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Stress-on-stress responses of a marine mussel, *Perna canaliculus*: food limitation reduces the ability to cope with heat stress in juveniles

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ABSTRACT: The marine environment is ever-changing, with daily and seasonal variations in factors such as food availability and seawater temperature. These stressors can affect physiological processes in aquatic organisms, resulting in sub-lethal or lethal consequences. This study assessed the effects of food limitation (i.e. fasting) on heat-stress responses in juveniles (~1.3 mm in shell length) of the green-lipped mussel *Perna canaliculus*. Fasting for up to 24 h did not have a significant effect on oxidative damage (protein carbonyls and lipid hydroperoxide accumulation) or the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) after exposure to heat stress. However, fasting for 54 h and subsequent heat stress resulted in increased oxidative damage and decreased activity of antioxidant enzymes in juvenile mussels. Gene expression of 70 kDa heat shock protein (*hsp70*) was only significantly affected by heat shock, not nutritional status. Tissue carbohydrate and protein levels were significantly depleted by 54 h of fasting; as these proximate components represent key energy substrates for bivalves, it is suggested that energy limitation contributes to compromised antioxidant activity and predisposition to oxidative damage.

KEY WORDS: Green-lipped mussel · *Hsp70* · ROS · Oxidative stress · Antioxidants · Fasting · Thermal shock · Proximate composition

1. INTRODUCTION

Marine invertebrates inhabiting intertidal and shallow subtidal environments experience fluctuations in environmental conditions such as temperature and food availability, among many others, which can trigger a series of physiological responses (Solan & Whiteley 2016). Multiple stressors can have individual, additive, synergistic or antagonistic physiological effects that can have sub-lethal or lethal consequences on an organism (Coors & De Meester 2008, Crain et al. 2008, Firth & Williams 2009). Determining the impacts and interactions between common stressors is important to understand and manage marine resources, and is critical to conservation and sustainable aquaculture practices alike. In this study, we assessed the sub-lethal physiological effects of food limitation and its interaction with heat stress, a combination of stressors that is becoming increasingly important due to global climate change and the increased frequency of heatwave events (Oliver et al. 2018).

Under stress, a group of reactive molecules including free radicals and peroxides is generated and collectively known as reactive oxygen species (ROS). The accumulation of ROS in cells can result in damage to macromolecules (i.e. oxidative damage) and potentially cause cell death (Burritt & Lamare 2016, Lewis & Santos 2016). In response to cell damage caused by ROS, organisms employ a suite of mechanisms to repair and protect their cells from further

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damage (Lesser 2006, El-Bahr 2013, Oksala et al. 2014). The most studied protective mechanisms are the upregulation of heat shock proteins (HSPs) and enzymatic and non-enzymatic antioxidants, which help to stabilise proteins and limit oxidative damage to proteins, lipids and DNA, respectively (Feder & Hofmann 1999, Buttemer et al. 2010, Burritt & Lamare 2016). The upregulation of these mechanisms can be energetically costly (Somero 2002, Jeno & Brokordt 2014). Therefore, food limitation may play a crucial role in the ability of marine invertebrates to cope with additional stressors through HSPs and antioxidants (Jeno & Brokordt 2014, Sacristán et al. 2016).

Food limitation is a common stressor for marine organisms, especially for those with limited mobility such as most filter-feeders that are exposed to natural fluctuations of food in the water column. The abundance, diversity and nutritional content of phytoplankton changes seasonally due to fluctuations in nutrient availability and hydrodynamic conditions (Chang et al. 2003, Ramadyan 2017). These food fluctuations can directly affect the nutritional status and fitness of marine invertebrates and can consequently impact their response to other environmental stressors (Carton et al. 2007, Jeno & Brokordt 2014, Wagner et al. 2015, Sacristán et al. 2016). For example, barnacles *Amphibalanus amphitrite* and mussels *Mytilus edulis* can grow under moderately elevated pCO_2 levels when food is abundant, but their growth is reduced under food limitation (Thomsen et al. 2013, Pansch et al. 2014). Similarly, periods of enforced fasting in larval *M. edulis* and juvenile *Perna canaliculus* reduced the juvenile performance of both species (Phillips 2002, Carton et al. 2007).

Another common stressor affecting marine invertebrates is variation in seawater temperature, due to its effects on molecular, biochemical and physiological processes (Willmer 2000, Somero 2012, Sokolova 2013). Seawater temperature changes daily and seasonally; however, more sporadic changes like heatwaves and climate phenomena such as El Niño–Southern Oscillation can significantly affect the overall performance of invertebrates, and even compromise their survival (Gutiérrez-Fonseca et al. 2018, Smale et al. 2019, Thomsen et al. 2019). In marine invertebrates, changes in temperature can affect traits such as development, growth, reproduction (Delorme & Sewell 2013, 2014, 2016a,b), behaviour (Książkiewicz-Parulska 2017) and survival (Byrne 2011, Byrne & Przeslawski 2013, Dunphy et al. 2013, 2015, 2018). Therefore, understanding how food availability changes the effects of temperature variations on the physiology of marine organisms is vital in the context of a globally changing climate (Hobday et al. 2018, Smale et al. 2019, Thomsen et al. 2019).

The green-lipped mussel *Perna canaliculus* (Gmelin, 1791) is an iconic species in New Zealand. It is a foundation species that controls biodiversity on rocky reefs and in soft-sediment habitats (Jeffs et al. 1999, Menge et al. 2007, McLeod et al. 2014), and has cultural and commercial importance as the most significant aquaculture species in the country (Jeffs et al. 2018). While many aspects of the physiological biology and ecology of *P. canaliculus* have received significant research attention (e.g. Alfaro 2006, Carton et al. 2007, Petes et al. 2007, Fenton 2012, Young et al. 2015, Powell et al. 2017, Zamora et al. 2019), very little is known about physiological stress responses in its juveniles. This knowledge gap is particularly relevant because natural wild beds in soft-sediment habitats have been massively reduced by dredging operations, and their recovery is, in part, prevented by poor survival of juveniles <10 mm in shell length (Wilcox et al. 2018). Furthermore, the productivity of the aquaculture industry is presently challenged by substantial losses of juveniles that occur in the first few months of sea-based aquaculture (South et al. 2019, 2020). A better understanding of the ways juvenile *P. canaliculus* respond to stressors in the marine environment is critical to improving the sustainability and productivity of wild *P. canaliculus* beds and the socio-economically important aquaculture industry.

