Conservation Physiology

Volume 4 • 2016 10.1093/conphys/cow066

SEB Society for Experimental Biology

Research article

Physiological responses of three species of unionid mussels to intermittent exposure to elevated carbon dioxide

Kelly D. Hannan, Jennifer D. Jeffrey, Caleb T. Hasler and Cory D. Suski*

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

***Corresponding author:** 401C Turner Hall, 1102 South Goodwin Avenue, Urbana, IL 61801, USA. Tel.: +1-217-244-2237. Email: suski@illinois.edu

Freshwater systems are at risk owing to increasing carbon dioxide (CO_2) levels, and one of the possible reasons for these elevations is the deployment of non-physical fish barriers to prevent invasive fish movements. Carbon dioxide barriers have the potential to create short, chronic and intermittent exposures of CO_2 for surrounding freshwater biota. Although intermittent exposures to a stressor may be more ecologically relevant, the majority of laboratory tests use chronic or short-term time periods to determine how organisms will respond to an environmental stressor. Measurements of the physiological responses of three species of unionid mussel, giant floaters (*Pyganodon grandis*), threeridge (*Amblema plicata*) and plain pocketbook (*Lampsilis cardium*), exposed to control pCO_2 (~1000 μatm) or intermittent conditions of pCO_2 (ranging from ~1000 to ~55 000 μatm) 12 times per day over a 28 day period were gathered. There was no indication of recovery in the physiological responses of mussels between applications of CO_2, suggesting that the recovery time between CO_2 pulses (1.5 h) was not sufficient for recovery from the CO_2 exposure period (0.5 h). Observations of acid–base and stress responses were consistent with what has been observed in chronic studies of freshwater mussels exposed to elevated pCO_2 (i.e. elevations in HCO_3^-, Ca^{2+}, Na^+ and glucose, and decreases in Mg^{2+} and Cl^-). However, species differences were observed across almost all variables measured, which emphasizes the need for multispecies studies.

Key words: Acid–base regulation, bivalve, freshwater acidification, ions

Editor: Steven Cooke

Received 2 May 2016; Revised 27 October 2016; Editorial Decision 15 November 2016; Accepted 16 November 2016

Cite as: Hannan KD, Jeffrey JD, Hasler CT, Suski CD (2016) Physiological responses of three species of unionid mussels to intermittent exposure to elevated carbon dioxide. *Conserv Physiol* 4(1): cow066; doi:10.1093/conphys/cow066.

Introduction

Environmental levels of carbon dioxide (CO_2) that are commonly found in freshwater ecosystems have the potential to act as both continuous and intermittent stressors for aquatic organisms. Over the past several decades, levels of CO_2 in the atmosphere have been increasing as a result of the anthropogenic burning of fossil fuels, which has led to a concomitant increase in the partial pressure of CO_2 gas (pCO_2) in marine ecosystems (Shirayama and Thornton, 2005). Unlike marine systems, there is no consensus regarding how pCO_2 will change in freshwater as a result of climate change (Hasler *et al.*, 2016). In freshwater, pCO_2 can vary across and within watersheds (Butman and Raymond, 2011), as well as episodically and on seasonal and diel cycles within water bodies (Maberly, 1996). In a review of ~7000 global rivers and streams, the average median value for pCO_2 was ~3100 μatm (Raymond *et al.*, 2013), and in another global review of 47 large rivers the means varied from

679 ± 543 to 35 617 ± 46 757 μatm, with means in the USA ranging from 679 ± 543 to 9475 ± 993 μatm (Cole and Caraco, 2001). In addition to these natural sources of elevated pCO_2, recent work has shown that zones of elevated CO_2 can act as non-physical fish barriers, thereby providing a management tool to prevent the movement and spread of invasive fish species (Kates *et al.*, 2012; Noatch and Suski, 2012). Although a specific method for the use of CO_2 barriers to deter fish movement has not yet been defined, one potential application is the intermittent addition of CO_2 into a navigational lock or approach channel at vulnerable times (i.e. when lock doors are open; United States Army Corps of Engineers, 2014a), resulting in downstream pulses of CO_2-rich water. Thus, downstream fluctuations in CO_2 might occur, making CO_2 a potential intermittent stressor for freshwater organisms.

A taxonomic group of freshwater organisms that may be particularly at risk to CO_2 stressors are freshwater mussels (Order Unionoida). Mussels serve many important ecological functions, influence many ecosystem processes (Vaughn and Hakenkamp, 2001) and are often used as indicators of ecosystem health (Williams *et al.*, 1993). Although North American freshwater ecosystems contain the highest diversity of freshwater mussels in the world (Williams *et al.*, 1993; Bogan, 2008), more than half (71%) are listed as endangered, threatened or of special concern, largely as a result of anthropogenic stressors, such as habitat alteration and degradation (Williams *et al.*, 1993; Ricciardi *et al.*, 1998). Additionally, while mussels are generally considered a homogeneous group of sessile animals, there are four main tribes of mussels in North America (Quadrulini, Lampsilini, Pleurobemini and Amblemini) that all vary in morphology, physiology and reproductive strategies and may thus respond differently to environmental stressors.

At present, there is a paucity of research on the effects of elevated pCO_2 on freshwater invertebrates, particularly unionid mussels. Hannan *et al.* (2016a, b) found that mussels experience acid–base regulation in response to short- and long-term exposures to elevated pCO_2, and a stress response to long-term exposure to elevated pCO_2. Previous studies on marine bivalves indicate that elevated pCO_2 causes internal acidosis (Michaelidis *et al.*, 2005; Bibby *et al.*, 2008) that is often buffered by increasing HCO_3^- in the fluids (Pörtner *et al.*, 2004). Both marine (Michaelidis *et al.*, 2005) and freshwater mussels (Hannan *et al.*, 2016a, b) can increase haemolymph HCO_3^- by using $CaCO_3$ released from the shell as a result of decreased pH and elevated CO_2 (i.e. increases both haemolymph HCO_3^- and Ca^{2+}) or by reducing the activity of the Cl^-–HCO_3^- exchanger to retain HCO_3^- at the cost of Cl^- uptake (Byrne and Dietz, 1997; Hannan *et al.* 2016a, b). Another strategy to buffer acidosis is to alter the activity of Na^+–H^+ exchangers to increase removal of H^+ ions, thus also increasing Na^+ uptake (Byrne and Dietz, 1997; Lannig *et al.*, 2010, Hannan *et al.* 2016b). Exposure to a chronic elevation in pCO_2 also appears to initiate the general stress response in mussels, because a decrease in haemolymph Mg^{2+} and an increase in haemolymph glucose have been observed in unionid mussels (Hannan *et al.* 2016a, b). More importantly, previous studies (i.e. studies described above) that have quantified CO_2 stressors in mussels have used a continuous application of CO_2 rather than one that was intermittent as might be expected downstream of a CO_2 barrier, and differences may exist between the continuous application of a stressor relative to one applied intermittently (exacerbation, attenuation or no change; Reinert *et al.*, 2002).

Based on this background, the goal of the present study was to quantify the physiological impacts of intermittent exposures to elevated pCO_2 on three species of freshwater mussels each belonging to a different tribe, *Pyganodon grandis* (tribe Anodontini), *Amblema plicata* (tribe Amblemini) and *Lampsilis cardium* (tribe Lampsilini). To accomplish this goal, over a 28 day period the mussels were exposed to either control pCO_2 or intermittent increases in pCO_2 and then sampled for a suite of physiological parameters related to acid–base status and physiological stress. The results of this study help to clarify further how different exposures to elevated pCO_2 affect the acid–base and stress responses of various freshwater mussel species in habitats where pCO_2 fluctuates.

