therefore, mitochondrial traits were assessed in the gills, which are the main organs of gas exchange directly exposed to O_2 fluctuations. Energy-related metabolites and enzymes of the aerobic/anaerobic branchpoint were assessed in hepatopancreas, which is a metabolically active tissue and a major organ for the glycogen storage in bivalves. Indices related to protein turnover, NKA activity and protein expression were measured in the adductor muscle.

Mitochondrial assays

Mitochondria were isolated from gills as described elsewhere (Sokolova, 2004; Kurochkin et al., 2011). Briefly, 2–4 g of gills were pooled from two to three animals and homogenized in an ice-cold buffer [100 mmol l^{-1} sucrose, 200 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl, 8 mmol l^{-1} ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 30 mmol l^{-1} 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), pH 7.5] using several passes of a Potter–Elvenhjem homogenizer at 200 r.p.m. The homogenate was centrifuged at 4°C and 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 pellet was resuspended in ice-cold assay medium containing 150 mmol l^{-1} sucrose, 250 mmol l^{-1} KCl, 10 mmol l^{-1} glucose, 10 mmol l^{-1} KH_2PO_4, 1% bovine serum albumin (fatty acid free) and 30 mmol l^{-1} HEPES, pH 7.2. Protein concentrations were measured using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with 0.1% Triton X-100 to solubilize mitochondrial membranes.

Respiration and mitochondrial membrane potentials ($\Delta\psi$) of mitochondrial suspensions (4 mg ml^{-1} protein) were determined simultaneously in a temperature-stabilized four-port chamber (World Precision Instruments, Sarasota, FL, USA) at 20°C using fiber-optic O_2 sensors and tetraphenyl phosphonium (TPP^+)-selective electrodes (Kurochkin et al., 2011). Succinate (5 mmol l^{-1}) was used as a substrate because it is transported into mitochondria non-electrogenically and does not affect $\Delta\psi$ (Hafner et al., 1990; Brand, 1998). $\Delta\psi$ values were determined using a TPP^+-selective electrode (KWIKTPP-2) and a Super Dri-Ref reference electrode (World Precision Instruments) connected to a pH meter (Jenco Instruments, San Diego, CA, USA) (Kurochkin et al., 2011). Because mitochondrial Δp consists of the electrical membrane potential ($\Delta\psi$) and the pH gradient across the inner mitochondrial membrane, we added the H^+/K^+ exchanger nigericin (123 nmol l^{-1}) to the assay medium to convert all of Δp into the electrical gradient ($\Delta\psi$). Therefore, throughout this study we refer to $\Delta\psi$ as a measure of Δp. Oxygen sensors were calibrated at 0% and 100% of air saturation, and the TPP^+ electrode was calibrated using stepwise additions of TPP^+ (2–10 µmol l^{-1}). Corrections for the non-specific binding of TPP^+ were conducted after fully collapsing $\Delta\psi$ with 400 µmol l^{-1} of KCN (Lötscher et al., 1980; Chamberlin, 2004b). TPP^+ concentrations were monitored using Logger Pro 3.2 with a Vernier LabPro interface (Vernier Software and Technology, Beaverton, OR, USA). $\Delta\psi$ was calculated using the Nernst equation using a mitochondrial matrix volume of 1 µl mg^{-1} protein and corrections for non-specific TPP^+ binding as described for marine molluscs (Kurochkin et al., 2011).

State 3 (ADP-stimulated) respiration was determined following addition of 150 nmol ADP, and state 4 (resting) respiration was measured after the depletion of ADP followed by addition of 2 µg ml^{-1} oligomycin, a specific inhibitor of mitochondrial F_o,F_1-ATPase. Respiration rates were corrected for non-mitochondrial O_2 consumption and sensor drift by subtracting the residual O_2 consumption in the presence of 400 µmol l^{-1} KCN. Respiratory control ratios (RCRs) were calculated as the ratio of state 3 over state 4 respiration rates.

The kinetic responses of the SOX, LEAK and PHOS subsystems were assessed by changes in O_2 consumption in response to the experimentally induced changes in $\Delta\psi$ (Brand, 1997, 1998; Kurochkin et al., 2011). For the LEAK and PHOS subsystems, a change in $\Delta\psi$ was achieved by titration with malonate, which affects $\Delta\psi$ via inhibition of the SOX subsystem but does not affect the LEAK or PHOS subsystems. For the SOX subsystem, manipulation of $\Delta\psi$ was achieved by addition of a mitochondrial uncoupler {[(3-chlorophenyl)hydrazono]malononitrile (CCCP)} that affects $\Delta\psi$ via stimulation of the proton leak but does not directly affect the SOX subsystem. For each treatment and each subsystem, five to eight mitochondrial isolates were used.

Oxidative lesions

Protein conjugates of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) were measured in mitochondria (*N*=5–8) using the MDA OxiSelect™ MDA adduct ELISA Kit and the HNE OxiSelect™ HNE-His adduct ELISA Kit, respectively, according to the manufacturer's protocols (Cell Biolabs, San Diego, CA, USA).

Metabolites

Approximately 250 mg of hepatopancreas were ground under liquid nitrogen and extracted with ice-cold perchloric acid (0.6 mol l^{-1}) containing 150 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) (Bagwe et al., 2015). Concentrations of L-alanine, acetate, glycogen, glucose and adenylates were measured using standard enzymatic assays as described elsewhere (Bagwe et al., 2015). Succinate concentration was measured with a succinic acid kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Lipid content was determined using a chloroform extraction method (Bagwe et al., 2015), and protein content was measured in hepatopancreas homogenates using the method described below for immunoblotting. The sample size was five to 12, with each replicate representing tissues from an individual mollusc.

Immunoblotting

Muscle tissues were homogenized in an ice-cold buffer containing 100 mmol l^{-1} Tris, pH 7.4, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 1% Triton-X 100, 10% glycerol, 0.1% sodium dodecylsulfate, 0.5% deoxycholate, 0.5 µg ml^{-1} leupeptin, 0.7 µg ml^{-1} pepstatin, 40 µg ml^{-1} phenylmethylsulfonyl fluoride and 0.5 µg ml^{-1} aprotinin, sonicated three times for 10 s each (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA), and centrifuged for 10 min at 20,000 ***g*** and 4°C. Protein content was measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Samples (20 µg protein per lane for EIF-2α, phospho-EIF-2α, HSP60, HSP69 and HSP72/78, or 50 µg for AMPKα, phospho-AMPKα and HIF-1α) were loaded onto 8% polyacrylamide gels and run at 100 mA for 2 h at room temperature. The proteins were transferred onto a polyvinylidene fluoride (PVDF) (for HSP60 and HSP70) or nitrocellulose membrane (for all other proteins) in 96 mmol l^{-1} glycine, 12 mmol l^{-1} Tris and 20% methanol (v/v) using a Trans-Blot semi-dry cell (Thermo

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Fisher Scientific, Portsmouth, NH, USA). Equal loading was verified with Amido Black staining. The membranes were blocked overnight in 5% non-fat milk in Tris-buffered saline, pH 7.6, and probed with primary monoclonal antibodies against phospho-EIF-2α (Ser51) (no. 07-760, Millipore, Temecula, CA, USA), EIF-2α (no. AHO1182, Life Technology, Grand Island, NY, USA), AMPKα and phospho-AMPKα (Thr172) (nos. 2793 and 2535, respectively, Cell Signaling Technology, Danvers, MA, USA), HIF-1α (MAB5382, EMD Millipore, Billerica, MA, USA), HSP70 (MA3-007, Affinity Bioreagents, Golden, CO, USA) and HSP60 (SPA-805, Stressgen Bioreagents, Ann Arbor, MI, USA). After washing off the primary antibody, membranes were probed with the polyclonal secondary antibodies 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). All antibodies produced bands of the expected molecular size (Fig. S1). Densitometric analysis of the signal was performed using the GelDoc 2000 System with Quantity One 1D Analysis Software (Bio-Rad Laboratories). Each blot included the same control sample as an internal standard. The sample size was five, with each replicate representing tissues from an individual mollusc.

Enzyme activities

Activities of PK (EC 2.7.1.40) and PEPCK (EC 4.1.1.31) were measured in hepatopancreas using standard spectrophotometric assays (Sokolova and Pörtner, 2001). Activities of NAD^+-ME (EC 1.1.1.38) and $NADP^+$-ME (EC 1.1.1.40) were determined in the same homogenates in an assay containing 45 mmol l^{-1} Tris HCl (pH 7.4), 3 mmol l^{-1} malate, 10 mmol l^{-1} $MnCl_2$ and 400 µmol l^{-1} of either β-$NADP^+$ or β-NAD^+, and monitored at 340 nmol l^{-1} as an increase in absorbance of NADPH or NADH, respectively. Activities of PK, PEPCK and ME were expressed as U g^{-1} protein.

Activities of 26S proteasome and NKA (EC 3.6.3.9) were determined in the adductor muscle. NKA activity [nmol l^{-1} phosphate (P_i) min^{-1} mg^{-1} protein] was measured as described earlier (Ramnanan and Storey, 2006) using a higher concentration of ouabain (30 mmol l^{-1}) to ensure complete inhibition of NKA. Release of P_i was determined using the Phosphate Assay Kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. Trypsin-like, chymotrypsin-like and caspase-like proteasome activities of the 26S proteasome were measured as described elsewhere (Götze et al., 2014) using specific fluorogenic substrates Boc-Leu-Arg-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC and Ac-Gly-Pro-Leu-Asp-AMC (Bachem, Torrance, CA, USA). Enzyme activities were measured at 20°C. The sample size was five to seven, except for hypoxia-exposed scallops where N=4. Each replicate represented tissues from an individual mollusc.