This study evaluated the stress-on-stress responses of juvenile *P. canaliculus* by manipulating food supply and subsequent heat stress. We determined the nutritional status of the juveniles using proximate composition analyses. Stress responses were determined by quantifying gene expression of heat shock protein 70 kDa (*hsp70*), oxidative stress biomarkers (protein carbonyls [PCs], lipid hydroperoxides [LPs] and 8-hydroxydeoxyguanosine [8-OHdG]) and enzymatic antioxidant profiles (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx] and glutathione reductase [GR]).

2. MATERIALS AND METHODS

2.1. Experimental design

Juvenile *Perna canaliculus* (average ± SD shell length: 1.3 ± 0.5 mm, n = 169) were provided by a local commercial hatchery (SPATnz) located adjacent to the laboratory used for these trials, at the Cawthron Aquaculture Park, Nelson, New Zealand. After arrival at the laboratory, 3 g of juvenile mussels were

placed into one of 12 cylindrical sieves (200 µm, 80 mm diameter, 50 mm deep) that were separately held in 2 l plastic tanks. The tanks were set up in a temperature-controlled room (20°C) and each tank was aerated and supplied filtered (5 µm) seawater (20.2 ± 0.1°C) at a rate of ~150 ml min^{-1}. All tanks were supplied with a mixture of cultured microalgae (*Chaetoceros calcitrans*, *C. muelleri* and *Pavlova lutheri*; total cell count: $1.1 \times 10^6 \pm 8.3 \times 10^3$ cells ml^{-1}; n = 3) as a source of food during the first 24 h after collection, as a recovery period before experimentation. The animals were then allocated to the respective fasting treatments before being exposed to the sub-lethal heat shock as explained below.

2.1.1. Food limitation simulation

After the recovery period, experimental tanks were randomly allocated to 4 fasting treatments: 0 h (control; fed as above throughout the experiment), 6 h fasting, 24 h fasting and 54 h fasting, with 3 replicate tanks per treatment. The duration of the fasting treatments provided a gradient of sub-lethal food limitation, with the 54 h period being dictated by the requirement for sub-lethal, rather than lethal, stress. For the fasting treatments, the food supply was removed, leaving only 5 µm filtered flowing seawater and aeration in the tanks. From each treatment, subsamples of ~130 mg of juvenile mussels (wet weight), equivalent to ~100 mg of fresh tissue, were collected in 1.8 ml cryo-vials, flash frozen in liquid nitrogen and stored at −80°C until analyses. Subsamples were collected for proximate composition analyses (protein, lipid and carbohydrate), gene expression of *hsp70*, oxidative damage analyses (PCs, LPs, 8-OHdG) and antioxidant analyses (i.e. SOD, CAT, GPx, GR).

2.1.2 Heat shock

To thermally stress the juvenile mussels, an aluminium block holding 20 ml glass scintillation vials was connected to a water bath set at 27°C. Scintillation vials were filled with 5 µm filtered seawater 1 h prior to the thermal stress to allow the water to reach 27°C. Juvenile mussels (~130 mg) from each fasting treatment and replicate tank were then placed in the scintillation vials at 27°C for 2 h (sub-lethal heat shock). After this time, the samples were placed into 1.8 ml cryo-vials, flash frozen and stored at −80°C until analyses. Mortality was not assessed in this experiment because all juvenile mussels were sampled for analyses. However, the treatments were considered to be sub-lethal based on our personal observations, therefore mortality was likely to have been negligible due to the treatments applied in this experiment.

2.2. Macromolecule extractions and proximate composition

Proximate analyses (i.e. water content, ash, total organic matter [TOM], lipid, protein and carbohydrate) were quantified in different subsamples of ~130 mg of pooled juvenile mussels. The ash and TOM content of the mussels coming from the different treatments was quantified first by oven-drying the mussels at 100°C until constant weight was achieved (24 h) to measure the water content. Then the dried mussels were placed in a furnace for 6 h at 450°C to ensure complete combustion of organic matter; the TOM content was then calculated by subtracting the ash weight from the dried weight (Byers et al. 1978). Both ash and TOM content were expressed as a proportion of the dry weight of juvenile mussels.

Protein for proximate analyses were extracted by adding 900 µl of ice-cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM magnesium chloride, 5 mM monosodium phosphate, 40 mM HEPES; pH 7.4) with the addition of a protease inhibitor cocktail (cOmplete™, EDTA-free; Roche) to the highest concentration according to manufacturer's recommendations. Each sample was then homogenized for 30 s at 1500 rpm (1600 MiniG®, SPEX®) using zirconia/silica beads (Biospec) and a pre-chilled (−80°C) cryo-block (SPEX®). After homogenization, the sample was centrifuged for 15 min at 17 000 × *g* at 4°C (Prism™R; Labnet). The supernatant was then collected, placed in a new 1.5 ml microtube (Eppendorf) and stored at −80°C until further analysis. Total protein was determined using the bicinchoninic acid method for a 96 well plate assay following manufacturer's instructions (Sigma-Aldrich). Samples were analysed in duplicate, incubated at 37°C for 30 min inside a plate reader (EnSpire; Perkin Elmer) and absorbance measured at 562 nm. Protein content in each sample was calculated from a standard curve using BSA (0–2000 µg ml^{-1}; $r^2 = 0.996$) and expressed as a proportion of the dry weight of juvenile mussels.

Lipid for proximate analysis was extracted using a modified Bligh & Dyer (1959) solvent method by adding 600 µl of methanol:chloroform (2:1 v/v) and homogenized as described above. Homogenate was

left to stand for 5 min and 400 µl of chloroform were added and vortexed for 30 s. Then, 400 µl of distilled water (Milli-Q®; Merck) were added and the sample was mixed again for 30 s before centrifugation at 17000 × *g* (Prism R™; Labnet) at ambient temperature for 30 s to separate the aqueous/methanol phase from the chloroform phase. Finally, the chloroform phase (bottom layer) was removed and transferred to a new 1.5 ml microtube. The total amount of lipid was quantified gravimetrically by placing the lipid extract in a tared 4 ml glass tube; the extract was dried in a stream of N_2 gas and then the remaining dried lipid in the tube was weighed. Lipid content in each sample was expressed as a proportion of the dry weight of juvenile mussels.