Materials and methods

Mussel collection and husbandry

Plain pocketbook (*L. cardium*) and threeridge mussels (*A. plicata*) were collected by benthic grab from the Mississippi River, Cordova, IL, USA, in July 2015. Giant floater mussels (*P. grandis*) were collected by benthic grab from a barrow pit near Champaign, IL, USA, in August 2015. Mussels were taken to the Aquatic Research Facility at the University of Illinois, Champaign-Urbana, IL, USA in coolers (travel time <3 h for *L. cardium* and *A. plicata* and <1 h for *P. grandis*). Upon arrival at the Aquatic Research Facility, all mussels were cleaned of epibionts and tagged for individual identification with a permanent marker (Neves and Moyer, 1988). Once tagged, mussels were placed in three tubs (1136 litres) supplied with water from a 0.04 ha natural, earthen-bottom pond, where they remained for at least 1 week to recover from transport stressors and to acclimate to laboratory conditions (Dietz, 1974; Horohov *et al.*, 1992; Dietz *et al.*, 1994). All tubs were equipped with a Teco 500 aquarium chiller (TECO-US, Aquarium Specialty, Columbia, SC, USA) and a low-pressure air blower (Sweetwater, SL24H Pentair, Apopka, FL, USA) to maintain aeration. Fifty per cent water changes using pond water were performed weekly to maintain water quality. Mussels were fed a commercial shellfish diet of the following consituents: *Nannochloropsis* sp. 1–2 μm and a mixed diet of *Isochrysis*, *Pavlova*, *Thalassiosira* and *Tertraselmis* spp. 5–12 μm (Instant Algae, Reed Mariculture Inc., Campbell, CA, USA) every other day (American Society of Testing and Materials, 2006; Wang *et al.*, 2007), although mussels did not receive supplemental food for 24 h prior to sampling. Temperature and dissolved oxygen (DO) were recorded daily

across all holding tanks with a portable meter (YSI 550A, Yellow Springs Instruments, Irvine, CA, USA) and averaged 22°C (21.7 ± 0.1°C, mean ± SEM) and 7.50 mg l^{-1} (7.60 ± 0.06 mg l^{-1}). Water pH was measured using a handheld meter (WTW pH 3310 meter, Germany) that was calibrated regularly, and averaged 8.55 ± 0.01 throughout the acclimation period. Dissolved CO_2 and total alkalinity (TA) concentrations were measured using digital titration kits and averaged 4.86 ± 0.04 mg l^{-1} and 1093.0 ± 27.0 µmol kg^{-1}, respectively (Hach Company, Loveland, CO, USA; Titrator model 16,900 catalogue no. 2272700 and catalogue no. 2271900 for CO_2 and TA, respectively).

Fluctuating CO_2 exposure

To define the impacts of fluctuating CO_2 on mussel physiology, mussels (*L. cardium*, *A. plicata* and *P. grandis*; $n = 28$) were separated into two recirculating treatment systems (92 litres), each with nine 5 litre tanks (adapted from Hohn and Petrie-Hanson, 2007). Systems were maintained as stated above with the exception that one system received a CO_2 treatment. In the CO_2 treatment system, pCO_2 was turned on every 1.5 h, and increased from ambient (~1000 µatm, 1355 ± 119 µatm; pH = 7.85 ± 0.02) to ~55 000 µatm (56 492 ± 1342 µatm; pH = 6.62 ± 0.03) by bubbling CO_2 gas into the water through an air stone (see Supplementary material, Fig. S1). Elevated pCO_2 was held constant at ~55 000 µatm for 0.5 h, for a total of 12 fluctuations per day. Thus, animals were held at elevated pCO_2 levels for 0.5 h and returned to control levels during the 1.5 h recovery period and then raised back up to elevated conditions for 0.5 h, repeatedly during the course of the experiment. A level of 55 000 µatm was targeted because this level has previously been defined as being a potential target CO_2 level that could deter the movement of fishes (Donaldson *et al.*, 2016) and will possibly be the target level of a CO_2 barrier. Twelve fluctuations per day represents the historical lock usage of Brandon Road Lock (41.5054°N, 88.0996°W), a possible site for deployment of a CO_2 barrier within the Des Plaines River, IL, USA (United States Army Corps of Engineers, 2014a, 2015). The target pCO_2 was maintained with a pH controller (PINPOINT®, American Marine Inc., CT, USA) that automatically bubbled CO_2 into the tank system through an air stone should the pH rise above a target level during exposure (Reynaud *et al.*, 2003; Riebesell *et al.*, 2010). The level of CO_2 was then returned to ~1000 µatm by bubbling in air though an air stone to off-gas excess CO_2. An identical recirculating system was used as a control, and mussels in this control system were treated in the same way as animals receiving CO_2, except that infused CO_2 gas was replaced with compressed air such that mussels were held continuously at ambient ~1000 µatm (876 ± 108 µatm; pH = 8.13 ± 0.02) pCO_2. A digital timer (DT620 Heavy Duty Digital Timer, Intermatic Inc Spring Grove, IL, USA) was used to control additions of CO_2 and air. A modified infrared probe was used to measure pCO_2 (Vaisala GMP220 and GMT221, Vantaa, Finland; Johnson *et al.*, 2010), along with a CO_2 titration kit to determine the concentration of CO_2 (Hach Company, catalogue no. 2272700, Loveland, CO, USA). Before and after the 12.00 h exposure, temperature (21.7 ± 0.1°C) and DO (7.60 ± 0.07 mg l^{-1}) were measured as stated above, and the temperature, pH (see above) and TA (2566.3 ± 252.9 µmol kg^{-1}) were entered into CO2calc to verify pCO_2 (Robbins *et al.*, 2010).

Individual mussels were non-lethally and repeatedly sampled for haemolymph on day 1, 4, 7, 14, 21 or 28 of exposure to fluctuating pCO_2 or control conditions. Mussels were sampled during the 1.5 h period when CO_2 was at ambient levels, not during the 0.5 h when CO_2 levels were elevated. Prior to starting this study, it was not known whether sampling mussels immediately prior to the increase in CO_2 or immediately after the period of increased CO_2 would be optimal to define the impacts of CO_2 on physiological parameters. Therefore, mussels were sample during both intervals, and $n = 7$ animals were sampled immediately prior to the increase in CO_2, whereas a second $n = 7$ animals were sampled immediately following the increase in pCO_2, once pCO_2 returned to control values. All samples were collected around the 12.00 h CO_2 exposure to standardize any potential for diel variation in physiological parameters.

Haemolymph (0.5 ml for *L. cardium* and *A. plicata*; and 0.25 ml for *P. grandis*) was extracted from the anterior adductor muscle with a 1 ml syringe and 26 gauge needle (Gustafson *et al.*, 2005) and then centrifuged at 12 000*g* for 2 min. After centrifugation, the supernatant was removed, flash frozen in liquid nitrogen and stored at −80°C until processing. Mussels were sampled for haemolymph only once per sampling day, and were randomly sampled before or after the CO_2 exposure on each sampling day over the 28 day period. On day 28 of exposure, mussels were sampled for haemolymph as stated above and then lethally sampled. Lethal mussel sampling included measurements for length, width, depth of the whole mussel using digital callipers (traceable digital carbon fiber calipers, Fisher Scientific, Pittsburg, PA, USA), and weight of the whole mussel (tissue + shell) was collected to the nearest 0.01 g using a balance (HL-300WP, A&D, Ann Arbor, MI, USA). Soft tissue dry weight (in milligrams) was determined by taking mussel soft tissues and drying them at 99°C for 24 h before weighing (Widdows *et al.*, 2002). If possible, sex was determined for *L. siliquoidia* and *P. grandis* using both their external sexual dimorphism and by examination of the gills for glochidia (Trdan, 1981).

