

# Responses to elevated CO<sub>2</sub> exposure in a freshwater mussel, *Fusconaia flava*

Jennifer D. Jeffrey<sup>1</sup> · Kelly D. Hannan<sup>1</sup> · Caleb T. Hasler<sup>1</sup> · Cory D. Suski<sup>1</sup>

Received: 7 April 2016 / Revised: 29 June 2016 / Accepted: 19 July 2016  
© Springer-Verlag Berlin Heidelberg 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<sub>2</sub> (*p*CO<sub>2</sub>). The present study quantified the impacts of acute (6 h) and chronic (up to 32 days) exposures to elevated *p*CO<sub>2</sub> on genes associated with shell formation (chitin synthase; *cs*) and the stress response (heat shock protein 70; *hsp70*) in *Fusconaia flava*. Oxygen consumption (MO<sub>2</sub>) was also assessed over the chronic CO<sub>2</sub> exposure period. Although mussels exhibited an increase in *cs* following an acute exposure to elevated *p*CO<sub>2</sub>, 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 *p*CO<sub>2</sub>. In response to an acute elevation in *p*CO<sub>2</sub>, 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 *p*CO<sub>2</sub>. A chronic elevation in *p*CO<sub>2</sub> also increased mussel MO<sub>2</sub>. This overall increase in *hsp70* mRNA levels and MO<sub>2</sub> in *F. flava* indicates that exposure to elevated *p*CO<sub>2</sub> 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 *p*CO<sub>2</sub> 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

## 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 (*p*CO<sub>2</sub>). In the context

Communicated by H.V. Carey.

✉ Jennifer D. Jeffrey  
jjeffrey@illinois.edu

<sup>1</sup> Department of Natural Resources and Environmental Science, University of Illinois at Urbana-Champaign, 1102 South Goodwin Avenue, Urbana, IL 61801, USA

of climate change and rising atmospheric CO<sub>2</sub>, the impact of ocean acidification (i.e., elevated *p*CO<sub>2</sub>) on marine calcifying organisms has been investigated to a large extent (reviewed by Fabry et al. 2008; Gazeau et al. 2013); however, virtually nothing is known about the responses of freshwater bivalves to increased *p*CO<sub>2</sub> (Hasler et al. 2016). Upon entering freshwater, CO<sub>2</sub> results in a decrease in water pH due to the production of carbonic acid (H<sub>2</sub>CO<sub>3</sub>), leading to the release of H<sup>+</sup> and, thus, the weak acidification of water. Levels of freshwater *p*CO<sub>2</sub> 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<sub>2</sub> levels that can fluctuate both seasonally and daily, and that can exceed atmospheric levels (i.e., water bodies can be supersaturated with CO<sub>2</sub>). River environments can thus experience a wide range of *p*CO<sub>2</sub> 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 *p*CO<sub>2</sub> may increase as a result of increased atmospheric CO<sub>2</sub>, 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<sub>2</sub> 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<sub>2</sub> levels would likely dissipate as the distance from the CO<sub>2</sub> infusion site increases, thus mussels may be exposed to a gradient of CO<sub>2</sub> depending on their proximity to the barrier. Together, freshwater mussels may experience periods of elevated *p*CO<sub>2</sub> due to both natural and anthropogenic sources, and with *p*CO<sub>2</sub> 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 *p*CO<sub>2</sub> 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<sub>3</sub>) and an organic matrix (1–5 %). Chitin is an insoluble polysaccharide that forms the highly structured organic framework of mollusc shells within which CaCO<sub>3</sub> 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 *p*CO<sub>2</sub> (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 *p*CO<sub>2</sub>, on shell formation in adult freshwater mussels.

The impacts of elevated *p*CO<sub>2</sub> 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<sub>2</sub> as a potential stressor in freshwater mussels.

In addition to the impacts of elevated *p*CO<sub>2</sub> on cellular function, elevations in *p*CO<sub>2</sub> 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  $\mu\text{atm}$  (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  $\mu\text{atm}$ ), 15,000 or 200,000  $\mu\text{atm}$   $pCO_2$ . 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  $\mu\text{atm}$ ) or 20,000  $\mu\text{atm}$   $pCO_2$  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,  $27.0 \pm 1.3$  g; length,  $50.2 \pm 0.8$  mm; mean  $\pm$  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  $\text{min}^{-1}$  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  $8.0 \pm 0.6$   $\text{mg L}^{-1}$  (mean  $\pm$  SEM) and  $17.5 \pm 0.2$   $^{\circ}\text{C}$ , 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  $\mu\text{atm}$  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 ( $273 \pm 30$   $\mu\text{atm}$ ), 15,000  $\mu\text{atm}$  ( $14,772 \pm 1685$ ), or 200,000  $\mu\text{atm}$  ( $188,114 \pm 12,669$ )  $pCO_2$  as described in Hannan et al.

