

## RESEARCH ARTICLE

# Effects of intermittent hypoxia on cell survival and inflammatory responses in the intertidal marine bivalves *Mytilus edulis* and *Crassostrea gigas*

Halina Falfushynska<sup>1,2</sup>, Helen Piontkivska<sup>3</sup> and Inna M. Sokolova<sup>1,4,\*</sup>**ABSTRACT**

Hypoxia is a major stressor in estuarine and coastal habitats, leading to adverse effects in aquatic organisms. Estuarine bivalves such as blue mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*) can survive periodic oxygen deficiency but the molecular mechanisms that underlie cellular injury during hypoxia-reoxygenation are not well understood. We examined the molecular markers of autophagy, apoptosis and inflammation during short-term (1 day) and long-term (6 days) hypoxia and post-hypoxic recovery (1 h) in mussels and oysters by measuring the lysosomal membrane stability, activity of a key autophagic enzyme (cathepsin D) and mRNA expression of the genes involved in the cellular survival and inflammation, including caspase 2, 3 and 8, Bcl-2, BAX, TGF- $\beta$ -activated kinase 1 (TAK1), nuclear factor kappa B1 (NF- $\kappa$ B) and NF- $\kappa$ B activating kinases IKK $\alpha$  and TBK1. *Crassostrea gigas* exhibited higher hypoxia tolerance, as well as blunted or delayed inflammatory and apoptotic response to hypoxia and reoxygenation as shown by the later onset and/or the lack of transcriptional activation of caspases, BAX and the inflammatory effector NF- $\kappa$ B, compared with *M. edulis*. Long-term hypoxia resulted in upregulation of Bcl-2 in the oysters and mussels, implying activation of anti-apoptotic mechanisms. Our findings indicate the potential importance of the cell survival pathways in hypoxia tolerance of marine bivalves, and demonstrate the utility of the molecular markers of apoptosis and autophagy for the assessment of sublethal hypoxic stress in bivalve populations.

**KEY WORDS:** Apoptosis, Autophagy, Caspases, Hypoxia tolerance, Hypoxia-reoxygenation, Inflammation

**INTRODUCTION**


Hypoxia (i.e. oxygen deficiency) and anoxia (the lack of oxygen) are major stressors for aquatic organisms that experience fluctuations in the oxygen availability due to the natural oxygen cycles as well as the human-driven deoxygenation of aquatic habitats caused by eutrophication and warming (Breitbart et al., 2018). In estuaries and coastal habitats, hypoxia is common and can last from a few hours (such as during the tidal emersion or diel

cycles of photosynthesis and respiration) to weeks to months (such as during seasonal hypoxia in eutrophicated estuaries) (Breitbart et al., 2018). Prolonged or permanent hypoxia is lethal for most metazoans, so that hypoxia is considered a major driver of biodiversity loss in aquatic habitats (Chu et al., 2018; Levin et al., 2009). Sessile benthic organisms are especially sensitive to the coastal hypoxia because of their inability to escape the deoxygenated bottom water and sediments (Chu et al., 2018; Levin et al., 2009). However, some benthic marine organisms, including intertidal bivalves, are exceptionally well adapted to survive periodic oxygen deficiency and can withstand days to weeks of hypoxia and anoxia (Babarro and De Zwaan, 2008; Diaz and Rosenberg, 2008). A major adaptive strategy for hypoxia survival in these organisms is an ability to suppress the rate of the ATP turnover well below the normoxic level, sometimes down to <5% of the standard aerobic metabolic rate (Guppy and Withers, 1999; Shick et al., 1983; Sokolova et al., 2000; Storey and Storey, 2004). This metabolic avoidance strategy of coordinated suppression of ATP consumption and ATP production, called metabolic rate depression, slows down accumulation of potentially toxic end products and helps to conserve the energy reserves (Hochachka et al., 1996; Hochachka and Guppy, 1987; Hochachka and Mommsen, 1983). These adaptations can slow down, but do not fully prevent, the deterioration of cellular homeostasis, especially during prolonged hypoxia.

Reduction of the metabolic rate is effective in extending the survival time during hypoxia and anoxia. However, it is a time-limited situation that requires the return of oxygen for an organism to recover and complete its life cycle. Post-hypoxic reoxygenation presents additional problems to aerobic organisms because of the energy costs associated with reinstatement of cellular homeostasis and increased biosynthesis to replenish energy reserves (Bayne, 2017; Ellington, 1983; Lewis et al., 2007). Furthermore, reoxygenation can cause oxidative damage via a surge of reactive oxygen species (ROS) from the mitochondrial electron transport system (ETS) (Andrienko et al., 2017; Jastroch et al., 2010). Oxidative stress is considered a hallmark of the hypoxia-reoxygenation (H-R) injury in hypoxia-sensitive organisms such as terrestrial mammals and, if left unchecked, leads to the accumulation of cellular damage and eventually cell death (Cadenas, 2018; Groehler et al., 2018; Ham and Raju, 2016; Hernansanz-Agustin et al., 2014). Studies in hypoxia-tolerant animals, such as freshwater turtles, crucian carp, and intertidal mollusks and fish, uncovered several putative mitochondrial mechanisms that can mitigate oxidative stress during H-R. These include upregulation of antioxidants, suppression of the pathways channeling electrons to ubiquinone, and upregulation of the mitochondrial quality control mechanisms (including mitochondrial proteases and heat shock proteins) that can repair

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the damage or prevent aggregation of the damaged proteins (Sokolova, 2018; Sokolova et al., 2019b). However, it remains unknown to what degree these mechanisms are effective in limiting the cellular and tissue damage caused by H-R and preventing the negative outcomes of excessive oxidative stress (such as apoptosis and inflammation) in hypoxia-tolerant organisms.

Studies in model organisms, such as terrestrial mammals, *Drosophila* and *Caenorhabditis elegans* highlight the important role of autophagy and apoptosis in the cellular response to H-R, which may be beneficial or detrimental depending on the intracellular conditions and the extent of autophagy and apoptosis (Ham and Raju, 2016; Lin et al., 2018; Sciarretta et al., 2011; Zhou et al., 2017). Autophagy is a catabolic process involved in recycling of long-lived proteins and organelles in the cell during normal cell maintenance (Bialik et al., 2018). Under hypoxic conditions, when the cells experience energy and nutrient deficiency, the autophagic processes are activated, providing substrates for anaerobic ATP production and removing superfluous or damaged intracellular structures (Lin et al., 2018; Solaini et al., 2010). However, during reoxygenation, oxidative stress, calcium overload and mitochondrial dysfunction lead to a major increase of autophagy which may result in tissue injury in hypoxia-sensitive organisms such as terrestrial mammals (Ham and Raju, 2016; Lin et al., 2018; Sciarretta et al., 2011). Apoptosis (or Type I cell death) also plays a key role in the tissue injury caused by H-R stress. Apoptosis is induced during reoxygenation in response to the oxidative damage caused by the elevated ROS production (Eefting et al., 2004; Wu et al., 2018). Apoptotic cell death mitigates tissue inflammation (which might be caused if a cell dies in a less controlled way via necrosis) but results in the cell loss and functional impairment of the tissue, so that the extent of tissue apoptosis negatively correlates with the survival and recovery in hypoxia-sensitive organisms (Blomgren et al., 2003; Eefting et al., 2004). Regulation of autophagy and apoptosis during H-R therefore appears to be an important tolerance mechanism and a potential target for selection in the organisms such as intertidal bivalves that are adapted to frequent oxygen fluctuations. However, the effects of fluctuating oxygen conditions on tissue levels of apoptosis and autophagy are not yet well understood in hypoxia-tolerant intertidal invertebrates.

Marine intertidal bivalves such as the Pacific oyster *Crassostrea gigas* and the blue mussel *Mytilus edulis* are common organisms in the intertidal, estuarine and shallow coastal habitats in the northern hemisphere where they can be exposed to frequent oxygen fluctuations ranging from near-anoxia to hyperoxia during diurnal and tidal cycles, as well as to seasonal hypoxia (Breitburg et al., 2015; de Zwaan and Putzer, 1985; Diaz and Rosenberg, 2008; Richards, 2011). Of these two species, the Pacific oysters are considered more tolerant to abiotic stressors, including prolonged hypoxia, than the blue mussels (David et al., 2005; Le Moullac et al., 2007; Meng et al., 2018; Zhang et al., 2012, 2016). Our recent study showed that *C. gigas* survives longer in severe hypoxia and is better at maintaining the intracellular homeostasis of intermediate metabolites (including the free amino acids, urea cycle and purine metabolism intermediates) during H-R than *M. edulis* (Haider et al., 2020). Therefore, we hypothesized that the higher tolerance to H-R stress of *C. gigas* will be reflected in lower activation of autophagic, apoptotic and inflammatory pathways compared with the less stress-tolerant *M. edulis*. To test this hypothesis, we determined the effects of short-term (1 day) and long-term (6 days) severe hypoxia and post-hypoxic recovery (1 h) on the lysosomal membrane stability, activity of a key autophagic enzyme (cathepsin D) and mRNA expression of the genes involved in the cellular survival and

inflammation pathways, including caspase 2, 3 and 8, Bcl-2, BAX, TGF- $\beta$ -activated kinase 1 (TAK1), nuclear factor kappa B-1 (NF- $\kappa$ B), and two NF- $\kappa$ B activating kinases – the inhibitor of NF- $\kappa$ B kinase subunit  $\alpha$  (IKK $\alpha$ ) and serine/threonine-protein kinase TBK1-like (TBK1), in *M. edulis* and *C. gigas*. Our study demonstrates that the more tolerant of the two studied species (*C. gigas*) shows blunted or delayed inflammatory and apoptotic response to hypoxia and reoxygenation compared with the less hypoxia-tolerant *M. edulis*. These findings indicate the importance of regulation of the cell survival pathways in tolerance to intermittent hypoxia in marine bivalves, and demonstrate the utility of the molecular markers of apoptosis, inflammation and autophagy in sentinel marine bivalves for monitoring of the hypoxia-induced stress in estuarine and coastal habitats.

