J Comp Physiol B (2017) 187:87–101
DOI 10.1007/s00360-016-1023-z

ORIGINAL PAPER

Responses to elevated CO_2 exposure in a freshwater mussel, *Fusconaia flava*

Jennifer D. Jeffrey[1] · Kelly D. Hannan[1] · Caleb T. Hasler[1] · Cory D. Suski[1]

Received: 7 April 2016 / Revised: 29 June 2016 / Accepted: 19 July 2016 / Published online: 29 July 2016

Abstract Freshwater mussels are some of the most imperiled species in North America and are particularly susceptible to environmental change. One environmental disturbance that mussels may encounter that remains understudied is an increase in the partial pressure of CO_2 (pCO_2). The present study quantified the impacts of acute (6 h) and chronic (up to 32 days) exposures to elevated pCO_2 on genes associated with shell formation (chitin synthase; *cs*) and the stress response (heat shock protein 70; *hsp70*) in *Fusconaia flava*. Oxygen consumption (MO_2) was also assessed over the chronic CO_2 exposure period. Although mussels exhibited an increase in *cs* following an acute exposure to elevated pCO_2, long-term exposure resulted in a decrease in *cs* mRNA abundance, suggesting that mussels may invest less in shell formation during chronic exposure to elevated pCO_2. In response to an acute elevation in pCO_2, mussels increased *hsp70* mRNA abundance in mantle and adductor muscle and a similar increase was observed in the gill and adductor muscle in response to a chronic elevation in pCO_2. A chronic elevation in pCO_2 also increased mussel MO_2. This overall increase in *hsp70* mRNA levels and MO_2 in *F. flava* indicates that exposure to elevated pCO_2 initiates activation of the general stress response and an increased energy demand. Together, the results of the present study suggest that freshwater mussels respond to elevated pCO_2 by increasing processes necessary to 'deal with' the stressor and, over the long-term, may reduce their investment in non-essential processes such as shell growth.

Keywords Chitin synthase · Heat shock protein 70 · Metabolic rate · Bivalve

Communicated by H.V. Carey.

✉ Jennifer D. Jeffrey
jjeffrey@illinois.edu

[1] Department of Natural Resources and Environmental Science, University of Illinois at Urbana-Champaign, 1102 South Goodwin Avenue, Urbana, IL 61801, USA

Introduction

Freshwater mussels have their highest abundance and diversity in North America, and provide many important ecological functions (Williams et al. 1993; Bogan 2008). For example, freshwater mussels filter large volumes of water daily, remove bacteria and particles from the water column, and generate nutrient-rich areas (Vaughn and Hakenkamp 2001; Hauer and Lamberti 2007). In addition, freshwater mussels provide an important resource as food for other aquatic and terrestrial animals (Vaughn and Hakenkamp 2001; Hauer and Lamberti 2007). Notably, freshwater mussel populations are on the decline, in both species richness and biomass (Williams et al. 1993; Lydeard et al. 2004; Regnier et al. 2009). Alterations in flow regimes, land-use changes, invasive species such as zebra mussels, and climate change are all thought to have contributed to these declines (Strayer et al. 2004; Vaughn 2010). With only a small percentage of stable freshwater mussel populations remaining (Williams et al. 1993) and continued degradation of freshwater ecosystems, there is an increased need to understand the vulnerabilities of these animals to environmental stressors, and the mechanisms underlying their physiological responses to these stressors (e.g., Jeffrey et al. 2015).

One environmental stressor that is currently understudied in the freshwater environment is the impact of elevations in the partial pressure of carbon dioxide (pCO_2). In the context

of climate change and rising atmospheric CO_2, the impact of ocean acidification (i.e., elevated pCO_2) on marine calcifying organisms has been investigated to a large extent (reviewed by Fabry et al. 2008; Gazeau et al. 2013); however, virtually northing is known about the responses of freshwater bivalves to increased pCO_2 (Hasler et al. 2016). Upon entering freshwater, CO_2 results in a decrease in water pH due to the production of carbonic acid (H_2CO_3), leading to the release of H^+ and, thus, the weak acidification of water. Levels of freshwater pCO_2 can vary for a variety of reasons including, terrestrial productivity, precipitation, and local geology (Cole et al. 1994; Maberly 1996; Butman and Raymond 2011), resulting in CO_2 levels that can fluctuate both seasonally and daily, and that can exceed atmospheric levels (i.e., water bodies can be supersaturated with CO_2). River environments can thus experience a wide range of pCO_2 over the course of a year (from less than 100 to over 15,000 µatm), with higher values being observed in warmer, dryer periods (Cole and Caraco 2001). Although less well understood than for the marine environment, freshwater pCO_2 may increase as a result of increased atmospheric CO_2, greater terrestrial primary productivity, increased precipitation, and longer periods of dry conditions—although the magnitude of change is not known (Phillips et al. 2015; Hasler et al. 2016; Perga et al. 2016). Levels of freshwater CO_2 can also be intentionally elevated in the context of generating non-physical barriers to deter the movement of invasive fishes (Noatch and Suski 2012). The form that such a non-physical barrier may take has not yet been well defined, but CO_2 levels would likely dissipate as the distance from the CO_2 infusion site increases, thus mussels may be exposed to a gradient of CO_2 depending on their proximity to the barrier. Together, freshwater mussels may experience periods of elevated pCO_2 due to both natural and anthropogenic sources, and with pCO_2 expected to rise in the future, this necessitates a need for a better understanding of the consequences for freshwater mussels.

In the marine environment, a major consequence of exposure to elevated pCO_2 for bivalves is a reduction in both shell growth and biomineralization (reviewed by Gazeau et al. 2013). The mollusc shell provides an important external structure to support living tissues, protect against predators, and exclude mud and sand from the mantle cavity of burrowing species (Gazeau et al. 2013). Changes in the integrity of the shell have occurred due to exposure to conditions of ocean acidification, and dissolution of the shell as a result can have consequences for the health and survival of bivalves (reviewed by Gazeau et al. 2013). The mantle, a thin secretory epithelial tissue lining the inner surface of the shell, is responsible for mollusc shell formation, and shell calcification occurs in a small compartment (i.e., extrapallial cavity) located between the calcifying outer mantle and the shell (Wilbur and Saleuddin 1983). The shell is comprised of a mineral phase (95–99 % predominantly calcium carbonate; $CaCO_3$) and an organic matrix (1–5 %). Chitin is an insoluble polysaccharide that forms the highly structured organic framework of mollusc shells within which $CaCO_3$ minerals are deposited (Weiner et al. 1984; Levi-Kalisman et al. 2001; Weiss and Schonitzer 2006). The enzymes involved in chitin synthesis are important not only for mechanical strength and toughness of the shell, but also for coordination of mineralization processes and shell formation (Schonitzer and Weiss 2007). Chitin synthase (CS) is a key enzyme involved in the synthesis of chitin (Fang et al. 2011), and inhibition of CS during early development has been shown to negatively affect rates of shell development, solubility of the shell, and survival in *Mytilus galloprovincialis* larvae (Schonitzer and Weiss 2007). Furthermore, changes in the mRNA abundance of *cs* occurred in mantle of adult *Laternula elliptica*, a marine bivalve, in response to changes in environmental pCO_2 (and thus pH) (Cummings et al. 2011). Due to its importance in the biological control of shell formation and evidence of its regulation in response to situations of ocean acidification, *cs* provides a useful target to assess the impact of environmental stressors, such as elevated pCO_2, on shell formation in adult freshwater mussels.

The impacts of elevated pCO_2 on other cellular functions, such as mediators of cellular stress, have also been investigated to some extent in marine bivalves (e.g., Cummings et al. 2011). Heat shock proteins (HSPs) are among the most evolutionarily conserved proteins, and are induced by a number of factors beyond heat-stress that affect cell protein structure and functioning (Feder and Hofmann 1999; Sørensen et al. 2003). A key role of HSPs is to protect and repair cellular proteins damaged by exposure to stressors, and to minimize protein aggregation (Feder and Hofmann 1999). Heat shock protein 70 (HSP70) is the most abundant family of HSPs, and consists of the constitutively expressed HSC70 and inducible HSP70 that are ubiquitously distributed in eukaryotic cells (Feder and Hofmann 1999). The key role of HSPs in mechanisms of cellular protection renders them good markers of the stress status of an organism. In this way, HSPs provide information about the general condition and health of an organism as well as the sub-lethal effects (i.e., early warning signs) of a stressor, before more complex functions are compromised (reviewed by Fabbri et al. 2008). The inducible HSP70 is widely up-regulated in response to a variety of stressors in bivalves (e.g., Franzellitti and Fabbri 2005; Toyohara et al. 2005; Cellura et al. 2006; Cummings et al. 2011; Chen et al. 2014; Luo et al. 2014) and may represent a useful biomarker in examining elevated CO_2 as a potential stressor in freshwater mussels.

In addition to the impacts of elevated pCO_2 on cellular function, elevations in pCO_2 also have the potential

to affect whole-animal energetics and metabolism. In response to acute hypercapnia, metabolic depression is an adaptive response used by shelled molluscs to conserve energy, and is likely driven by decreased extracellular pH (reviewed by Pörtner et al. 2004; Gazeau et al. 2013). Acute responses to hypercapnia are time-dependent however, and long-term depressions in metabolic rate (rate of oxygen consumption; MO_2) due to chronic hypercapnia can be lethal (Gazeau et al. 2013). Interestingly, although some studies have observed decreases in metabolic rate due to exposure to elevated pCO_2 (e.g., Michaelidis et al. 2005; Fernández-Reiriz et al. 2011; Liu and He 2012; Navarro et al. 2013), results from other studies on marine bivalves have been mixed (reviewed by Gazeau et al. 2013). For instance, elevations in MO_2 in response to increased pCO_2 have been observed (e.g., Beniash et al. 2010; Lannig et al. 2010; Thomsen and Melzner 2010; Cummings et al. 2011; Parker et al. 2012) suggesting that shelled molluscs may be able to at least partially compensate for the energetic costs of acidosis (Wicks and Roberts 2012; Gazeau et al. 2013). Moderate elevations in pCO_2 below 1200 µatm (approximately three times current ocean pCO_2), on the other hand, have had minimal effects on MO_2 (e.g., Matoo et al. 2013). To date, no studies have quantified the impact of elevated pCO_2 on the MO_2 of freshwater bivalves, which is an important biomarker of the overall energetic status of an organism.