**Table 1** Water chemistry for short- and long-term  $p\text{CO}_2$  exposures

Experiment	$\text{CO}_2$ level	Temperature ( $^{\circ}\text{C}$ )	Dissolved $\text{O}_2$ ( $\text{mg L}^{-1}$ )	pH	Total alkalinity ( $\text{mg L}^{-1}$ )	Dissolved $\text{CO}_2$ ( $\text{mg L}^{-1}$ )	$p\text{CO}_2$ ( $\mu\text{atm}$ )
Short term	Ambient (300 $\mu\text{atm}$ )	$17.8 \pm 0.2$	$8.98 \pm 0.12$	$8.65 \pm 0.05$	$211 \pm 5$	$13.5 \pm 0.5$	$272.8 \pm 30.2$
	15,000 $\mu\text{atm}$	$17.9 \pm 0.25$	$8.76 \pm 0.26$	$6.99 \pm 0.02$	$202.6 \pm 14.6$	$36.9 \pm 4.7$	$14,772.6 \pm 1685.3$
	200,000 $\mu\text{atm}$	$17.8 \pm 0.2$	$8.56 \pm 1.21$	$6.01 \pm 0.01$	$268 \pm 12$	$255.5 \pm 0.5$	$188,114 \pm 12,669.6$
Long term	Ambient (1000 $\mu\text{atm}$ )	$17.6 \pm 0.2$	$8.05 \pm 0.04$	$8.42 \pm 0.02$	$211.1 \pm 2.3$	$16.2 \pm 0.7$	$994.2 \pm 61.8$
	20,000 $\mu\text{atm}$	$17.5 \pm 0.2$	$7.99 \pm 0.07$	$7.24 \pm 0.06$	$255.1 \pm 8.8$	$40.79 \pm 3.1$	$22,712.0 \pm 2482.5$

Data are presented as mean  $\pm$  SEM

(2016). A  $p\text{CO}_2$  of 200,000  $\mu\text{atm}$  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  $\text{CO}_2$  level of 15,000  $\mu\text{atm}$  was chosen as this level may be expected downstream of a  $\text{CO}_2$  barrier and thus affect mussels not residing immediately within the  $\text{CO}_2$  addition zone. A level of 15,000  $\mu\text{atm}$  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  $\text{CO}_2$  levels were achieved by the common method of bubbling compressed  $\text{CO}_2$  gas (commercial grade, 99.9 % purity) into the central reservoir through an air stone (Riebesell et al. 2010). Levels of  $\text{CO}_2$  were maintained within the central reservoir with a pH controller (PINPOINT<sup>®</sup>, American Marine Inc., CT, USA) that adds  $\text{CO}_2$  if the pH rises above a target level ( $7.00 \pm 0.10$  and  $6.00 \pm 0.10$  pH, for 15,000 and 200,000  $\mu\text{atm}$  treatment, respectively) during the exposure period. Mussels were exposed to one of the three  $\text{CO}_2$  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 ( $24.8 \pm 1.5$  g) and length ( $49.2 \pm 1.0$  mm), were measured as well as the dry mass of the soft tissue ( $1.05 \pm 0.06$  g; see ‘Oxygen consumption’) and no significant effect of  $\text{CO}_2$  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  $4^{\circ}\text{C}$  prior to storage at  $-80^{\circ}\text{C}$ .

### Long-term exposure to elevated $p\text{CO}_2$

Mussels ( $N = 48$ ) were separated into one of two recirculating systems (as described above) and held at either ambient ( $994 \pm 62$   $\mu\text{atm}$ ) or 20,000  $\mu\text{atm}$  ( $22,712 \pm 2482$   $\mu\text{atm}$ )  $p\text{CO}_2$  for 4, 8, and 32 days. Note that the difference in