Quantitative real-time PCR

RNA extraction and cDNA synthesis were conducted as described elsewhere (Sanni et al., 2008). Gene fragments for mitochondrial

Table 1. Effects of exposure conditions (control, hypoxia and reoxygenation) and species (scallops versus clams) on the studied bioenergetic and oxidative stress markers

	ANOVA factor effect		
	Species	Conditions	Species×Conditions
Energy stores			
Glycogen	**$F_{1,44}$=22.56, P<0.0001**	$F_{2,44}$=0.619, P=0.543	$F_{2,44}$=0.372, P=0.692
Glucose	$F_{1,44}$=0.145, P=0.706	$F_{2,44}$=2.17, P=0.127	$F_{2,44}$=0.209, P=0.812
Lipids	$F_{1,46}$=0.73, P=0.399	**$F_{2,46}$=4.14, P=0.022**	$F_{2,46}$=1.22, P=0.304
Proteins	**$F_{1,29}$=9.612, P=0.0043**	*$F_{2,29}$=2.58, P=0.093*	$F_{2,29}$=0.64, P=0.536
Metabolites			
ATP	$F_{1,40}$=0.301, P=0.586	$F_{2,40}$=0.636, P=0.534	$F_{2,40}$=0.101, P=0.904
ADP	**$F_{1,41}$=7.189, P=0.0104**	$F_{2,41}$=1.533, P=0.228	$F_{2,41}$=0.498, P=0.612
AMP	**$F_{1,42}$=21.191, P<0.0001**	**$F_{2,42}$=3.211, P=0.050**	$F_{2,42}$=0.495, P=0.613
L-alanine	**$F_{1,42}$=16.93, P=0.0002**	$F_{2,42}$=1.91, P=0.161	$F_{2,42}$=0.17, P=0.844
Acetate	**$F_{1,38}$=16.42, P=0.0002**	$F_{2,38}$=1.28, P=0.289	$F_{2,38}$=1.48, P=0.241
Succinate	**$F_{1,41}$=16.68, P=0.0002**	$F_{2,41}$=1.77, P=0.183	$F_{2,41}$=0.13, P=0.881
Energy-related indices			
Σ adenylates	**$F_{1,42}$=12.05, P=0.0012**	**$F_{2,42}$=3.35, P=0.045**	$F_{2,42}$=1.50, P=0.235
AEC	**$F_{1,42}$=21.27, P<0.0001**	$F_{2,42}$=0.12, P=0.892	$F_{2,42}$=0.005, P=0.995
ADP/ATP	**$F_{1,38}$=15.39, P=0.0003**	$F_{2,38}$=0.622, P=0.542	$F_{2,38}$=0.211, P=0.811
AMP/ATP	**$F_{1,37}$=20.86, P<0.0001**	$F_{2,37}$=0.919, P=0.407	$F_{2,37}$=0.255, P=0.777
Enzyme activities			
PK	**$F_{1,30}$=5.43, P=0.027**	**$F_{2,30}$=4.26, P=0.024**	$F_{2,30}$=0.66, P=0.524
PEPCK	**$F_{1,35}$=15.77, P=0.0003**	$F_{2,35}$=0.46, P=0.632	$F_{2,35}$=2.45, P=0.101
NAD^+-ME	$F_{1,30}$=0.631, P=0.433	**$F_{2,30}$=4.56, P=0.019**	$F_{2,30}$=0.834, P=0.444
$NADP^+$-ME	$F_{1,30}$=2.08, P=0.160	$F_{2,30}$=1.51, P=0.236	$F_{2,30}$=0.49, P=0.614
NKA	**$F_{1,24}$=31.47, P<0.0001**	**$F_{2,24}$=4.55, P=0.021**	$F_{2,24}$=0.74, P=0.488
Proteasome activity			
Caspase-like activity of 26S proteasome	$F_{1,23}$=0.30, P=0.592	*$F_{2,23}$=3.20, P=0.059*	$F_{2,23}$=0.36, P=0.702
Trypsin-like activity of 26S proteasome	$F_{1,22}$=1.70, P=0.205	*$F_{2,22}$=3.30, P=0.056*	$F_{2,22}$=2.32, P=0.122
Chymotrypsin-like activity of 26S proteasome	**$F_{1,24}$=4.65, P=0.043**	$F_{2,24}$=0.46, P=0.637	$F_{2,24}$=0.39, P=0.681
Oxidative lesions			
MDA-protein conjugates	**$F_{1,32}$=1415, P<0.0001**	$F_{2,32}$=0.01, P=0.991	$F_{2,32}$=0.833, P=0.444
HNE-protein conjugates	**$F_{1,32}$=39.39, P<0.0001**	$F_{2,32}$=0.32, P=0.727	$F_{2,32}$=0.40, P=0.672
mRNA expression			
Mitochondrial aconitase	**$F_{1,53}$=10.75, P=0.002**	**$F_{2,53}$=10.14, P=0.0002**	**$F_{2,53}$=9.01, P=0.0004**
Lon protease	*$F_{1,52}$=3.91, P=0.053*	$F_{2,52}$=1.12, P=0.335	$F_{2,52}$=1.52, P=0.229

Significant effects (P<0.05) are highlighted in bold; marginally significant effects (0.05<P<0.10) are shown in italics.

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Table 2. Effects of exposure conditions (control, hypoxia and reoxygenation) on protein expression in scallops and clams

	Scallops	Clams
AMPKα	n.d.	**$F_{2,14}$=4.96, P=0.027**
pAMPKα	**$F_{2,6.5}$=5.02*, P=0.048**	$F_{2,13}$=1.03, P=0.388
EIF-2a	$F_{2,13}$=1.99, P=0.182	$F_{2,7.4}$=0.623*, P=0.562
pEIF-2a	$F_{2,14}$=1.71, P=0.223	**$F_{2,5.4}$=13.49*, P=0.008**
HIF-1α	n.d.	**$F_{2,14}$=16.75, P=0.0003**
HSP60	$F_{2,5.7}$=2.05*, P=0.213	**$F_{2,4.7}$=11.53*, P=0.015**
HSP69	–	$F_{2,14}$=1.41, P=0.282
HSP72/78	$F_{2,14}$=0.39, P=0.685	$F_{2,7.32}$=0.99*, P=0.413

Total AMPK and HIF-1α expression were measured in clams only. Asterisks mark the instances in which Welch's ANOVA was used instead of the GLM ANOVA because of heteroscedasticity of variances. ANOVA could not be run on HSP69 data for scallops because there was no HSP69 expression in the control group. Significant effects (P<0.05) are highlighted in bold. n.d., not determined.

aconitase and Lon protease (NCBI accession nos KT897897 to KT897910) were isolated using degenerate primers from Sanni et al. (2008), cloned and sequenced to confirm identity. Sequences for β-actin (used as a reference gene) were obtained from NCBI (accession nos. CB417135 and GO915201 for scallops and clams, respectively). Transcript levels were quantified in the adductor muscle tissues by quantitative real-time PCR (qRT-PCR) using a 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) and SYBR Green PCR kit (Life Technologies, Bedford, MA, USA). The following specific primers were used: for scallops, aconitase FWD 5′-GAC AGG ATG TAA AGA AGG GTG AG-3′, REV 5′-GCT GTC TGT CTC TGG GTT AAA G-3′, Lon protease FWD 5′-GGT GCT TAT TGA TGA GGT GGA-3′, REV 5′-GCT CTG GAA TGG TGT CTG TTA-3′, and actin FWD 5′-TCC ACG AAA CCA CAT ACA ACA-3′, REV 5′-GAT TTC TTT CTG CAT ACG GTC A-3′; for clams, aconitase FWD 5′-CTC CAG TCA GTT GAT TCC TTA CTT-3′, REV 5′-TCT CCT AGA GAT GCC AGT ACT TAT-3′, Lon protease FWD 5′-GCA AGG GTT ACC AAG GTG AT-3′, REV 5′-TCC ATC CTA TCT TTC AAT GGT TCT-3′, and actin FWD 5′-GAC CGT CTG GGA GTT CGT AG-3′, REV 5′-AGC GTG GTT ACT CCT TCA CC-3′. The qRT-PCR conditions were as described in previous studies (Sanni et al., 2008); the annealing and read temperatures were 55°C and 72°C, respectively, for all primer pairs. A single cDNA sample from scallops or clams was used as an internal cDNA standard to test for run-to-run amplification variability. Serial dilutions of the internal standard were amplified in each run to determine amplification efficiency and calculate relative mRNA expression of the target genes (Pfaffl, 2001). The sample size for mRNA expression was five to 14, each replicate sample representing mRNA from an individual mollusc.