Carbohydrate content for each sample (µg of carbohydrates per mg of ash-free dry weight) was calculated by subtracting the lipid and protein content from the TOM content and expressed as a proportion of the dry weight of juvenile mussels.

2.3. Oxidative damage

Total protein extraction for oxidative stress biomarkers was performed on ice by adding 900 µl of ice-cold enzyme extraction buffer (100 mM potassium phosphate [pH 7.5] containing 50 mM NaCl, 0.1 mM Na_2EDTA, 1% polyvinylpyrrolidone-40, 2 mM phenylmethylsulfonyl fluoride and 0.1% TritonX-100) and homogenized as described above. The supernatant (i.e. protein extract) was then purified using ultrafiltration and purification columns (AMICON) according to the manufacturer's instructions. The purified protein extract was then washed and reconstituted with 250 µl of 50 mM potassium phosphate buffer (pH 7.2), placed in a 1.5 ml microtube and stored at −80°C until analyses. Protein content was determined by the Lowry protein assay (Fryer et al. 1986), with samples being diluted as required with potassium phosphate buffer before analysis. The level of PCs in the reconstituted protein extracts were determined via reaction with 2.4-dinitrophenylhydrazine as described by Reznick & Packer (1994) and expressed as nmols of carbonyls per mg protein.

Total lipid extraction for oxidative stress biomarkers was carried out as described in Section 2.2, and the levels of LPs in the samples were determined by absorbance at 500 nm using the ferric thiocyanate method described by Mihaljević et al. (1996), adapted for measurement in a microtitre plate reader. A calibration curve with t-butyl hydroperoxide was used and the LPs content calculated as nmol of lipid hydroperoxide per g of fresh (wet) weight of juvenile mussels.

Assays for PCs and LPs were carried out using a Victor 1420 Multilabel plate reader (Perkin Elmer Wallac) fitted with a temperature control cell (set to 25°C) and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer).

DNA extraction was performed by using an ISOLATE II Genomic DNA Kit (Bioline), following the manufacturer's instructions with one minor modification. After addition of the pre-lysis buffer, the samples were crushed and homogenized using a tube pestle. The final DNA extracts were placed in a 1.5 ml Eppendorf tube and stored at −80°C until analyses. The level of oxidised DNA was calculated by quantifying the amount of 8-OHdG present using HPLC followed by UV detection of guanine and electrochemical detection (coulometric) of 8-OHdG as described previously (Gale et al. 2014), with minor modifications.

2.4. Enzymatic antioxidants

Total protein extracted for PCs measurement (as outlined in Section 2.3) was used to perform antioxidant enzyme assays for SOD, CAT, GPx and GR activity. SOD was determined using a Cayman Chemicals superoxide dismutase assay kit, according to the manufacturer's instructions, and the activity was expressed as units of SOD per mg of protein. CAT was assayed using the chemiluminescent method of Maral et al. (1977), as adapted by Janssens et al. (2000) for 96 well microplates, and the activity expressed as µmol per min per mg of protein. Briefly, 50 µl of extract, diluted extract or standard (purified bovine liver CAT; Sigma-Aldrich, in homogenization buffer) was mixed with 100 µl of 100 mM phosphate buffer (pH 7.0) containing 100 mM of Na_2EDTA and 50 µl of 1 mM H_2O_2. Samples were then incubated at 25°C for 30 min, after which 50 µl of a solution containing 20 mM luminol and 11.6 U ml^{-1} of horseradish peroxidase (Sigma-Aldrich) was injected into each well. Light emission was proportional to the amount of H_2O_2 remaining in the mixture. CAT activities were calculated using the standard detailed above.

GPx activity was measured according to the spectrophotometric method described by Paglia & Valentine (1967) and expressed as nmol per min per mg of protein. Briefly, 20 µl of extract, diluted extract or standard (GPx purified from bovine erythrocytes; Sigma-Aldrich) was mixed with 170 µl of assay buf-

fer. The assay buffer contained 50 mM of Tris-HCl buffer (pH 7.6), 5 mM of Na_2EDTA, 0.14 mM of NADPH, 1 mM of glutathione (GSH) and 3 U ml^{-1} of GR (from wheat germ; Sigma-Aldrich). The reaction was initiated by the addition of 20 µl of t-butyl hydroperoxide to give a final concentration of 0.2 mM. The consumption of nicotinamide adenine dinucleotide phosphate was monitored at 340 nm (A_{340}) every 30 s for 3 min, with the plate shaken automatically before each reading. GPx activities were calculated using the standard detailed above.

GR activity was assayed using the method of Cribb et al. (1989) with minor modifications (Gale et al. 2014) and activity expressed as nmol per min per mg of protein. Briefly, 50 µl of extract, diluted extract or standard (GR from wheat germ; Sigma-Aldrich, in homogenization buffer) was mixed with 150 µl of 100 mM sodium phosphate buffer (pH 7.6) containing 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 10 µl of NADPH (10 mg ml^{-1}; 12 mM). The reaction was initiated by the injection of 10 µl of oxidized glutathione (1 mg ml^{-1}; 3.25 mM) and the absorbance at 415 nm (A_{415}) was measured every 30 s for 3 min, with the plate shaken automatically before each reading. GR activities in the extracts were calculated using the standard detailed above.

All enzymatic assays were carried out using a Perkin Elmer Wallac Victor 1420 multilabel counter as detailed above (Section 2.3).