the ambient  $\text{CO}_2$  levels between the short- and long-term exposures was due to natural fluctuations in the  $\text{CO}_2$  levels of the pond water. Target  $\text{CO}_2$  pressures were again achieved by bubbling  $\text{CO}_2$  gas into the reservoir through an air stone, and maintained using a pH controller set to a pH of  $7.20 \pm 0.10$ , as described above. A level of 20,000  $\mu\text{atm}$  was chosen as this may represent a level that mussels downstream of a  $\text{CO}_2$  barrier may experience, and it was unknown whether mussels would survive long-term exposure to a higher level of  $\text{CO}_2$  (i.e., like the upper level used in the ‘short-term’ experiment). Water quality measurements including temperature, DO, pH, alkalinity, concentration of  $\text{CO}_2$ ,  $p\text{CO}_2$  in  $\mu\text{atm}$  (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  $\text{CO}_2$  conditions. No significant effect of  $\text{CO}_2$  treatment or time-point were found for wet mass ( $25.4 \pm 1.7$  g) and length ( $49.7 \pm 1.0$  mm), as well as the dry mass of the soft tissue ( $1.02 \pm 0.06$  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<sup>®</sup>, 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  $\mu\text{g}$  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	<i>cs</i>	Forward—TGT GCT ACA ATG TGG CAC GA	
		Reverse—TAC CAC ACC ATC GGA CCT GA	
	<i>hsp70</i>	Forward—CCA TTG CCT ATG GTC TGG A	
		Reverse—TTG CTG AGA CGA CCT TTG TC	
	<i>18s</i>	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	<i>cs</i>	Forward—GAG TCG ATT GGC CCA AGA CA	104
		Reverse—CCA CCT GTT CGT CGA GTT CA	
	<i>hsp70</i>	Forward—GAG CAT CAC CAG GGC AAG AT	103
		Reverse—TGG CTT GTC CAT CTT GGC AT	
	<i>18s</i>	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  $\geq 0.85$ ). 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  $\mu$ l, of which 5  $\mu$ l was the RealMasterMix SYBR ROX. For *cs* and *hsp70*, cDNA was diluted 20-fold, and 1000-fold for *18s*. Primer concentrations were 0.1  $\mu$ M. 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<sup>®</sup> 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  $p\text{CO}_2$ ' experiment were assessed repeatedly for  $\text{MO}_2$  at 4, 8, and 32 days of exposure. The  $\text{MO}_2$  was determined using computerized intermittent-flow respirometry (Steffensen 1989). Briefly, the system consisted of four glass chambers (143 mm length  $\times$  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  $\text{MO}_2$  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  $\text{MO}_2$  ( $\text{mg O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) for each mussel was calculated as:

$$\text{MO}_2 = \alpha V_{\text{resp}} \beta M_b^{-1}$$

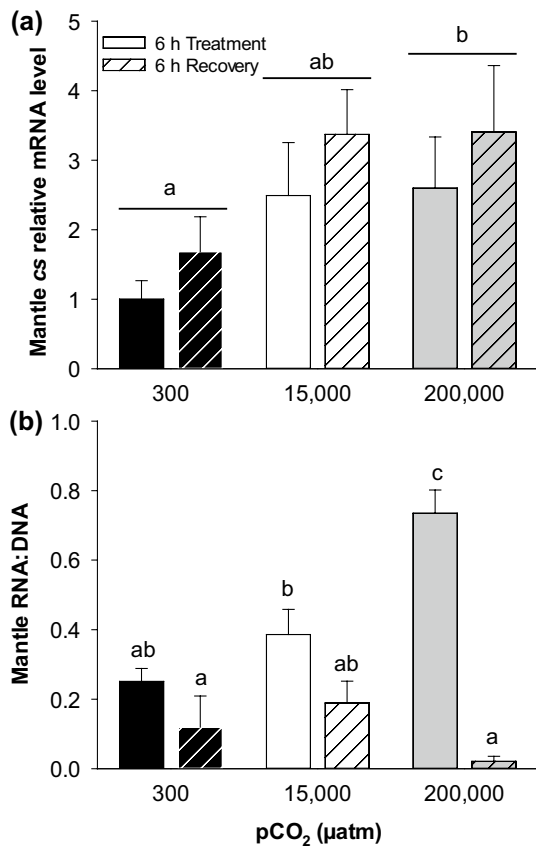
where  $V_{\text{resp}}$  is the volume of each chamber minus the volume of the mussel ( $L$ ),  $\beta$  is the oxygen solubility (adjusted daily for the barometric pressure and temperature), and  $M_b$  is the dry weight of the mussel ( $g$ ; see below). For each trial, the coefficient of determination ( $r^2$ ) 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  $\text{O}_2$  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  $\text{CO}_2$  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  $p\text{CO}_2$  level, sampling time, and their interaction ( $p\text{CO}_2 \times$  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  $p\text{CO}_2$  on mussel  $\text{MO}_2$  during long-term exposure. Main effects, including  $p\text{CO}_2$  treatment, sampling time (4, 8, and 32 days), as well as the interactions of treatment  $\times$  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  $p\text{CO}_2$ . Mussels were exposed to one of three treatments: ambient (300  $\mu\text{atm}$ ), 15,000 or 200,000  $\mu\text{atm}$   $p\text{CO}_2$  for 6 h followed by exposure to 6 h at ambient conditions (recovery). Data are presented as mean  $\pm$  SEM ( $N = 7-8$ ). All mRNA data were normalized to the mRNA abundance of *18s* and expressed relative to the 6-h ambient (300  $\mu\text{atm}$ ) treatment group. Treatment groups that do not share a letter are significantly different from one another. For a, only a significant effect of  $p\text{CO}_2$  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 ( $\alpha$ ) was 0.05.

## Results

### The effect of exposure to a short-term elevation in $p\text{CO}_2$

Mantle *cs* mRNA abundance was significantly increased by the exposure to a short-term elevation in  $p\text{CO}_2$  relative to controls for those mussels exposed to the highest  $p\text{CO}_2$  (200,000  $\mu\text{atm}$ ), 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  $p\text{CO}_2$  (200,000  $\mu\text{atm}$ ) relative to mussels held

at ambient conditions, levels that returned to control levels following 6 h at ambient conditions (300  $\mu\text{atm}$ ; Fig. 1b; Table 3).