Data analysis and statistics

We used a two-way generalized linear model (GLM) ANOVA to test for the effects of species (scallops and clams) and oxygen level (control, hypoxia and reoxygenation), as well as the two-way factor interactions on bioenergetic and oxidative traits (Table 1). For immunoblotting analyses, one-way ANOVA was used to test for the effect of oxygen level (control, hypoxia and reoxygenation) on protein expression (Table 2). Protein expression was not compared among species because of the possible species-specific differences in the antibody affinity and signal strength. For traits that showed variance heteroscedasticity by Levene's test, Welch's ANOVA for unequal variance was used. All factors were treated as fixed. Differences between various pairs of means of interest were tested

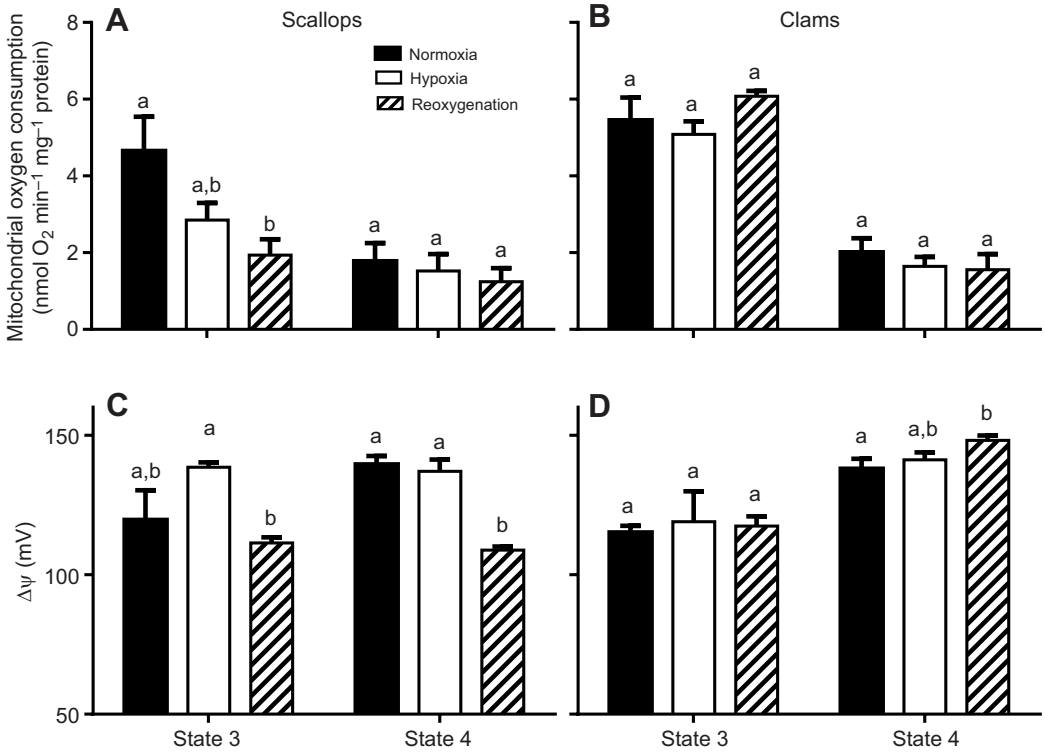

Fig. 1. Effects of hypoxia/reoxygenation (H/R) on ADP-stimulated (state 3) and resting (state 4) respiration and mitochondrial membrane potential (Δψ) of mitochondria from gills of scallops and clams. (A,B) Mitochondrial oxygen consumption, (C,D) Δψ. N=4–5 for scallops (A,C) and 6–8 for clams (B,D). Different letters indicate values that are significantly different among the treatments (P<0.05).

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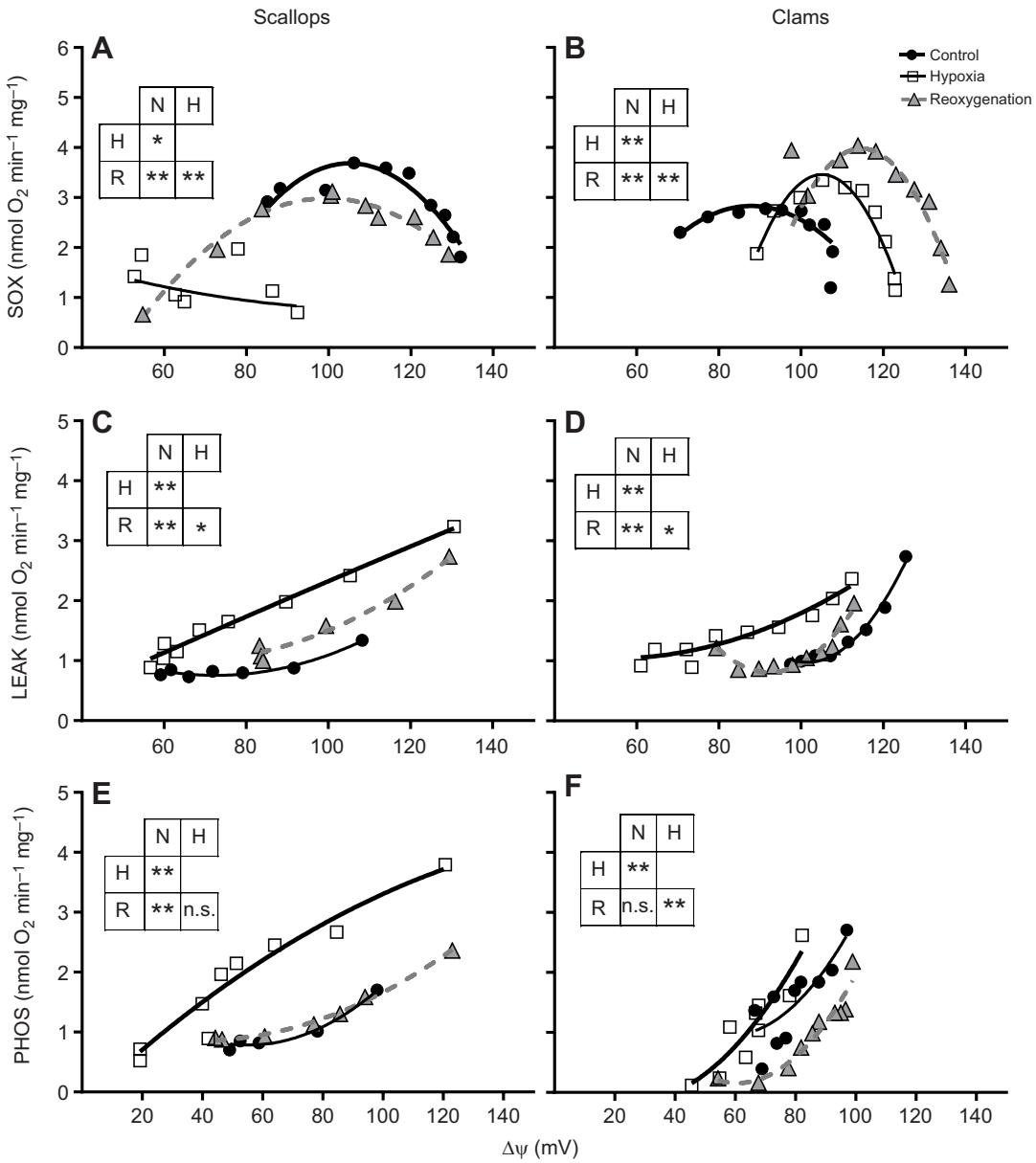

Fig. 2. Kinetic responses of mitochondrial subsystems to changes in Δψ. (A,C,E) Scallops, (B,D,F) clams. Subsystems: (A,B) substrate oxidation (SOX), (C,D) proton leak (LEAK) and (E,F) phosphorylation (PHOS). Each data point reflects an average of five to seven mitochondrial isolations. The significance of the differences between the kinetic curves (N, control; H, hypoxia; R, reoxygenation) is given in the insets: **P*<0.01; ***P*<0.001; n.s., not significant. For the sake of clarity, error bars are omitted from the graph. Means±s.e.m. for respiration and Δψ are given in Table S1, and *P*-values for statistical comparison of the kinetic curves are given in Table S2.

with Fisher's least significant difference (LSD) test for unequal sample size or non-parametric Wilcoxon test for the traits with unequal variance.

The data from the kinetic analyses (i.e. plots of the oxygen consumption rates versus Δψ for each subsystem) were described using second-order polynomials (Kurochkin et al., 2011). To test for significant differences between the kinetic responses in different treatment groups, we used contrasts generated for the polynomial curves for each of the three subsystems using the GLM procedure of SAS (SAS Institute, 1992). These contrasts tested the collective differences between the curves (including both the intercepts and slopes, with three degrees of freedom for the quadratic polynomials). Separate statistical comparisons of intercepts and slopes of the respective curves were not conducted, because we were interested in the overall differences in the kinetic responses of different subsystems between the treatments, rather than in the individual estimated parameters of the empirical curves. The polynomial regressions were also used to calculate elasticities and the flux control coefficients (Brand et al., 1988; Hafner et al., 1990). For calculation of elasticities and flux control coefficients, oxygen consumption of the PHOS subsystem was corrected for LEAK at the respective Δψ (Brand et al., 1988; Hafner et al., 1990). The flux control coefficients could not be calculated at the same Δψ for all treatments because assumption of the additivity of fluxes was fulfilled at different Δψ, reflecting differences in the mitochondrial steady states among the treatments (Brand et al., 1988; Hafner et al., 1990). Therefore, the distribution of control over the respiratory flux was analyzed at different Δψ corresponding to the apparent steady state for each experimental condition. Flux rates of different subsystems were calculated at a common Δψ across all experimental treatments (105 mV in scallops and 115 mV in