2.5. *Hsp70* gene expression

Total RNA was isolated by adding 1 ml of TriReagent® (Sigma-Aldrich) solution to the ZR BashingBead lysis tubes (0.1 and 0.5 mm; Zymo Research) containing the juvenile mussel samples. The samples were then homogenized at 1500 rpm for 90 s (1600 MiniG®, SPEX®) and centrifuged for 30 s at 3000 × *g* at 4°C. RNA was extracted following the manufacturer's instructions and then purified using a RNeasy kit (Qiagen). The quality and purity of the isolated RNA in all samples were checked using a spectrophotometer (Eppendorf). Trace DNA molecules carried over in RNA extracts were eliminated by 2 sequential DNase treatments as described in Langlet et al. (2013). To confirm the absence of DNA in RNA eluents, the droplet digital PCR (ddPCR) assays (*18S* and *28S*) described below were run on each RNA sample after DNase treatment. The DNase-treated RNA was transcribed into cDNA, using the SuperScript® III reverse transcriptase (Life Technologies). The various extract products (RNA and cDNA) were separated into aliquots and stored at −20°C (for cDNA) and −80°C (for pure RNA and DNase-treated RNA samples) until analyses.

The ddPCR was conducted in an automated droplet generator (QX200 Droplet Digital PCR System™; BioRad) to determine the relative expression of *hsp70*. Copy numbers of the gene were measured in all samples using primers specific to *P. canaliculus* with reference to known *hsp70* as designed by Callander (2012): *hsp70* forward primer, 5′-TTG AGT TGA CAG GAA TCC CAC C-3′; *hsp70* reverse primer, 5′-TTC TTT GCT TAG TCG TCC TTT GTC-3′. Expression of 2 stable reference genes, *18S* and *28S*, was also quantified (primer sequences acquired from Callander 2012).

Each ddPCR reaction included 1 µl of 450 nM of each primer, 10 µl BioRad ddPCR Supermix for Evagreen, 1 µl DNA and sterile water for a total reaction volume of 21 µl. The BioRad QX200 droplet generator partitioned each reaction mixture into nanodroplets by combining 20 µl of the reaction mixture with 70 µl of BioRad droplet oil. After processing, this resulted in a total nanodroplet volume of 40 µl, which was transferred to a PCR plate for amplification using the following cycling protocol for *hsp70*: hold at 95°C for 3 min, 40 cycles of 95°C for 10 s, 57°C 10 s, 72°C for 20 s and a final enzyme deactivation step at 72°C for 5 min. For the reference genes (*18S* and *28S*), the amplification protocol followed a 3 min hold at 95°C, followed by 40 cycles of 95°C for 10 s, 56°C for 15 and 72°C for 20 s.

The plate was then analysed on the QX200 instrument. For each ddPCR plate run, at least one negative control (RNA/DNA-free water; Life Technologies), and one positive control (genomic DNA extracted from *P. canaliculus*) were included. Target gene expression was normalized over the geometric mean of *18S* and *28S* (Vandesompele et al. 2002). Results were then converted to *hsp70* relative copy number per ml using the following formula: number of copies per µl × 21 µl (the initial volume of the PCR reaction) × 60 µl (the final elution volume used).

2.6. Statistical analyses

All data were checked for normality and homoscedasticity using Shapiro-Wilks and Levene's tests, respectively. Proximate composition percent data were arcsine transformed before analysis. Water content data violated normality, but ANOVA was used for analysis since the data met the homoscedasticity assumption (Quinn & Keough 2002). Untransformed

Table 1. Proximate composition of *Perna canaliculus* juveniles exposed to different fasting treatments and a subsequent thermal stress. Ash, total organic matter (TOM), protein, lipid and carbohydrate are represented as percent of dry weight of juvenile mussels. All data correspond to mean percent ± SE (n = 3). Results of Tukey's pair-wise comparisons tests are shown as superscripts next to values; significant differences ($p < 0.05$) are denoted by different letters for the main effect of fasting and asterisks for the main effect of temperature. No significant interactions ($p > 0.05$) were observed for all proximate components

Treatment	Fasting (h)	Water %	Ash %	TOM %	Protein %	Lipid %	Carbohydrate %
Control temperature (20°C)	0	74.9 ± 0.6	81.3 ± 0.8^{a}	18.7 ± 0.8^{a}	3.4 ± 0.4^{a*}	2.9 ± 0.2*	12.4 ± 0.4^{a}
	6	74.2 ± 0.5	80.1 ± 1.3^{a}	20.0 ± 1.3^{a}	3.0 ± 0.1^{a*}	3.6 ± 0.3*	13.3 ± 1.4^{a}
	24	74.9 ± 0.6	85.3 ± 0.9^{b}	14.7 ± 0.9^{b}	2.5 ± 0.2^{b*}	3.2 ± 0.5*	9.0 ± 0.2^{b}
	54	76.4 ± 0.6	86.2 ± 0.6^{b}	13.8 ± 0.6^{b}	2.5 ± 0.2^{b*}	2.2 ± 0.2*	9.2 ± 0.5^{ab}
Heat shock (27°C × 2 h)	0	76.4 ± 1.6	79.8 ± 1.2^{a}	20.2 ± 1.2^{a}	2.5 ± 0.1^{a}	2.5 ± 0.3	15.2 ± 0.9^{a}
	6	75.0 ± 1.4	81.0 ± 1.1^{a}	19.1 ± 1.1^{a}	2.8 ± 0.1^{a}	2.4 ± 0.1	13.9 ± 1.2^{a}
	24	77.3 ± 1.5	85.4 ± 2.8^{b}	14.6 ± 2.8^{b}	2.4 ± 0.1^{b}	1.8 ± 0.1	10.4 ± 2.9^{b}
	54	77.1 ± 1.0	83.8 ± 1.2^{b}	16.2 ± 1.2^{b}	2.2 ± 0.1^{b}	2.2 ± 0.5	11.8 ± 1.6^{ab}

data of *hsp70*, oxidative damage and antioxidant biomarkers were used in the analyses. All data were analysed with 2-way ANOVA using fasting time and temperature as fixed factors. In all analyses, significant differences among levels of a factor were identified using Tukey's pair-wise comparisons tests ($\alpha = 0.05$). Analyses were run using the statistical software Sigma Plot 14.0 (SYSTAT Software).