Mantle and adductor muscle *hsp70* mRNA levels were not affected following 6 h of exposure to 15,000  $\mu\text{atm}$   $p\text{CO}_2$ , but these levels were significantly elevated 6 h post-stressor (Fig. 2a, b; Table 3). Exposure to the highest  $p\text{CO}_2$  (200,000  $\mu\text{atm}$ ) for 6 h resulted in a significant elevation in *hsp70* mRNA levels in the adductor muscle relative to mussels held at ambient  $p\text{CO}_2$ , which subsequently returned to control levels after an additional 6-h period at ambient conditions (300  $\mu\text{atm}$ ; Fig. 2b; Table 3). In the gill and foot, *hsp70* mRNA levels were largely unaffected by short-term exposure to elevated  $p\text{CO}_2$  (Fig. 2c, d; Table 3), with the exception that exposure to 200,000  $\mu\text{atm}$   $p\text{CO}_2$  caused a decrease in *hsp70* mRNA levels in gill that returned to control levels following 6 h at ambient conditions (300  $\mu\text{atm}$ ; Fig. 2c; Table 3).

### The effect of exposure to a long-term elevation in $p\text{CO}_2$

Contrary to the impact of a short-term elevation in  $p\text{CO}_2$ , *cs* mRNA levels were significantly reduced during long-term exposure to 20,000  $\mu\text{atm}$   $p\text{CO}_2$  in  $\text{CO}_2$ -treated mussels relative to control mussels (held at 1000  $\mu\text{atm}$ ), with no significant effect of sampling time (Fig. 3a; Table 4). Additionally, the ratio of RNA:DNA in mantle tissue was relatively unaffected by  $\text{CO}_2$  treatment at 20,000  $\mu\text{atm}$  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  $\mu\text{atm}$   $p\text{CO}_2$  resulted in an overall increase in *hsp70* mRNA levels in the gill of  $\text{CO}_2$ -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  $\text{CO}_2$ -treatment at 4 and 8 days in the adductor muscle; however, after 32 days of exposure to elevated  $p\text{CO}_2$  these levels were no longer different from mussels held at 1000  $\mu\text{atm}$  for the same period of time (Fig. 4b; Table 4). Mantle and foot *hsp70* mRNA levels were not significantly affected by long-term  $p\text{CO}_2$  treatment (Fig. 4a, d; Table 4).

Exposure to 20,000  $\mu\text{atm}$   $p\text{CO}_2$  also resulted in an increase in  $\text{MO}_2$  compared to mussels held at ambient conditions (1000  $\mu\text{atm}$ ), with no significant effects of sampling time (Fig. 5; Table 5).

## Discussion

The present study provides evidence that exposure to elevations in  $p\text{CO}_2$  may result in changes in shell formation

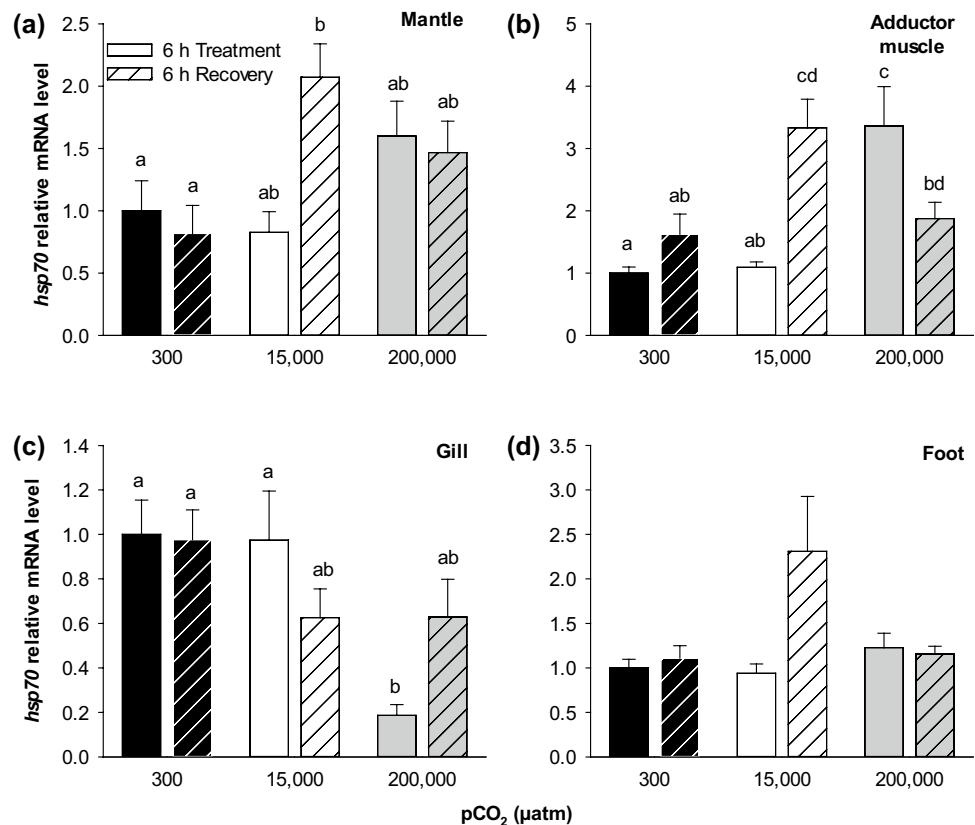
**Table 3** Results of two-way ANOVA for the effects of short-term exposure to elevated  $p\text{CO}_2$  in *Fusconaia flava*