The goals of the present study were to define the impacts of elevated pCO_2 on the biological control of shell formation, as well as stress status at the cellular and whole-animal levels, in freshwater mussels. To accomplish these goals, adult Wabash pigtoe mussels (*Fusconaia flava*) were first exposed to a short-term elevation in pCO_2 (6 h) that was followed by a 6-h post-stressor period at ambient conditions. In this experiment, mussels were exposed to either ambient (300 µatm), 15,000 or 200,000 µatm pCO_2. In a second experiment, and to quantify the impacts of an extended exposure to elevated pCO_2, *F. flava* were exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. In both experiments, *cs* mRNA levels and RNA:DNA ratio (as an indicator of total protein synthesis) were quantified in mantle tissue as indicators of shell formation, and *hsp70* mRNA levels were assessed across a number of tissues. In addition, MO_2 was assessed repeatedly in mussels exposed to the long-term CO_2 exposure.

Materials and methods

Experimental animals

Adult *F. flava* (wet mass, 27.0 ± 1.3 g; length, 50.2 ± 0.8 mm; mean ± standard error of the mean, SEM) were collected by benthic grab from Big Four Ditch, Paxton, IL. Mussels were transported in coolers (<1-h transport time) to the Aquatic Research Facility at the University of Illinois, Champaign-Urbana, IL, cleaned of epibionts, and individually tagged (Queen Marking Kit tags; The Bee Works, Orillia, ON, CA). Mussels were then placed into one of three 128.6 L recirculating systems, each consisting of three tanks with 5 cm of sand, supplied with water from a 0.04 ha naturalized, earthen-bottom pond, a UV Sterilizer (TMC Vecton 8 W, 11 L min^{-1} flow rate, Pentair, Apopka, FL, USA), a heater/chiller (TECO-US, Aquarium Specialty, Columbia, SC, USA), and a low pressure air blower (Sweetwater, SL24H Pentair, Apopka, FL, USA). Mussels were held for at least 1 week prior to the onset of experiments and were fed a commercial diet (*Nannochloropsis* sp. 1–2 microns and *Isochrysis*, *Pavlova*, *Thalassiosira*, and *Tertraselmis* spp. 5–12 microns; Instant Algae, Reed Mariculture Inc., Campbell, CA, USA) every other day for the duration of the study period, with the exception of the 48-h period prior to the onset of sampling. Dissolved oxygen (DO) and temperature were monitored daily with a portable meter (YSI 550A, Yellow Springs Instruments, Irvine, CA, USA) and averaged 8.0 ± 0.6 mg L^{-1} (mean ± SEM) and 17.5 ± 0.2 °C, respectively. Fifty percent water changes were performed weekly and no mussel mortality occurred throughout the experiment.

Short-term exposure to elevated pCO_2

Mussels ($N = 48$) were removed from recirculating systems and transferred to individual 0.71 l containers within a recirculating system. Each system consisted of a raceway with eight containers (i.e., held eight mussels at a time), and a central reservoir. Pond water was pumped from the central reservoir into individual containers and allowed to overflow into the raceway and return to the reservoir forming a closed system. Individual containers were supplied with an air stone to maintain DO levels. Temperature and DO were monitored (see above), and pH was measured using a handheld meter (WTW pH 3310 m, Germany), calibrated regularly throughout the study (Moran 2014). Free CO_2 and total alkalinity (TA) concentrations were measured using digital titration kits (Hach Company, Titrator model 16900, cat. no. 2272700 for CO_2 and cat. no. 2271900 for TA). Water temperature, TA, and pH values were then used to determine the pCO_2 in µatm using CO2calc where all default parameters were used with the exception that 'Set of constants' was set to 'Salinity = 0 (freshwater) K1; K2 from Millero 1979' (Robbins et al. 2010) (Table 1).

Following a 24-h period in individual containers, mussels were exposed to either ambient (273 ± 30 µatm), 15,000 µatm (14,772 ± 1685), or 200,000 µatm (188,114 ± 12,669) pCO_2 as described in Hannan et al.

Table 1 Water chemistry for short- and long-term pCO_2 exposures

Experiment	CO_2 level	Temperature (°C)	Dissolved O_2 (mg L^{-1})	pH	Total alkalinity (mg L^{-1})	Dissolved CO_2 (mg L^{-1})	pCO_2 (µatm)
Short term	Ambient (300 µatm)	17.8 ± 0.2	8.98 ± 0.12	8.65 ± 0.05	211 ± 5	13.5 ± 0.5	272.8 ± 30.2
	15,000 µatm	17.9 ± 0.25	8.76 ± 0.26	6.99 ± 0.02	202.6 ± 14.6	36.9 ± 4.7	14,772.6 ± 1685.3
	200,000 µatm	17.8 ± 0.2	8.56 ± 1.21	6.01 ± 0.01	268 ± 12	255.5 ± 0.5	188,114 ± 12,669.6
Long term	Ambient (1000 µatm)	17.6 ± 0.2	8.05 ± 0.04	8.42 ± 0.02	211.1 ± 2.3	16.2 ± 0.7	994.2 ± 61.8
	20,000 µatm	17.5 ± 0.2	7.99 ± 0.07	7.24 ± 0.06	255.1 ± 8.8	40.79 ± 3.1	22,712.0 ± 2482.5

Data are presented as mean ± SEM

(2016). A pCO_2 of 200,000 µatm was chosen as it represents a conservative target for a non-physical fish barrier due to its efficacy at deterring the movement of several juvenile fish species, including bigheaded carp (Kates et al. 2012). A second CO_2 level of 15,000 µatm was chosen as this level may be expected downstream of a CO_2 barrier and thus affect mussels not residing immediately within the CO_2 addition zone. A level of 15,000 µatm may also potentially arise in some freshwater systems as a result of climate change (Phillips et al. 2015; Hasler et al. 2016; Perga et al. 2016). Target CO_2 levels were achieved by the common method of bubbling compressed CO_2 gas (commercial grade, 99.9 % purity) into the central reservoir through an air stone (Riebesell et al. 2010). Levels of CO_2 were maintained within the central reservoir with a pH controller (PINPOINT®, American Marine Inc., CT, USA) that adds CO_2 if the pH rises above a target level (7.00 ± 0.10 and 6.00 ± 0.10 pH, for 15,000 and 200,000 µatm treatment, respectively) during the exposure period. Mussels were exposed to one of the three CO_2 treatments for 6 h and sampled either directly following the 6-h treatment, or after being held for an additional 6 h at ambient conditions (i.e., recovery period). Mussel wet mass (24.8 ± 1.5 g) and length (49.2 ± 1.0 mm), were measured as well as the dry mass of the soft tissue (1.05 ± 0.06 g); see 'Oxygen consumption') and no significant effect of CO_2 treatment or time-point were found (see Hannan et al. 2016). Mussels ($N = 8$) were sampled for mantle, gill, foot, and adductor muscle tissues that were placed in 1 ml of RNAlater Stabilization Solution (Ambion, cat. no. AM7020, Life Technologies, Carlsbad, CA, USA) and stored overnight at 4 °C prior to storage at −80 °C.

Long-term exposure to elevated pCO_2

Mussels ($N = 48$) were separated into one of two recirculating systems (as described above) and held at either ambient (994 ± 62 µatm) or 20,000 µatm (22,712 ± 2482 µatm) pCO_2 for 4, 8, and 32 days. Note that the difference in the ambient CO_2 levels between the short- and long-term exposures was due to natural fluctuations in the CO_2 levels of the pond water. Target CO_2 pressures were again achieved by bubbling CO_2 gas into the reservoir through an air stone, and maintained using a pH controller set to a pH of 7.20 ± 0.10, as described above. A level of 20,000 µatm was chosen as this may represent a level that mussels downstream of a CO_2 barrier may experience, and it was unknown whether mussels would survive long-term exposure to a higher level of CO_2 (i.e., like the upper level used in the 'short-term' experiment). Water quality measurements including temperature, DO, pH, alkalinity, concentration of CO_2, pCO_2 in µatm (calculated by CO2Calc) were monitored using the same methods as described in the short-term experiment (Table 1). Mussels ($N = 8$) were sampled as above after 4, 8, and 32 days of exposure to either ambient or elevated CO_2 conditions. No significant effect of CO_2 treatment or time-point were found for wet mass (25.4 ± 1.7 g) and length (49.7 ± 1.0 mm), as well as the dry mass of the soft tissue (1.02 ± 0.06 g) (see Hannan et al. 2016).

RNA and first-strand cDNA synthesis

Total RNA was extracted from 50 to 100 mg of tissue using TRI Reagent (Ambion, cat. no. AM9738, Life Technologies) according to the manufacturer's protocol. Tissues were disrupted and homogenized with a mechanical homogenizer (Tissue-Tearor®, Biospec Products Inc., model no. 935370, Bartlesville, OK, USA). Extracted RNA was quantified using a Nanodrop ND-1000 UV–Vis spectrophotometer (Peqlab, Erlangen, Germany) and 1 µg of RNA was treated with deoxyribonuclease I (Amplification Grade, DNase; cat. no. 18068015, Invitrogen, Life Technologies). To synthesize cDNA, MultiScribe Reverse Transcriptase, RNase inhibitor, and random primers were used according to the manufacturer's protocol (High-Capacity cDNA Reverse Transcription kit; Applied Biosystems, cat. no. 4374966, Life Technologies).