3. RESULTS

3.1. Proximate composition

Water content was ~76% overall across all juvenile mussels with no differences among treatments (Tables 1 & 2). Ash content increased by around 6% in the fasted juveniles exposed to both control temperature and heat shock (Tables 1 & 2). TOM followed the opposite trend, decreasing by around 24% in fasted juveniles (Tables 1 & 2).

Protein content in the juveniles changed significantly among fasting treatments and after temperature stress, with no significant interaction between factors (Tables 1 & 2). Juvenile mussels that were fed throughout the experiment (0 h of fasting) had, on average, 27% more protein content than juveniles that were fasted for 24 and 54 h ($p = 0.018$ and 0.005, respectively; Tables 1 & 2). Similarly, juveniles fasted for 6 h had 17% more protein than juveniles fasted for 24 and 54 h ($p = 0.039$ and 0.012, respectively; Tables 1 & 2). Protein content decreased on average 14% in juveniles that were exposed to a heat shock compared to those held at the control temperature ($p = 0.007$; Tables 1 & 2).

Lipid content in the juveniles did not vary among fasting treatments, but changed significantly after temperature stress, and no interaction between factors was observed (Tables 1 & 2). Lipid content of juveniles tended to decrease with increased fasting time, with juveniles fasted for 54 h having 24 and

Table 2. Statistical analyses of proximate composition data for *Perna canaliculus* juveniles exposed to different fasting treatments and a subsequent thermal stress. Significant p-values ($p < 0.05$) are shown in bold

	df	MS	*F*	p
Water				
Fasting	3	2.144	1.376	0.286
Temperature	1	5.396	3.464	0.081
Fasting × temperature	3	0.398	0.255	0.856
Residual	16	1.558		
Ash				
Fasting	3	26.084	7.522	**0.002**
Temperature	1	1.633	0.471	0.502
Fasting × temperature	3	2.039	0.588	0.632
Residual	16	3.468		
Total organic matter (TOM)				
Fasting	3	26.084	7.522	**0.002**
Temperature	1	1.633	0.471	0.502
Fasting × temperature	3	2.039	0.588	0.632
Residual	16	3.468		
Protein				
Fasting	3	2.068	8.319	**0.001**
Temperature	1	2.400	9.657	**0.007**
Fasting × temperature	3	0.515	2.071	0.144
Residual	16	0.249		
Lipid				
Fasting	3	2.306	2.467	0.100
Temperature	1	10.760	11.514	**0.004**
Fasting × temperature	3	2.116	2.265	0.120
Residual	16	0.935		
Carbohydrate				
Fasting	3	22.264	4.827	**0.014**
Temperature	1	14.462	3.135	0.096
Fasting × temperature	3	1.225	0.266	0.849
Residual	16	4.612		

12% lower lipid content compared to unfasted juveniles exposed to control temperature and after a heat shock, respectively (Table 1). On average, juveniles exposed to the control temperature had 27% more lipid than those that experienced a 27°C heat shock (p = 0.004; Tables 1 & 2).

Carbohydrate content in the juveniles was significantly different among fasting treatments, but it was not affected by subsequent temperature stress, and no interaction between factors was observed (Tables 1 & 2). Carbohydrate content in juveniles tended to decrease with fasting time, with juveniles fasted for 54 h having 26 and 22% lower carbohydrate content compared to unfasted juveniles exposed to control temperature and after a heat shock, respectively (Table 1). However, only juveniles that fasted for 24 h had an average of 30% less carbohydrate content than unfasted juveniles and juveniles that fasted for 6 h at both temperature treatments (p = 0.035 and 0.047, respectively; Tables 1 & 2).

3.2. Oxidative damage

PCs showed significant changes with fasting regime and subsequent temperature stress, with no interaction between factors (Table 3, Fig. 1A). On average, juveniles that were fasted for 54 h had 40% more PCs than juveniles that were either unfasted (0 h) or fasted for 6 and 24 h (p < 0.001 for all comparisons; Fig. 1A). Similarly, juveniles that were exposed to a heat shock had 20% more PCs compared to those held at the control temperature (p = 0.002; Fig. 1A).

LPs also showed significant differences among fasting regimes and subsequent temperature stress, but there was no interaction between factors (Table 3, Fig. 1B). Juveniles that had been fasted for 54 h had, on average, 19% more LPs than those fasted for 6 h (p = 0.021; Fig. 1B). Juvenile mussels exposed to a heat shock showed 26% more LPs than those held at the control temperature (p < 0.001; Fig. 1B).

DNA damage measured through the 8-OHdG showed significant differences among fasting treatments and subsequent temperature stress, with a significant interaction between factors (Table 3, Fig. 1C). At control temperature, fasting did not have a significant effect on 8-OHdG levels (Fig. 1C). However, 8-OHdG significantly increased by 24 and 22% after a heat shock in juveniles that had been fasted for 6 and 54 h, respectively, compared to the same fasting treatment at control temperature (6 h: p = 0.015; 54 h: p = 0.005; Fig. 1C). In addition, 8-OHdG levels were between 22 and 31% higher in juveniles that had been fasted for 54 h compared to juveniles that had been fasted for 0, 6 and 24 h following a temperature shock (p = 0.002, 0.027 and 0.022, respectively; Fig. 1C).

Table 3. Statistical analyses of *hsp70* gene expression, oxidative damage and antioxidant biomarkers data for *Perna canaliculus* juveniles exposed to different fasting treatments and a subsequent thermal stress. Significant p-values (p < 0.05) are shown in bold