Variable	Main effects	Degrees of freedom	Sum of squares	F value	P
Mantle <i>cs</i>	$p\text{CO}_2$	2	65.70	3.817	<b>0.030</b>
	Time	1	17.50	2.032	0.161
	$p\text{CO}_2 \times \text{time}$	2	0.20	0.010	0.990
Mantle RNA:DNA	$p\text{CO}_2$	2	0.28	4.736	<b>0.014</b>
	Time	1	1.46	50.137	<b>&lt;0.001</b>
	$p\text{CO}_2 \times \text{time}$	2	0.80	13.806	<b>&lt;0.001</b>
Mantle <i>hsp70</i>	$p\text{CO}_2$	2	7.13	4.216	<b>0.022</b>
	Time	1	1.25	1.484	0.230
	$p\text{CO}_2 \times \text{time}$	2	6.43	3.802	<b>0.031</b>
Adductor <i>hsp70</i>	$p\text{CO}_2$	2	1916.40	13.415	<b>&lt;0.001</b>
	Time	1	491.60	6.882	<b>0.012</b>
	$p\text{CO}_2 \times \text{time}$	2	2396.3	16.773	<b>&lt;0.001</b>
Gill <i>hsp70</i>	$p\text{CO}_2$	2	3.63	7.625	<b>0.002</b>
	Time	1	0.01	0.036	0.851
	$p\text{CO}_2 \times \text{time}$	2	1.64	3.432	<b>0.042</b>
Foot <i>hsp70</i>	$p\text{CO}_2$	2	239.00	0.658	0.523
	Time	1	523.00	2.877	0.097
	$p\text{CO}_2 \times \text{time}$	2	434.00	1.193	0.314

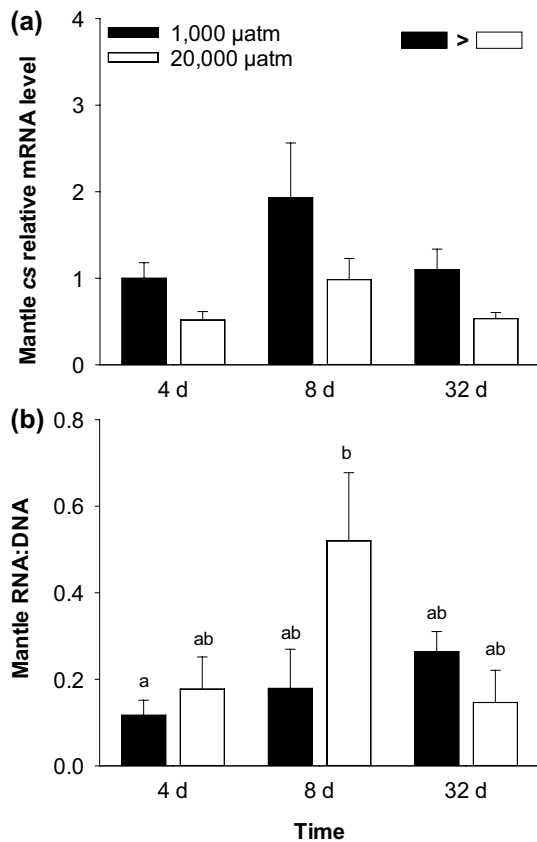
*cs* chitin synthase, *hsp70* heat shock protein 70

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  $p\text{CO}_2$ . Mussels were exposed to one of three treatments: ambient (300  $\mu\text{atm}$ ), 15,000 or 200,000  $\mu\text{atm}$   $p\text{CO}_2$  for 6 h followed by exposure to 6 h at ambient conditions (recovery). Data are presented as mean  $\pm$  SEM ( $N = 7-8$ ). All data were normalized to the mRNA abundance of *18s* and expressed relative to the 6-h ambient (300  $\mu\text{atm}$ ) treatment group. Treatment groups that do not share a letter are significantly different from one another. Neither  $p\text{CO}_2$  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  $p\text{CO}_2$ . Mussels were exposed to either ambient (1000  $\mu\text{atm}$ ) or 20,000  $\mu\text{atm}$   $p\text{CO}_2$  for 4, 8, or 32 days. Data are presented as mean  $\pm$  SEM ( $N = 7\text{--}8$ ). All mRNA data were normalized to the mRNA abundance of *18s* and expressed relative to the 4 days ambient (1000  $\mu\text{atm}$ ) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **a** only a significant effect of  $p\text{CO}_2$  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  $p\text{CO}_2$  resulted in an overall elevation in *cs* mRNA that was significant for mussels exposed to the highest  $p\text{CO}_2$  (200,000  $\mu\text{atm}$ ). Previous work on the Antarctic bivalve, *L. elliptica* exposed to elevated  $p\text{CO}_2$  (~800  $\mu\text{atm}$ ), 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  $p\text{CO}_2$  (up to 32 days) at 20,000  $\mu\text{atm}$ , resulted in approximately a twofold decrease in *cs* mRNA levels compared to mussels held at ambient  $p\text{CO}_2$  conditions