Gene sequences

For the purpose of developing primers for quantitative real-time RT-PCR (qPCR; see below), partial sequences were generated for *cs*, *hsp70*, and *18s* from cDNA synthesized from mantle tissue. Gene-specific primers (Table 2) were designed based on conserved regions of sequences from several bivalve species using Primer3plus (primer3plus.com). For *18s*, primer forward 2 was nested within the product from primers forward 1 and reverse 1 to extend the sequence; a single set of primers was sufficient to generate a partial sequence for *hsp70* and *cs*. Primers for *cs* were based on conserved regions in *Atrina rigida* (DQ081727), *L. elliptica* (HQ186262), *M. galloprovincialis* (EF535882), *Pinctada fucata* (AB290881), and *Septifer virgatus* (AB613818). Primers for *hsp70* were based on conserved regions in *Argopecten irradians* (AY485261), *A. purpuratus* (FJ839890), *Chlamys farreri* (AY206871), *Corbicula fluminea* (KJ461738), *Crassostrea ariakensis* (AY172024), *C. gigas* (AF144646), *C. hongkongensis* (FJ157365), *C. virginica* (AJ271444), *Cristaria plicata* (HQ148706), *Hyriopsis cumingii* (KJ123764), *L. elliptica* (EF198332), *Meretrix meretrix* (HQ256748), *Mizuhopecten yessoensis* (AY485262), *M. coruscus* (KF322135), *M. galloprovincialis* (AB180909), *P. fucata* (EU822509), *Paphia undulata* (JX885711), *Pteria penguin* (EF011060), *Ruditapes philippinarum* (KJ569079), *Sinonovacula constricta* (JF748730), and *Tegillarca granosa* (JN936877). Primers for *18s* were based on conserved regions in *Anodonta cygnea* (AM774476), *Elliptio complanata* (AF117738), *Lampsilis cardium* (AF120537), *Psilunio littoralis* (AF120536), and *Unio pictorum* (AM774477).

All PCR reactions were performed using an Eppendorf Mastercycler. Reaction compositions (total volume 25 µl) were as follows; 2 µl cDNA, 0.2 µM primer, and 5 µl Taq 5X Master Mix (cat. no. M0258L, New England BioLabs, Ipswich, MA, USA). In each case, cycling conditions were 95 °C (30 s), 55 °C (30 s), and 68 °C (30 s) for 38 cycles. Resulting amplicons were run on 1.5 % agarose gels with ethidium bromide and extracted using a QIAquick gel extraction kit (cat. no. 28704, QIAGEN, Valencia, CA, USA). Amplicons were cloned using a PCR cloning kit (cat. no. 231122, QIAGEN) and Sub-cloning Efficiency DH5α Competent Cells (Invitrogen, cat. no. 18265017, Life Technologies) following the manufacturers' protocols, with the exception that cloning reactions were scaled to 5 µl rather than 10 µl. Plasmids were extracted using a QIAprep Spin Miniprep Kit (cat. no. 27104, QIAGEN) and were sequenced by Core DNA Sequencing Facility (University of Illinois at Urbana-Champaign, Urbana, IL, USA) resulting in partial sequences: 712 bp for *cs* (KX342020), 958 bp for *hsp70* (KX342019), and 1240 bp for *18s* (KX342024). These partial sequences were sufficient to generate primers for qPCR (see below). Partial sequences were also generated for *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*; KX342023), *elongation factor 1-α* (*ef1-α*; KX342022), and *β-actin* (KX342021) for use as alternate normalization genes; however, *18s* was chosen as it varied the least across individuals and treatment groups when analyzed by qPCR.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was used to assess the relative abundance of *cs* and *hsp70* mRNA. Oligonucleotide primers were generated using Primer3plus (primer3plus.com) for the target genes as well as the reference gene *18s* (Table 2), and their specificity was verified by sequencing

Table 2 Oligonucleotide primer sets used for gene cloning and quantitative real-time RT-PCR (qPCR) in *Fusconaia flava*

Purpose	Gene	Primers (5′–3′)	Product size (bp)
Gene cloning	*cs*	Forward—TGT GCT ACA ATG TGG CAC GA	
		Reverse—TAC CAC ACC ATC GGA CCT GA	
	hsp70	Forward—CCA TTG CCT ATG GTC TGG A	
		Reverse—TTG CTG AGA CGA CCT TTG TC	
	18s	Forward 1—GGT TCC GCT GGT GAA TCT GA	
		Reverse 1—CAC CAC CCA CCG AAT CAA GA	
		Forward 2—CTT GGA TCG CCG TAA GAC GA	
		Reverse 2—CCT TCC GGG TAA GGG CAA AT	
qPCR	*cs*	Forward—GAG TCG ATT GGC CCA AGA CA	104
		Reverse—CCA CCT GTT CGT CGA GTT CA	
	hsp70	Forward—GAG CAT CAC CAG GGC AAG AT	103
		Reverse—TGG CTT GTC CAT CTT GGC AT	
	18s	Forward—GCT CGT AGT TGG ATC TCG GG	76
		Reverse—CCA GGA GGT AGG TCA GGA CA	

cs chitin synthase, *hsp70* heat shock protein 70

the product from each primer set. To optimize reaction compositions, standard curves were generated for each primer set using cDNA pooled from individuals across treatment groups (efficiencies were ≥0.85). Real-time PCR was carried out using RealMasterMix SYBR ROX (cat. no. 22008800, 5 Prime) and ABI 7900HT Fast Real-Time PCR System (Life Technologies) with the following cycling condition: 95 °C for 15 s and 60 °C for 60 s over 40 cycles. For all reactions, manufacturer's instructions were followed with the exceptions that reactions were scaled to 10 µl, of which 5 µl was the RealMasterMix SYBR ROX. For *cs* and *hsp70*, cDNA was diluted 20-fold, and 1000-fold for *18s*. Primer concentrations were 0.1 µM. The abundance of each gene was calculated relative to the 'control 6-h treatment' group for the short-term experiment and to the 'control 4 d' group for the long-term experiment using the modified delta–delta Ct method (Pfaffl 2001) with *18s* as the normalizing gene.

Ratio of RNA to DNA

The ratio of RNA:DNA has been used as a measure of protein synthesis and overall mussel health in previous studies (e.g., Norkko et al. 2006; Menge et al. 2007). In the present study, the ratio of RNA:DNA was assessed in the mantle tissue. Total RNA and DNA were extracted from the same piece of mantle tissue using the AllPrep DNA/RNA Mini Kit as in Tsangaris et al. (2010) following the manufacturer's protocol. Levels of RNA and DNA were then determined using a Qubit® 3.0 Fluoremeter (Fisher Scientific, Hanover Park, IL, USA) and expressed as the ratio of the RNA to DNA.

Oxygen consumption

An additional set of mussels ($N = 16$) from the 'long-term exposure to elevated pCO_2' experiment were assessed repeatedly for MO_2 at 4, 8, and 32 days of exposure. The MO_2 was determined using computerized intermittent-flow respirometry (Steffensen 1989). Briefly, the system consisted of four glass chambers (143 mm length × 45 mm diameter) that were each connected to two pumps, one for recirculation, and one for flushing ambient oxygenated water into the chamber. The total volume of the set-up, including the glass chamber and all associated tubing was 0.248 L. The MO_2 in each individual chamber was quantified within twelve 70 min cycles consisting of a 55 min measurement period, a 14 min flush period, and a 1 min wait period prior to commencing the subsequent cycle. The MO_2 (mg O_2 g^{-1} h^{-1}) for each mussel was calculated as:

$$MO_2 = \alpha\, V_{resp} \beta\, M_b^{-1}$$

where V_{resp} is the volume of each chamber minus the volume of the mussel (L), β is the oxygen solubility (adjusted daily for the barometric pressure and temperature), and M_b is the dry weight of the mussel (g; see below). For each trial, the coefficient of determination (r^2) for all slope measurements was >0.85. Calibration of the fiber optic oxygen probes with oxygen-free and fully saturated water was performed regularly throughout the experiments. Data were recorded using AutoResp software (Version 1.4), and background O_2 levels were collected and adjusted for during each trial (Steffensen 1989). At the completion of the 32 days repeated sampling period, mussel soft tissues were excised and dried at 99 °C (Widdows et al. 2002) for 24 h to determine dry weight.

Statistical analysis

The effects of CO_2 exposure on the mRNA abundance of *cs* and *hsp70* and the RNA:DNA ratio were assessed using a two-way analysis of variance (ANOVA) with pCO_2 level, sampling time, and their interaction (pCO_2 × sampling time) entered as fixed effects. If at least one of the main effects, or the interaction term, was significant, a Tukey–Kramer honestly significant difference (HSD) post hoc test was applied to separate means.

A general linear mixed effect model (GLMEM) was used to assess the impact of elevated pCO_2 on mussel MO_2 during long-term exposure. Main effects, including pCO_2 treatment, sampling time (4, 8, and 32 days), as well as the interactions of treatment × time, were treated as fixed effects, and mussel ID was treated as a random effect. The use of a random effect was necessary because multiple measurements were taken from each animal across trials, thus, each measurement was not independent (Laird and Ware 1982; Lindstrom and Bates 1990). The GLMEM was fit using 'lmer' from the R package lme4 (Bates 2010), and coefficients were estimated using restricted maximum likelihood. To define the importance of the fixed effects, the sim function ('arm' package in R) was used to generate $N = 1000$ posterior simulations of each fixed effect. The resulting posterior distribution of effect estimates were evaluated and those that did not overlap zero at the 95 %-level were considered significant.