The dry weight and length of individuals within each species was not different between control and fluctuating CO_2 treatment groups (Student's unpaired *t*-test, $P > 0.05$; Table 1). Additionally, mortalities were limited over the exposure period, but occurred for two and five *P. grandis* from the control and fluctuating pCO_2 treatments, respectively, and for two *A. plicata* and one *L. cardium* exposed to the fluctuating pCO_2 treatment.

Laboratory analyses

Haemolymph Cl^-, Mg^{2+} and Ca^{2+} concentrations were assayed in duplicate using commercially available kits (QuantiChrom assay kits Cl^-, catalogue no. DICL-250; Mg^{2+}, catalogue

Table 1: Results of Student's unpaired *t*-test examining the impact of dry weight and length on different pCO_2 treatments

Measured variable	Species	d.f.	*t*	*P*-value
Dry weight (g)	Threeridge	17.26	−1.247	0.229
Length (cm)		21.65	0.526	0.604
Dry weight (g)	Pocketbook	22.23	0.673	0.508
Length (cm)		20.70	0.218	0.830
Dry weight (g)	Giant floater	16.82	−0.617	0.545
Length (cm)		18.95	0.350	0.730

No significant effects were detected.

Table 2: Results of two-way ANOVA examining the impact of fluctuating exposure to elevated pCO_2 on *Pyganodon grandis* exposed to one of two different pCO_2 treatments [~1000 µatm (ambient); intermittent at ~55 000 µatm] for 28 days

Measured variable	Main effects	Sum of squares	d.f.	*F*	*P*-value
HCO_3^- (mmol l^{-1})	Treatment	4.84	1	115.59	**<0.001**
	Day	1.29	4	7.71	**<0.001**
	Treatment × day	2.18	4	12.99	**<0.001**
	Residuals	4.61	110		
Ca^{2+} (mg ml^{-1})	Treatment	40 708	1	61.50	**<0.001**
	Day	40 630	4	15.34	**<0.001**
	Treatment × day	22 285	4	8.42	**<0.001**
	Residuals	79 450	120		
Cl^- (mg ml^{-1})	Treatment	16 163	1	21.43	**<0.001**
	Day	59 308	4	19.66	**<0.001**
	Treatment × day	17 084	4	5.66	**<0.001**
	Residuals	90 518	120		
Na^+ (g l^{-1})	Treatment	3033	1	3.87	0.0517
	Day	48 876	4	15.58	**<0.001**
	Treatment × day	9883	4	3.15	**0.0170**
	Residuals	87 826	112		
Mg^{2+} (mg ml^{-1})	Treatment	0.001	1	46.21	**<0.001**
	Day	0.002	4	15.28	**<0.001**
	Treatment × day	0.002	4	18.33	**<0.001**
	Residuals	0.003	120		
Glucose (µM)	Treatment	2656	1	2.23	0.1385
	Day	12 606	4	2.64	**0.0373**
	Treatment × day	10 084	4	2.11	0.0838
	Residuals	137 268	115		

Bold *P*-values indicate statistical significance across treatment groups within a measured variable.

no. DIMG-250; Ca^{2+}, catalogue no. DICA-500; BioAssay Systems, Hayward, CA, USA). Haemolymph HCO_3^- and Na^+ levels were measured by the diagnostic clinical pathology laboratory at the University of Illinois Urbana-Champaign using a Beckman chemistry analyser (Beckman Coulter AU680, Beckman Coulter, Brea, CA, USA). Quality control testing for

this analyser was performed at least every 24 h. Haemolymph glucose concentrations were assayed in duplicate according to the method of Bergmeyer (1974) using a 96-well microplate and a plate spectrophotometer (Molecular Devices, SpectraMax Plus 384, Sunnyvale, CA, USA). For all assays, the inter- and intra-assay coefficients of variability were <10%.

Statistical analyses

The effects of CO_2 exposure on haemolymph ion levels and glucose concentrations were quantified using a two-way analysis of variance (ANOVA), with pCO_2 (fluctuating or control), sampling day and their interaction (pCO_2 × sampling day) entered into each model as fixed effects. Individual mussel identification number (ID), time point (i.e. sampling before or after pCO_2), length, dry weight and sex (if applicable) were initially included in the models as cofactors to quantify their potential influence on response variables, but were removed because they had no significant effect on model outputs (Engqvist, 2005; Zuur *et al.*, 2009). If at least one of the main effects in the ANOVA model was significant, or if the interaction term was significant, a Tukey–Kramer honestly significant difference (HSD) *post hoc* test was applied to separate means (Rohlf and Sokal, 1995). Finally, a separate Student's unpaired *t*-test was run on each species to quantify differences in dry weight and length across different pCO_2 treatments.

For all statistical analyses, analysis of fitted residuals using a quantile–quantile plot (Anscombe and Tukey, 1963) was used to assess normality, while a Hartley's F_{max} test (Hartley, 1950), combined with visual inspection of the distribution of fitted residuals, was used to assess homogeneity of variances. If either normality or homogeneity of variance assumptions were violated (Siegel and Castellan, 1988), data were rank transformed

Table 3: Results of two-way ANOVA examining the impact of fluctuating exposure to elevated pCO_2 on *Amblema plicata* exposed to one of two different pCO_2 treatments [~1000 µatm (ambient); intermittent at ~55 000 µatm] for 28 days

Measured variable	Main effects	Sum of squares	d.f.	*F*	*P*-value
HCO_3^- (mmol l^{-1})	Treatment	8.73	1	118.33	**<0.001**
	Day	3.12	4	10.57	**<0.001**
	Treatment × day	2.54	4	8.61	**<0.001**
	Residuals	9.37	127		
Ca^{2+} (mg ml^{-1})	Treatment	0.02	1	16.06	**<0.001**
	Day	0.02	4	4.99	**<0.001**
	Treatment × day	0.02	4	4.14	**0.003**
	Residuals	0.13	127		
Cl^- (mg ml^{-1})	Treatment	1676	1	1.44	0.232
	Day	47 572	4	10.12	**<0.001**
	Treatment × day	16 923	4	3.63	**0.008**
	Residuals	148 094	127		
Na^+ (g l^{-1})	Treatment	521	1	211.07	**<0.001**
	Day	26.5	4	2.68	**0.0345**
	Treatment × day	126.8	4	12.85	**<0.001**
	Residuals	313.5	127		
Mg^{2+} (mg ml^{-1})	Treatment	0.0005	1	16.41	**<0.001**
	Day	0.0015	4	11.49	**<0.001**
	Treatment × day	0.0021	4	15.68	**<0.001**
	Residuals	0.0041	125		
Glucose (µM)	Treatment	1.09	1	8.75	**0.004**
	Day	0.32	4	0.65	0.627
	Treatment × day	0.49	4	0.99	0.416
	Residuals	15.77	127		

Bold *P*-values indicate statistical significance across treatment groups within a measured variable.

and then re-analysed within the same parametric model described above, and the assumptions of both normality and equal variances were confirmed (Conover and Iman, 1981; Iman *et al.*, 1984; Potvin and Roff, 1993). All data are presented as means ± SEM where appropriate, all tests were performed using R (version 3.2.2), and differences were considered significant if α was <0.05. For all variables and species, there was no effect of sampling before vs. after CO_2 application (i.e. time point) for either treatment (control or fluctuating), so data from mussels sampled before and after CO_2 application were combined.