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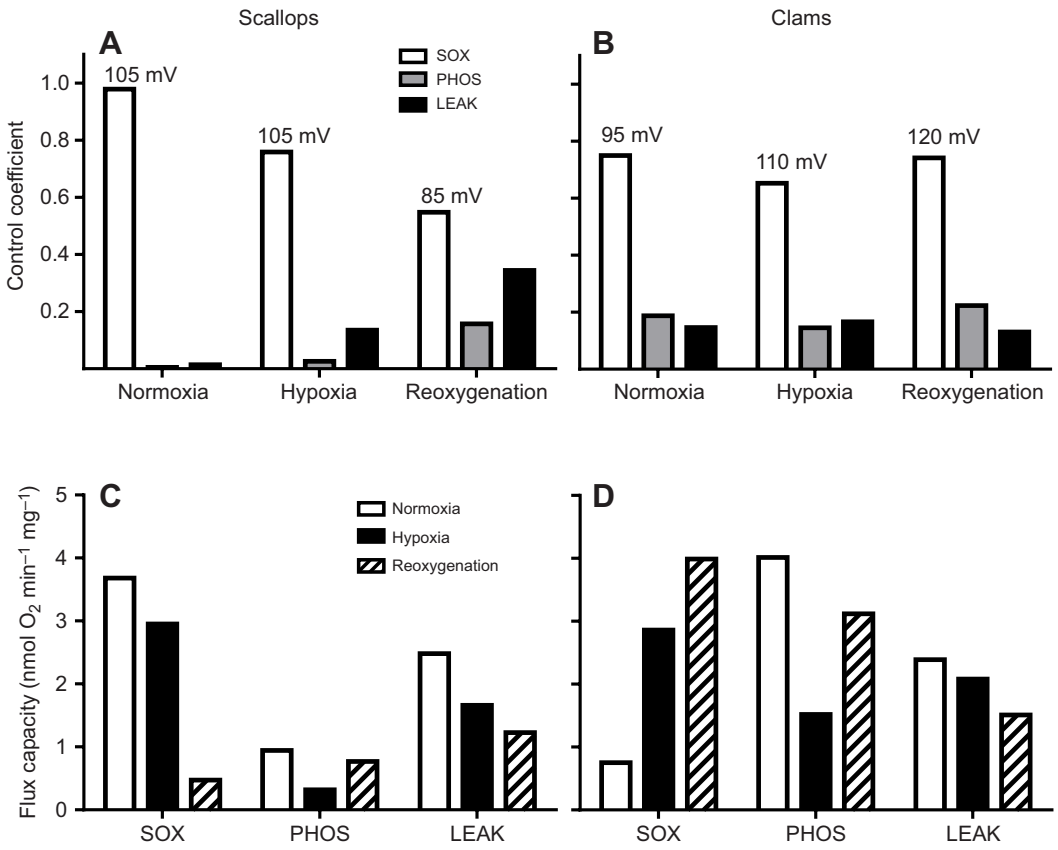

Fig. 3. Flux control coefficients and flux capacity of the SOX, LEAK and PHOS subsystems in mitochondria from scallops and clams. (A,B) Control over OXPHOS flux in state 3 mitochondria. The control coefficients are calculated at the highest $\Delta\psi$ (shown in graphs) where the fluxes were additive as expected in steady state in each treatment. The value of the control coefficient reflects the degree of control each subsystem exerts over the overall oxygen consumption of state 3 mitochondria. (C,D) Flux capacities of different subsystems calculated at a common $\Delta\psi$ (105 mV for scallops and 115 mV for clams).

clams), chosen based on the overlap of the kinetic curves for different treatments.

All differences were considered significant if the probability of Type I error (P) was less than 0.05. Data are expressed as means± s.e.m.

RESULTS

Mitochondrial function

In scallops, hypoxia did not significantly affect mitochondrial respiration and $\Delta\psi$, while reoxygenation resulted in a significant decrease of state 3 respiration and depolarization of state 3 and 4 mitochondria (Fig. 1). In contrast, mitochondria of clams maintained respiratory activity and normal or slightly elevated $\Delta\psi$ during hypoxia and reoxygenation (Fig. 1). The most pronounced difference in mitochondrial response to H/R stress between scallops and clams was detected at the level of the SOX subsystem. In scallops, the flux capacity of the SOX subsystem was maintained during hypoxia but plummeted (by ~8-fold when compared at a common $\Delta\psi$ of 105 mV) during reoxygenation (Figs 2, 3). In contrast, the SOX flux capacity of clam mitochondria increased by ~4-fold during hypoxia and by ~5-fold during reoxygenation (compared with the control at 115 mV; Figs 2, 3).

Responses of the LEAK and PHOS subsystems to H/R stress were similar in scallops and clams (Figs 2, 3). The flux capacities of the LEAK subsystem decreased after 18 h of hypoxia and remained suppressed during reoxygenation, indicating lower proton conductance of the inner mitochondrial membrane in scallops and clams. The flux capacity of the PHOS subsystem also decreased in hypoxia in both studied species; it remained suppressed after 1 h of reoxygenation in scallops but almost fully recovered during reoxygenation in clams.

Top-down MCA showed that H/R stress has a stronger effect on the distribution of control over oxidative phosphorylation (OXPHOS) flux in mitochondria of scallops than in clams (Fig. 3). Under the control conditions, the SOX subsystem exerted the highest degree of control over state 3 respiration in mitochondria of scallops and clams. H/R stress had little effect on the control over state 3 respiration in clam mitochondria. In scallops, hypoxia exposure decreased the degree of control exerted by the SOX subsystem over the mitochondrial state 3 respiration and increased the control exerted by the LEAK subsystem. This trend was further enhanced during reoxygenation, resulting in a strong increase of the share of control over the OXPHOS flux by the LEAK and PHOS subsystems and a decrease of control exerted by the SOX subsystem (Fig. 3).

Anaerobic metabolism

In scallops, there was a trend of decreasing PK activity and increasing PEPCK activity during hypoxia and reoxygenation, which resulted in a significantly reduced PK/PEPCK ratio, indicating increased channeling of the glycolytic substrates into anaerobic pathways (Fig. 4). Activity of NAD^+-dependent ME also increased during reoxygenation in scallops, consistent with the increased flux of pyruvate into anaerobic pathways (Fig. 4). In clams, activity of PK slightly decreased during hypoxia, whereas

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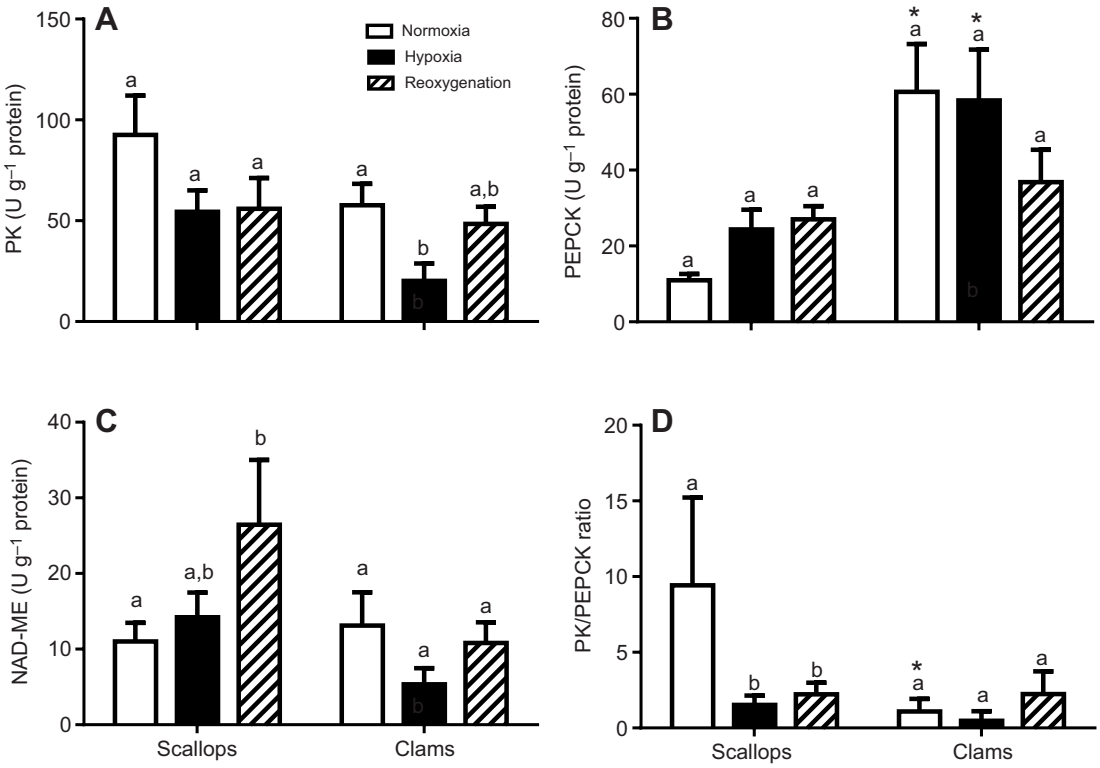

Fig. 4. Effects of H/R stress on specific activities of key enzymes at aerobic/anaerobic branchpoint. (A) Pyruvate kinase (PK), (B) phosphoenolpyruvate carboxykinase (PEPCK), (C) mitochondrial NAD^+-dependent malic enzyme (NAD-ME) and (D) PK/PEPCK activity ratio. *N*=4–7. Asterisks indicate values that are significantly different between scallops and clams under the same treatment conditions, and different letters indicate values that are significantly different among the treatments within each species ($P<0.05$). Activity of $NADP^+$-ME was 51.6±8.0 and 31.8±9.9 U g^{-1} protein (*N*=17–19) in scallops and clams, respectively, and was not significantly affected by hypoxia or reoxygenation in either species (ANOVA and LSD tests: $P>0.05$).

PEPCK and ME activities and the PK/PEPCK ratio remained stable throughout the H/R challenge (Fig. 4).

Short-term hypoxia (18 h) led to a slight decrease in tissue glycogen stores (Fig. 5) and accumulation of anaerobic end products (L-alanine, succinate and acetate) in scallops and clams (Fig. 6). However, these trends were not statistically significant, indicating low rates of anaerobic glycolysis in both studied species. Tissue levels of lipids and proteins remained unchanged during H/R exposure in scallops and were slightly elevated during hypoxia in clams, possibly reflecting a slow-down in oxidative catabolism of these substrates (Fig. 5).