## MATERIALS AND METHODS

### Chemicals

Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Thermo Fisher Scientific (Schwerte, Germany) and were of analytical grade or higher.

### Animal collection and care

Blue mussels, *Mytilus edulis* Linnaeus 1758, were from submerged piles in an tidal zone near Warnemünde in the Baltic Sea (54°10' 49.602"N, 12°05'21.991"E). The mussels are permanently submerged in this habitat. Pacific oysters, *Crassostrea gigas* (also *Magallana gigas* Thunberg 1793), were gathered in the low intertidal zone of the island of List/Sylt in the German Wadden Sea (55°02'54.4"N 8°27'12.6"E). Depending on the tidal phase, these animals experience 1–3 h of emersion per day (M. Wegner, personal communication). All animals were transported within 12 h of collection to the University of Rostock in coolers lined with seawater-soaked paper towels. The shells were cleaned from epibionts, and the bivalves were kept in aquaria with aerated artificial sea water (ASW) (Instant Ocean®, Aquarium Systems, Sarrebourg, France) at 15±1°C for at least 4 weeks prior to the experiments. Acclimation temperature was similar to the habitat water temperatures (12–16°C). During acclimation and all exposures, salinity was maintained at 15±1 and 30±1 practical salinity units for the mussels and oysters, respectively, which was within 2–4 salinity units of the habitat salinities of these species at the time of collection. Mollusks were fed *ad libitum* by continuous addition of a commercial algal blend (DT's Live Marine Phytoplankton, CoralSands, Wiesbaden, Germany) to experimental tanks according to the manufacturer's instructions.

The experimental exposures were conducted as described in our earlier work (Haider et al., 2020). Briefly, bivalves were subjected to severe hypoxia (>0.5% air saturation, >0.01% O<sub>2</sub>) in air-tight plastic chambers filled with ASW at the respective acclimation salinity (five bivalves in 6 liters of ASW). The water in the chambers was bubbled with nitrogen until oxygen levels dropped below 0.5% of air saturation. The oxygen content of seawater was measured using a fiber optic oxygen sensor connected to a FireStingO<sub>2</sub> Optical Oxygen Meter (PyroScience GmbH, Aachen, Germany). The chamber was submerged in the temperature-controlled aquarium to maintain the temperature at 15±0.5°C. The animals were exposed to a short-term (1 day) or long-term (6 days) hypoxia. Extreme hypoxia such as used in the present study, leads to the valve closure and cessation of feeding in bivalves; therefore, the phytoplankton blend was not added during hypoxia exposure to prevent excessive bacterial growth. For reoxygenation, a

subset of mollusks exposed to 1 and 6 days of hypoxia was placed into a fully aerated aquarium for 1 h prior to tissue isolation. Control animals were maintained in normoxia (>95% air saturation). No mortality was found in the mussels or oysters maintained under the normoxic (control) conditions. All mussels survived 1 day of hypoxic exposure, and 25% of mussels died after 6 days of severe hypoxia ( $N=40$ ). In oysters, no mortality was observed during 1–6 days of hypoxia exposure ( $N=40$ ).

After experimental exposure, control animals as well as those exposed to hypoxia or reoxygenation were dissected on ice. Hemolymph was withdrawn from the anterior adductor muscle sinus to assess the lysosomal membrane stability of hemocytes as a general stress marker (Moore et al., 2006). The digestive gland was dissected, shock-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analyses, and the digestive gland tissues were collected for analyses of the biomarkers of lipid peroxidation, activity of cathepsin D, and mRNA expression of the apoptotic and inflammatory genes. The digestive gland was chosen as a key organ involved in the nutrient uptake and energy storage of marine bivalves that plays a crucial role in whole-organism bioenergetics (Gosling, 1992; Kennedy et al., 1996). Individual mussels were used as biological replicates in all subsequent analyses ( $N=6$ ).

### Lysosomal membrane stability

Lysosomal membrane stability was determined in hemocyte suspension by the Neutral Red retention (NRR) assay, which is based on the incorporation of dye into the lysosomes of living cells (Borenfreund and Puerner, 1985). Hemocytes were collected by centrifugation at 700 g for 10 min, washed using a hemocyte (HC) buffer containing 20 mmol l<sup>-1</sup> Hepes, 436 mmol l<sup>-1</sup> NaCl, 53 mmol l<sup>-1</sup> MgSO<sub>4</sub>, 10 mmol l<sup>-1</sup> CaCl<sub>2</sub> and 10 mmol l<sup>-1</sup> KCl, pH 7.3, and enumerated using a BrightLine hemocytometer. The hemocytes were resuspended in HC buffer and diluted to  $1 \times 10^7$  cells ml<sup>-1</sup>. The hemocyte suspensions were incubated for 2 h with 0.04% Neutral Red and collected by centrifugation (10 min at 700 g). The dye was extracted from the hemocytes using 200  $\mu\text{l}$  of an acetic acid: ethanol solution (1:1 v:v). The absorbance of the extract was measured at 550 nm and standardized to  $10^6$  cells.

### Lipid peroxidation

Lipid peroxidation (LPO) was determined in the digestive gland homogenate (1:10 w:v) by the production of thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). The absorbance was determined at 532 nm using a SpectraMax M2 microplate reader (Molecular Devices GmbH, Biberach-an-der-Riß, Germany) with LightPath correction. TBARS concentration was calculated using a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  and expressed as nmol g<sup>-1</sup> wet mass.

### Cathepsin D activity

Cathepsin D (EC 3.4.23.5) activity was determined following incubation of the digestive gland homogenate in 0.2 mol l<sup>-1</sup> sodium acetate, pH 3.8, with 1% hemoglobin as a substrate (Dingle et al., 1971). The resulting acid-soluble peptides were detected spectrophotometrically at 280 nm using a SpectraMax M2 microplate reader (Molecular Devices) with LightPath correction. Free cathepsin D activity was assessed in the crude homogenate of the digestive gland tissue without detergent addition, whereas the total cathepsin D activity was measured in an aliquot of the same homogenate treated with Triton X-100 (0.25%) to release cathepsin D from the lysosomes. Activities were determined using a standard curve with tyrosine. Enzyme activities were referred to the

protein content of the enzyme fraction as nmole tyrosine min<sup>-1</sup> g<sup>-1</sup> wet mass.

### mRNA expression of the target genes

Total RNA was extracted from the digestive gland tissue using TRI Reagent<sup>®</sup> (Sigma, St Louis, MO) according to the manufacturer's protocol. The tissue to TRI reagent ratio was kept below 1:10 (w:v). RNA samples (280/260 absorbance ratio >2.0) were cleaned up from possible DNA contamination using a TURBO DNA-free Kit (Thermo Fisher Scientific, Berlin, Germany) according to the manufacturer's instructions. cDNA was obtained from 2  $\mu\text{g}$  of the total RNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR was carried out using StepOnePlus<sup>™</sup> Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Thermo Fisher Scientific) and Biozym Blue S'Green qPCR Mix Separate ROX kit (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) using gene-specific primers (Table 1). Reaction mixtures containing 10  $\mu\text{l}$  of 2 $\times$  qPCR S'Green BlueMix and ROX additive mixture, 1.6  $\mu\text{l}$  of each forward and reverse primer (to a final concentration of 0.4  $\mu\text{mol l}^{-1}$ ), 4.8  $\mu\text{l}$  PCR grade water and 2  $\mu\text{l}$  cDNA sample were added to the wells of 96 well PCR plates, sealed (RT-PCR Seal foil, Roth, Karlsruhe, Germany) and centrifuged to collect the contents and eliminate air bubbles. The cycling parameters were as follows: 95 $^{\circ}\text{C}$  for 10 min to activate the polymerase followed by 40 cycles of 15 s at 95 $^{\circ}\text{C}$  and 60 s at 60 $^{\circ}\text{C}$ . Following the amplification, a melt curve analysis was performed to ensure that only a single PCR product was amplified. In each run, serial dilutions of a cDNA standard were amplified to determine the apparent amplification efficiency  $E_a$  (Pfaffl, 2001). In a pilot study, we tested three potential housekeeping genes [eukaryotic elongation factor 1 (eEF1), tubulin and  $\beta$ -actin] in both studied species and chose the reference gene that showed the least variation among and within the experimental treatment groups (eEF1 for *M. edulis* and  $\beta$ -actin for *C. gigas*). Gene-specific primers for the target and housekeeping genes were designed based on the published sequences from *Mytilus* spp. and *C. gigas* (Table 1). Putative homology between oyster and mussel sequences for the respective genes identified through the TBLASTN search was confirmed by phylogenetic analysis using neighbor-joining tree based on the maximum composite likelihood distance (Kumar et al., 2008). The expression of the target genes [caspase 2, caspase 3, caspase 8, Bcl-2, BAX, TGF- $\beta$ -activated kinase 1 (TAK1), nuclear factor kappa B-1 (NF- $\kappa$ B), inhibitor of NF- $\kappa$ B kinase subunit  $\alpha$  (IKK $\alpha$ ) and serine/threonine-protein kinase TBK1-like (TBK1)] was normalized against the expression of a housekeeping gene (eEF1 or  $\beta$ -actin in the mussels and oysters, respectively) using the gene-specific  $E_a$  values as described elsewhere (Pfaffl, 2001; Sanni et al., 2008).