Results

There was a significant interaction between treatment and day for all three species of mussels for haemolymph HCO_3^- (Tables 2–4). At 14 days of exposure to fluctuating pCO_2, *P. grandis* (treatment × day, $F = 13.0$, $P < 0.001$; Fig. 1A) and *A. plicata* (treatment × day, $F = 8.61$, $P < 0.001$; Fig. 1D) had approximately a 2-fold increase in haemolymph HCO_3^- relative to mussels held at ambient pCO_2, and these concentrations remained significantly elevated for the duration of the exposure period. For *L. cardium*, haemolymph HCO_3^- was significantly elevated beginning at 4 days of exposure compared with control mussels and throughout the rest of the exposure period (treatment × day, $F = 0.52$, $P < 0.001$; Fig. 1G).

A significant interaction of treatment and day was also found for all three species of mussels with respect to haemolymph Ca^{2+} concentrations(Tables 2–4). *Pyganodon grandis* had a significant elevation in haemolymph Ca^{2+} concentrations beginning at 14 days of exposure to fluctuating levels of CO_2 relative to control mussels; however, it should be noted that

Table 4: Results of two-way ANOVA examining the impact of fluctuating exposure to elevated pCO_2 on *Lampsilis cardium* exposed to one of two different pCO_2 treatments [~1000 µatm (ambient); intermittent at ~55 000 µatm] for 28 days

Measured variable	Main effects	Sum of squares	d.f.	*F*	*P*-value
HCO_3^- (mmol l^{-1})	Treatment	7.28	1	141.70	**<0.001**
	Day	0.47	4	2.27	0.0653
	Treatment × day	2.10	4	0.52	**<0.001**
	Residuals	6.48	126		
Ca^{2+} (mg ml^{-1})	Treatment	0.171	1	377.5	**<0.001**
	Day	0.024	4	13.36	**<0.001**
	Treatment × day	0.022	4	12.20	**<0.001**
	Residuals	0.058	127		
Cl^- (mg ml^{-1})	Treatment	479	1	0.35	0.553
	Day	30 987	4	5.73	**<0.001**
	Treatment × day	4442	4	0.821	0.514
	Residuals	169 113	125		
Na^+ (g l^{-1})	Treatment	369.3	1	134.96	**<0.001**
	Day	10.6	4	0.97	0.4273
	Treatment × day	33.4	4	3.05	**0.0194**
	Residuals	344.8	126		
Mg^{2+} (mg ml^{-1})	Treatment	0.001	1	46.24	**<0.001**
	Day	0.0003	4	2.62	**0.038**
	Treatment × day	0.0008	4	7.31	**<0.001**
	Residuals	0.004	127		
Glucose (µM)	Treatment	483	1	0.81	0.369
	Day	1938	4	0.81	0.517
	Treatment × day	2369	4	1.00	0.411
	Residuals	69 399	117		

Bold *P*-values indicate statistical significance across treatment groups within a measured variable.

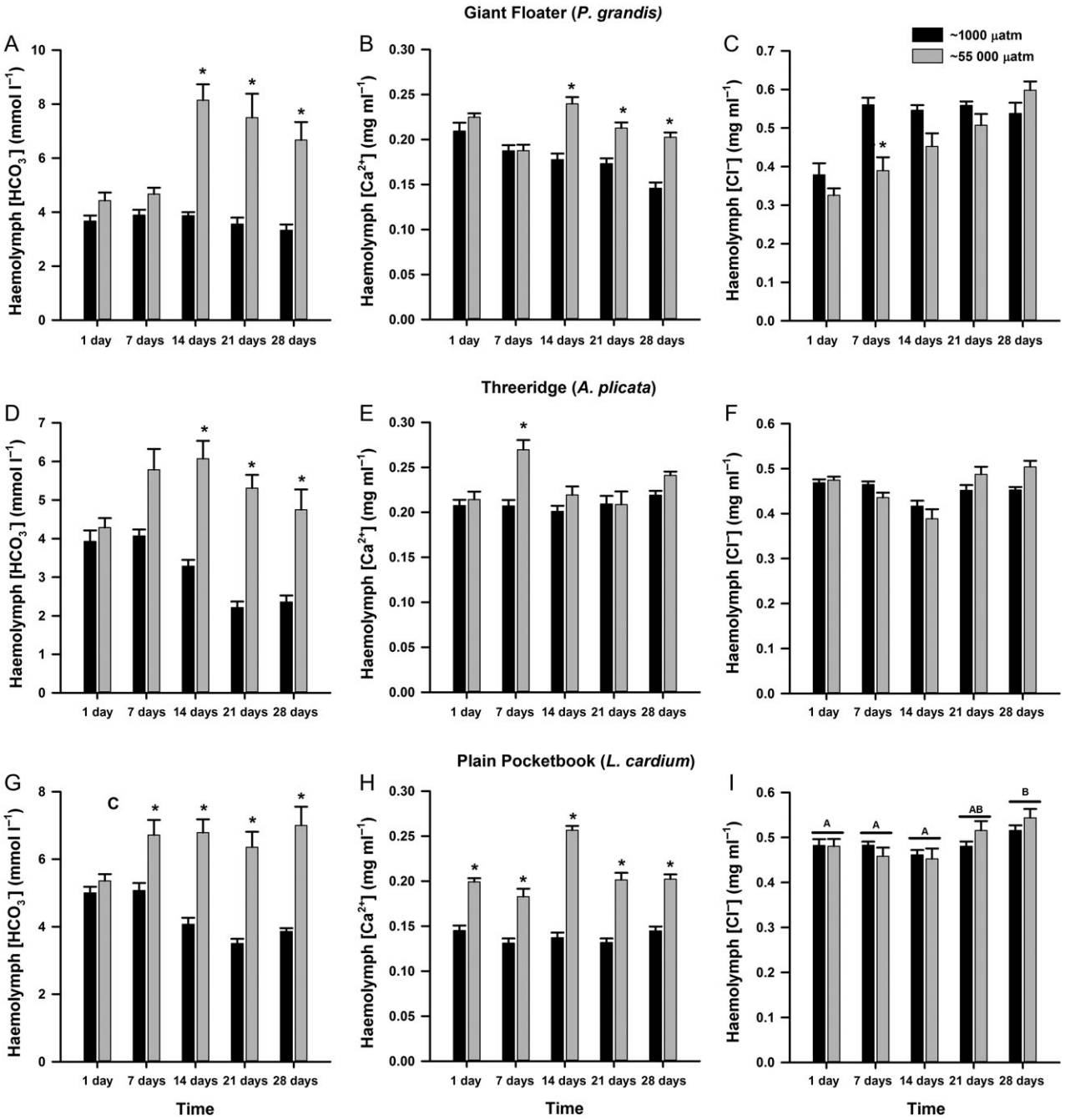

Figure 1: Concentrations of HCO_3^-, Ca^{2+} and Cl^- in the haemolymph of *Pyganodon grandis* (n = 9–14; **A–C**), *Amblema plicata* (n = 12–14; **D–F**) and *Lampsilis cardium* mussels (n = 13–14; **G–I**) exposed to two treatments of pCO_2, ~1000 µatm (control) or intermittent increase at ~55 000 µatm for 1, 7, 14, 21 or 28 days. Data are presented as means + SEM. *Groups that were significantly different from the control treatment within a time point (two-way ANOVA; see Tables 2–4). For (I), there was no significant interaction between pCO_2 treatment and sampling day; a bar above the treatments of a day represents a significant effect of time (two-way ANOVA).

control mussels also experienced a decrease in haemolymph Ca^{2+} concentrationsat 28 days compared with 1 day of treatment (treatment × day, $F = 8.42$, $P < 0.001$; Fig. 1B). For *A. plicata*, haemolymph Ca^{2+} in mussels exposed to fluctuating levels of CO_2 was significantly elevated compared with control mussels at 7 days of exposure (treatment × day, $F = 4.14$, $P = 0.003$; Fig. 1E). A similar increase in haemolymph Ca^{2+} in *L. cardium* occurred in response to fluctuating pCO_2, where

levels were significantly elevated compared with control mussels for the entire period of exposure (treatment × day, $F = 12.2$, $P < 0.001$; Fig. 1H).