In clams, expression of hypoxia-inducible factor 1α (HIF-1α) did not change in hypoxia but increased during reoxygenation (Fig. 7). No data for HIF-1α expression could be obtained for scallops because of the lack of the antibody's cross-reactivity.

Cellular energy status

In scallops, H/R exposure had no significant effects on tissue concentrations of adenylates (ATP, ADP and AMP) (Fig. 6). In clams, hypoxia led to a significant increase in [ADP] levels, and reoxygenation resulted in a slight but significant decrease in [AMP] concentrations, while [ATP] levels were maintained during H/R exposure (Fig. 6). In both studied species, ratios of ADP/ATP and AMP/ATP (Table 1) and expression of activated pAMPKα (Fig. 7, Table 2) were not affected by H/R stress.

Protein homeostasis

Expression of the total and phosphorylated EIF-2α was not affected by H/R exposure in scallops (Fig. 7, Table 2). In clams, levels of total EIF-2α were not affected by H/R stress but expression of pEIF-2α increased by ∼25-fold during hypoxia (Fig. 7).

Mitochondrial HSP60 was elevated during hypoxia in clams and during reoxygenation in scallops (Fig. 7). Expression of a cytosolic chaperone HSP69 was induced by H/R in two out of five scallops (Fig. 7), while no HSP69 expression was detected in five control scallops; however, these differences were not statistically significant because of the small sample size (LSD: $P>0.05$). The levels of a constitutive chaperone HSP72/78 was not affected by H/R exposure in scallops. In clams, expression levels of HSP69 and HSP72/78 were not affected by H/R stress (Fig. 7, Table 2).

Exposure to hypoxia led to a dramatic suppression of the trypsin-like and caspase-like (but not the chemotrypsin-like) activity of the 26S proteasome in clams (Fig. 8). These changes were rapidly reversed after 1 h of reoxygenation. In contrast, trypsin-, chemotrypsin- and caspase-like activities of the 26S proteasome were not affected by H/R exposure in scallops.

NKA activity

Activity of NKA dropped to non-detectable levels in hypoxia-exposed clams and was quickly restored after 1 h of reoxygenation (Fig. 8). In scallops, NKA activity did not significantly change in response to H/R.

Oxidative markers

H/R stress had no effect on the concentrations of MDA- and NHE-protein adducts in mitochondria of scallops and clams (Fig. 9). In scallops, hypoxia led to a strong upregulation of mRNA expression of mitochondrial aconitase and Lon protease (Fig. 9); this trend was quickly reversed during reoxygenation. In clams, H/R

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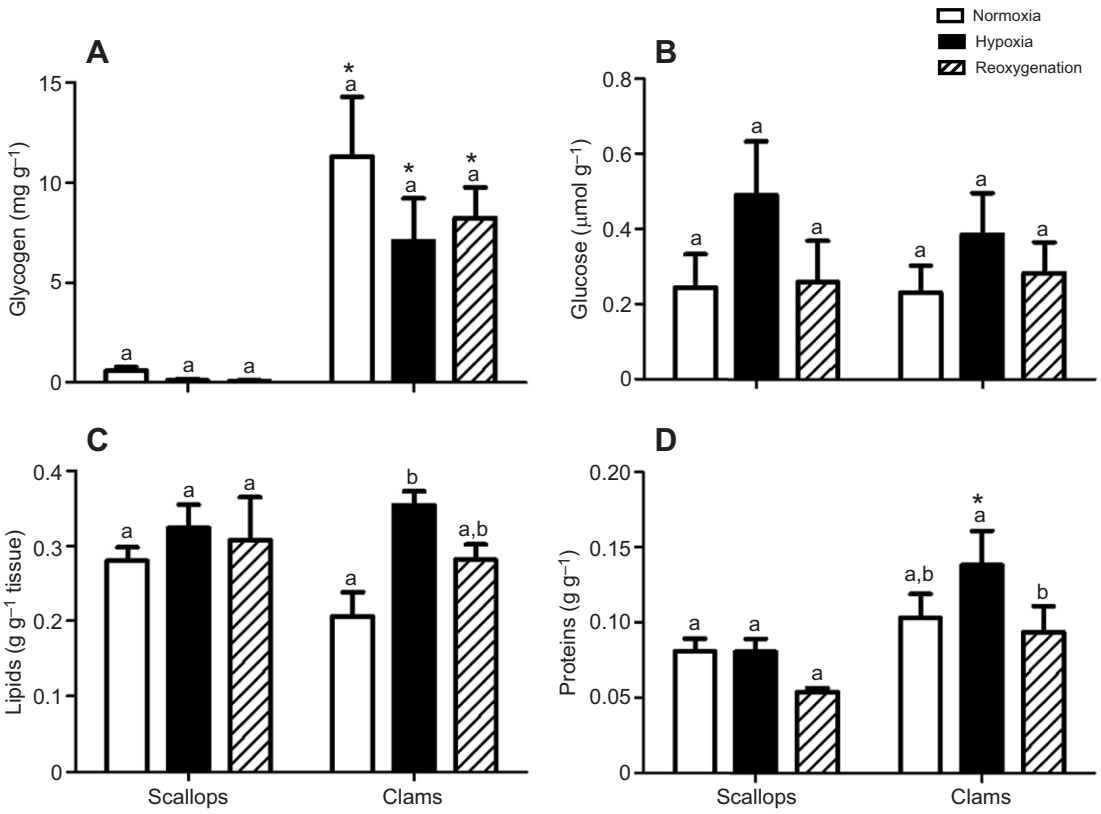

Fig. 5. Energy stores in the hepatopancreas of scallops and clams during H/R stress. (A) Glycogen levels (N=5–11), (B) tissue concentration of glucose (N=5–11), (C) lipid content (N=6–12) and (D) protein content (N=5–7). Asterisks indicate values that are significantly different between scallops and clams under the same treatment conditions, and different letters indicate values that are significantly different among the treatments within each species (P<0.05).

stress had no significant effect on mRNA expression of aconitase and Lon protease.

DISCUSSION

H/R-induced mitochondrial reorganization correlates with hypoxia tolerance

H/R stress has a strong effect on mitochondrial functions and metabolic control in scallops and clams. The comparison of a hypoxia-sensitive mollusc, the bay scallop, with the better studied mammalian models (Honda et al., 2005; Gorr et al., 2010; Di Lisa et al., 2011; Kadenbach et al., 2011; Hüttemann et al., 2012; Pamenter, 2014) reveals similarities of the H/R-induced mitochondrial pathology in hypoxia-sensitive organisms, including the loss of ETS function, protein damage and collapse of the normal control over the mitochondrial OXPHOS flux. Thus, the mitochondrial OXPHOS capacity of scallops quickly and dramatically decreased during reoxygenation, as reflected in the loss of the ETS activity and mitochondrial depolarization. Similar deterioration of OXPHOS and ETS activity accompanied by dissipation of $\Delta\psi$ and Ca^{2+} overload occurs during H/R stress in mitochondria of hypoxia-sensitive mammals, leading to massive mitochondrial injury, oxidative damage and, eventually, to cell death (Sadek et al., 2003; Honda et al., 2005; Kalogeris et al., 2014). A decrease in OXPHOS flux rate was also found after 2 h of exposure to hypoxia (~3% O_2) in a hypoxia-sensitive fish, the shovelnose ray, *Aptychotrema rostrata* (Hickey et al., 2012). In stark contrast, exposure to H/R stress strongly enhanced the mitochondrial SOX capacity in hypoxia-tolerant clams. A similar increase in SOX capacity was found in hypoxia-tolerant intertidal oysters *Crassostrea virginica* exposed to 6 days of hypoxia (<0.5% O_2) or to 6 days of hypoxia followed by 1 h of reoxygenation (Ivanina et al., 2012). Mitochondria of a hypoxia-tolerant vertebrate, the epaulette shark, *Hemiscyllum ocellatum*, were also capable of maintaining high SOX flux after exposure to short-term hypoxia (2 h at ~3% O_2) and reoxygenation (Hickey et al., 2012). It is worth noting that epaulette sharks can survive only ~3.5 h at 24°C (Hickey et al., 2012) and thus are considerably less anoxia-tolerant than clams and oysters, which can withstand the lack of oxygen for up to 2 weeks at 20°C (Savage, 1976; Vaquer-Sunyer and Duarte, 2011). Overall, H/R-induced changes in SOX capacity appear to correlate with hypoxia tolerance so that the SOX capacity declines during hypoxia and reoxygenation in hypoxia-sensitive species (e.g. terrestrial mammals, shovelnose rays and scallops), remains unchanged in a vertebrate species with moderate hypoxia tolerance (the epaulette shark) and increases in exceptionally hypoxia-tolerant intertidal invertebrates (oysters and clams).