For all statistical analyses, a visual analysis of fitted residuals using a normal probability plot (Anscombe and Tukey 1963) and a Shapiro–Wilk normality test were used to assess normality. A Levene's test, in combination with visual inspection of fitted residuals, was used to assess the homogeneity of variances. If either the assumption of normality or the homogeneity of variance were violated, data were ranked transformed and run with the same parametric model provided that the assumptions were met (Conover and Iman 1981; Iman et al. 1984; Potvin and Roff 1995).

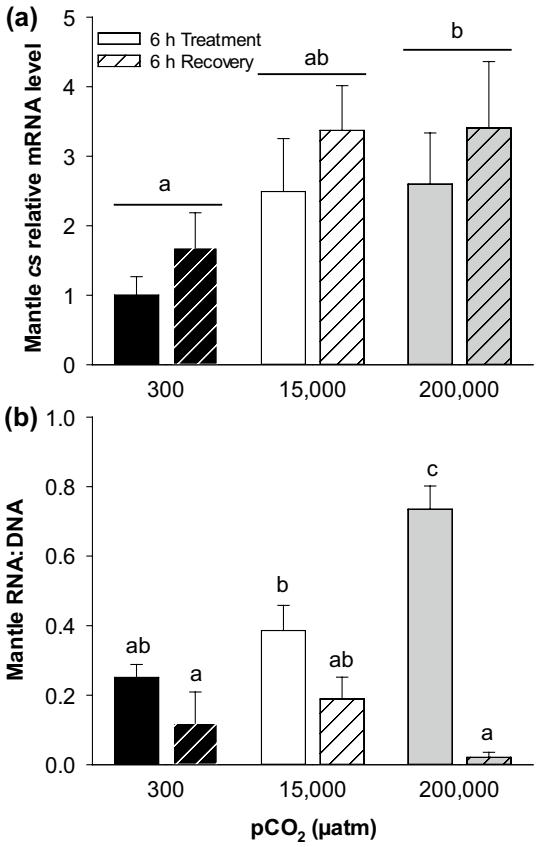

Fig. 1 Mantle (**a**) relative mRNA abundance of *chitin synthase* (*cs*) and **b** ratio of RNA to DNA in *Fusconaia flava* exposed to a short-term elevation in pCO_2. Mussels were exposed to one of three treatments: ambient (300 µatm), 15,000 or 200,000 µatm pCO_2 for 6 h followed by exposure to 6 h at ambient conditions (recovery). Data are presented as mean ± SEM ($N = 7$–8). All mRNA data were normalized to the mRNA abundance of *18s* and expressed relative to the 6-h ambient (300 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **a**, only a significant effect of pCO_2 treatment occurred, with no significant effect of sampling time (two-way ANOVA; see Table 3)

Statistical analyses were performed using R version 3.2.2 and the level of significance (α) was 0.05.

Results

The effect of exposure to a short-term elevation in pCO_2

Mantle *cs* mRNA abundance was significantly increased by the exposure to a short-term elevation in pCO_2 relative to controls for those mussels exposed to the highest pCO_2 (200,000 µatm), with no significant effect of sampling time (Fig. 1a; Table 3). In mantle tissue, the RNA:DNA ratio was also significantly elevated by 6 h of exposure to the highest pCO_2 (200,000 µatm) relative to mussels held at ambient conditions, levels that returned to control levels following 6 h at ambient conditions (300 µatm; Fig. 1b; Table 3).

Mantle and adductor muscle *hsp70* mRNA levels were not affected following 6 h of exposure to 15,000 µatm pCO_2, but these levels were significantly elevated 6 h post-stressor (Fig. 2a, b; Table 3). Exposure to the highest pCO_2 (200,000 µatm) for 6 h resulted in a significant elevation in *hsp70* mRNA levels in the adductor muscle relative to mussels held at ambient pCO_2, which subsequently returned to control levels after an additional 6-h period at ambient conditions (300 µatm; Fig. 2b; Table 3). In the gill and foot, *hsp70* mRNA levels were largely unaffected by short-term exposure to elevated pCO_2 (Fig. 2c, d; Table 3), with the exception that exposure to 200,000 µatm pCO_2 caused a decrease in *hsp70* mRNA levels in gill that returned to control levels following 6 h at ambient conditions (300 µatm; Fig. 2c; Table 3).

The effect of exposure to a long-term elevation in pCO_2

Contrary to the impact of a short-term elevation in pCO_2, *cs* mRNA levels were significantly reduced during long-term exposure to 20,000 µatm pCO_2 in CO_2-treated mussels relative to control mussels (held at 1000 µatm), with no significant effect of sampling time (Fig. 3a; Table 4). Additionally, the ratio of RNA:DNA in mantle tissue was relatively unaffected by CO_2 treatment at 20,000 µatm and was only significantly elevated after 8 days of treatment compared to control mussels at 4 days, but not compared to control mussels held for the same duration (i.e., 8 days; Fig. 3b; Table 4).

Long-term exposure to 20,000 µatm pCO_2 resulted in an overall increase in *hsp70* mRNA levels in the gill of CO_2-treated mussels relative to mussels held at ambient conditions, with no significant effect of sampling time (Fig. 4c; Table 4). Similarly, *hsp70* mRNA levels were significantly elevated by CO_2-treatment at 4 and 8 days in the adductor muscle; however, after 32 days of exposure to elevated pCO_2 these levels were no longer different from mussels held at 1000 µatm for the same period of time (Fig. 4b; Table 4). Mantle and foot *hsp70* mRNA levels were not significantly affected by long-term pCO_2 treatment (Fig. 4a, d; Table 4).

Exposure to 20,000 µatm pCO_2 also resulted in an increase in MO_2 compared to mussels held at ambient conditions (1000 µatm), with no significant effects of sampling time (Fig. 5; Table 5).

Discussion

The present study provides evidence that exposure to elevations in pCO_2 may result in changes in shell formation

Table 3 Results of two-way ANOVA for the effects of short-term exposure to elevated pCO_2 in *Fusconaia flava*

Variable	Main effects	Degrees of freedom	Sum of squares	*F* value	*P*
Mantle *cs*	pCO_2	2	65.70	3.817	**0.030**
	Time	1	17.50	2.032	0.161
	pCO_2 × time	2	0.20	0.010	0.990
Mantle RNA:DNA	pCO_2	2	0.28	4.736	**0.014**
	Time	1	1.46	50.137	**<0.001**
	pCO_2 × time	2	0.80	13.806	**<0.001**
Mantle *hsp70*	pCO_2	2	7.13	4.216	**0.022**
	Time	1	1.25	1.484	0.230
	pCO_2 × time	2	6.43	3.802	**0.031**
Adductor hsp70	pCO_2	2	1916.40	13.415	**<0.001**
	Time	1	491.60	6.882	**0.012**
	pCO_2 × time	2	2396.3	16.773	**<0.001**
Gill *hsp70*	pCO_2	2	3.63	7.625	**0.002**
	Time	1	0.01	0.036	0.851
	pCO_2 × time	2	1.64	3.432	**0.042**
Foot hsp70	pCO_2	2	239.00	0.658	0.523
	Time	1	523.00	2.877	0.097
	pCO_2 × time	2	434.00	1.193	0.314

cs chitin synthase, *hsp70* heat shock protein 70

Significant *P* values are bolded and variables in italics are run on ranks

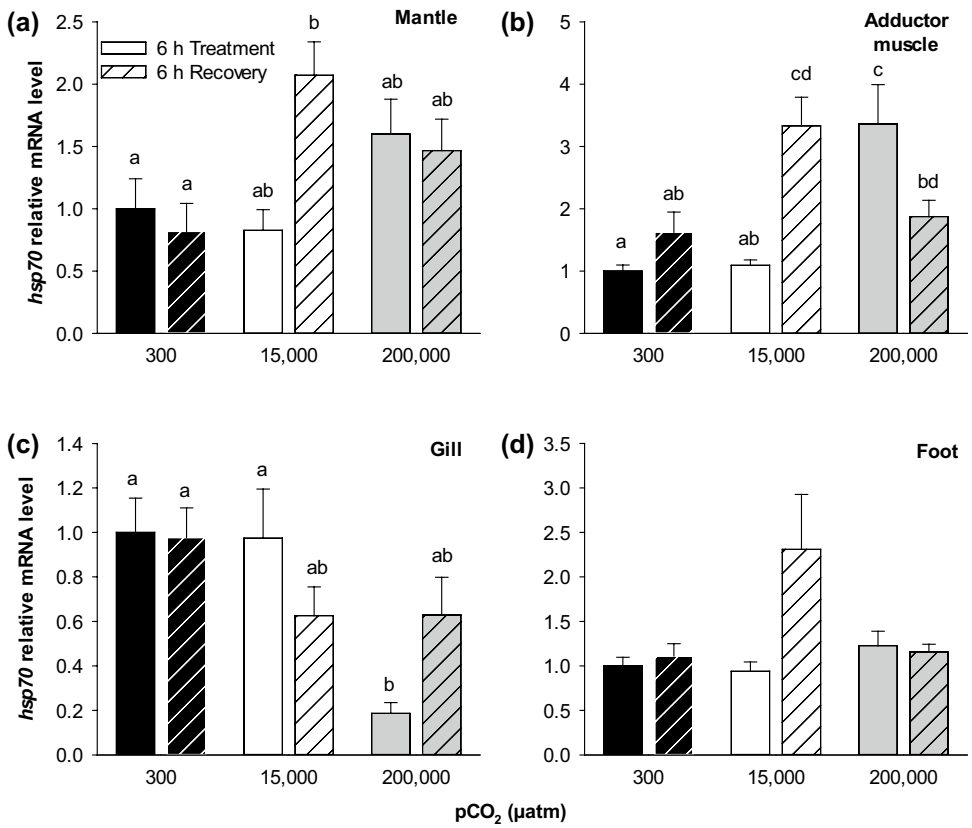

Fig. 2 Relative mRNA abundance of *heat shock protein 70* (*hsp70*) in the **a** mantle, **b** adductor muscle, **c** gill, and **d** foot of *Fusconaia flava* exposed to a short-term elevation in pCO_2. Mussels were exposed to one of three treatments: ambient (300 µatm), 15,000 or 200,000 µatm pCO_2 for 6 h followed by exposure to 6 h at ambient conditions (recovery). Data are presented as mean ± SEM ($N = 7$–8). All data were normalized to the mRNA abundance of *18s* and expressed relative to the 6-h ambient (300 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. Neither pCO_2 treatment nor sampling time had a significant effect on the *hsp70* mRNA level in the foot (two-way ANOVA; see Table 3)