For *P. grandis* and *A. plicata* (Tables 2 and 3), there was a significant interaction between treatment and day for haemolymph Cl^- concentrations, but only a significant effect of day for *L. cardium* (Table 4). Haemolymph Cl^- was lower in *P. grandis* exposed to fluctuating pCO_2 at 7 days of exposure relative to control mussels; however, it is important to note that this might have been attributable to a significant increase in haemolymph Cl^- concentrations in control mussels at 7 days compared with control mussels on day 1 of treatment (treatment × day, $F = 5.66$, $P < 0.001$; Fig. 1C). In *A. plicata*, although there was a significant interaction of treatment and day, haemolymph Cl^- was not significantly different in mussels exposed to fluctuating levels of CO_2 and control mussels at any point throughout the exposure (treatment × day, $F = 3.63$, $P = 0.008$; Fig. 1F). In *L. cardium*, no significant effect of CO_2 treatment was detected, and haemolymph Cl^- increased overall at 28 days of treatment (day, $F = 5.73$, $P < 0.001$; Fig. 1I).

With respect to haemolymph Na^+, a significant interaction between treatment and day was detected for all three species of mussels (Tables 2–4). *Pyganodon grandis* exposed to fluctuating pCO_2 had a significant elevation in haemolymph Na^+ at 28 days of exposure compared with control mussels (treatment × day, $F = 3.15$, $P = 0.017$; Fig. 2A). Haemolymph Na^+ for both *A. plicata* (treatment × day, $F = 12.85$, $P < 0.001$; Fig. 2B) and *L. cardium* (treatment × day, $F = 3.05$, $P = 0.0194$; Fig. 2C) exposed to the fluctuating pCO_2 were significantly elevated compared with control mussels beginning at 4 days and throughout the duration of the exposure period.

For haemolymph Mg^{2+}, a significant interaction between treatment and day was also found for all three species of mussels (Tables 2–4). Haemolymph Mg^{2+} was significantly reduced at 14 and 21 days of exposure to fluctuating pCO_2 compared with control mussels for *P. grandis* (treatment × day, *P. grandis*, $F = 28.33$, $P < 0.001$; Fig. 3A) and *A. plicata* (treatment × day, $F = 15.68$, $P< 0.001$; Fig. 3B), but these concentrations were no longer different from control mussels at 28 days of exposure. Likewise, haemolymph Mg^{2+} in *L. cardium* exposed to fluctuating CO_2 levels was significantly decreased compared with control mussels on 7 and 14 days but returned to control values after 21 days of exposure (treatment × day, $F = 7.31$, $P < 0.001$; Fig. 3C).

For haemolymph glucose, there was no significant interaction of treatment and day for any species of mussel (Tables 2–4). Haemolymph glucose concentrations of *P. grandis* and *L. cardium* were unaffected by pCO_2 exposure (treatment, $P > 0.05$; Fig. 4A and C). Haemolymph glucose of *A. plicata* was significantly affected by fluctuating pCO_2 treatment, but not sampling day, and was elevated throughout the exposure period compared with control mussels (treatment, $F = 8.75$, $P = 0.004$; Fig. 4B).

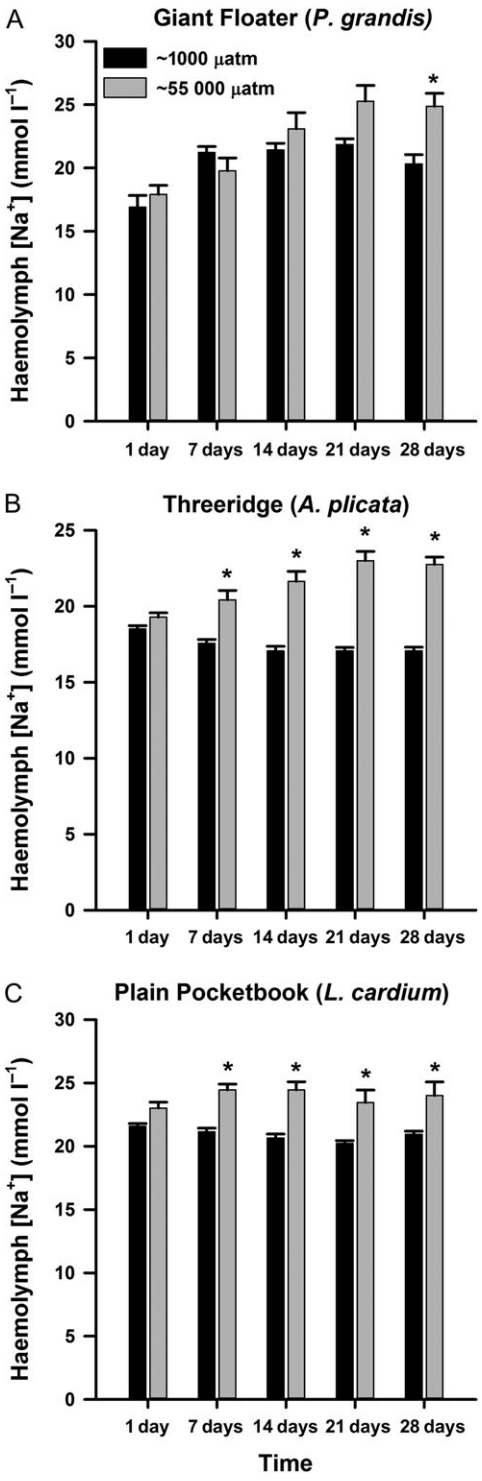

Figure 2: Concentrations of Na^+ in the haemolymph of *Pyganodon grandis* (n = 9–14; **A**), *Amblema plicata* (n = 12–14; **B**) and *Lampsilis cardium* mussels (n = 13–14; **C**) exposed to two treatments of pCO_2, ~1000 µatm (control) or intermittent increase at ~55 000 µatm for 1, 7, 14, 21 or 28 days. Data are presented as means + SEM. *Groups that were significantly different from the control treatment within a time point (two-way ANOVA; see Tables 2–4).