### Statistics

Data were tested for the normal distribution and homogeneity of variances using Shapiro–Wilk and Levine tests, respectively. In the case of non-normal distribution, data were normalized using the Box-Cox transformation. Within each species, effects of hypoxia and reoxygenation on the studied traits were tested using one-way ANOVA with the experimental exposure regime as a fixed factor. Tukey's honest significant difference (HSD) test was used to determine which pairs of means are significantly different from each other. Principal component analysis (PCA) was used on the raw (non-transformed) data to reduce the dimensionality of the data set and compare the integrated biomarker profiles in different experimental groups. The PCA allows integration and visualization



**Table 1. Primers used for RT-PCR of the target stress-related genes in *Mytilus edulis* and *Crassostrea gigas***

Gene	Forward primer (3'–5')	Reverse primer (3'–5')	NCBI accession no.
<b><i>Mytilus edulis</i></b>			
Caspase 2	ACAAGTGCAGATGCTGTGTTG	ACACCTCTCACATTGTCGGC	HQ424449.1
Caspase 3	ACGACAGCTAGTTCACCAGG	CCACCAGAAGAGGAGTTCCG	HQ424453.1
Caspase 8	AATGTCGGTACCCACGATG	CGTGTATGAACCATGCCCT	HQ424450.1
Bcl-2	CGGTGGTTGGCAAGGATTTG	CGCCATTGCGCCTATTACAC	KC545829.1
BAX	TAAGTGGGACGTGTAGGCA	CCAGGGGGCGACATAATCTG	KC545830.1
TAK1	CACCAAACCGAACTGGACCT	GGAACTGCTGTGATCCGACA	KF015298.1
NF-κB	TGGATGATGAGGCCAAACCC	TGAAGTCCACCATGTGACGG	KF051275.1
IKKα	GTGGCCACCAGTCAAGTGAT	TAAGGCTGCAGCTTGCTGAT	KF015301.1
TBK1	TGCAGGAGCCGATAAGCAA	CCGCCGGAACAAAATTCCAT	KF015302.1
EF1	GACAGCAAAAACGCCACC	TTCTCCAGGGTGGTTCAGGA	AF063420
<b><i>Crassostrea gigas</i></b>			
Caspase 2	ACAAACGTGGCCAGATCCAT	ATGATGAGGACCCTGCCTCT	XM_011451515.2
Caspase 3	GACAGGCTCTTTGACGGACA	ATTTCTCCATTTCGGAGCCC	KR559684.1
Caspase 8	GTCACATGGTGTGCTCCAGA	CCC GCCAGTCTGTACATTT	XM_011447144.2
Bcl-2	AGATTACCGTGCCCTTGTGG	CGGCTGGAACACTTTGTTG	EU678310.1
BAX	GGATTTACAAGACCCCGGCA	TCATGGTTTGCACCTGGGGT	XM_011426180.2
TAK1	GGGAGGAGCACGAGTTTGA	ACAGTTCTGCTGGCCTCCTC	XM_011457394.2
NF-κB	GCTACGAGTGTGAGGGGAGATCA	GGGAAACTGATGACGTTGGTGT	XM_020066072
IKKα	ACCAGGCCGTGAAAAGTCAA	TATACAGCTTCTGCCACGC	XM_011450699.2
TBK1	CCAGGACATATACGTCGCC	TCCCTCGAACAGACCTCTA	XM_011437045.2
β-Actin	TCCGGAATCCATGAAACATCA	TCCTTTTGCATACGGTCAGC	NM_001308859.1

Bcl-2, B-cell lymphoma 2; BAX, Bcl-2-associated X protein; TAK1, TGF-β-activated kinase 1; NF-κB, nuclear factor κB (p100/p105); IKKα, inhibitor of NF-κB kinase subunit α; TBK1, serine/threonine-protein kinase TBK1; EF1, eukaryotic elongation factor 1α. Because *M. edulis* does not yet have a completely sequenced genome assembly, we used homologous sequences from either *M. galloprovincialis* or *M. edulis* to generate primers.

of the patterns of variation and similarities within a multi-variable data set by transforming the set of correlated variables into a smaller number of the orthogonal (uncorrelated) variables called principal components (Ringnér, 2008). All statistical analyses were conducted using IBM SPSS Statistics v. 22.0.0.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v. 8.02 (GraphPad Software Inc., La Jolla, CA, USA) software. The data are shown as means±s.e.m. The effects were considered significant if the probability of the Type I error (*P*) was less than 0.05.

## RESULTS

### Lysosomal membrane stability

Exposure to short-term (1 day) and long-term (6 days) hypoxia resulted in the reduced stability of lysosomal membranes of mussels and oysters as shown by the lower Neutral Red retention in their hemocytes (Fig. 1). After 1 h of reoxygenation, the lysosomal membrane stability partially recovered but remained below the respective control levels (Fig. 1A,B) except for oysters recovering after 6 days of hypoxia, where the lysosomal membrane stability of hemocytes returned to the baseline levels (Fig. 1A,B).

### Oxidative lesions

Tissue levels of TBARS increased during the short-term hypoxia in the mussels and decreased in oysters, recovering back to the control levels after 1 h of reoxygenation (Fig. 1C,D). After long-term (6 days) hypoxia, the TBARS levels were suppressed in the mussels and elevated in oysters. One hour of reoxygenation after long-term hypoxia was insufficient to restore the TBARS concentrations back to the baseline levels in either of the two studied species (Fig. 1C,D).

### Cathepsin D activity

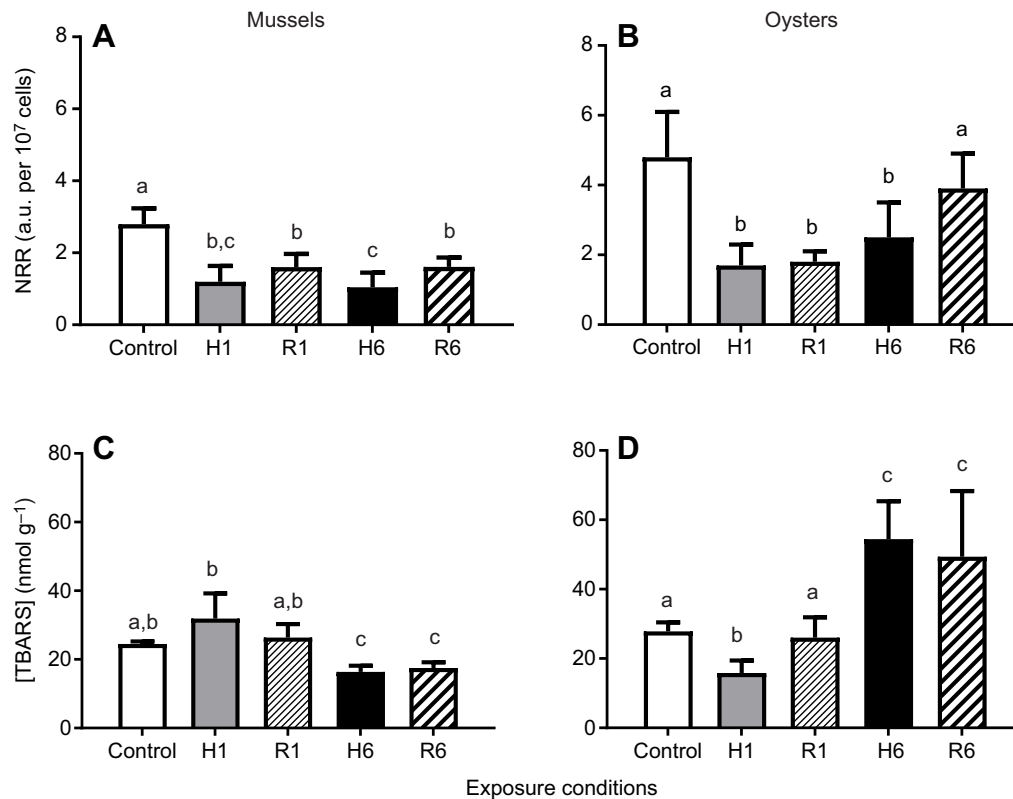
Total and free cathepsin D activity generally remained near baseline levels after 1 day of hypoxia and subsequent reoxygenation in mussels and oysters (Fig. 2) although a slight increase was found in the total cathepsin D activity after 1 day of hypoxia in mussels (Fig. 2A). Long-term (6 days) hypoxia strongly elevated total and

free cathepsin D activity in the digestive gland of mussels and oysters (Fig. 2). Notably, the free cathepsin D activity partially recovered after 1 h of reoxygenation following long-term hypoxia in oysters but continued to increase in mussels (Fig. 2C,D).