The anticipatory increase in the SOX capacity during hypoxia in clams raises intriguing questions about the possible mechanisms of this phenomenon. Allosteric effects of ADP or kinetic effects of substrate availability on ETS complexes can be ruled out as an explanation because the mitochondrial parameters were measured at high ADP/ATP ratios and saturating levels of an electron donor (succinate). Therefore, the H/R-induced increase in SOX capacity in clam mitochondria must reflect intrinsic properties of the ETS such as substrate affinity, catalytic efficiency and/or the amount of active ETS complexes. Among the likely candidates responsible for enhanced SOX capacity of clams during hypoxia are post-translational modifications of existing ETS complexes, such as reversible phosphorylation. This mechanism plays a key role in the regulation of the anaerobic metabolic flux during hypoxia in

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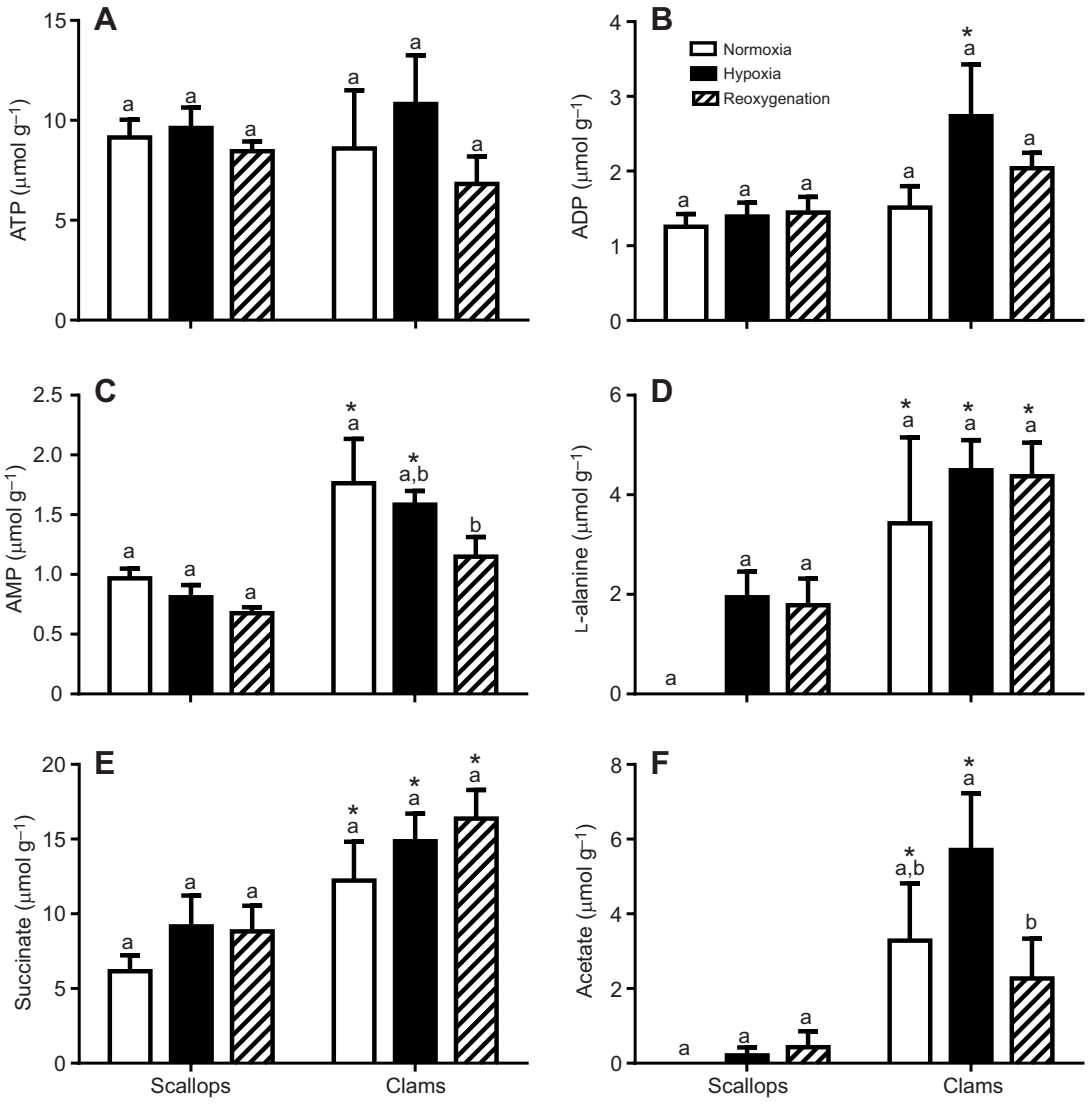

Fig. 6. Effects of H/R stress on cellular energy status and accumulation of anaerobic end products in hepatopancreas of scallops and clams. (A–C) Cellular levels of adenylates, (D) L-alanine, (E) succinate and (F) acetate. Asterisks indicate values that are significantly different between scallops and clams under the same treatment conditions, and different letters indicate values that are significantly different among the treatments within each species (P<0.05). N=7–8.

molluscs (Russell and Storey, 1995; Brooks and Storey, 1997; Fernández et al., 1998; Lama et al., 2013) and might also be involved in regulation of aerobic metabolism. This hypothesis is consistent with a study on Pacific oysters, *Crassostrea gigas*, where exposure to 3–12 h of hypoxia (~3% O_2) led to a significant increase in the activity of Complex IV of the ETS even though the amount of Complex IV (assessed by the concentration of cytochrome *a*) did not change (Sussarellu et al., 2013). Other possibilities such as the *de novo* synthesis of additional ETS proteins seem less likely because hypoxia leads to an arrest of protein synthesis in hypoxia-tolerant molluscs (Storey and Storey, 2004; Larade and Storey, 2007). Future studies are required to identify the molecular mechanisms responsible for the upregulation of ETS capacity in molluscan mitochondria during hypoxia and assess the role of post-translational protein modifications in these mechanisms. Regardless of the exact molecular mechanisms, hypoxia-induced activation of ETS could promote high ATP production rates and rapid restoration of energy homeostasis of intertidal molluscs during frequent H/R cycles in the intertidal zone.

Unlike the SOX subsystem, the capacity of the PHOS subsystem (encompassing the F_O,F_1-ATPase, phosphate transporter and ADP-ATP translocase) was suppressed by ~60–70% during hypoxia in scallops and clams. This is in agreement with previous studies on hypoxia-tolerant animals such as hibernating frogs and aestivating snails, which show a strong suppression of F_O,F_1-ATPase activity during prolonged hypoxia (Bishop and Brand, 2000; St-Pierre et al., 2000c; Bishop et al., 2002; Boutilier and St-Pierre, 2002). In hypoxia, mitochondrial F_O,F_1-ATPase acts in the reverse direction and becomes an ATP consumer hydrolyzing ATP to prevent the collapse of $\Delta\psi$ (St-Pierre et al., 2000b). This may lead to a drop of the ATP concentrations below the levels required to support the function of other cellular ATPases (including ion pumps) (Sokolova et al., 2000a; St-Pierre et al., 2000b), resulting in dissipation of the transmembrane ion gradients and cell death. Thus, suppression of the PHOS subsystem may be an adaptive response in hypoxia-tolerant animals (such as intertidal bivalves, terrestrial snails and hibernating frogs) to prevent ATP wastage during hypoxia (Brand et al., 2000; St-Pierre et al., 2000b; present study). Notably, although the magnitude

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of the hypoxia-induced suppression of the PHOS capacity was similar in clams and scallops, the PHOS subsystem of clam mitochondria showed fast recovery within 1 h of reoxygenation, unlike scallop mitochondria, in which the PHOS capacity remained suppressed. This ability of the PHOS subsystem to quickly rebound during reoxygenation is consistent with adaptations of clams to intermittent hypoxia in the intertidal zone.

Exposure to H/R stress suppressed the proton conductance of the inner mitochondrial membrane of the studied bivalves, as indicated by the lower flux capacity of the LEAK subsystem. Suppression of the mitochondrial proton leak rates during hypoxia have also been found in other hypoxia-tolerant organisms such as hibernating frogs and aestivating terrestrial snails (Brand et al., 2000). However, the mechanisms responsible for the reduction of the proton leak appear