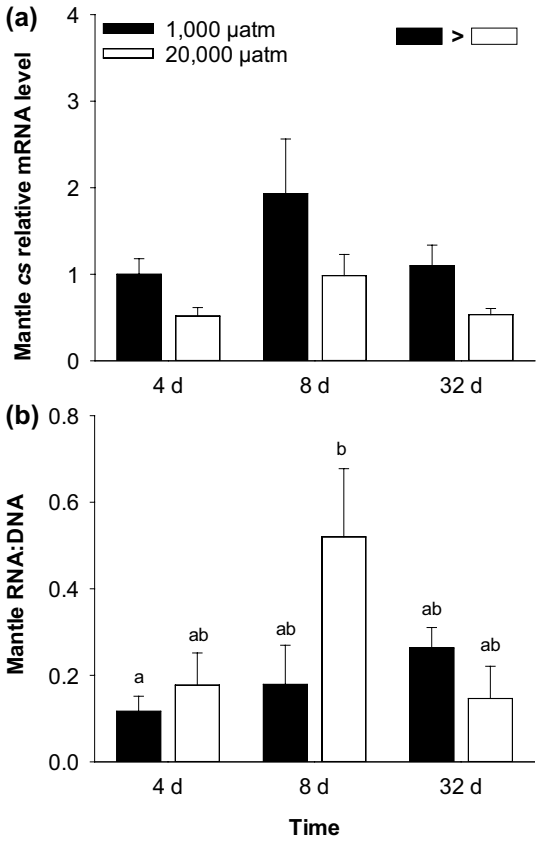

Fig. 3 Mantle (**a**) relative mRNA abundance of *chitin synthase* (*cs*) and **b** ratio of RNA to DNA in *Fusconaia flava* exposed to a long-term elevation in pCO_2. Mussels were exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. Data are presented as mean ± SEM ($N = 7$–8). All mRNA data were normalized to the mRNA abundance of *18s* and expressed relative to the 4 days ambient (1000 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **a** only a significant effect of pCO_2 treatment occurred, with no significant effect of sampling time (two-way ANOVA; see Table 4)

in freshwater mussels, particularly if the exposure is prolonged. Chitin plays an important structural role in the formation of mussel shells, and thus changes in the expression of *cs*, the enzyme that synthesizes chitin, may have consequences for shell formation (Weiss et al. 2006; Schonitzer and Weiss 2007). In the present study, short-term exposure to elevated pCO_2 resulted in an overall elevation in *cs* mRNA that was significant for mussels exposed to the highest pCO_2 (200,000 µatm). Previous work on the Antarctic bivalve, *L. elliptica* exposed to elevated pCO_2 (~800 µatm), but for a longer period of time (21 days), showed a similar increase in *cs* mRNA that was attributed to increased effort in the calcification of the shell (Cummings et al. 2011). Interestingly, in the present study, a longer exposure to elevated pCO_2 (up to 32 days) at 20,000 µatm, resulted in approximately a twofold decrease in *cs* mRNA levels compared to mussels held at ambient pCO_2 conditions (1000 µatm). It is possible that *F. flava* exposed to a relatively short elevation in pCO_2 (i.e., 6 h) may up-regulate processes to maintain normal shell formation in a compromising environment (i.e., elevated pCO_2 and concomitant decrease in pH), while prolonged exposure to pCO_2 may result in resources being diverted away from non-critical functions (e.g., shell formation) to other more vital functions (e.g., stress response). Additionally, as bivalves utilize $CaCO_3$ stores released from the shell to buffer against the acidosis experienced as a result of exposure to elevated pCO_2 (e.g., Crenshaw 1972; Michaelidis et al. 2005; Hannan et al. 2016), investing further in additional shell growth may be futile if shells are to be degraded to buffer acidosis. In addition to *cs*, the ratio of RNA to DNA in the mantle provides a measurement of total protein synthesis of processes likely to be associated with shell formation (Norkko et al. 2006). Similar to *cs* mRNA levels, the ratio of RNA to DNA in mantle was significantly elevated by short-term exposure to 200,000 µatm, but was largely unaffected by long-term exposure to 20,000 µatm pCO_2. Thus, processes associated with shell formation may be up-regulated during an acute exposure to elevated pCO_2, but not during an extended exposure to elevated pCO_2. Following a 6-h post-stressor period, mantle RNA:DNA fell to baseline levels, suggesting that this increase in protein synthesis in the mantle is transient. Though measurements of *cs* mRNA and mantle RNA:DNA are not direct assessments of calcification, changes in these factors may affect the structure and formation of the shell (Schonitzer and Weiss 2007; Cummings et al. 2011; Fang et al. 2011). The results of the present study provide evidence for regulation of the biological control of shell formation in response to changes in pCO_2, and provide potential biomarkers for further assessments of biomineralization in freshwater mussels.

The induction of HSPs by environmental stimuli has been widely documented, and the HSP70 family is frequently used as an indicator of the physiological mechanisms used by bivalves (and other animals) to cope with environmental disturbances (reviewed by Fabbri et al. 2008). The occurrence of the inducible *hsp70* mRNA under unstressed conditions is consistent with the ability of mussels to thrive in environments with fluctuating physical and chemical variables (Franzellitti and Fabbri 2005). In the present study, *hsp70* mRNA was present in *F. flava* held at control/ambient conditions in all of the tissues examined (e.g., gill, adductor muscle, mantle, and foot). This basal transcript level of *hsp70* is in agreement with the expression profiles of other bivalve species (e.g., Franzellitti and Fabbri 2005; Cellura et al. 2006; Cummings et al. 2011; Chen et al. 2014; Ivanina et al. 2014) and may help to minimize the effects of moderate environmental stressors. The synthesis of *hsp70* transcripts can be further increased in response to increased cellular stress, although this appears

Table 4 Results of two-way ANOVA for the effects of long-term exposure to elevated pCO_2 in *Fusconaia flava*

Variable	Main effects	Degrees of freedom	Sum of squares	*F* value	*P*
Mantle cs	pCO_2	1	1323	9.914	**0.003**
	Time	2	446	1.670	0.201
	pCO_2 × time	2	257	0.962	0.391
Mantle RNA:DNA	pCO_2	1	77	0.479	0.493
	Time	2	385	1.205	0.310
	pCO_2 × time	2	1624	5.081	**0.011**
Mantle hsp70	pCO_2	1	70	0.368	0.547
	Time	2	26	0.069	0.934
	pCO_2 × time	2	111	0.294	0.747
Adductor hsp70	pCO_2	1	4144	42.920	**<0.001**
	Time	2	248	1.284	0.288
	pCO_2 × time	2	765	3.960	**0.027**
Gill *hsp70*	pCO_2	1	15.245	44.204	**<0.001**
	Time	2	0.379	0.550	0.581
	pCO_2 × time	2	0.847	1.228	0.303
Foot hsp70	pCO_2	1	280	1.791	0.189
	Time	2	805	2.573	0.090
	pCO_2 × time	2	408	1.306	0.283