### mRNA expression of target genes

In *M. edulis*, H-R stress strongly elevated mRNA expression of apoptotic initiator (caspase 2 and caspase 8) and executor (caspase 3) caspases compared with control (normoxic) levels (Fig. 3A,C,E). Expression levels of caspase 2 increased ~310-fold after 1 day of hypoxia and ~2100-fold after 1 h of reoxygenation in *M. edulis*, compared with the control levels (Fig. 3A). The upregulation was significant but less pronounced after 6 days of hypoxia and subsequent reoxygenation in *M. edulis* resulting in ~140-fold and ~40-fold increase in the levels of caspase 2 transcripts, respectively (Fig. 3A). In *C. gigas*, caspase 2 mRNA remained at the baseline levels after 1 day of hypoxia and subsequent reoxygenation, and increased by ~1.7-fold after 6 days of hypoxia and reoxygenation (Fig. 3B). Caspase 3 mRNA expression in *M. edulis* was ~2.3-fold higher than baseline (normoxic) levels following 1 h of recovery from short-term hypoxia, and ~1.8- and 1.7-fold higher than the baseline after long-term hypoxia and subsequent reoxygenation (Fig. 3C). In *C. gigas*, no change in the caspase 3 transcript levels were observed during H-R exposures (Fig. 3D). Compared to normoxia, caspase 8 mRNA levels increased by ~2.9-fold in *M. edulis* exposed to 1 h of reoxygenation after the short-term hypoxia but did not change during the long-term hypoxia and reoxygenation (Fig. 3E). In *C. gigas*, short-term hypoxia and reoxygenation had no effect on caspase 8 mRNA levels, and the long-term hypoxia and reoxygenation led to a ~2.2- and 1.8-fold increase in the levels of caspase 8 transcript, respectively (Fig. 3F).

Transforming growth factor-β-activated kinase 1 (TAK1) mRNA expression did not change in response to H-R exposure in mussels (Fig. 4A). In oysters, there was a slight (~1.7-fold) but significant elevation of TAK1 mRNA levels after 6 days of hypoxia compared with the normoxic control (Fig. 4B). In *M. edulis*, expression levels



**Fig. 1. Effects of hypoxia and reoxygenation on lysosomal membrane stability and levels of lipid peroxidation in the digestive gland of *Mytilus edulis* and *Crassostrea gigas*.** (A,B) Neutral Red retention (NRR) as an index of lysosomal membrane stability in hemocytes. (C,D) Tissue levels of TBARS. Experimental groups: H1, short-term (1 day) hypoxia; R1, 1 h reoxygenation following short-term (1 day) hypoxia; H6, long-term (6 days) hypoxia; R6, 1 h reoxygenation following long-term (6 days) hypoxia. Different letters above columns indicate the values that are significantly different from each other ( $P < 0.05$ ). If two columns share a letter, they are not significantly different ( $P > 0.05$ ). Data are shown as means  $\pm$  s.d.  $N = 6$ .

of Bcl-2 mRNA increased  $\sim 1.9$ -fold after 6 days of hypoxia compared with the normoxic baseline but did not change in other experimental treatments (Fig. 4C). In *C. gigas*, long-term (but not short-term) hypoxia and reoxygenation led to elevated mRNA levels of Bcl-2 ( $\sim 2.4$ - and  $2.0$ -fold, respectively, above the normoxic baseline) (Fig. 4D). Reoxygenation after short-term hypoxia as well as long-term hypoxia and reoxygenation led to increased levels of BAX mRNA ( $\sim 1.9$ -,  $1.6$ - and  $1.8$ -fold, respectively) in *M. edulis* (Fig. 4E) but not in *C. gigas* (Fig. 4F).

Nuclear factor NF- $\kappa$ B mRNA expression increased during short-term and long-term hypoxia ( $\sim 3.7$ - and  $4.6$ -fold, respectively) and subsequent reoxygenation ( $\sim 6.0$ - and  $3.3$ -fold, respectively) in *M. edulis* (Fig. 5A) but not in *C. gigas* (Fig. 5B). In *M. edulis*, short- and long-term hypoxia suppressed mRNA expression of NF- $\kappa$ B inhibitor kinase subunit  $\alpha$  (IKK $\alpha$ )  $\sim 4.0$ - and  $2.2$ -fold below the normoxic baseline, respectively (Fig. 5C). In *M. edulis*, mRNA levels of IKK $\alpha$  were also suppressed  $\sim 5.3$ -fold below the normoxic baseline after 1 h of reoxygenation following short-term hypoxia. In *C. gigas*, mRNA levels of IKK $\alpha$  were slightly ( $\sim 1.6$ -fold) but significantly elevated after 6 days of hypoxia but not in any other treatment (Fig. 5D). mRNA levels of serine/threonine kinase TBK1 increased after 6 days of hypoxia in *M. edulis* ( $\sim 1.9$ -fold) and *C. gigas* ( $1.8$ -fold) but remained close to the control levels in other treatments (Fig. 5E,F).

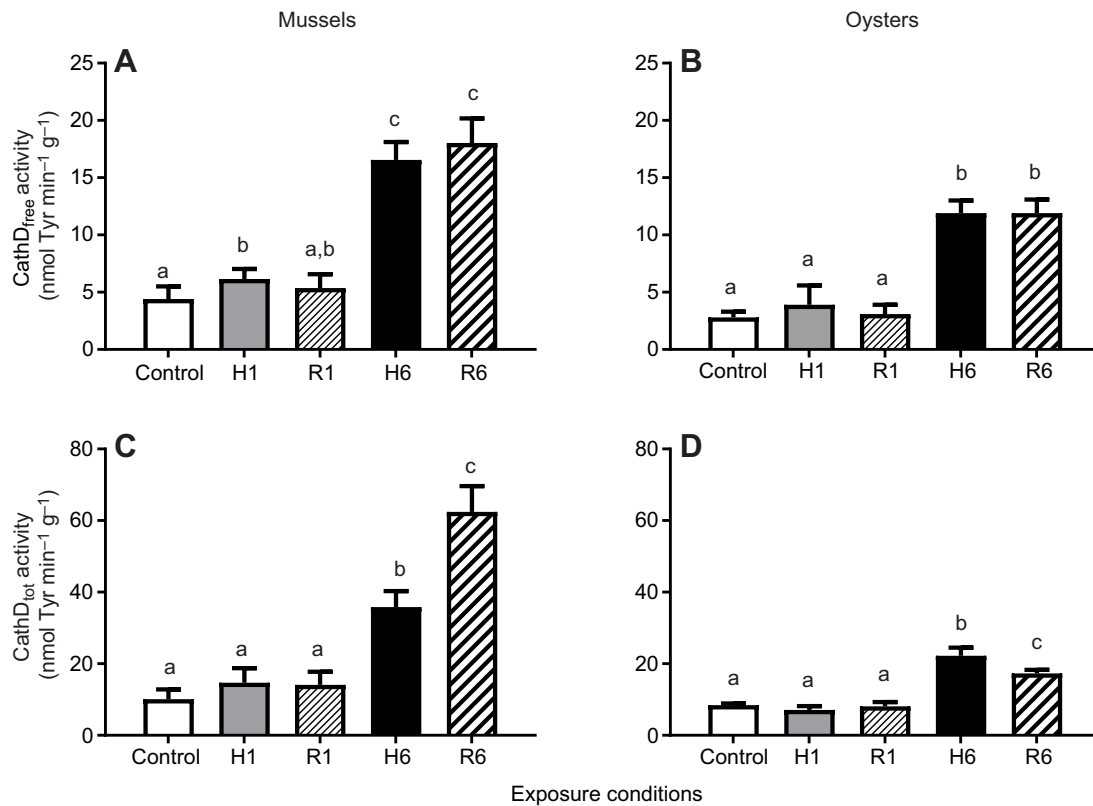
#### Data integration

The PCA analysis integrating all the studied stress biomarkers in the digestive gland of *M. edulis* identified two principal components (PC) jointly explaining 56% of the variation in the data set (Table S1). The 1st PC (34% of the total variation) had high negative loadings ( $< -0.5$ ) for most of the studied apoptotic markers (caspase 3, caspase 8, Bcl-2 and BAX), as well as for TAK1, NF- $\kappa$ B and TBK1, corresponding to strong negative correlations of the

respective variables with PC1. The 2nd PC had high positive loadings ( $> 0.5$ ) of the lysosomal membrane stability marker NRR. The 2nd principal component (22% of the total variation) had high negative loadings of the caspase 2 mRNA expression and tissue TBARS content, and high positive loadings of IKK $\alpha$  and free and total cathepsin D activity (Table S1).

In *M. edulis*, the control and hypoxia-exposed groups were clearly separated in the plane of the two first principal components (Fig. 6A). After a day of hypoxic exposure, the biomarker profile showed a strong shift of the hypoxia-exposed group towards more negative values along the PC2 mostly associated with the elevated levels of TBARS and caspase 2 mRNA (Fig. 6B). After 1 h of recovery following the short-term (1 day) hypoxia, the biomarker profile of mussels did not return to control levels (Fig. 6A); rather, a strong shift along the PC2 was observed, reflecting higher expression of multiple apoptotic and inflammatory markers (Fig. 6B). After 6 days of hypoxia, the position of the hypoxia-exposed group shifted to the more positive values of PC1 and more negative values of PC2 compared with the control (Fig. 6A), reflecting an increase in the expression of apoptotic, inflammatory and autophagic markers (Fig. 6B). Similar to the situation found after short-term hypoxia, recovery after the long-term (6 days) hypoxia did not result in the recovery of the baseline (control) biomarker profile in the mussels (Fig. 6A).