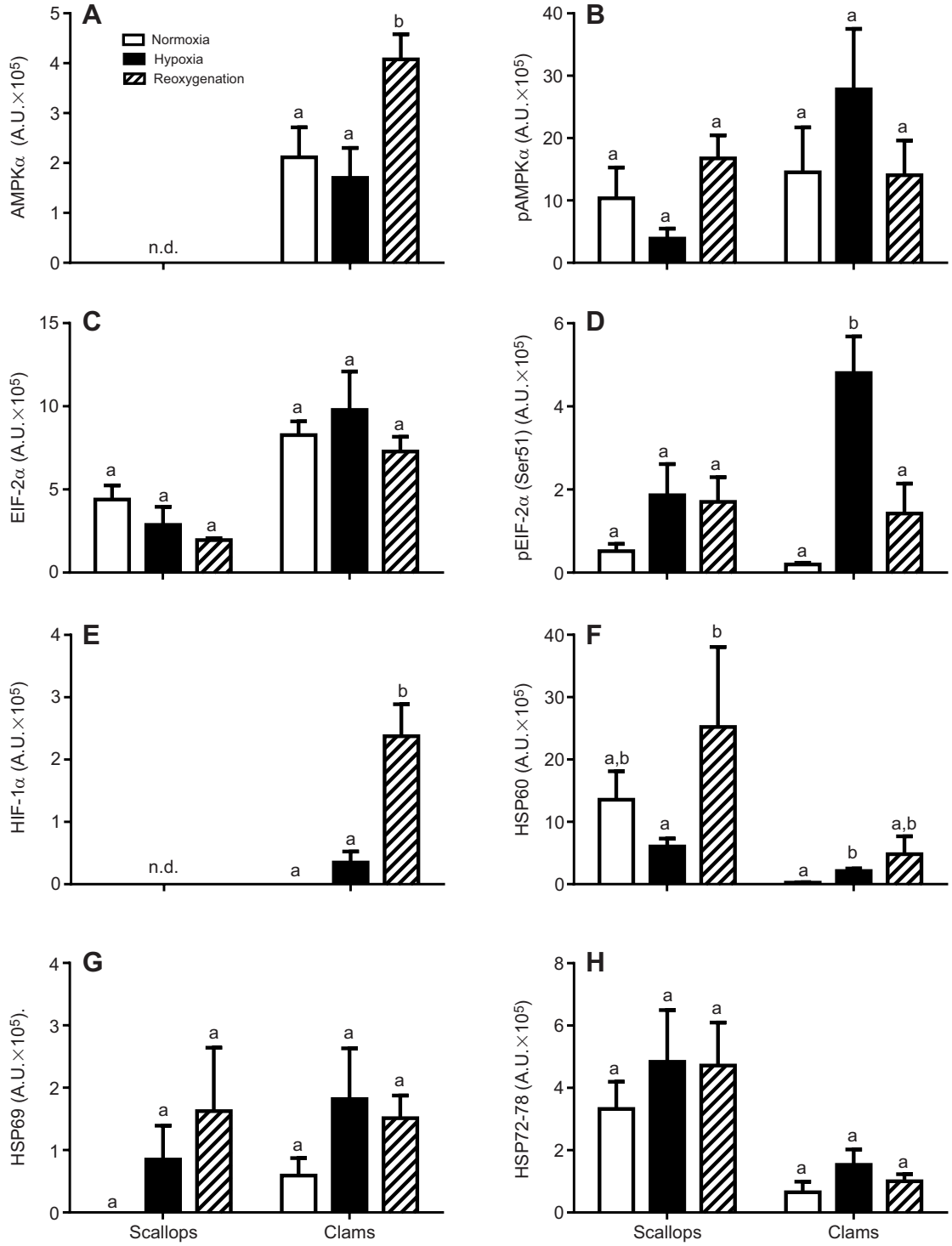

Fig. 7. Effects of H/R stress on protein expression in the muscle of scallops and clams. (A,B) Total and phosphorylated AMPKα, (C,D) total and phosphorylated EIF-2α, (E) HIF-1α and (F–H) heat shock proteins. Different letters indicate values that are significantly different among the treatments within each species (P<0.05). N=5. Because of the lack of antibody specificity, HIF-1α and total AMPKα were not determined in scallops (n.d.).

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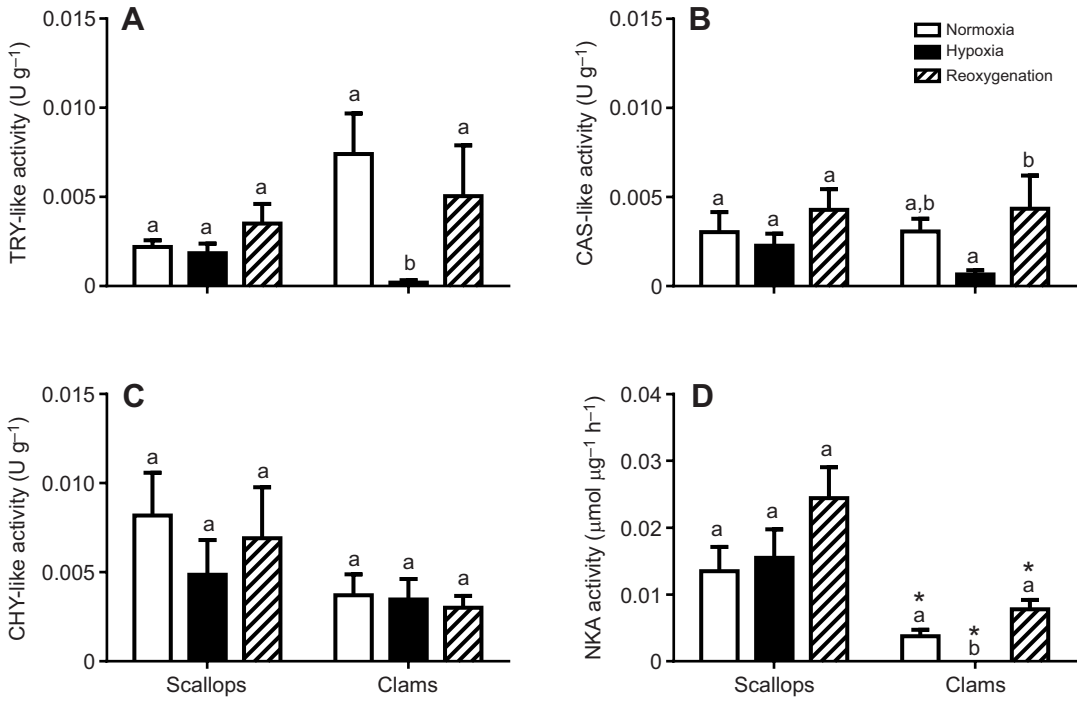

Fig. 8. Effects of H/R stress on activity of the 26S proteasome and Na^+/K^+-ATPase (NKA) in scallops and clams. (A–C) Trypsin-like, caspase-like and chymotrypsin-like activity of 26S proteasome, respectively. (D) NKA. Asterisks indicate values that are significantly different between scallops and clams under the same treatment conditions, and different letters indicate values that are significantly different among the treatments within each species (P<0.05). N=5.

to differ in different hypoxia-tolerant animals. Unlike in bivalves, where H/R exposure decreased the mitochondrial proton conductance (present study), suppression of the proton cycling in hypoxia-tolerant frogs and land snails was primarily driven by a decrease in $\Delta\psi$ (Bishop and Brand, 2000; Brand et al., 2000; St-Pierre et al., 2000a; Bishop et al., 2002; Boutilier and St-Pierre, 2002). Regardless of the mechanisms, a coordinated decrease in the mitochondrial proton cycling and activity of F_O,F_1-ATPase may diminish the energy cost of conservation of mitochondrial $\Delta\psi$ in hypoxia-tolerant organisms (Brand et al., 2000), thus helping to preserve the integrity of mitochondria during prolonged hypoxia. In contrast, in hypoxia-sensitive terrestrial mammals, H/R stress leads to a progressive increase in the leakiness of the inner mitochondrial membrane and, eventually, collapse of the mitochondrial $\Delta\psi$ (Honda et al., 2005).

The state 3 (OXPHOS) flux in the control mitochondria of scallops and clams was predominantly controlled by the SOX subsystem, as shown by its high control coefficients (0.75–0.98). Similarly, a high degree of control by SOX over the state 3 respiration has also been found in oysters (Kurochkin et al., 2011; Ivanina et al., 2012), insects (Chamberlin, 2004a,b), plants (Kesseler et al., 1992; Kesseler and Brand, 1994a,b) and rodents (Brown et al., 2007; Ciapaite et al., 2009). Notably, the distribution of control over the OXPHOS flux was preserved during H/R exposure in clams. This contrasts a progressive decline in the degree of control exerted by the SOX subsystem and an increase in the control by the PHOS and LEAK subsystems during H/R stress in scallops. Interestingly, earlier studies showed that mitochondrial dysfunction caused by other stressors (such as exposure to toxic metals Cd^{2+} and Cu^{2+} and/or elevated temperatures) is also associated with the alteration of the control structure over the OXPHOS flux (Kesseler and Brand, 1994a; Chamberlin, 2004b; Ciapaite et al., 2009; Kurochkin et al., 2011; Ivanina et al., 2012). Taken together, these findings suggest that the coordinated changes in the capacities of different functional subsystems that preserve the normal structure of control over OXPHOS respiration may contribute to the resilience to H/R stress in intertidal bivalves.

Metabolic rate depression and anaerobic capacity

Metabolic rate depression, involving a coordinated downregulation of ATP-consuming and ATP-producing processes, is considered a major adaptive mechanism in hypoxia-tolerant organisms (Hochachka and Lutz, 2001; Storey and Storey, 2007). In agreement with these earlier findings, our study shows a stronger suppression of ATP-consuming processes during hypoxia in clams compared with scallops. In clams, hypoxia led to a dramatic suppression of NKA activity, a major cellular ATP consumer responsible for 30–60% of the normoxic ATP turnover (Buck and Hochachka, 1993; Hand and Hardewig, 1996). Suppression of NKA activity has also been shown in other hypoxia-tolerant organisms such as turtles, crustaceans and molluscs during hypoxia- and anoxia-induced hypometabolism (Buck and Hochachka, 1993; Ramnanan and Storey, 2006; Staples and Buck, 2009; Hand et al., 2011). Furthermore, an ~25-fold increase in the levels of pEIF-2α in hypoxia-exposed clams shows inactivation of this essential translation initiation factor by reversible phosphorylation, consistent with translational arrest (Larade and Storey, 2002). A hypoxia-induced decrease in protein synthesis (including transcription and translation) is commonly found in hypoxia-tolerant organisms (Guppy and Withers, 1999; Fraser et al., 2001; Larade and Storey, 2002, 2007; Storey and Storey, 2004). In contrast to clams, NKA activity and pEIF-2α expression were not affected by hypoxia in scallops. While we cannot rule out a possibility of suppression of other energy-consuming processes, our findings demonstrate that at least one major ion-motive pump