cs chitin synthase, *hsp70* heat shock protein 70

Significant *P* values are bolded and variables in italics are run on ranks

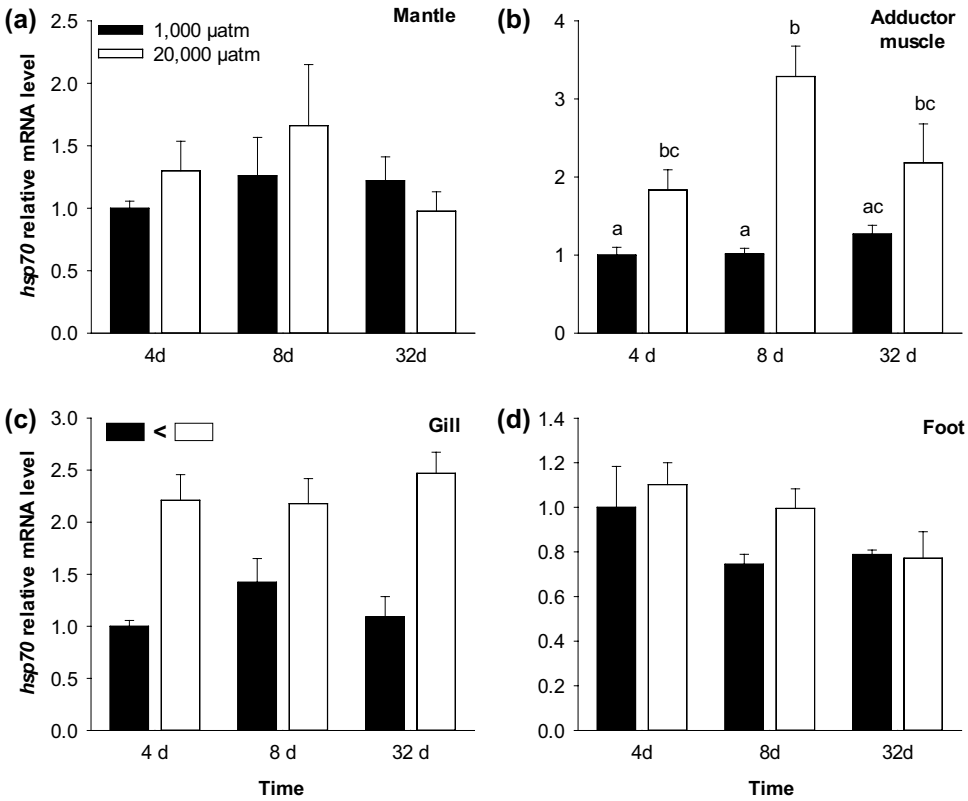

Fig. 4 Relative mRNA abundance of *heat shock protein 70* (*hsp70*) in the **a** mantle, **b** adductor muscle, **c** gill, and **d** foot in *Fusconaia flava* exposed to a long-term elevation in pCO_2. Mussels were exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. Data are presented as mean ± SEM ($N = 8$). All data were normalized to the mRNA abundance of *18s* and expressed relative to the 4 days ambient (1000 µatm) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **c**, only a significant effect of pCO_2 treatment occurred, with no significant effect of sampling time (two-way ANOVA; see Table 4). Neither pCO_2 treatment nor sampling time had a significant effect on the *hsp70* mRNA level in the mantle or foot (two-way ANOVA; see Table 4)

to occur in a tissue-, time-, and stressor-specific manner in bivalves (reviewed by Fabbri et al. 2008), as was observed in the present study.

The dynamics of *hsp70* mRNA expression were dependent on the severity of the CO_2 stressor during an acute exposure. An increase in *hsp70* mRNA levels in response

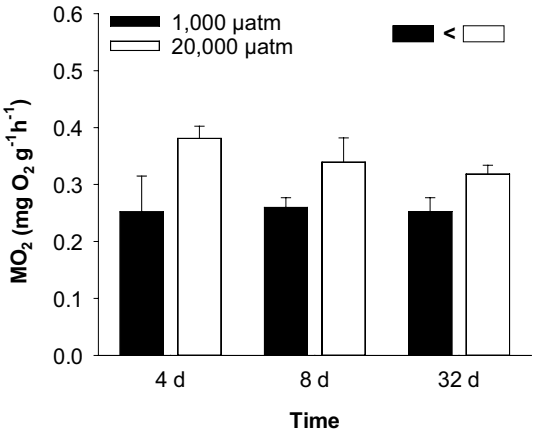

Fig. 5 Oxygen consumption (MO_2) of *Fusconaia flava* exposed to either ambient (1000 µatm) or 20,000 µatm pCO_2 for 4, 8, or 32 days. Mussels were repeatedly sampled for MO_2 over the course of the long-term exposure to elevated pCO_2. Data are presented as mean ± SEM ($N = 7$–8). Only a significant effect of pCO_2 occurred, with no significant effect of sampling time (general linear mixed effect model; see Table 5)

Table 5 Results of the general linear mixed effect model used to evaluate the effect of long-term pCO_2 exposure on oxygen consumption rate (MO_2) in *Fusconaia flava*

Parameter	Mean	95 % credible interval
Intercept (Ambient, 4 days)	0.25	0.18, 0.32
pCO_2 treatment	0.13	**0.02, 0.23**
8 days	0.01	−0.08, 0.11
32 days	0.00	−0.10, 0.10
pCO_2 × 8 days	−0.05	−0.19, 0.09
pCO_2 × 32 days	−0.06	−0.21, 0.08

to 6 h at 15,000 µatm pCO_2 was only evident in mantle and adductor muscle following an additional 6 h at ambient conditions (300 µatm). Thus, at moderate elevations in pCO_2 (15,000 µatm), a longer post-stressor period following an acute exposure may be necessary to visualize changes in *hsp70* mRNA levels and is in line with previous studies (e.g., Piano et al. 2004; Franzellitti and Fabbri 2005). Interestingly, although an increase in *hsp70* mRNA levels did not occur until 6 h post-stressor at 15,000 µatm, an increase in *hsp70* mRNA in the adductor muscle occurred following the 6-h CO_2 exposure at the highest CO_2 level of 200,000 µatm, and these levels began to return to pre-exposure levels following an additional 6 h at ambient conditions. The more rapid increase in *hsp70* mRNA levels in the adductor muscle (i.e., following the 6-h stressor) in response to 200,000 µatm compared to 15,000 µatm pCO_2 may reflect a response to a more severe stressor. In addition, in response to a higher level of CO_2 exposure (200,000 µatm), gill *hsp70* mRNA levels fell following the 6-h exposure period and began to increase to baseline levels 6 h post-stressor. A similar transient decrease in *hsp70* mRNA occurred in the digestive gland of *M. galloprovincialis* 1 h following an acute heat stressor, levels that subsequently increased above baseline 3 h post-stressor (Franzellitti and Fabbri 2005). It was suggested that the transient decrease in *hsp70* mRNA may reflect regulation of RNA metabolism during the heat shock response (Yost et al. 1990; Fabbri et al. 2008), and may help to explain the decrease in gill *hsp70* mRNA observed in the present study in response to the highest CO_2 exposure level (i.e., 200,000 µatm). The subsequent increase in *hsp70* mRNA to basal levels in the gill 6 h post-stressor may also reflect an increase in transcript production that may have exceeded control levels with a longer recovery period, as seen in previous studies (e.g., Piano et al. 2004; Franzellitti and Fabbri 2005). Together, these results suggest that acute exposure to elevated pCO_2 results in activation of the cellular stress response, and that this response is dependent on the tissue and severity of the stressor.

Chronic exposure to elevated pCO_2 also resulted in cellular responses to the stressor that were dependent on the tissue and duration of treatment. In gill, although *hsp70* mRNA levels were unaffected by short-term exposure to 15,000 µatm, they were significantly elevated throughout a 32-day exposure at 20,000 µatm. The delay in the increase of *hsp70* mRNA in response to elevations in pCO_2 further supports the idea that an extended exposure (or post-stressor period following a short-term stressor) may be necessary for this response to develop completely (reviewed by Fabbri et al. 2008). For the adductor muscle, a transient response was observed over the long-term exposure of *F. flava* to 20,000 µatm pCO_2, where *hsp70* mRNA levels were elevated at 4 and 8 days of CO_2 treatment but were no longer different from mussels held at ambient conditions for the same period of time at 32 days. A return to control levels of the mRNA abundance of *hsp70* at 32 days suggests that responses to a CO_2 stressor in the adductor muscle may be desensitized over a long-term exposure to elevated pCO_2. A similar transient response to Hg^{2+} exposure in *M. galloprovincialis* was observed, where *hsp70* mRNA in the digestive gland was elevated following 1 day of exposure but returned to basal levels by 6 days of exposure (Franzellitti and Fabbri 2005). In this study, Franzellitti and Fabbri (2005) found that mRNA abundances of the constitutive *hsc70* were inversely related to *hsp70*, and suggested that *hsp70* may be involved in the shorter-term response, whereas *hsc70* may be involved in longer-term cytoprotection (Franzellitti and Fabbri 2005). Although *hsc70* mRNA levels were not assessed in the present study, the potential short- and long-term roles of *hsp70* and *hsc70*, respectively, may also occur in the adductor muscle of *F. flava*

in response to elevations of pCO_2, though further investigation on this topic is required. Although mantle *hsp70* mRNA levels were elevated by short-term pCO_2 exposure at 15,000 µatm in *F. flava* (present study), and in the marine bivalve *L. elliptica* following 21 days at ~800 µatm (Cummings et al. 2011), exposure to 20,000 µatm for up to 32 days had no significant impact. Overall, exposure to elevated pCO_2 at any level or duration assessed in the present study had no effect on foot *hsp70* mRNA levels, indicating that the foot may be less affected, or is not responding in the same way as other tissues during CO_2 exposure. Together, the results of the present study suggest that *hsp70* responses to exposure to elevated pCO_2 are transient in some (e.g., mantle, adductor muscle) but not all tissues (e.g., gill), and some tissues may be more robust (e.g., foot) to changes in pCO_2. Although not consistent across all tissues and treatments, elevations in *hsp70* mRNA are indicative of an increase in the general stress response, a response that is likely to be energetically costly (Sørensen et al. 2003); however, an evaluation of variables beyond *hsp70* would be necessary to gain a more complete understanding of the stress status of mussels in response to elevated pCO_2 (e.g., glycogen stores, other HSPs, oxidative stress genes, etc.).