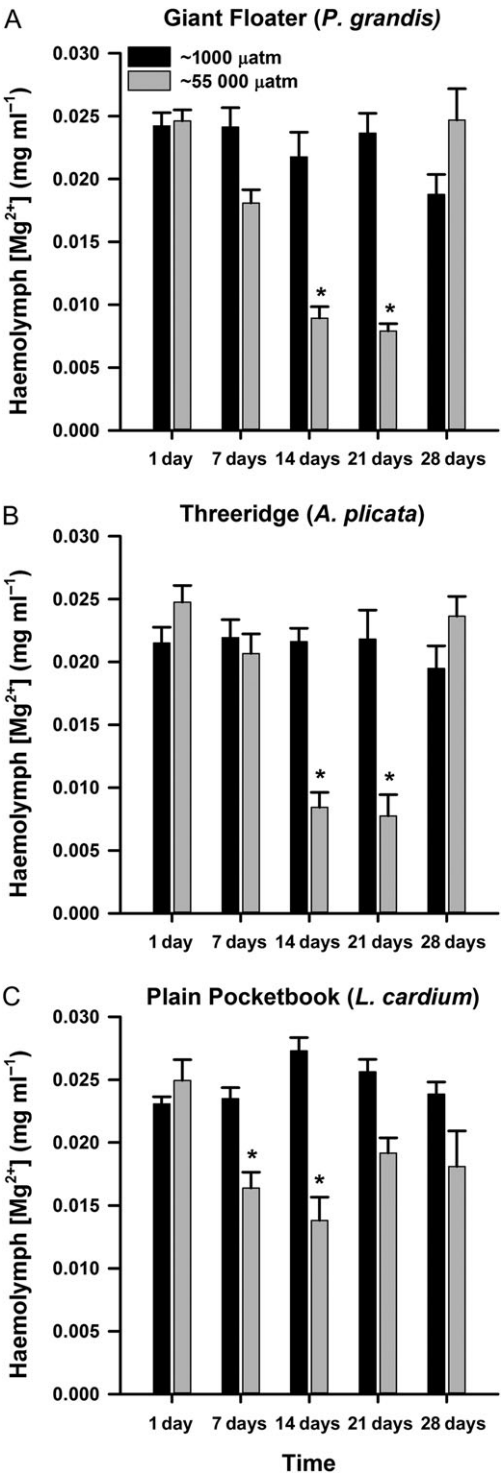

Figure 3: Concentrations of Mg^{2+} in the haemolymph of *Pyganodon grandis* (n = 9–14; **A**), *Amblema plicata* (n = 12–14; **B**) and *Lampsilis cardium* mussels (n = 13–14; **C**) exposed to two treatments of pCO_2, ~1000 µatm (control) or intermittent increase at ~55 000 µatm for 1, 7, 14, 21 or 28 days. Data are presented as means + SEM. *Groups that were significantly different from the control treatment within a time point (two-way ANOVA; see Tables 2–4).

Discussion

Following exposure to fluctuating elevated pCO_2, all three mussel species demonstrated physiological changes indicative of disturbance in acid–base regulation. Exposure to high CO_2 often causes the acidification of internal fluids in aquatic animals (Pörtner *et al.*, 2004), and one strategy for animals to buffer this internal acidosis is to increase HCO_3^- concentrations (Pörtner *et al.*, 2004; Pörtner, 2008). Marine mussels are thought to increase haemolymph HCO_3^- by using $CaCO_3$ released from the shell (Michaelidis *et al.*, 2005; Bibby *et al.*, 2008). In the present study, all three mussel species may have used this buffering strategy during intermittent pCO_2 exposure, as both haemolymph HCO_3^- and Ca^{2+} were elevated. Haemolymph HCO_3^- can also be increased by reducing activity of Cl^-–HCO_3^- exchangers, thus increasing the retention of HCO_3^- in the haemolymph but at the cost of Cl^- uptake (Byrne and Dietz, 1997). A reduction in haemolymph Cl^-, which appears to be a short-term response to elevated pCO_2 (Hannan *et al.*, 2016a, b), was observed only in *P. grandis*; however, this difference may have been due to rising Cl^- concentrations in the haemolymph of control mussels rather than a decrease in Cl^- of CO_2-treated mussels. A third strategy that mussels use to buffer acidosis is to mediate activity of Na^+–H^+ exchangers to increase excretion of H^+ ions, thus also increasing Na^+ uptake (Byrne and Dietz, 1997; Lannig *et al.*, 2010; Hannan *et al.* 2016b). Increases in haemolymph Na^+ were observed for all species of mussels exposed to fluctuating pCO_2, but the timing of the elevation in haemolymph Na^+ was species specific, as haemolymph Na^+ concentrations for *A. plicata* and *L. cardium* mussels increased after 4 days and for *P. grandis* after 28 days of exposure. Together, the results of the present study suggest that the three species of mussels used similar mechanisms to deal with acidosis to marine mussels (i.e. manipulating haemolymph HCO_3^- and H^+ concentrations); however, species-specific differences in these responses occurred.

In addition to an acid–base disturbance, the results of our study indicate that the stress response was also activated. An indicator of stress in freshwater mussels is declining Mg^{2+} of the haemolymph, which has been associated with stressors such as elevated temperature (Fritts *et al.*, 2015a), exposure to heavy metals (Hemelraad *et al.*, 1990) and chronic exposures to elevated pCO_2 (Hannan *et al.*, 2016a, b). Haemolymph Mg^{2+} concentrations decreased by ~2-fold in all mussel species exposed to the fluctuating pCO_2 treatment, but returned to control values after 28 days of exposure. In contrast, Hannan *et al.* (2016a) did not observe a return of Mg^{2+} to control values in *Fusconaia flava* exposed to ~20 000 µatm pCO_2 for 32 days. In addition, *Lampsilis siliquoidea* but not *A. plicata* exposed to either 20 000 or 55 000 µatm pCO_2 showed a decrease in haemolymph Mg^{2+} during 28 days of exposure, and these values returned to baseline once the CO_2 stressor was removed (Hannan *et al.* 2016b). Although the pCO_2 and the species of mussels were not the same in our study and those of Hannan *et al.* (2016a, b), these data suggest that

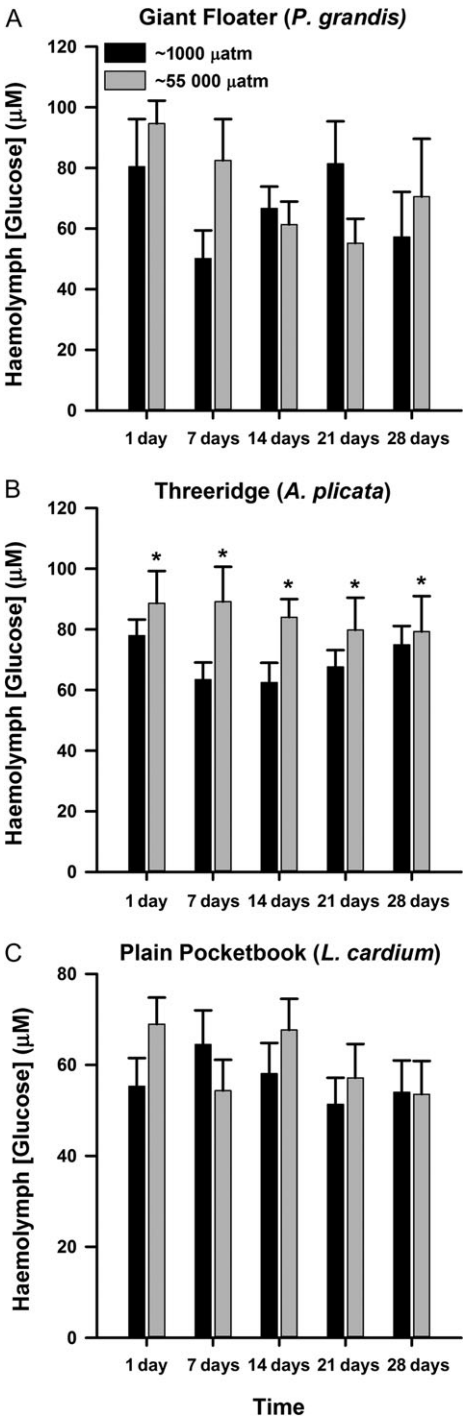

Figure 4: Concentrations of glucose in the haemolymph of *Pyganodon grandis* (n = 9–14; **A**), *Amblema plicata* (n = 12–14; **B**) and *Lampsilis cardium* mussels (n = 13–14; **C**) exposed to two treatments of pCO_2, ~1000 µatm (control) or intermittent increase at ~55 000 µatm for 1, 7, 14, 21 or 28 days. Data are presented as means + SEM. For (B), there was no significant interaction between pCO_2 treatment and sampling day; *significant effect of pCO_2 treatment between mussels exposed to fluctuating ~55 000 µatm and those exposed to ~1000 µatm (two-way ANOVA; see Table 3).