PCA analysis of the stress biomarkers in the digestive gland of *C. gigas* identified two principal components (PC) jointly explaining 70% of the variation in the data set (Table S2). The PC1 (55% of the total variation) had high negative loadings ( $< -0.5$ ) of caspase 2, caspase 8, Bcl-2, BAX, TAK1, NF- $\kappa$ B, IKK $\alpha$ , TBK1, free and total cathepsin D activity and TBARS. PC2 (15% of the total variation) had high negative loadings of BAX and NF- $\kappa$ B mRNA expression and high positive loadings of tissue TBARS content (Table S2).



**Fig. 2. Effects of hypoxia and reoxygenation on cathepsin D activity in the digestive gland of *M. edulis* and *C. gigas*.** (A,B) Activity of free cathepsin D (CathD<sub>free</sub>). (C,D) Total activity of cathepsin D (CathD<sub>tot</sub>). Experimental groups are as described in Fig. 1. Different letters above columns indicate the values that are significantly different from each other ( $P < 0.05$ ). If two columns share a letter, they are not significantly different ( $P > 0.05$ ). Data are shown as means  $\pm$  s.d.  $N = 6$ .

In *C. gigas*, short-term (1 day) hypoxia did not result in a major shift in the biomarker profile shown by a large overlap in the position of the control group and the group exposed to 1 day of hypoxia (Fig. 6C). One hour of reoxygenation following the short-term hypoxia led to the recovery of the biomarker profile in the digestive gland of oysters to the baseline (control) levels (Fig. 6C). After 6 days of hypoxia exposure, the biomarker profile of the oysters shifted towards the more negative values along the PC1 axis (Fig. 6C), indicating elevated expression of apoptotic, inflammatory and autophagic biomarkers (Fig. 6D). One hour of reoxygenation following 6 days of hypoxia was not sufficient to fully restore the normal biomarker profile in *C. gigas*, but a partial recovery occurred, shown by a shift towards the control (baseline) values (Fig. 6).

## DISCUSSION

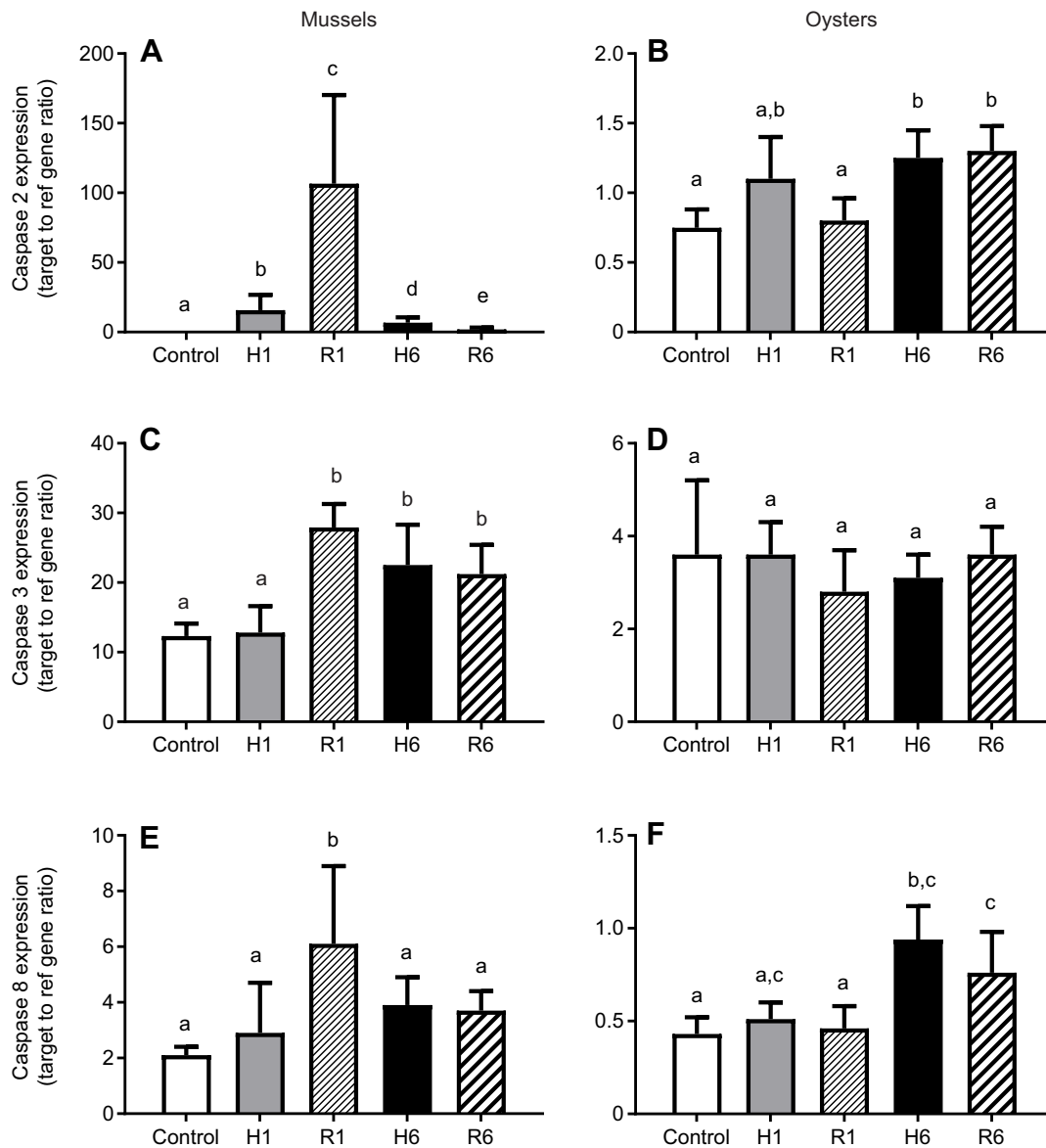
Severe short-term (1 day) and long-term (6 days) hypoxia and subsequent reoxygenation induced different responses in the autophagic, apoptotic and inflammatory pathways in the two studied species of marine bivalves (*M. edulis* and *C. gigas*) with the different degree of hypoxia tolerance. The multivariate PCA analysis showed that in *M. edulis*, both periods of hypoxia led to a major shift in the profile of the cellular stress biomarker compared with the control, and this shift was further exaggerated during reoxygenation (Fig. 6). Unlike the mussels, 1 day of hypoxia in the oysters led to a minor change on the profile of the apoptotic, inflammatory and autophagic biomarkers that quickly recovered after reoxygenation. Furthermore, while the long-term hypoxia induced a shift in the biomarker profile in the oysters, detailed

analysis of individual biomarkers (discussed below) indicates that this shift largely reflects a protective cellular response rather than accumulated damage. One hour of reoxygenation was sufficient to ensure a partial recovery of the normal biomarker profile in the oysters even after the long-term (6 days) hypoxia. These data indicate that the oysters are more resistant to the induction of apoptosis, inflammation and tissue injury during hypoxia, which may contribute to higher hypoxia tolerance of the oysters compared with the mussels.

### Autophagy during hypoxia in the mussels and the oysters

H-R stress activated autophagy in the mussels and oysters, as shown by an increase in the activity of the cathepsin D in the digestive gland. However, the time course and the extent of autophagy activation was different in the two studied species. In the mussels, a significant increase in the activity of the free cathepsin D during the short-term (1 day) hypoxia was found despite the lack of an increase in the total cathepsin D activity. This indicates a release of existing cathepsin D from the mussel lysosomes during short-term hypoxia, which was not recovered during subsequent reoxygenation. Unlike the mussels, no release of cathepsin D was found after short-term hypoxia and reoxygenation in oysters.

The long-term (6 days) hypoxia led to a considerable increase in the total and free cathepsin D activity in the mussels and the oysters, indicating stimulation of autophagic processes. This finding is consistent with earlier reports on hypoxia-sensitive organisms, such as mammals and insects, that show activation of autophagy for energy supply during prolonged hypoxia/ischemia (Bialik et al., 2018; Ham and Raju, 2016; Sciarretta et al., 2011). While mild

