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 (CO₂) 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 CO₂ 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 pCO₂ (~1000 µatm) or intermittent conditions of pCO₂ (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 CO₂, suggesting that the recovery time between CO₂ pulses (1.5 h) was not sufficient for recovery from the CO₂ 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 pCO₂ (i.e. elevations in HCO₃⁻, Ca²⁺, Na⁺ and glucose, and decreases in Mg²⁺ 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

Introduction

Environmental levels of carbon dioxide (CO₂) 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 CO₂ 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 CO₂ gas (pCO₂) in marine ecosystems (Shirayama and Thornton, 2005). Unlike marine systems, there is no consensus regarding how pCO₂ will change in freshwater as a result of climate change (Hasler et al., 2016). In freshwater, pCO₂ 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 pCO₂ 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 μtm, with means in the USA ranging from 679 ± 543 to 9475 ± 993 μtm (Cole and Caraco, 2001). In addition to these natural sources of elevated pCO2, recent work has shown that zones of elevated CO2 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; Noacht and Suski, 2012). Although a specific method for the use of CO2 barriers to deter fish movement has not yet been defined, one potential application is the intermittent addition of CO2 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 CO2-rich water. Thus, downstream fluctuations in CO2 might occur, making CO2 a potential intermittent stressor for freshwater organisms.

A taxonomic group of freshwater organisms that may be particularly at risk to CO2 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 (Quadrunlini, Lampislini, 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 pCO2 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 pCO2, and a stress response to long-term exposure to elevated pCO2. Previous studies on marine bivalves indicate that elevated pCO2 causes internal acidosis (Michaelidis et al., 2005; Bibby et al., 2008) that is often buffered by increasing HCO3− in the fluids (Pörtner et al., 2004). Both marine (Michaelidis et al., 2005) and freshwater mussels (Hannan et al., 2016a, b) can increase haemolymph HCO3− by using CaCO3 released from the shell as a result of decreased pH and elevated CO2 (i.e. increases both haemolymph HCO3− and Ca2+) or by reducing the activity of the Cl−–HCO3− exchanger to retain HCO3− 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 pCO2 also appears to initiate the general stress response in mussels, because a decrease in haemolymph Mg2+ 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 CO2 stressors in mussels have used a continuous application of CO2 rather than one that was intermittent as might be expected downstream of a CO2 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 pCO2 on three species of freshwater mussels each belonging to a different tribe, Pyganodon grandis (tribe Anodontini), Ambeloma plica (tribe Amblemini) and Lampisus cardium (tribe Lampislini). To accomplish this goal, over a 28 day period the mussels were exposed to either control pCO2 or intermittent increases in pCO2 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 pCO2 affect the acid-base and stress responses of various freshwater mussel species in habitats where pCO2 fluctuates.

Materials and methods
Mussel collection and husbandry

Plain pocketbook (L. cardium) and three ridge 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. Materials and methods. 2006

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 pCO2 also appears to initiate the general stress response in mussels, because a decrease in haemolymph Mg2+ 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 CO2 stressors in mussels have used a continuous application of CO2 rather than one that was intermittent as might be expected downstream of a CO2 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 pCO2 on three species of freshwater mussels each belonging to a different tribe, Pyganodon grandis (tribe Anodontini), Ambeloma plica (tribe Amblemini) and Lampisus cardium (tribe Lampislini). To accomplish this goal, over a 28 day period the mussels were exposed to either control pCO2 or intermittent increases in pCO2 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 pCO2 affect the acid-base and stress responses of various freshwater mussel species in habitats where pCO2 fluctuates.

Materials and methods
Mussel collection and husbandry

Plain pocketbook (L. cardium) and three ridge 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 (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 constituents: Nannochloropsis sp. 1–2 μm and a mixed diet of Isochrysis, Pavlova, Thalassiosira and Tetraselmis 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 CO₂ 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 CO₂ and TA, respectively).

Fluctuating CO₂ exposure

To define the impacts of fluctuating CO₂ 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 CO₂ treatment. In the CO₂ treatment system, pCO₂ 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 CO₂ gas into the water through an air stone (see Supplementary material, Fig. S1). Elevated pCO₂ 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 pCO₂ 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 CO₂ level that could deter the movement of fishes (Donaldson et al., 2016) and will possibly be the target level of a CO₂ barrier. Twelve fluctuations per day represents the historical low usage of Brandon Road Lock (41.5054°N, 88.0996°W), a possible site for deployment of a CO₂ barrier within the Des Plaines River, IL, USA (United States Army Corps of Engineers, 2014a, 2015). The target pCO₂ was maintained with a pH controller (PIPOINTER®, American Marine Inc., CT, USA) that automatically bubbled CO₂ 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 CO₂ was then returned to ~1000 µatm by bubbling in air though an air stone to off-gas excess CO₂. An identical recirculating system was used as a control, and mussels in this control system were treated in the same way as animals receiving CO₂, except that infused CO₂ gas was replaced with compressed air such that mussels were held continuously at ambient ~1000 µatm (876 ± 108 µatm; pH = 8.13 ± 0.02) pCO₂. A digital timer (DT620 Heavy Duty Digital Timer, Intermatic Inc Spring Grove, IL, USA) was used to control additions of CO₂ and air. A modified infrared probe was used to measure pCO₂ (Vaisala GMP220 and GMT221, Vantaa, Finland; Johnson et al., 2010), along with a CO₂ titration kit to determine the concentration of CO₂ (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 pCO₂ (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 pCO₂ or control conditions. Mussels were sampled during the 1.5 h period when CO₂ was at ambient levels, not during the 0.5 h when CO₂ levels were elevated. Prior to starting this study, it was not known whether sampling mussels immediately prior to the increase in CO₂ or immediately after the period of increased CO₂ would be optimal to define the impacts of CO₂ on physiological parameters. Therefore, mussels were sample during both intervals, and n = 7 animals were sampled immediately prior to the increase in CO₂, whereas a second n = 7 animals were sampled immediately following the increase in pCO₂, once pCO₂ returned to control values. All samples were collected around the 12.00 h CO₂ 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 000g 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 CO₂ 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 and one tissue (tissue + shell) was collected around the 12.00 h CO₂ exposure to standardize any potential for diel variation in physiological parameters.