	df	MS	*F*	p
Protein carbonyls (PCs)				
Fasting	3	260.624	24.343	**<0.001**
Temperature	1	142.009	13.264	**0.002**
Fasting × temperature	3	8.620	0.805	0.509
Residual	16	10.706		
Lipid hydroperoxides (LPs)				
Fasting	3	90.158	4.313	**0.021**
Temperature	1	901.233	43.108	**<0.001**
Fasting × temperature	3	8.292	0.397	0.757
Residual	16	20.906		
8-Hydroxydeoxyguanosine (8-OHdG)				
Fasting	3	30.685	6.435	**0.005**
Temperature	1	42.029	8.814	**0.009**
Fasting × temperature	3	19.823	4.157	**0.023**
Residual	16	4.768		
Superoxide dismutase (SOD)				
Fasting	3	50.226	5.209	**0.011**
Temperature	1	921.940	95.621	**<0.001**
Fasting × temperature	3	59.988	6.222	**0.005**
Residual	16	9.642		
Catalase (CAT)				
Fasting	3	26.766	3.611	**0.036**
Temperature	1	572.815	77.290	**<0.001**
Fasting × temperature	3	23.549	3.177	0.053
Residual	16	7.411		
Glutathione peroxidase (GPx)				
Fasting	3	58.457	14.403	**<0.001**
Temperature	1	500.415	123.294	**<0.001**
Fasting × temperature	3	24.823	6.116	**0.006**
Residual	16	4.059		
Glutathione reductase (GR)				
Fasting	3	2.153	12.288	**<0.001**
Temperature	1	12.717	72.565	**<0.001**
Fasting × temperature	3	1.367	7.801	**0.002**
Residual	16	0.175		
Hsp70				
Fasting	3	4.334	1.541	0.243
Temperature	1	37.460	13.315	**0.002**
Fasting × temperature	3	4.774	1.697	0.208
Residual	16	2.813		

3.3. Enzymatic antioxidants

SOD activities changed significantly with fasting treatment and subsequent temperature stress, with

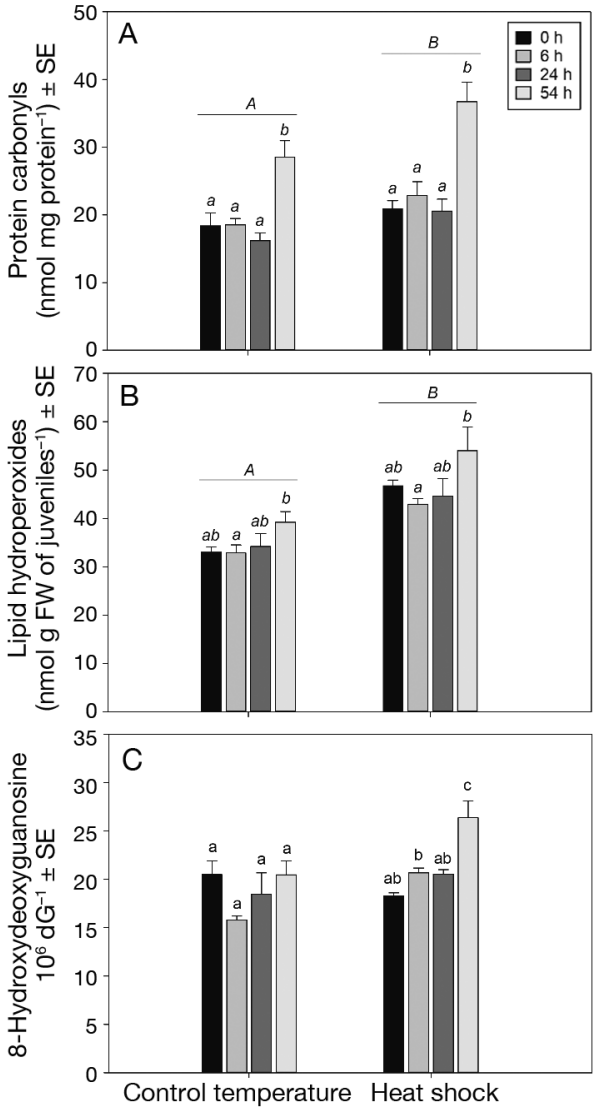

Fig. 1. Oxidative damage biomarkers of *Perna canaliculus* juveniles exposed to different fasting treatments (0, 6, 24 and 54 h) and a subsequent thermal stress. (A) Protein carbonyls; (B) lipid hydroperoxides; (C) 8-hydroxydeoxyguanosine (8-OHdG). Control temperature: 20°C; heat shock: 27°C for 2 h. Data correspond to mean concentration ± SE (n = 3). Tukey's pair-wise comparisons tests shown above bars ($p < 0.05$); significant differences in main effects of fasting and temperature are denoted respectively by different lower- and uppercase letters in italics above bars. Significant interactions ($p < 0.05$) terms in 8-OHdG are denoted by lowercase letters above bars. No significant interactions ($p < 0.05$) were observed for protein carbonyls and lipid hydroperoxides

a significant interaction among factors (Table 3, Fig. 2A). At the control temperature, fasting time did not affect the activity levels of SOD in juvenile mussels (Fig. 2A). However, a heat shock resulted in an increase in SOD activity levels in all fasting treatments (compared to the same fasting treatment in juveniles held at control temperatures), except in juveniles that fasted for 54 h (0 h: $p < 0.001$; 6 h: $p < 0.001$; 24 h: $p < 0.001$; 54 h: $p = 0.135$; Fig. 2A). In addition, juveniles from 0, 6 and 24 h of fasting showed an average of 29% greater SOD activity levels compared to juveniles that were fasted for 54 h following a temperature shock ($p = 0.006$, 0.005 and <0.001, respectively; Fig. 2A).

CAT activities changed significantly with fasting treatment and subsequent temperature stress, but no interaction between factors was observed (Table 3, Fig. 2B). CAT activity levels in unfasted juveniles were 23% greater than in juveniles fasted for 54 h ($p = 0.029$; Fig. 2B). In addition, CAT activity levels were, on average, 39% greater in juveniles that were exposed to a heat shock compared to those exposed to control temperatures only ($p < 0.01$; Fig. 2B).

GPx activities were significantly affected by fasting treatment and subsequent temperature stress, with a significant interaction between factors (Table 3, Fig. 2C). At control temperature, there were no differences in the activity levels of GPx among fasting treatments (Fig. 2C). In contrast, after the heat shock, juveniles from 0, 6 and 24 h of fasting showed 38% more GPx activity than juveniles that were fasted for 54 h ($p = 0.001$, 0.004 and <0.001, respectively; Fig. 2C). Juveniles that fasted for 54 h and where then exposed to a heat shock showed similar GPx activity levels to juveniles that were held at control temperature (Fig. 2C).