fluctuating exposures to elevated pCO_2 have a different effect on the Mg^{2+} response of unionid mussels compared with a chronic exposure. Haemolymph glucose concentrations, another indicator of stress in freshwater mussels (Patterson *et al.*, 1999; Fritts *et al.*, 2015a), were elevated only in *A. plicata*. Increasing glucose in response to stress comes at a cost to non-vital functions, such as growth, reproduction and movement (Patterson *et al.*, 1999; Fritts *et al.*, 2015a). Although the interaction of pCO_2 and sampling time was not significant in the model, the elevation in glucose in *A. plicata* appeared to return to control levels following 28 days of exposure to fluctuating pCO_2, suggesting that *A. plicata* recovers in terms of this stress marker by the end of the exposure period. A similar increase in haemolymph glucose was also observed for *A. plicata* exposed to a chronic elevation in pCO_2 at 55 000 µatm over a 28 day period (Hannan *et al.* 2016b), suggesting that fluctuating and long-term exposure to pCO_2 may have similar effects on haemolymph glucose in this species. Taken together, changes in haemolymph Mg^{2+} and glucose concentrations suggest that all three species of mussel experienced physiological stress during exposure to fluctuating pCO_2; however, desensitization, acclimation or recovery might have occurred over extended exposure to the intermittent CO_2 stressor.

Physiological changes, such as acid–base and stress responses, experienced by animals following a stressor are energetically challenging, and long-term upregulation or maintenance of these responses can lead to less energy availability for non-vital functions, such as growth and reproduction (Wendelaar Bonga, 1997). Following exposure to intermittent or repeated stressors, animals may respond to subsequent exposures in different ways (i.e. exacerbation, attenuation or no change; Reinert *et al.*, 2002). Our results suggest that the duration and CO_2 concentration used in the present study did not permit recovery between pulses of high pCO_2, evidenced by the fact that mussels sampled before and after the CO_2 exposure were not statistically different from each other. Additionally, the responses of mussels to intermittent pCO_2 exposure (i.e. elevations of Ca^{2+} and Na^+ and reduction in Mg^{2+}) were similar to those observed in unionid mussels exposed to a chronically elevated pCO_2 (Hannan *et al.*, 2016a, b), suggesting that mussels react to the intermittent and chronic CO_2 exposures in a similar way. However, differences in the responses of these variables during intermittent (present study) and chronic exposures (Hannan *et al.*, 2016a, b) did arise during the later stages of the 28 and 32 days exposure period, respectively. For instance, as mentioned above, the concentration of Mg^{2+} returned to control values by the end of the intermittent CO_2 exposure, whereas in previous studies using either chronic exposure to elevated CO_2 (Hannan *et al.*, 2016a, b) or elevated temperature (Fritts *et al.*, 2015b), Mg^{2+} remained reduced throughout the exposure period. In addition, haemolymph Ca^{2+} (*P. grandis* and *L. cardium*) and Na^+ (all three mussel species) remained elevated for the intermittent exposure to 55 000 µatm pCO_2, whereas these ions returned to control values by 32 days of chronic exposure to ~20 000 µatm pCO_2 for *F. flava* (Hannan *et al.*, 2016a). This sustained increase in

haemolymph Ca^{2+} and Na^{+}, as well as the difference in the dynamics of the haemolymph Mg^{2+} response in at least two of the mussels species, may suggest that mussels respond differently to intermittent and chronic CO_2 exposure. These responses also do not exclude the possibility that the differences might be species specific or driven by the difference in pCO_2 used in these two studies. The present study suggests that exposure to intermittent elevations in pCO_2 do result in acid–base disturbances and stress responses in unionid mussels that are both attenuated (e.g. Mg^{2+}) and exacerbated (Ca^{2+} and Na^{+}).

Species-specific responses observed in the present study might have resulted from a combination of differences in the physiology and behaviour of the three mussel species examined. Haemolymph Ca^{2+} was elevated in both *P. grandis* and *L. cardium* for more than half of the treatment period, whereas Ca^{2+} concentrations were elevated only on day 7 of exposure in *A. plicata*, suggesting that these species may rely differently on shell $CaCO_3$ stores. In addition, a decrease in haemolymph Cl^{-} was observed only in *P. grandis* and did not occur in either *L. cardium* or *A. plicata*. These differences in the studied unionid mussels suggest that they may use different strategies to retain HCO_3^{-} for acid–base regulation. Finally, similar elevations in haemolymph Na^{+} throughout nearly the entire pCO_2 exposure period were observed in *L. cardium* and *A. plicata*, whereas haemolymph Na^{+} in *P. grandis* was elevated only at 28 days of exposure. This difference in haemolymph Na^{+} concentrations in response to pCO_2 exposure suggests that *L. cardium* and *A. plicata* may rely on increased regulation of the Na^{+}–H^{+} exchanger to buffer acidosis, a mechanism that may be less important for *P. grandis* until CO_2 exposure is extended. In terms of measures of the stress response, a similar transient decrease in Mg^{2+} was observed across all species; however, haemolymph glucose was elevated only in *A. plicata*. Taken together, similar responses to intermittent elevation in pCO_2 were observed across the three species examined, and the species differences that arose highlight the importance of considering multiple species when testing an organism's reaction to a stressor.

Results obtained in our study increase the understanding of responses of freshwater unionid mussels to fluctuating exposures of elevated pCO_2, as modelled after a CO_2 barrier to invasive fish movement. There is evidence that, like marine mussels, if freshwater unionid mussels are exposed to elevated pCO_2 at either chronically high levels (Hannan *et al.*, 2016a, b) or intermittent elevations (pres study), they will experience acid–base disturbances. If unionid mussels were to be exposed to intermittent elevations in pCO_2 for an extended period of time, populations might be negatively affected owing to the increased energy demand of acid–base regulation and stress responses that may come at the expense of growth and reproduction. Additionally, resident mussel species may be affected differently, as evidenced by the observed species-specific responses to elevated pCO_2, which may arise because of differences in their behaviour and physiology. It is also important to consider that fluctuating elevations in pCO_2 may have similar but potentially also differential impacts compared with chronic exposures of elevated pCO_2 and that, generally, exposure time and duration between applications of a stressor are important aspects to consider for study design. Taken together, the results of our study suggest that the duration and manner of pCO_2 exposure (i.e. chronic vs. intermittent), as well as the species characteristics of resident unionid mussels that may be impacted, are all important factors to consider when designing, implementing and assessing the potential impacts of a CO_2 barrier.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

Acknowledgements

The authors would like to acknowledge Jeremy Tiemann, Kevin Cummings, Eric Schneider and Josh Sherwood, who provided valuable help collecting mussels. We would also like to thank Adam Wright for providing valuable help with mussel husbandry and laboratory assistance.