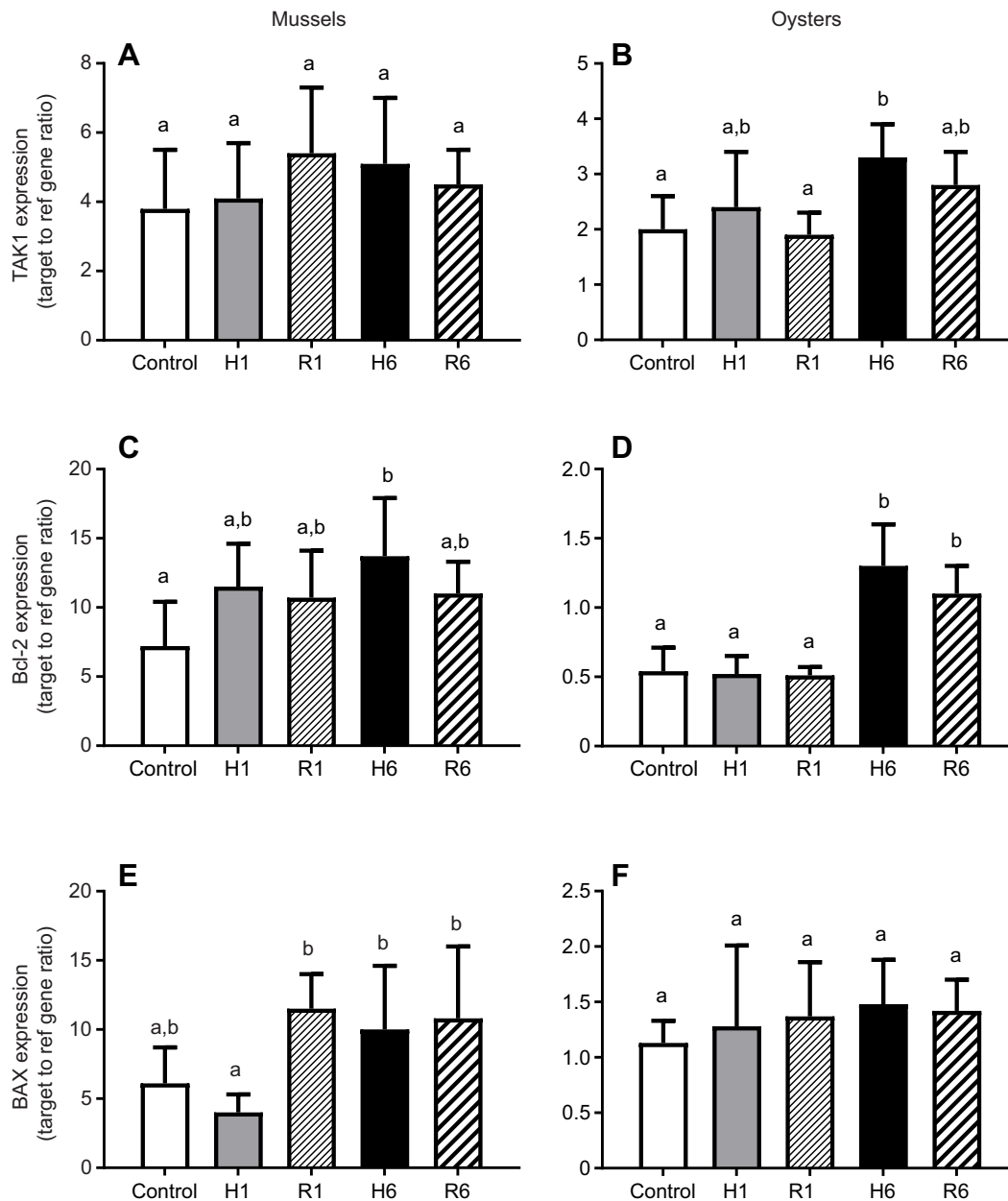


**Fig. 3. Effects of hypoxia and reoxygenation on mRNA expression of caspase family genes in the digestive gland of *M. edulis* and *C. gigas*.** Expression of (A,B) caspase 2, (C,D) caspase 3 and (E,F) caspase 8 relative to reference gene. Experimental groups are as described in Fig. 1. Different letters above columns indicate the values that are significantly different from each other ( $P < 0.05$ ). If two columns share a letter, they are not significantly different ( $P > 0.05$ ). Data are shown as means  $\pm$  s.d.  $N=6$ .

stimulation of autophagy is considered protective during H-R stress (Samokhvalov et al., 2008; Zhang et al., 2017), a massive increase in the autophagic activity is detrimental and can lead to apoptosis and autophagy-dependent cell death (Bialik et al., 2018; Sciarretta et al., 2011; Solaini et al., 2010). The latter appears to be the case in the mussels, where prolonged hypoxia resulted in  $\sim 3.5$ - and  $\sim 6$ -fold increase in the total cathepsin D activity after 6 days of hypoxia and subsequent reoxygenation, respectively, reaching  $36$ – $62$  nmol Tyr  $\text{min}^{-1} \text{g}^{-1}$  (compared with  $10$  nmol Tyr  $\text{min}^{-1} \text{g}^{-1}$  in the control). By contrast, the hypoxia-induced increase in the total cathepsin D was less pronounced in the oysters, showing a  $\sim 2.6$ -fold increase (to  $22$  nmol Tyr  $\text{min}^{-1} \text{g}^{-1}$ ) after 6 days of hypoxia, and a partial recovery after 1 h of reoxygenation. This smaller increase in the autophagic enzyme activity might indicate less severe energy deficiency and/or lower degree of tissue damage induced by prolonged hypoxia and reoxygenation in the oysters.

### Effects of severe hypoxia and reoxygenation on apoptotic pathways

While the activity thresholds separating beneficial from detrimental stimulation of autophagy are not known in marine bivalves, analysis of the apoptosis induction patterns are consistent with the notion of the greater tissue damage during H-R in the mussels compared with the oysters. In *M. edulis*, short-term hypoxia led to a dramatic ( $\sim 310$ -fold) increase in the expression of the stress-induced initiator caspase 2 above the normoxic baseline, indicating induction of the pathways of intrinsic (stress-induced) apoptosis. This response was further enhanced upon reoxygenation indicated by a  $\sim 2100$ -fold upregulation of caspase 2 expression and a  $\sim 2.3$ - to  $2.9$ -fold upregulation of caspase 3 and caspase 8 mRNA above the normoxic levels. These findings indicate that post-hypoxic reoxygenation acts as a major activator of both the intrinsic (stress-induced) and extrinsic (death-receptor-induced) apoptosis pathways in the mussels. A



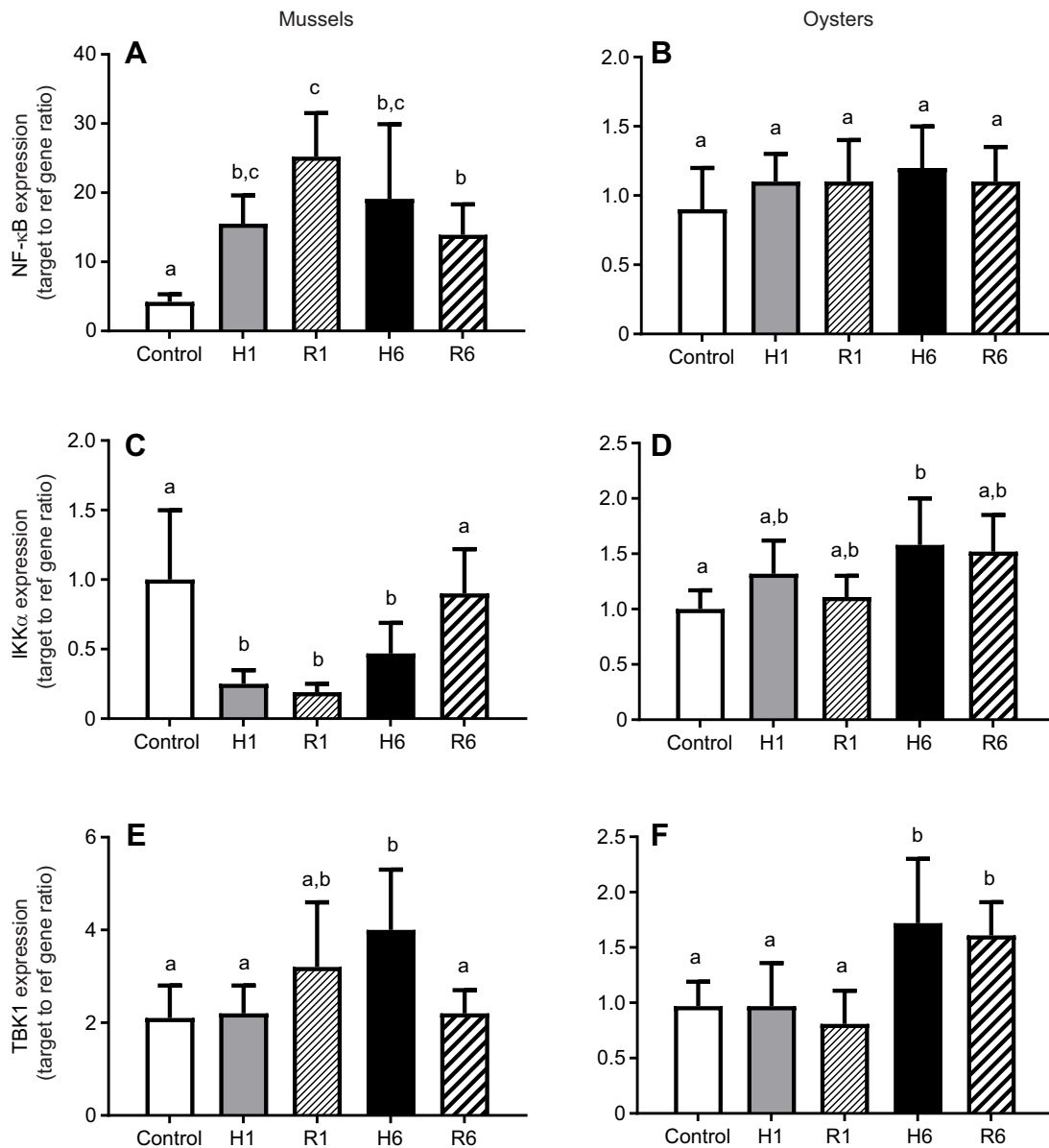
**Fig. 4. Effects of hypoxia and reoxygenation on mRNA expression of the target genes involved in apoptosis pathways in the digestive gland of *M. edulis* and *C. gigas*.** Expression of (A,B) TAK1, (C,D) Bcl-2 and (E,F) BAX relative to reference gene. Experimental groups are as described in Fig. 1. Different letters above columns indicate the values that are significantly different from each other ( $P < 0.05$ ). If two columns share a letter, they are not significantly different ( $P > 0.05$ ). Data are shown as means  $\pm$  s.d.  $N=6$ .

stronger activation of apoptotic pathways during reoxygenation (compared with hypoxia/ischemia) is commonly observed in hypoxia-sensitive organisms and tissues such as brain, heart and retina of terrestrial vertebrates (Qin et al., 2004; Shao et al., 2011; Singh et al., 2001; Thornton et al., 2017; Vanden Hoek et al., 2003).