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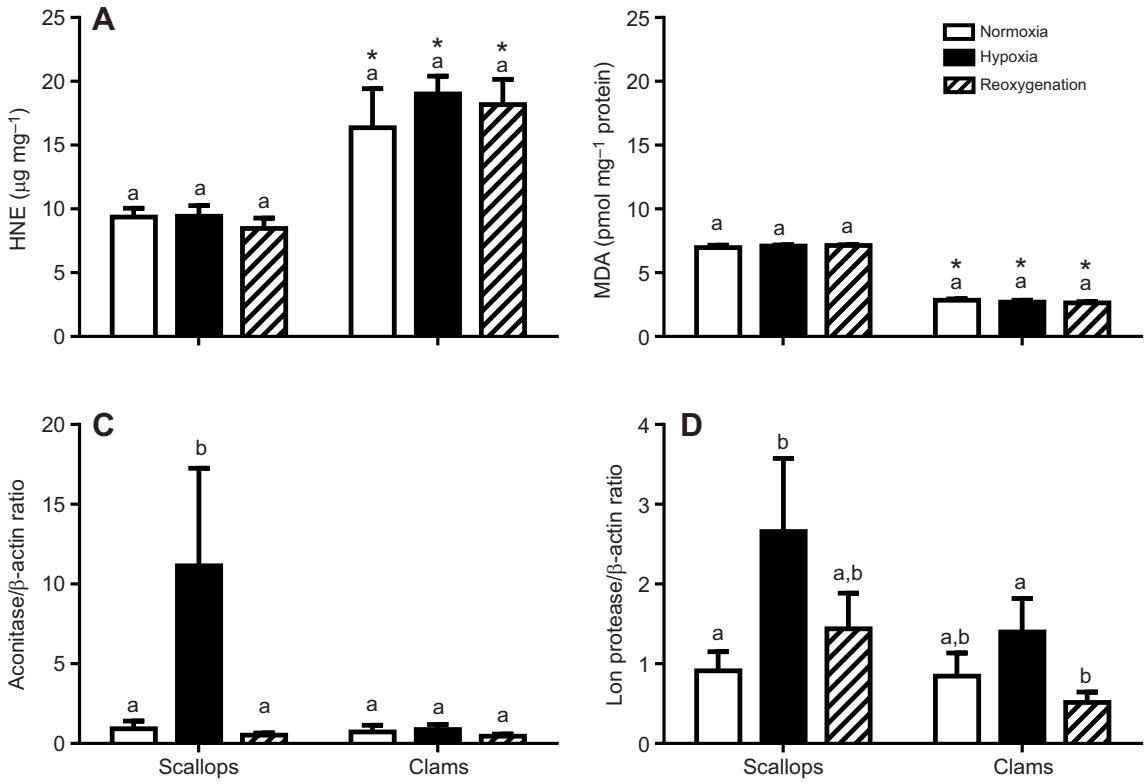

Fig. 9. Effects of H/R stress on oxidative lesions in mitochondria and mRNA expression of aconitase and Lon protease in scallops and clams. (A) 4-Hydroxynonenal (HNE)-protein conjugates, (B) malondialdehyde (MDA)-protein conjugates, (C) aconitase and (D) Lon protease. Asterisks indicate values that are significantly different between scallops and clams under the same treatment conditions, and different letters indicate values that are significantly different among the treatments within each species (P<0.05). N=5–8 for oxidative lesions and 5–14 for mRNA expression.

(NKA) remains active and that translational arrest via pEIF-2α mechanism is not engaged during hypoxia in scallops. Taken together, these data indicate that the energy savings due to the stronger suppression of NKA activity and protein synthesis during hypoxia may contribute to the greater hypoxia tolerance of clams compared with scallops.

Notably, suppression of the protein synthesis was also accompanied by inhibition of the activity of the 26S proteasome in hypoxia-exposed clams, indicating a decrease in the protein breakdown. The 26S proteasome complex carries out ATP-dependent degradation of damaged proteins via three catalytic subunits with different preferential cleaving mechanisms (trypsin-like, chymotrypsin-like and caspase-like activities) (Voges et al., 1999; Glickman and Ciechanover, 2002). In hypoxia-exposed clams, activities of two subunits responsible for the trypsin-like and caspase-like proteolysis were suppressed by ~40- and 5-fold, respectively. The coordinated suppression of protein synthesis and degradation in clams may act to conserve energy during hypoxia and can also extend the life of existing proteins to ensure the maintenance of essential cellular functions until normal protein synthesis is restored. In hypoxia-sensitive scallops, the activity of the 26S proteasome was maintained at similar levels throughout H/R exposure, while in terrestrial mammals, hypoxia stimulated the activity of the proteasome (Caron et al., 2009). Taken together, these data show that a stronger ability to reduce protein degradation during energy-limited conditions correlates with higher hypoxia tolerance.

Anaerobic glycolysis, which plays a key role in long-term hypoxic survival of facultative anaerobes including molluscs (Grieshaber et al., 1994; Ivanina et al., 2010, 2011, 2012), does not appear to contribute to differential survival of scallops and clams during relatively short-term (18 h) hypoxia. Anaerobic pathways were only stimulated in the less tolerant scallops (as shown by an increase in the PK/PEPCK ratio), consistent with the less pronounced metabolic rate depression in this species. However, the rates of anaerobic metabolism were low in both studied species, as indicated by the lack of significant depletion of the glycogen stores or accumulation of metabolic wastes (L-alanine, succinate or acetate). This agrees with previous findings in oysters showing that significant accumulation of anaerobic end products occurs only after prolonged hypoxia (3–6 days at <0.5% O_2) (Kurochkin et al., 2009). Notably, both studied species were capable of maintaining the cellular energy status during H/R exposure, as indicated by relatively small changes in the levels of high-energy phosphates (ATP, ADP and AMP), constant ADP/ATP and AMP/ATP ratios, and lack of activation of AMPKα, supporting the conclusion that 18 h of hypoxic exposure does not result in cellular energy deficiency in these species.

An intriguing finding of our present study is the lack of accumulation of HIF-1α during hypoxia and its strong upregulation during reoxygenation in clams. HIF-1α is a highly conserved transcriptional factor that undergoes rapid O_2-dependent degradation in normoxia, but accumulates in hypoxia (Semenza, 2004). In mammals, accumulation of HIF-1α transcriptionally regulates a coordinated response to hypoxia including upregulation of systemic O_2 delivery, stimulation of glycolysis and attenuation of mitochondrial function by the expression of more efficient isoforms of ETS proteins (Lahiri et al., 2006; Semenza, 2007). Such changes would be maladaptive during prolonged hypoxia in intertidal molluscs because they would prevent the metabolic rate depression, a major adaptive strategy to survive prolonged O_2 deprivation (Hochachka and Lutz, 2001; Storey and Storey, 2007). The genomic

targets of HIF-1α in hypoxia-tolerant organisms such as molluscs are not known and may differ from those in mammals, but the present study suggests that HIF-1α stabilization may not be essential for adaptive cellular response to hypoxia in molluscs. It is also unclear which mechanisms prevent HIF-1α accumulation in clams during hypoxia; one possible mechanism may involve suppression of HIF-1α transcription as shown in hypoxia-exposed eastern oysters (Ivanina et al., 2010; Piontkivska et al., 2011). In contrast to hypoxia, reoxygenation led to increased levels of HIF-1α in clams. This increase might reflect decreased cellular O_2 concentrations as expected from the combined effects of elevated ETS activity and the high ATP demand during reoxygenation (Ellington, 1983). An increase in HIF-1α levels during reoxygenation was also found in *Caenorhabditis elegans*, where it plays a key role in mitochondrial recovery (Ghose et al., 2013). Notably, in *C. elegans*, HIF-1α is not involved in adaptive mitochondrial reorganization during anoxia, similar to our finding of substantial functional reorganization of mitochondria from hypoxia-exposed clams in the absence of HIF-1α accumulation.

Oxidative damage and unfolded protein response

Oxidative damage is considered a hallmark of H/R-induced stress that plays a key role in mitochondrial and cellular injury (Hermes-Lima and Zenteno-Savín, 2002; Korge et al., 2015). Our study shows that mitochondria of clams and scallops are protected against oxidative damage to the membranes, demonstrated by the lack of accumulation of MDA and HNE during H/R stress. In contrast, proteins appear more susceptible to the H/R-induced damage, as indicated by upregulation of the mitochondrial chaperone HSP60 during hypoxia in clams and reoxygenation in scallops, as well as a notable (albeit statistically not significant) induction of HSP69 during H/R stress in scallops. Furthermore, massive upregulation of mRNA expression of mitochondrial aconitase and Lon protease are suggestive of oxidative stress during H/R exposure in mitochondria of scallops (but not in clams). Aconitase is an ROS-sensitive enzyme that becomes inactivated because of the loss of Fe^{2+} from its active center during exposure to ROS (Bulteau et al., 2003). In animals, aconitase inactivation is among the first signs of the mitochondrial oxidative stress (Bulteau et al., 2003). Transcriptional upregulation of aconitase in hypoxia-induced scallops may, therefore, be a compensatory mechanism to counteract aconitase inactivation by ROS. A strong upregulation of mRNA expression of mitochondrial Lon protease in hypoxia-induced scallops may also reflect higher demand for degradation of oxidatively damaged proteins including aconitase, a major target for Lon-dependent degradation (Bota and Davies, 2002; Smakowska et al., 2014). Transcriptional changes in aconitase and Lon protease expression are consistent with higher levels of the oxidative damage protein due to H/R exposure in hypoxia-sensitive scallops compared with clams, but future studies are needed to test this hypothesis by measuring the enzyme activities.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

I.M.S. and A.V.I. designed the research. A.V.I., I.M.S. and E.P.S. carried out the experiments. I.M.S., I.N., A.V.I. and L.L. conducted the data analyses. I.M.S. wrote the first draft of the manuscript, and all the authors participated in the discussion and revisions of the manuscript.

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Data availability

Partial gene sequences for mitochondrial aconitase and Lon protease from clams and scallops have been submitted to the NCBI database, accession numbers KT897897 to KT897910.

Supplementary information

Supplementary information available online at
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