(1000  $\mu\text{atm}$ ). It is possible that *F. flava* exposed to a relatively short elevation in  $p\text{CO}_2$  (i.e., 6 h) may up-regulate processes to maintain normal shell formation in a compromising environment (i.e., elevated  $p\text{CO}_2$  and concomitant decrease in pH), while prolonged exposure to  $p\text{CO}_2$  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  $\text{CaCO}_3$  stores released from the shell to buffer against the acidosis experienced as a result of exposure to elevated  $p\text{CO}_2$  (e.g., Crenshaw 1972; Michaelidis et al. 2005; Hanan 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  $\mu\text{atm}$ , but was largely unaffected by long-term exposure to 20,000  $\mu\text{atm}$   $p\text{CO}_2$ . Thus, processes associated with shell formation may be up-regulated during an acute exposure to elevated  $p\text{CO}_2$ , but not during an extended exposure to elevated  $p\text{CO}_2$ . 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  $p\text{CO}_2$ , 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

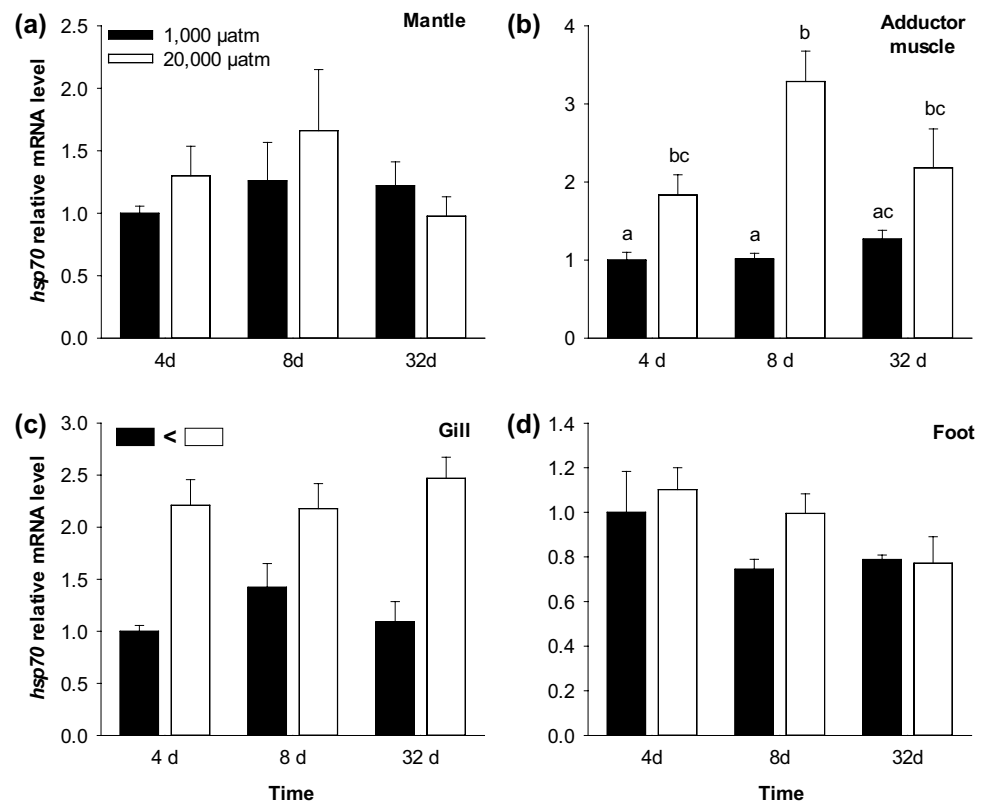
**Table 4** Results of two-way ANOVA for the effects of long-term exposure to elevated  $p\text{CO}_2$  in *Fusconaia flava*