Caspase 2 appears to be the key caspase implicated in apoptosis induced by the short-term hypoxia and reoxygenation in the blue mussels *M. edulis*, similar to the earlier reports on the H-R stress in hypoxia-sensitive vertebrate models (Fava et al., 2012; Qin et al., 2004; Shao et al., 2011; Thornton et al., 2017; Vanden Hoek et al., 2003; Xie et al., 2008). Caspase 2 is a highly conserved initiator caspase activated in response to metabolic imbalance,  $Ca^{2+}$  overload or endoplasmic reticulum stress (Fava et al., 2012; Movassagh and Foo, 2008). In *M. edulis*, upregulation of caspase 2 mRNA can also

be caused by toxic stress such as exposure to ZnO nanomaterials (Falfushynska et al., 2019). Caspase 8 (a key initiator caspase of the extrinsic apoptosis initiated by death receptor ligands such as the damage activated molecular patterns released from injured cells), caspase 3 (the main executor caspase on which the intrinsic and extrinsic apoptotic pathways converge) and a pro-apoptotic regulatory protein BAX are also upregulated during reoxygenation in *M. edulis* albeit to a lesser degree than caspase 2. No increase in the expression of an anti-apoptotic regulator Bcl-2, known for its protective effects during H-R stress (Grünenfelder et al., 2001), was found in *M. edulis* during the short-term hypoxia or reoxygenation. In contrast to results in mussels, no increase in the apoptotic markers was found during the short-term hypoxia and subsequent reoxygenation in the Pacific oyster *C. gigas*.

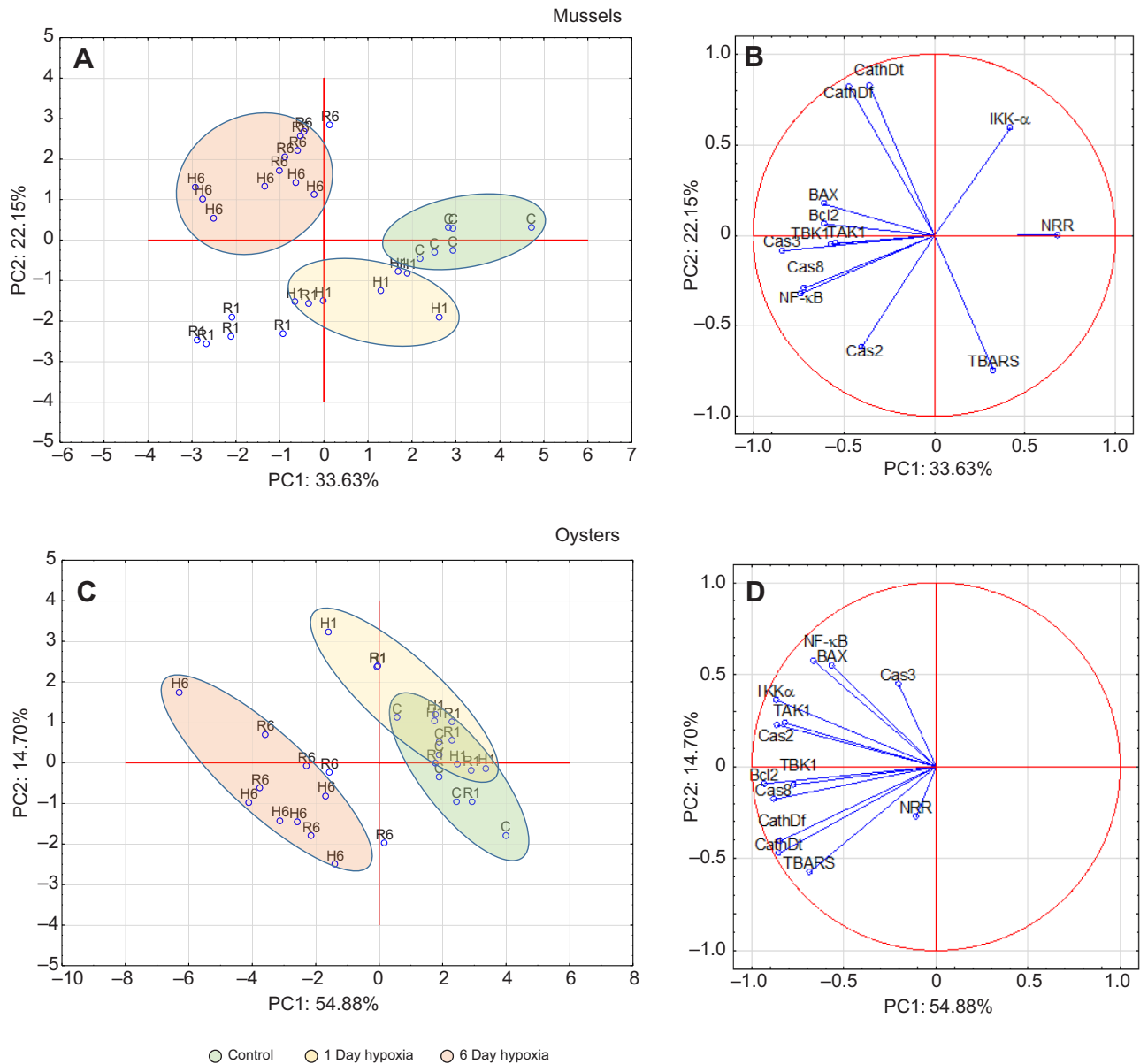




**Fig. 5. Effects of hypoxia and reoxygenation on mRNA expression of the target genes involved in inflammation signaling pathways in the digestive gland of *M. edulis* and *C. gigas*.** Expression of (A,B) NF- $\kappa$ B, (C,D) IKK $\alpha$  and (E,F) TBK1 relative to reference gene. Experimental pathways are as described in Fig. 1. Different letters above columns indicate the values that are significantly different from each other ( $P < 0.05$ ). If two columns share a letter, they are not significantly different ( $P > 0.05$ ). Data are shown as means  $\pm$  s.d.  $N = 6$ .

The apoptotic signature caused by long-term (6 days) hypoxia and subsequent reoxygenation in the mussels is qualitatively similar to that found during the short-term H-R stress, as shown by upregulation of mRNA expression of caspase 2, caspase 3 and a pro-apoptotic regulator BAX. However, an increase in caspase 2 expression was considerably less pronounced in *M. edulis* after long-term hypoxia and reoxygenation (~40- to 140-fold) than during the short-term H-R stress (~310- to 2100-fold). This might be due to the activation of some cellular protective mechanisms such as the elevated expression of the anti-apoptotic regulator Bcl-2 after prolonged hypoxia in *M. edulis*. Alternatively, it could reflect selective survival of the mussels that show blunted apoptotic response and thus less extensive tissue damage during prolonged hypoxia. Interestingly, the Pacific oysters also showed activation of apoptotic pathways during prolonged hypoxia

including a ~1.7- to 2.2-fold increase in the mRNA levels of the initiator caspases 2 and 8. It is not known whether such mild upregulation of the transcript levels has implications for the functional activities of caspases 2 and 8, but this increase indicates an induction of apoptotic pathways in the oysters during long-term hypoxia. Notably, mRNA levels of the executor caspase 3 and the pro-apoptotic regulator BAX did not increase during the prolonged hypoxia in oysters, whereas the mRNA levels of an anti-apoptotic Bcl-2 protein were ~2.4-fold elevated. These results show that the oyster tissues are well protected from apoptosis during prolonged hypoxia and indicate a potential role of Bcl-2 in this protection, similar to the findings in the vertebrate model systems (de Graaf et al., 2002; Gr nenfelder et al., 2001; Mattson and Kroemer, 2003; Strasser et al., 2000; Vukosavic et al., 2000). Reoxygenation after long-term hypoxia did not lead to further



**Fig. 6. Principal component analysis score plots and variable loading plots based on the studied biomarkers from *M. edulis* and *C. gigas* under hypoxia-reoxygenation stress.** (A,C) Position of the individual samples from different experimental groups in the coordinate plane of 1st two principal components (PC1 and PC2). Experimental groups: H1, short-term (1 day) hypoxia; R1, 1 h reoxygenation following short-term (1 day) hypoxia; H6, long-term (6 days) hypoxia; R6, 1 h reoxygenation following long-term (6 days) hypoxia. Colored ovals mark the clusters of samples from the control (green), 1 day of hypoxia (yellow) and 6 days of hypoxia (orange) groups. (B,D) variable loading plots showing how much weight different biomarkers have on each of the two 1st principal components. When the vectors corresponding to different variables (i.e. biomarker values) are close, the two variables are positively correlated. Orthogonal vectors (i.e. forming an angle close to 90 deg) indicate that the respective variables are not correlated. NRR, Neutral Red retention; CathDt, total cathepsin D; CathDf, free cathepsin D; Cas2, caspase 2; Cas3, caspase 3; Cas8, caspase 8; TAK1, TGF- $\beta$ -activated kinase 1; Bcl2, B-cell lymphoma 2, BAX, bcl-2-like protein 4; IKK $\alpha$ , inhibitor of NF- $\kappa$ B kinase subunit  $\alpha$ ; TBK1, serine/threonine-protein kinase TBK1-like; TBARS, thiobarbituric acid-reactive substances.

activation of the apoptotic pathways in the mussels or the oysters, and the gene expression pattern of the apoptosis-related genes during the first hour of recovery was similar to that found after the long-term (6 days) hypoxia.