Whole-animal MO_2 was elevated in *F. flava* in response to prolonged exposure to elevated pCO_2. When exposed to 20,000 µatm pCO_2 for up to 32 days, mussels displayed an overall increase in MO_2, indicating that mussels were consuming more oxygen, and likely also expending more energy, to deal with CO_2 exposure. Previous work evaluating the consequences for elevated pCO_2 on marine bivalves found that increases in pCO_2 also caused elevations in MO_2 (e.g., Beniash et al. 2010; Lannig et al. 2010; Thomsen and Melzner 2010; Cummings et al. 2011; Parker et al. 2012). Increases in MO_2 are thought to occur due to a higher energy allocation to homeostasis (Beniash et al. 2010), and these increases may allow for quicker and more complete compensation of homeostatic disturbances induced by elevated pCO_2 (Parker et al. 2012). Increases in metabolic rate may be one of the mechanisms responsible for higher resilience to elevations in pCO_2 in some marine bivalves (e.g., oysters; Parker et al. 2012) and to support processes such as ion and acid–base regulation (e.g., Hannan et al. 2016), protein synthesis, and growth (Pörtner 2008). This increase in metabolic rate in *F. flava* may thus be adaptive, provided that food availability is not restricted, and metabolic rate is not elevated beyond a level that is sustainable. Interestingly, other studies assessing the consequences of pCO_2 exposure on marine bivalves found that increases in pCO_2 had either minimal effects on MO_2 levels (Matoo et al. 2013) or resulted in a decrease in MO_2 (e.g., Michaelidis et al. 2005; Fernández-Reiriz et al. 2011; Liu and He 2012; Navarro et al. 2013). The variation in the metabolic responses of marine bivalves may stem from differences in their resilience to changes in environmental pCO_2 (Parker et al. 2012). To our knowledge, the present study is the first to assess the impacts of elevated pCO_2 on MO_2 in a freshwater mussel. The increase in MO_2 observed in *F. flava* was in response to a much higher CO_2 level (i.e., 20,000 µatm, approximately 20 times the current mean pCO_2 in freshwater systems) (Cole et al. 2007) compared to previous exposures of marine bivalves. The capacity of *F. flava* to sustain increases in MO_2 over a period of at least 32 days suggests that freshwater mussels may be more robust to environmental changes in pCO_2 than marine mussels. This potential increase in CO_2 tolerance may have arisen in freshwater mussels due to their exposure to the natural fluctuations in pCO_2 that occur in freshwater systems (Maberly 1996; Hasler et al. 2016). The increase in MO_2 in *F. flava* during exposure to elevated pCO_2 may reflect an increase in the energy demand of processes involved in the stress response, that is also supported by the observed increases in *hsp70* mRNA levels, although the measurement of additional energetic parameters (e.g., metabolites) are necessary to support this hypothesis. Overall, the increase in MO_2 observed in *F. flava* suggests that freshwater mussels may be resilient to large elevations in pCO_2; however, it remains unclear whether mobilization of energy resources will be sufficient to support processes associated with the stress response necessary for survival, as well as growth and reproduction if exposures are prolonged (i.e., beyond 32 days), which may have population level consequences.

Results from the present study suggest that *F. flava* respond to both acute and extended elevations in pCO_2 by increasing processes related to the stress response. During short bouts of increased pCO_2, mussels attempt to maintain normal physiological functions such as shell formation. However, during extended exposure to elevated pCO_2, investing in processes such as shell formation may become less important, and mussels may divert limited resources away from non-vital functions to processes necessary for survival such as the stress response (present study) and acid–base balance (reviewed by Gazeau et al. 2013; e.g., Hannan et al. 2016). This diversion of limited resources may have long-term consequences for the survival and fitness of mussel populations that are already imperiled (reviewed by Pörtner et al. 2004). Encouragingly, the increase in MO_2 observed in *F. flava* in response to elevations in pCO_2 suggests that adult freshwater mussels may have the capacity to regulate in situations of elevated CO_2, at least in the short term (i.e., up to 32 days). The present study also provides potential targets for assessing the physiological status of mussels. For instance, the mantle tissue may provide a useful target for assessing the biological control of shell formation during both acute and chronic exposure to elevated pCO_2; although a broader picture of the mechanisms underlying this control would be gained by

assessing multiple genes (in addition to *cs*) associated with shell formation. In addition, although foot tissue provides a useful tissue for non-lethal sampling in assessments of physiological condition (e.g., Fritts et al. 2015), its use in assessing the impacts of elevated pCO_2 may be less informative compared to other tissues such as the gill and adductor muscle. Together, the data presented in the current study suggest that in future situations where pCO_2 elevations are expected to occur (e.g., due to increased atmospheric CO_2 or the deployment of a CO_2 fish barrier), mussels would be expected to have an increased energy demand, and respond in a way that is indicative of stress.

Acknowledgments This work was supported by the Illinois Department of Natural Resources, and the United States Geological Survey, through funds provided by the United States Environmental Protection Agency's Great Lakes Restoration Initiative. Funding was also provided by USFWS Federal Aid in Sport Fish Restoration Project F-69-R, and the USDA National Institute of Food and Agriculture Hatch project ILLU-875-947. J. Tiemann, K. Cummings, J. Tix, C. Sullivan, and J. Sherwood provided valuable help collecting mussels. We would also like to thanks A. Wright for providing valuable help with mussel husbandry and laboratory assistance, as well as S. Adhikari for her assistance with molecular techniques.

References

Anscombe FJ, Tukey JW (1963) The examination and analysis of residuals. Technometrics 5:141–160

Bates DM (2010) lme4: mixed-effects modeling with R

Beniash E, Ivanina A, Lieb NS, Kurochkin I, Sokolova IM (2010) Elevated level of carbon dioxide affects metabolism and shell formation in oysters *Crassostrea virginica* (Gmelin). Mar Ecol Prog Ser 419:95–108. doi:10.3354/meps08841

Bogan AE (2008) Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. Hydrobiologia 595:139–147. doi:10.1007/s10750-007-9011-7

Butman D, Raymond PA (2011) Significant efflux of carbon dioxide from streams and rivers in the United States. Nat Geosci 4:839–842. doi:10.1038/ngeo1294

Cellura C, Toubiana M, Parrinello N, Roch P (2006) HSP70 gene expression in *Mytilus galloprovincialis* hemocytes is triggered by moderate heat shock and *Vibrio anguillarum*, but not by *V. splendidus* or *Micrococcus lysodeikticus*. Dev Comp Immunol 30:984–997. doi:10.1016/j.dci.2005.12.009

Chen H, Zha J, Liang X, Li J, Wang Z (2014) Effects of the human antiepileptic drug carbamazepine on the behavior, biomarkers, and heat shock proteins in the Asian clam *Corbicula fluminea*. Aquat Toxicol 155:1–8. doi:10.1016/j.aquatox.2014.06.001

Cole JJ, Caraco NF (2001) Carbon in catchments: connecting terrestrial carbon losses with aquatic metabolism. Mar Freshw Res 52:101–110

Cole JJ, Caraco NF, Kling GW, Kratz TK (1994) Carbon dioxide supersaturation in the surface waters of lakes. Science 265:1568–1570

Cole JJ, Prairie YT, Caraco NF, McDowell WH, Tranvik LJ, Striegl RG, Duarte CM, Kortelainen P, Downing JA, Middelburg JJ, Melack J (2007) Plumbing the global carbon cycle: integrating inland waters into the terrestrial carbon budget. Ecosystems 10:172–185. doi:10.1007/s10021-006-9013-8

Conover WJ, Iman RL (1981) Rank transformations as a bridge between parametric and nonparametric statistics. Am Stat 35:124–129

Crenshaw MA (1972) The inorganic composition of molluscan extrapallial fluid. Biol Bull 143:506–512

Cummings V, Hewitt J, Van Rooyen A, Currie K, Beard S, Thrush S, Norkko J, Barr N, Heath P, Halliday NJ, Sedcole R, Gomez A, McGraw C, Metcalf V (2011) Ocean acidification at high latitudes: potential effects on functioning of the Antarctic bivalve *Laternula elliptica*. PLoS One 6:e16069. doi:10.1371/journal.pone.0016069

Fabbri E, Valbonesi P, Frazellitti S (2008) HSP expression in bivalves. Invert Surviv J 5:135–161

Fabry VJ, Seibel BA, Feely RA, Orr JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES Int Counc Explor Sea Mar Sci Symp 65:414–432

Fang D, Xu G, Hu Y, Pan C, Xie L, Zhang R (2011) Identification of genes directly involved in shell formation and their functions in pearl oyster, *Pinctada fucata*. PLoS One 6:e21860. doi:10.1371/journal.pone.0021860

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282

Fernández-Reiriz MJ, Range P, Álvarez-Salgado XA, Labarta U (2011) Physiological energetics of juvenile clams *Ruditapes decussatus* in a high CO_2 coastal ocean. Mar Ecol Prog Ser 433:97–105. doi:10.3354/meps09062

Franzellitti S, Fabbri E (2005) Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. Biochem Biophys Res Commun 336:1157–1163. doi:10.1016/j.bbrc.2005.08.244

Fritts AK, Peterson JT, Wisniewski JM, Bringolf RB (2015) Evaluation of methods for assessing physiological biomarkers of stress in freshwater mussels. Can J Fish Aquat Sci 72:1450–1459. doi:10.1139/cjfas-2014-0565

Gazeau F, Parker LM, Comeau S, Gattuso J-P, O'Connor WA, Martin S, Pörtner H-O, Ross PM (2013) Impacts of ocean acidification on marine shelled molluscs. Mar Biol 160:2207–2245. doi:10.1007/s00227-013-2219-3

Hannan KD, Jeffrey JD, Hasler CT, Suski CD (2016) Physiological effects of short and long-term hypercarbia on a freshwater mussel, *Fusconaia flava*. Can J Fish Aquat Sci

Hasler CT, Butman D, Jeffrey JD, Suski CD (2016) Freshwater biota and rising pCO_2? Ecol Lett 19:98–108. doi:10.1111/ele.12549

Hauer FR, Lamberti GA (2007) Methods in stream ecology, vol 2. Academic Press, Amsterdam

Iman RL, Hors SC, Conover WJ (1984) Comparison of asymptotically distribution-free procedures for the analysis of complete blocks. J Am Stat Assoc 79:674–685