GR activities changed significantly with fasting treatment and subsequent temperature stress, with a significant interaction between factors (Table 3, Fig. 2D). At the control temperature, no significant differences were observed in GR activity levels among fasting treatments (Fig. 2D). By contrast, juveniles exposed to a heat shock had GR activity levels that were on average 43% greater in juveniles fasted for 0, 6 and 24 h compared to those fasted for 54 h ($p = 0.003$, <0.001 and 0.003, respectively; Fig. 2D).

3.4. *Hsp70* gene expression

Expression of the gene *hsp70* was only significantly affected by temperature (Table 3). Fasting treatments had no significant effects on gene expression and no significant interactions were observed between factors (Table 3). Expression of *hsp70* showed high variability (Fig. 3) and was significantly upregulated when juveniles were exposed to the heat shock, having on average 25% more *hsp70* copies than juveniles exposed to control temperature ($p = 0.002$;

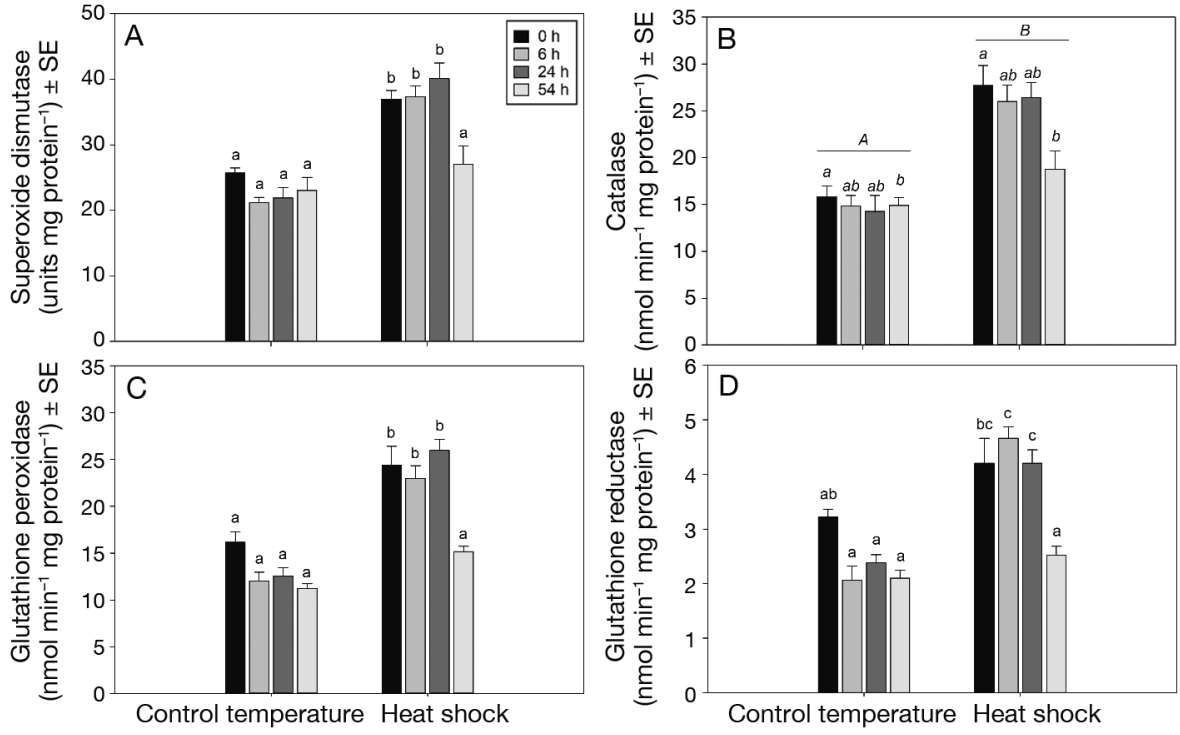

Fig. 2. Antioxidant biomarkers of *Perna canaliculus* juveniles exposed to different fasting treatments (0, 6, 24 and 54 h) and a subsequent thermal stress. (A) Superoxide dismutase (SOD); (B) catalase (CAT); (C) glutathione peroxidase (GPx); (D) glutathione reductase (GR). Control temperature: 20°C; heat shock: 27°C for 2 h. Data corresponds to mean enzyme activity ± SE (n = 3). Tukey's pair-wise comparisons tests shown above bars ($p < 0.05$); significant differences in main effects of fasting and temperature are denoted respectively by different lower- and uppercase letters in italics above bars. Significant interactions ($p < 0.05$) terms in SOD, GPx and GR are denoted by lowercase letters above bars. No significant interactions ($p < 0.05$) were observed in CAT concentrations

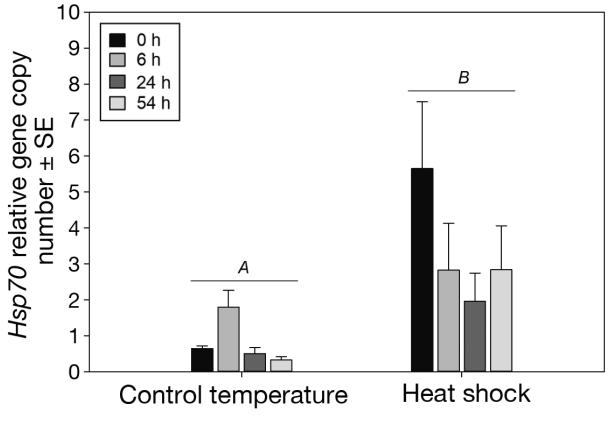

Fig. 3. *Hsp70* gene expression of *Perna canaliculus* juveniles exposed to different fasting treatments (0, 6, 24 and 54 h) and a subsequent thermal stress. Control temperature: 20°C; heat shock: 27°C for 2 h. Target gene expression was normalized over the geometric mean of *18S* and *28S*. Data corresponds to mean relative gene copy number ± SE (n = 3). Tukey's pair-wise comparisons tests shown above bars ($p < 0.05$); significant differences in main effects of temperature are denoted by different uppercase letters in italics above bars. No significant differences among fasting treatments and no significant interactions ($p < 0.05$) were observed for *hsp70* gene expression

Fig. 3). Although not significant, *hsp70* copy numbers tended to decrease with fasting (Fig. 3). Gene expression at the control temperature tended to increase during the first 6 h of fasting but then decreased by 49% in juveniles fasted for 54 h compared to those fasted for 0 h (Fig. 3). A similar trend was observed for juveniles exposed to the heat shock, where juveniles fasted for 54 h had 51% less upregulation of the *hsp70* gene compared to the juveniles fasted for 0 h (Fig. 3).