Variable	Main effects	Degrees of freedom	Sum of squares	F value	P
<i>Mantle cs</i>	$p\text{CO}_2$	1	1323	9.914	<b>0.003</b>
	Time	2	446	1.670	0.201
	$p\text{CO}_2 \times \text{time}$	2	257	0.962	0.391
Mantle RNA:DNA	$p\text{CO}_2$	1	77	0.479	0.493
	Time	2	385	1.205	0.310
	$p\text{CO}_2 \times \text{time}$	2	1624	5.081	<b>0.011</b>
<i>Mantle hsp70</i>	$p\text{CO}_2$	1	70	0.368	0.547
	Time	2	26	0.069	0.934
	$p\text{CO}_2 \times \text{time}$	2	111	0.294	0.747
<i>Adductor hsp70</i>	$p\text{CO}_2$	1	4144	42.920	<b>&lt;0.001</b>
	Time	2	248	1.284	0.288
	$p\text{CO}_2 \times \text{time}$	2	765	3.960	<b>0.027</b>
<i>Gill hsp70</i>	$p\text{CO}_2$	1	15.245	44.204	<b>&lt;0.001</b>
	Time	2	0.379	0.550	0.581
	$p\text{CO}_2 \times \text{time}$	2	0.847	1.228	0.303
<i>Foot hsp70</i>	$p\text{CO}_2$	1	280	1.791	0.189
	Time	2	805	2.573	0.090
	$p\text{CO}_2 \times \text{time}$	2	408	1.306	0.283

*cs* chitin synthase, *hsp70* heat shock protein 70

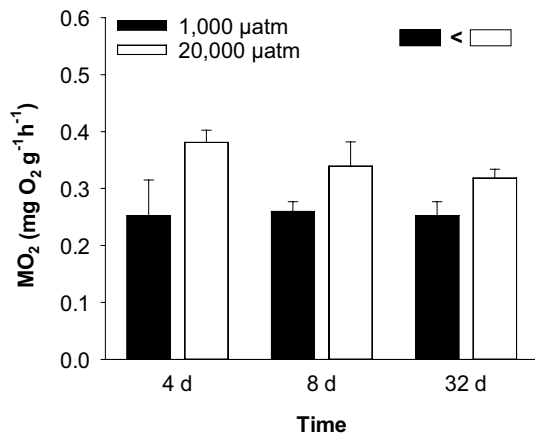
Significant *P* values are bolded and variables in italics are run on ranks

**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  $p\text{CO}_2$ . Mussels were exposed to either ambient (1000  $\mu\text{atm}$ ) or 20,000  $\mu\text{atm}$   $p\text{CO}_2$  for 4, 8, or 32 days. Data are presented as mean  $\pm$  SEM ( $N = 8$ ). All data were normalized to the mRNA abundance of *18s* and expressed relative to the 4 days ambient (1000  $\mu\text{atm}$ ) treatment group. Treatment groups that do not share a letter are significantly different from one another. For **c**, only a significant effect of  $p\text{CO}_2$  treatment occurred, with no significant effect of sampling time (two-way ANOVA; see Table 4). Neither  $p\text{CO}_2$  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  $\text{CO}_2$  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  $\mu\text{atm}$ ) or 20,000  $\mu\text{atm}$   $p\text{CO}_2$  for 4, 8, or 32 days. Mussels were repeatedly sampled for  $MO_2$  over the course of the long-term exposure to elevated  $p\text{CO}_2$ . Data are presented as mean  $\pm$  SEM ( $N = 7\text{--}8$ ). Only a significant effect of  $p\text{CO}_2$  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  $p\text{CO}_2$  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
$p\text{CO}_2$ treatment	0.13	<b>0.02, 0.23</b>
8 days	0.01	-0.08, 0.11
32 days	0.00	-0.10, 0.10
$p\text{CO}_2 \times 8$ days	-0.05	-0.19, 0.09
$p\text{CO}_2 \times 32$ days	-0.06	-0.21, 0.08

to 6 h at 15,000  $\mu\text{atm}$   $p\text{CO}_2$  was only evident in mantle and adductor muscle following an additional 6 h at ambient conditions (300  $\mu\text{atm}$ ). Thus, at moderate elevations in  $p\text{CO}_2$  (15,000  $\mu\text{atm}$ ), 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  $\mu\text{atm}$ , an increase in *hsp70* mRNA in the adductor muscle occurred following the 6-h  $\text{CO}_2$  exposure at the highest  $\text{CO}_2$  level of 200,000  $\mu\text{atm}$ , 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  $\mu\text{atm}$  compared to 15,000  $\mu\text{atm}$   $p\text{CO}_2$  may reflect a response to a more severe stressor. In addition, in response to a higher level of  $\text{CO}_2$