Ivanina AV, Hawkins C, Sokolova IM (2014) Immunomodulation by the interactive effects of cadmium and hypercapnia in marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Fish Shellfish Immunol 37:299–312. doi:10.1016/j.fsi.2014.02.016

Jeffrey JD, Hasler CT, Chapman JM, Cooke SJ, Suski CD (2015) Linking landscape-scale disturbances to stress and condition of fish: implications for restoration and conservation. Integr Comp Biol 55:618–630. doi:10.1093/icb/icv022

Kates D, Dennis C, Noatch MR, Suski CD, MacLatchy DL (2012) Responses of native and invasive fishes to carbon dioxide: potential for a nonphysical barrier to fish dispersal. Can J Fish Aquat Sci 69:1748–1759. doi:10.1139/f2012-102

Laird NM, Ware JH (1982) Random-effects models for longitudinal data. Biometrics 38:963–974

Lannig G, Eilers S, Portner HO, Sokolova IM, Bock C (2010) Impact of ocean acidification on energy metabolism of oyster,

Crassostrea gigas—changes in metabolic pathways and thermal response. Mar Drugs 8:2318–2339. doi:10.3390/md8082318

Levi-Kalisman Y, Falini G, Addadi L, Weiner S (2001) Structure of the nacreous organic matrix of a bivalve mollusk shell examined in the hydrated state using cryo-TEM. J Struct Biol 135:8–17. doi:10.1006/jsbi.2001.4372

Lindstrom MJ, Bates DM (1990) Nonlinear mixed effects models for repeated measures data. Biometrics 46:673–687

Liu W, He M (2012) Effects of ocean acidification on the metabolic rates of three species of bivalve from southern coast of China. Chin J Oceanol Limnol 30:206–211. doi:10.1007/s00343-012-1067-1

Luo Y, Li C, Landis AG, Wang G, Stoeckel J, Peatman E (2014) Transcriptomic profiling of differential responses to drought in two freshwater mussel species, the giant floater *Pyganodon grandis* and the pondhorn *Uniomerus tetralasmus*. PLoS One 9:e89481. doi:10.1371/journal.pone.0089481

Lydeard C, Cowie RH, Ponder WF, Bogan AE, Bouchet P, Clark SA, Cummings KS, Frest TJ, Gargominy O, Herbert DG, Hershler R, Perez KE, Roth B, Seddon M, Strong EE, Thompson FG (2004) The global decline of nonmarine mollusks. Bioscience 54:321–330

Maberly SC (1996) Diel, episodic and seasonal changes in pH and concentrations of inorganic carbon in a productive lake. Freshw Biol 35:579–598

Matoo OB, Ivanina AV, Ullstad C, Beniash E, Sokolova IM (2013) Interactive effects of elevated temperature and CO_2 levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). Comp Biochem Physiol A Mol Integr Physiol 164:545–553. doi:10.1016/j.cbpa.2012.12.025

Menge BA, Daley BA, Sanford E, Dahlhoff EP, Lubchenco J (2007) Mussel zonation in New Zealand: an integrative eco-physiological approach. Mar Ecol Prog Ser 345:129–141. doi:10.3354/meps06951

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar Ecol Prog Ser 293:109–118

Millero FJ (1979) The thermodynamics of the carbonate system in seawater. Geochimica et Cosmochimica Acta 43(10):1651–1661

Moran D (2014) The importance of accurate CO_2 dosing and measurement in ocean acidification studies. J Exp Biol 217:1827–1828. doi:10.1242/jeb.104414

Navarro JM, Torres R, Acuna K, Duarte C, Manriquez PH, Lardies M, Lagos NA, Vargas C, Aguilera V (2013) Impact of medium-term exposure to elevated pCO_2 levels on the physiological energetics of the mussel *Mytilus chilensis*. Chemosphere 90:1242–1248. doi:10.1016/j.chemosphere.2012.09.063

Noatch MR, Suski CD (2012) Non-physical barriers to deter fish movements. Environ Rev 20:71–82. doi:10.1139/a2012-001

Norkko J, Thrush SF, Wells RMG (2006) Indicators of short-term growth in bivalves: detecting environmental change across ecological scales. J Exp Mar Biol Ecol 337:38–48. doi:10.1016/j.jembe.2006.06.003

Parker LM, Ross PM, O'Connor WA, Borysko L, Raftos DA, Pörtner H-O (2012) Adult exposure influences offspring response to ocean acidification in oysters. Glob Change Biol 18:82–92. doi:10.1111/j.1365-2486.2011.02520.x

Perga M-E, Maberly SC, Jenny J-P, Alric B, Pignol C, Naffrechoux E (2016) A century of human-driven changes in the carbon dioxide concentration of lakes. Glob Biogeochem Cycles 30:93–104. doi:10.1002/2015GB005286

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Phillips J, McKinley G, Bennington V, Bootsma H, Pilcher D, Sterner R, Urban N (2015) The potential for CO_2-induced acidification in freshwater: a great lakes case study. Oceanography 25:136–145. doi:10.5670/oceanog.2015.37

Piano A, Valbonesi P, Fabbri E (2004) Expression of cytoprotective proteins, heat shock protein 70 and metallothioneins, in tissues of *Ostrea edulis* exposed to heat and heavy metals. Cell Stress Chaperones 9:134. doi:10.1379/483.1

Pörtner H (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol Prog Ser 373:203–217. doi:10.3354/meps07768

Pörtner HO, Langenbuch M, Reipschläger A (2004) Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. J Oceanogr 60:705–718

Potvin C, Roff DA (1995) Distribution-free and robust statistical methods viable alternatives to parametric statistics. Ecology 76:2000

Regnier C, Fontaine B, Bouchet P (2009) Not knowing, not recording, not listing: numerous unnoticed mollusk extinctions. Conserv Biol 23:1214–1221. doi:10.1111/j.1523-1739.2009.01245.x

Riebesell U, Fabry VJ, Hansson L, Gattuso J-P (2010) Guide to best practices for ocean acidification research and data reporting. Publications Office of the European Union, Luxembourg

Robbins LL, Hansen ME, Kleypas JA, Meylan SC (2010) CO2calc: A user-friendly seawater carbon calculator for Windows, Mac OS X, and iOS (iPhone). US Geological Survey, Reston

Schonitzer V, Weiss IM (2007) The structure of mollusc larval shells formed in the presence of the chitin synthase inhibitor Nikkomycin Z. BMC Struct Biol 7:71. doi:10.1186/1472-6807-7-71

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6:1025–1037. doi:10.1046/j.1461-0248.2003.00528.x

Steffensen JF (1989) Some errors in respirometry of aquatic breathers: how to avoid and correct for them. Fish Physiol Biochem 6:49–59

Strayer DL, Downing JA, Haag WR, King TL, Layzer JB, Newton TJ, Nichols SJ (2004) Changing perspectives on pearly mussels, North America's most imperiled animals. Bioscience 54:429–439

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar Biol 157:2667–2676. doi:10.1007/s00227-010-1527-0

Toyohara H, Hosoi M, Hayashi I, Kibota S, Hashimoto H, Yokoyama Y (2005) Expression of HSP70 in response to heat-shock and its cDNA cloning from Mediterranean blue mussel. Fish Sci 71:327–332

Tsangaris C, Kormas K, Strogyloudi E, Hatzianestis I, Neofitou C, Andral B, Galgani F (2010) Multiple biomarkers of pollution effects in caged mussels on the Greek coastline. Comp Biochem Physiol C Comp Pharmacol 151:369–378. doi:10.1016/j.cbpc.2009.12.009

Vaughn CC (2010) Biodiversity losses and ecosystem function in freshwaters: emerging conclusions and research directions. Bioscience 60:25–35. doi:10.1525/bio.2010.60.1.7

Vaughn CC, Hakenkamp CC (2001) The functional role of burrowing bivalves in freshwater ecosystems. Freshw Biol 46:1431–1446

Weiner W, Traub W, Parker SB (1984) Macromolecules in mollusc shells and their functions in biomineralization. Philos Trans R Soc Lond B Biol Sci 304:425–434

Weiss IM, Schonitzer V (2006) The distribution of chitin in larval shells of the bivalve mollusk *Mytilus galloprovincialis*. J Struct Biol 153:264–277. doi:10.1016/j.jsb.2005.11.006

Weiss IM, Schonitzer V, Eichner N, Sumper M (2006) The chitin synthase involved in marine bivalve mollusk shell

formation contains a myosin domain. FEBS Lett 580:1846–1852. doi:10.1016/j.febslet.2006.02.044

Wicks LC, Roberts JM (2012) Benthic invertebrates in a high-CO_2 world. In: Gibson RN, Atkinson RJA, Gordon JDM, Hughes RN, Hughes DJ, Smith IP (eds) Oceanography and marine biology: an annual review, vol 50. CRC Press, Boca Raton, pp 127–187

Widdows J, Donkin P, Staff RJ, Matthiessen P, Law RJ, Allen YT, Thain JE, Allchin CR, Jones BR (2002) Measurements of stress effects (scope for growth) and contaminant levels in mussels (*Mytilus edulis*) collected in the Irish sea. Mar Environ Res 53:327–356

Wilbur KM, Saleuddin ASM (1983) Shell formation. In: Saleuddin ASM, Wilbur KM (eds) The mollusca, vol 4. Academic Press, New York, pp 235–287

Williams JD, Warren ML, Cummings KS, Harris JL, Neves RJ (1993) Conservation status of freshwater mussels of the United States and Canada. Endanger Species 18:6–22

Yost HJ, Petersen RB, Lindquist S (1990) RNA metabolism: strategies for regulation in the heat shock response. Trends Genet 6:223–227