4. DISCUSSION

This study showed that different environmental stressors can have cumulative negative impacts on the physiology of a marine mussel under a stress-on-stress scenario. Increases in the duration of fasting periods caused increases in markers of oxidative stress, decreases in energy-rich proximate components and antioxidant activity in juvenile *Perna canaliculus*, indicating that even relatively short periods (54 h) of food deprivation are important stressors.

Further, the capacity of juvenile mussels to respond to subsequent stress was significantly modified by their fasting regime, with juveniles fasted for 54 h showing increased production of oxidative stress markers and an apparent inability to increase antioxidant activity.

In the wild, marine animals are naturally exposed to fluctuations in food availability (Chang et al. 2003). In this study, short-term fasting (up to 54 h) decreased the content of protein and carbohydrate in juvenile *P. canaliculus* to a similar extent (26% after 54 h of fasting). During periods of food scarcity, marine bivalves reduce their metabolism to basal levels and use their body storage reserves to fuel critical biological functions (Albentosa et al. 2007). This study suggests that protein and carbohydrate reserves may play an important role in maintaining basal metabolism during short-term (54 h) fasting in juvenile *P. canaliculus*, and are in accordance with longer studies that found carbohydrates, followed by proteins and lipids, to be the main source of energy during fasting in bivalves (Hawkins et al. 1985, Albentosa et al. 2007, Cordeiro et al. 2016). Acute heat stress decreased the protein and lipid content of juvenile *P. canaliculus*, indicating that reserves might also be used as fuel to cover energetic costs associated with a short-term stress response. However, it is possible that this pattern of reserve use changes depending on life stage and sex, as has been suggested for other bivalve species (Albentosa et al. 2007, da Costa et al. 2012). The juveniles used in the present study had previously been subjected to grading within a commercial hatchery and were therefore of a reasonably homogenous size. However, it would be a useful refinement for future studies to consider possible differences in mass gain, even during short-term trials, as this would allow not only assessment of acute growth responses but also the determination of absolute levels of each proximate component.

Fasting for 54 h reduced energy reserves in juvenile *P. canaliculus* and reduced resilience to additional stress. Food limitation can be energetically costly and impact protective mechanisms in many organisms (Somero 2002). For example, fasting decreased the ability of the gastropod *Concholepas concholepas* to upregulate and synthesize the protein hsp70 in response to fluctuations in seawater temperature or emersion (Jeno & Brokordt 2014). Similarly, long-term fasting reduced levels of the non-enzymatic antioxidant GSH in the juveniles of the crayfish *Cherax quadricarinatus* (Sacristán et al. 2016). It is likely that the greater oxidative damage observed after 54 h of fasting in juvenile *P. canaliculus* is a consequence of the lower protective capacity associated with reduced antioxidant enzyme activity (Cubillos et al. 2016, Lister et al. 2017, Delorme et al. 2019a,b). Further studies that correlate cellular damage, protective capacity and resilience across gradients of commonly occurring underlying stressors, such as food limitation, would provide much needed mechanistic insight into the capacity to respond to acute stress.

The antioxidant system of juvenile *P. canaliculus* did not respond to fasting regardless of its duration. Rather, heat shock was the most important trigger of antioxidant activity in this study. At shorter time scales (6–24 h), the *P. canaliculus* antioxidant system was balanced with fasting, neither increasing oxidative damage nor eliciting increased antioxidant activity in fasted and fed groups. However, this consistent pattern of antioxidant activity was disrupted by 54 h of fasting, when juveniles failed to increase antioxidant capacity that likely led to the increased ROS damage in these animals. The finding that juveniles that fasted for 24 h had similar antioxidant activity to fed mussels following heat shock suggests there is a clear window during which juvenile mussels are resilient to additional stressors. This is vital information for aquaculture and hatchery operations and suggests that stressful processes, such as transfer among growing systems or sites, should be carried out within 24 h of the last opportunity to feed, while the juveniles' antioxidant systems are in equilibrium.

The expression levels of *hsp70* were not affected by fasting up to 54 h in juvenile *P. canaliculus*. *Hsp70* has been widely considered to be a good general stress marker (Sørensen 2010, Morris et al. 2013, Valenzuela-Castillo et al. 2019). However, caution on the use of *hsp70* as a sole biomarker in stress studies is recommended because of tissue-specific upregulation and variation in timing of upregulation after stress exposure (Buckley et al. 2006, Fabbri et al. 2008, Morris et al. 2013). Gill tissue has been shown to have larger and more rapid upregulation compared to other tissues (Buckley et al. 2006, del Rey et al. 2011, Morris et al. 2013, Aleng et al. 2015). Thus, tissue homogenates of very small organisms, like the juvenile *P. canaliculus* used in this study, may mask significant *hsp70* tissue-specific upregulation (Morris et al. 2013). Future studies in *P. canaliculus* and other marine invertebrates should aim to determine expression levels of *hsp70* in different sized individuals exposed to longer starvation times, using larger replication and a suite of biomarkers, as presented in this study, in order to better understand the effect that certain stressors have on the organisms.

Overall, this study showed that fasting in juvenile *P. canaliculus* has detrimental effects on their biochemical and molecular performance and their ability to withstand additional stress. The long-term effects of stressors, such as fasting and subsequent temperature stress tested here, on the physiological performance (e.g. retention) and survival of juvenile *P. canaliculus* remain unknown and require further research. If survival of the juvenile mussels is not compromised under a stress-on-stress scenario, like the one presented in this work, it is possible that cellular damage, together with the poor nutritional status of the mussels, could have carry-over effects influencing their overall performance and behaviour later in life. Determining the relative and cumulative impacts of multiple stressors is critical to understanding the performance of juvenile mussels in the contexts of the wild population, aquaculture farming and a changing global climate.

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