exposure (200,000  $\mu\text{atm}$ ), 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  $\text{CO}_2$  exposure level (i.e., 200,000  $\mu\text{atm}$ ). 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  $p\text{CO}_2$  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  $p\text{CO}_2$  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  $\mu\text{atm}$ , they were significantly elevated throughout a 32-day exposure at 20,000  $\mu\text{atm}$ . The delay in the increase of *hsp70* mRNA in response to elevations in  $p\text{CO}_2$  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  $\mu\text{atm}$   $p\text{CO}_2$ , where *hsp70* mRNA levels were elevated at 4 and 8 days of  $\text{CO}_2$  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  $\text{CO}_2$  stressor in the adductor muscle may be desensitized over a long-term exposure to elevated  $p\text{CO}_2$ . A similar transient response to  $\text{Hg}^{2+}$  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  $p\text{CO}_2$ , though further investigation on this topic is required. Although mantle *hsp70* mRNA levels were elevated by short-term  $p\text{CO}_2$  exposure at 15,000  $\mu\text{atm}$  in *F. flava* (present study), and in the marine bivalve *L. elliptica* following 21 days at  $\sim 800$   $\mu\text{atm}$  (Cummings et al. 2011), exposure to 20,000  $\mu\text{atm}$  for up to 32 days had no significant impact. Overall, exposure to elevated  $p\text{CO}_2$  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  $\text{CO}_2$  exposure. Together, the results of the present study suggest that *hsp70* responses to exposure to elevated  $p\text{CO}_2$  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  $p\text{CO}_2$ . 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  $p\text{CO}_2$  (e.g., glycogen stores, other HSPs, oxidative stress genes, etc.).

Whole-animal  $\text{MO}_2$  was elevated in *F. flava* in response to prolonged exposure to elevated  $p\text{CO}_2$ . When exposed to 20,000  $\mu\text{atm}$   $p\text{CO}_2$  for up to 32 days, mussels displayed an overall increase in  $\text{MO}_2$ , indicating that mussels were consuming more oxygen, and likely also expending more energy, to deal with  $\text{CO}_2$  exposure. Previous work evaluating the consequences for elevated  $p\text{CO}_2$  on marine bivalves found that increases in  $p\text{CO}_2$  also caused elevations in  $\text{MO}_2$  (e.g., Beniash et al. 2010; Lannig et al. 2010; Thomsen and Melzner 2010; Cummings et al. 2011; Parker et al. 2012). Increases in  $\text{MO}_2$  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  $p\text{CO}_2$  (Parker et al. 2012). Increases in metabolic rate may be one of the mechanisms responsible for higher resilience to elevations in  $p\text{CO}_2$  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  $p\text{CO}_2$  exposure on marine bivalves found that increases in  $p\text{CO}_2$  had either minimal effects on  $\text{MO}_2$  levels (Matoo et al. 2013) or resulted in a decrease in  $\text{MO}_2$  (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  $p\text{CO}_2$  (Parker et al. 2012). To our knowledge, the present study is the first to assess the impacts of elevated  $p\text{CO}_2$  on  $\text{MO}_2$  in a freshwater mussel. The increase in  $\text{MO}_2$  observed in *F. flava* was in response to a much higher  $\text{CO}_2$  level (i.e., 20,000  $\mu\text{atm}$ , approximately 20 times the current mean  $p\text{CO}_2$  in freshwater systems) (Cole et al. 2007) compared to previous exposures of marine bivalves. The capacity of *F. flava* to sustain increases in  $\text{MO}_2$  over a period of at least 32 days suggests that freshwater mussels may be more robust to environmental changes in  $p\text{CO}_2$  than marine mussels. This potential increase in  $\text{CO}_2$  tolerance may have arisen in freshwater mussels due to their exposure to the natural fluctuations in  $p\text{CO}_2$  that occur in freshwater systems (Maberly 1996; Hasler et al. 2016). The increase in  $\text{MO}_2$  in *F. flava* during exposure to elevated  $p\text{CO}_2$  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  $\text{MO}_2$  observed in *F. flava* suggests that freshwater mussels may be resilient to large elevations in  $p\text{CO}_2$ ; 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  $p\text{CO}_2$  by increasing processes related to the stress response. During short bouts of increased  $p\text{CO}_2$ , mussels attempt to maintain normal physiological functions such as shell formation. However, during extended exposure to elevated  $p\text{CO}_2$ , 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  $\text{MO}_2$  observed in *F. flava* in response to elevations in  $p\text{CO}_2$  suggests that adult freshwater mussels may have the capacity to regulate in situations of elevated  $\text{CO}_2$ , 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  $p\text{CO}_2$ ; 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  $p\text{CO}_2$  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  $p\text{CO}_2$  elevations are expected to occur (e.g., due to increased atmospheric  $\text{CO}_2$  or the deployment of a  $\text{CO}_2$  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 thank 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  $\text{CO}_2$  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  $p\text{CO}_2$ ? *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, MacLachy 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-Kalishman 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 *Unio merus 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>-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<sub>2</sub> 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) CO<sub>2</sub>calc: A user-friendly seawater carbon calculator for Windows, Mac OS X, and iOS (iPhone). US Geological Survey, Reston
- Schönitzer 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, Stroglyoudi 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 mollusk shells and their functions in biomineralization. *Philos Trans R Soc Lond B Biol Sci* 304:425–434
- Weiss IM, Schönitzer 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, Schönitzer 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](https://doi.org/10.1016/j.febslet.2006.02.044)
- Wicks LC, Roberts JM (2012) Benthic invertebrates in a high-CO<sub>2</sub> 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