Funding

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.

References

American Society of Testing and Materials (2006) *Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels E2455-06*. Annual book of ASTM standards, Philadelphia, PA, USA.

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

Bergmeyer HU (1974) *Methods of Enzymatic Analysis*. Academic Press, New York, NY, USA.

Bibby R, Widdicombe S, Parry H, Spicer J, Pipe R (2008) Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. *Aquat Biol* 2: 67–74.

Bogan AE (2008) Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. *Hydrobiologia* 595: 139–147.

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

Byrne RA, Dietz TH (1997) Ion transport and acid–base balance in freshwater bivalves. *J Exp Biol* 200: 457–465.

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

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

Dietz TH (1974) Body fluid composition and aerial oxygen consumption in the freshwater mussel, *Ligumia subrostrata*: effects of dehydration and anoxic stress. *Biol Bull* 147: 560–572.

Dietz TH, Lessard D, Silverman H, Lynn J (1994) Osmoregulation in *Dreissena polymorpha*: the importance of Na, Cl, K, and particularly Mg. *Biol Bull* 187: 76–83.

Donaldson MR, Amberg J, Adhikari S, Cupp A, Jensen N, Romine J, Wright A, Gaikowski M, Suski CD (2016) Carbon dioxide as a tool to deter the movement of invasive bigheaded carps. *Trans Am Fish Soc* 145: 657–670.

Engqvist L (2005) The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. *Anim Behav* 70: 967–971.

Fritts AK, Peterson JT, Hazelton PD, Bringolf RB, MacLatchey D (2015a) Evaluation of methods for assessing physiological biomarkers of stress in freshwater mussels. *Can J Fish Aquat Sci* 72: 1–10.

Fritts AK, Peterson JT, Wisniewski JM, Bringolf RB, MacLatchy D (2015b) Nonlethal assessment of freshwater mussel physiological response to changes in environmental factors. *Can J Fish Aquat Sci* 72: 1460–1468.

Gustafson LL, Stoskopf MK, Bogan AE, Showers W, Kwak TJ, Hanlon S, Levine JF (2005) Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). *Dis Aquat Organ* 65: 159–165.

Hannan KD, Jeffrey JD, Hasler CT, Suski CD (2016a) Physiological effects of short- and long-term exposure to elevated carbon dioxide on a freshwater mussel, *Fusconaia flava*. *Can J Fish Aquat Sci* 73: 1538–1546.

Hannan KD, Jeffrey JD, Hasler CT, Suski CD (2016b) The response of two species of unionid mussels to extended exposure to elevated carbon dioxide. *Comp Biochem Physiol A Mol Integr Physiol* 201: 173–181.

Hartley HO (1950) The maximum F-ratio as a short-cut test for heterogeneity of variance. *Biometrika* 37: 308–312.

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

Hemelraad J, Holwerda DA, Wijnne HJA, Zandee DI (1990) Effects of cadmium in freshwater clams. I. Interaction with essential elements in *Anodonta cygnea*. *Arch Environ Contam Toxicol* 19: 686–690.

Hohn C, Petrie-Hanson L (2007) Low-cost aquatic lab animal holding system. *Zebrafish* 4: 117–122.

Horohov J, Silverman H, Lynn J, Dietz T (1992) Ion transport in the freshwater zebra mussel, *Dreissena polymorpha*. *Biol Bull* 183: 297–303.

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

Johnson MS, Billett MF, Dinsmore KJ, Wallin M, Dyson KE, Jassal RS (2010) Direct and continuous measurement of dissolved carbon dioxide in freshwater aquatic systems—method and applications. *Ecohydrology* 3: 68–78.

Kates D, Dennis C, Noatch MR, Suski CD, MacLatchy D (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.

Lannig G, Eilers S, Pörtner 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.

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

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.

Neves RJ, Moyer SN (1988) Evaluation of techniques for age determination of freshwater mussels (Unionidae). *Am Malacol Bull* 6: 179–188.

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

Patterson MA, Parker BC, Neves RJ (1999) Glycogen concentration in the mantle tissue of freshwater mussels (Bivalvia: Unionidae) during starvation and controlled feeding. *Am Malacol Bull* 15: 47–50.

Pörtner HO (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Mar Ecol Prog Ser* 373: 203–217.

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 (1993) Distribution-free and robust statistical methods: viable alternatives to parametric statistics. *Ecology* 74: 1617–1628.

Raymond PA, Hartmann J, Lauerwald R, Sobek S, McDonald C, Hoover M, Butman D, Striegl R, Mayorga E, Humborg C *et al.* (2013) Global carbon dioxide emissions from inland waters. *Nature* 503: 355–359.

Reinert KH, Giddings JM, Judd L (2002) Effects analysis of time-varying or repeated exposures in aquatic ecological risk assessment of agrochemicals. *Environ Toxicol Chem* 2: 1977–1992.

Reynaud S, Leclercq N, Romaine-Lioud S, Ferrier-Pagés C, Jaubert J, Gattuso J (2003) Interacting effects of CO_2 partial pressure and temperature on photosynthesis and calcification in a scleractinian coral. *Glob Chang Biol* 9: 1660–1668.

Ricciardi A, Neves RJ, Rasmussen JB (1998) Impending extinctions of North American freshwater mussels (Unionida) following zebra mussel (*Dressena polymorpha*) invasion. *J Anim Ecol* 67: 613–619.

Riebesell U, Fabry VJ, Hansson L, Gattuso JP (2010) *Guide to Best Practices for Ocean Acidification Research and Data Reporting*. Publications Office of the European Union, Luxembourg.

Robbins L, Hansen M, Kleypas J, Meylan S (2010) CO2calc—a user-friendly seawater carbon calculator for windows, Mac OS X, and iOS (iPhone). 1280, United States Geological Survey Open-File Report, Reston, VA, USA.

Rohlf FJ, Sokal RR (1995) *Statistical Tables*. W. H. Freeman and Company, New York, NY, USA.

Shirayama Y, Thornton H (2005) Effect of increased atmospheric CO_2 on shallow water marine benthos. *J Geophys Res-Oceans* 110: 1–7.

Siegel S, Castellan JN (1988) *Nonparametric Statistics for the Behavioral Sciences*. McGraw-HiU Book Company, New York, NY.

Trdan RJ (1981) Reproductive biology of *Lampsilis radiata siliquoidea* (Pelecypoda: Unionidae). *Am Midl Nat* 106: 243–248.

United States Army Corps of Engineers (2014a) *The Great Lakes Mississippi River interbasin study (GLIMRIS) report*. United States Army Corps of Engineers, Washington, DC, USA.

United States Army Corps of Engineers (2015) Lock performance monitoring system. http://corpslocks.usace.army.mil/lpwb/f?p=121:1:3908078497390.

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

Wang N, Ingersoll CG, Greer E, Hardesty DK, Ivey CD, Kunz JL, Brumbaugh WG, Dwyer FJ, Roberts AD, Augspurger T *et al.* (2007) Chronic toxicity of copper and ammonia to juvenile freshwater mussels (Unionidae). *Environ Toxicol Chem* 26: 2048–2056.

Wendelaar Bonga SE (1997) The stress response in fish. *Physiol Rev* 77: 591–625.

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

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

Zuur A, Ieno EN, Walker N, Saveliev AA, Smith GM 2009. *Mixed Effects Models and Extensions in Ecology with R*. Springer Science & Business Media, New York, NY, USA.