It is worth noting that oxidative injury is unlikely to be the main trigger of the apoptotic cell death in the digestive gland tissues of the mussels and the oysters exposed to H-R stress. There was no correlation between the accumulation of lipid peroxidation products (TBARS) and the expression of pro-apoptotic genes (including caspases and BAX) in the digestive gland tissue of the mussels shown by the different direction of the respective vectors in the PCA

analysis (Fig. 6B). In oysters, accumulation of the lipid peroxidation products coincides with an increase in the expression of apoptotic genes including caspase 2, caspase 8 and the anti-apoptotic gene Bcl-2. However, comparison across the two studied species does not support the hypothesis that the onset of apoptosis is driven by the oxidative stress, as the strongest upregulation of caspases 2, 3 and 8 (found after the short-time hypoxia and reoxygenation in the mussels) was not associated with accumulation of the oxidative lesions, while a major increase in TBARS (observed after the long-term hypoxia and reoxygenation in the oysters) was associated with a modest increase in caspase 2 and caspase 8, and no increase in

caspase 3 expression. Similarly, a decrease in the lysosomal membrane stability caused by H-R stress in the mussels and oysters does not correlate with the degree of stimulation of apoptosis or autophagy within and across the studied species. This indicates that traditional general stress biomarkers (such as lipid peroxidation or lysosomal membrane destabilization) may not be good markers of the tissue injury caused by H-R stress in marine bivalves.

### Inflammatory response to hypoxia-reoxygenation (H-R) stress

Apoptosis is considered an important mechanism of limiting inflammatory response to H-R stress (Haanen and Vermes, 1995); however, ineffective clearing of the apoptotic debris as well as accidental (unregulated) cell death can lead to the release of damage-activated molecular patterns (DAMPs), resulting in inflammation and bystander injury of surrounding cells (Rovere-Querini et al., 2008; Thornton et al., 2017). Common DAMPs released during H-R stress include DNA, heat shock proteins, ATP, urea or purine metabolites that act as inflammatory signals when found outside their normal cellular compartments (Rovere-Querini et al., 2008; Thornton et al., 2017). A comparative metabolomics study of *M. edulis* and *C. gigas* showed a stronger accumulation of potential DAMPs (such as purine catabolism and urea cycle intermediates) during H-R stress in the tissues of the mussels compared with the oysters (Haider et al., 2020). It is not known whether these metabolites are released from the affected cells during H-R stress, but if so, they might contribute to the elevated inflammatory response in the mussels. Thus, elevated mRNA expression of an inflammatory marker NF- $\kappa$ B during H-R stress found in our present study indicates onset of inflammation during the short- and long-term hypoxia and reoxygenation in the mussels, a response that was apparently absent in the oysters. The expression of the inflammatory regulator NF- $\kappa$ B was tightly linked with the induction of apoptosis in *M. edulis* and *C. gigas*, as shown by the positive correlation between caspase 2 and NF- $\kappa$ B transcript levels in the mussels ( $R=0.40$ ,  $P<0.05$ ) and oysters ( $R=0.65$ ,  $P<0.001$ ). This finding agrees with the notion of caspase 2 as a potent activator of NF- $\kappa$ B pathway in marine bivalves, including *M. edulis* and *C. gigas* (Falfushynska et al., 2019; Wang et al., 2018).

Transcript levels of NF- $\kappa$ B activating kinases, IKK $\alpha$  and TBK1, showed different responses to H-R stress in *M. edulis* and *C. gigas*. In model organisms such as mammals, IKK $\alpha$  and TBK1 induce an NF- $\kappa$ B-dependent inflammatory response and can stimulate or suppress apoptosis, depending on the nature of the stimulus (Adli et al., 2010; Chau et al., 2008; Kaltschmidt et al., 2000; Möser et al., 2015; Pomerantz and Baltimore, 1999). IKK $\alpha$  belongs to the catalytic core of the IKK complex, which activates NF- $\kappa$ B by phosphorylating and inactivating the I $\kappa$ B (inhibitor of kappa B) proteins (Adli et al., 2010). TBK-1 is a non-canonical IKK that activates NF- $\kappa$ B by phosphorylating an NF- $\kappa$ B activator TANK (Möser et al., 2015). Activation of NF- $\kappa$ B by IKKs initiates the transcriptional response of multiple genes involved in inflammation and apoptosis (Adli et al., 2010; Kaltschmidt et al., 2000). The molecular signaling pathways of IKK $\alpha$  and TBK1 are not well studied in marine bivalves, but recent analyses show that IKKs of *C. gigas* are highly conserved and structurally similar to their vertebrate homologues (Yu et al., 2018) and can activate NF- $\kappa$ B (Escoubas et al., 1999; Huang et al., 2019). Our present study shows that in the mussels, transcriptional upregulation of an inflammatory marker NF- $\kappa$ B during short-term (1 day) hypoxia and subsequent reoxygenation occurs despite the lack of increase in the TBK1 transcript and suppressed transcription of IKK $\alpha$ . During long-term

(6 days) hypoxia, NF- $\kappa$ B and TBK1 (but not IKK $\alpha$ ) mRNA increases in the mussels, suggesting propagation of the inflammatory signal. In *C. gigas*, IKK $\alpha$  and TBK1 mRNA levels remain unchanged during short-term hypoxia and show a modest increase during long-term hypoxia. However, no transcriptional activation of NF- $\kappa$ B is observed during H-R stress in the oysters. Overall, these data indicate an earlier onset of the inflammatory response during H-R stress in the mussels compared with the oysters, and suggest that transcriptional activation of the upstream (IKK $\alpha$  and TBK1) and downstream (NF- $\kappa$ B) components of the inflammatory cascade are not tightly correlated in these two species.

Expression of TAK1, a key intracellular kinase involved in the cross-talk between apoptotic pathways regulated by TGF- $\beta$  and the inflammatory, NF- $\kappa$ B-dependent pathways (Freudlsperger et al., 2012), was not affected by H-R stress in the mussels and was only slightly upregulated during the prolonged hypoxia in the oysters. No correlation was found between TAK1 and NF- $\kappa$ B mRNA expression in the mussels and oysters. This finding is in contrast to the coordinated upregulation of TAK1 and NF- $\kappa$ B mRNA found during exposure of *M. edulis* to ZnO nanoparticles and nanorods (Falfushynska et al., 2019). Taken together, our data and earlier published research indicate that the molecular mechanisms regulating inflammation are stressor-dependent in marine bivalves and may differ during exposure to natural stressors (such as H-R) and anthropogenic contaminants such as ZnO nanomaterials.

### Conclusions and outlook

The present study demonstrated strong differences in the patterns of induction of autophagy, apoptosis and inflammation between the two species of marine bivalves with different degree of hypoxia tolerance. The less hypoxia-tolerant of the two studied species, *M. edulis*, showed transcriptional upregulation of key pro-apoptotic and pro-inflammatory genes after one day of hypoxia, accompanied by a strong induction of autophagic enzyme activity after 6 days of hypoxia. In contrast, the more hypoxia-tolerant *C. gigas* showed a muted apoptotic and autophagic response, as well as a lack of increase in the inflammation marker NF- $\kappa$ B during severe hypoxia. A lack of correlation between the tissue oxidative injury and stimulation of the apoptotic and inflammatory pathways indicates that oxidative stress is not a major underlying mechanism of the cellular stress response in the studied species, and other mechanisms (such as energy deficiency or negative shifts in the metabolic homeostasis) might be implicated in the induction of apoptosis and inflammation during severe hypoxia. Interestingly, a metabolomics study using animals from the same exposures showed that *C. gigas* is better able to maintain the homeostasis of intermediate metabolites (such as the free amino acids, purine metabolites and urea cycle metabolites) during severe hypoxia and reoxygenation than *M. edulis* (Haider et al., 2020). This ability to maintain cellular homeostasis may contribute to the oysters' resistance to apoptosis and inflammation in hypoxia. Future studies using a broader, phylogenetically controlled comparative framework are needed to test this hypothesis and shed new light on the potential role of the resistance to apoptosis and inflammation in the evolution of hypoxia tolerance of marine bivalves. Our study also indicates that expression levels of apoptotic and inflammatory markers (especially the strongly responding genes such as caspase 2 and NF- $\kappa$ B) can serve as useful molecular markers of hypoxia-induced cellular injury in marine bivalves for assessment of the effects of sublethal hypoxic stress as well as the rate of recovery in the coastal populations subjected to periodic oxygen deficiency.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

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

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**Data availability**

The project metadata are available from the PANGAEA open access database (Sokolova et al., 2019a) at <https://doi.org/10.1594/PANGAEA.909733>.

**Supplementary information**

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.217026.supplemental>

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