The dry weight and length of individuals within each species was not different between control and fluctuating CO₂ 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 pCO₂ treatments, respectively, and for two A. plicata and one L. cardium exposed to the fluctuating pCO₂ treatment.

Laboratory analyses

Haemolymph Cl⁻, Mg²⁺ and Ca²⁺ concentrations were assayed in duplicate using commercially available kits (QuantiChrom assay kits Cl⁻, catalogue no. DICL-250; Mg²⁺, catalogue
no. DIMG-250; Ca²⁺, catalogue no. DICA-500; BioAssay Systems, Hayward, CA, USA). Haemolymph HCO₃⁻ 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

Table 1: Results of Student’s unpaired t-test examining the impact of dry weight and length on different pCO₂ 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 pCO₂ on Pyganodon grandis exposed to one of two different pCO₂ treatments [~1000 µatm (ambient); intermittent at ~55 000 µatm] for 28 days

| Measured variable | Main effects       | Sum of squares | d.f. | F      | P-value  |
|-------------------|--------------------|----------------|------|--------|----------|
| HCO₃⁻ (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  |        |          |
| Ca²⁺ (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  |        |          |
| Mg²⁺ (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.
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 CO₂ exposure on haemolymph ion levels and glucose concentrations were quantified using a two-way analysis of variance (ANOVA), with pCO₂ (fluctuating or control), sampling day and their interaction (pCO₂ × sampling day) entered into each model as fixed effects. Individual mussel identification number (ID), time point (i.e. sampling before or after pCO₂), 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 pCO₂ treatments.

Table 3: Results of two-way ANOVA examining the impact of fluctuating exposure to elevated pCO₂ on Amblema plicata exposed to one of two different pCO₂ treatments (~1000 µatm (ambient); intermittent at ~55 000 µatm) for 28 days

| Measured variable | Main effects | Sum of squares | d.f. | F     | P-value |
|-------------------|--------------|----------------|------|-------|---------|
| HCO₃⁻ (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  |        |         |
| Ca²⁺ (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  |        |         |
| Mg²⁺ (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 $\alpha$ was <0.05. For all variables and species, there was no effect of sampling before vs. after CO2 application (i.e. time point) for either treatment (control or fluctuating), so data from mussels sampled before and after CO2 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

| 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.
control mussels also experienced a decrease in haemolymph Ca\(^{2+}\) concentrations at 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 pCO₂ 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 = 3.63, P = 0.008; Fig. 1F). In *L. cardium*, no significant effect of CO₂ 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 pCO₂ 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 pCO₂ were significantly elevated compared with control mussels beginning at 4 days and throughout the duration of the exposure period.

For haemolymph Mg²⁺, a significant interaction between treatment and day was also found for all three species of mussels (Tables 2–4). Haemolymph Mg²⁺ was significantly reduced at 14 and 21 days of exposure to fluctuating pCO₂ compared with control mussels for *P. grandis* (treatment × day, *P. grandis*, F = 28.33, P < 0.001; Fig. 3A) and *A. plicata* (treatment × day, *A. plicata*, 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 Mg²⁺ in *L. cardium* exposed to fluctuating CO₂ 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, *L. cardium*, 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 pCO₂ exposure (treatment, P > 0.05; Fig. 4A and C). Haemolymph glucose of *A. plicata* was significantly affected by fluctuating pCO₂ 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 pCO₂ (~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 $p$CO$_2$, 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 $p$CO$_2$ 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 $p$CO$_2$ (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 $p$CO$_2$, 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 (Hemelaar et al., 1990) and chronic exposures to elevated $p$CO$_2$ (Hannan et al., 2016a, b). Haemolymph Mg$^{2+}$ concentrations decreased by ~2-fold in all mussel species exposed to the fluctuating $p$CO$_2$ 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 $p$CO$_2$ for 32 days. In addition, Lampsilis silicicola but not A. plicata exposed to either 20 000 or 55 000 µatm $p$CO$_2$ 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 $p$CO$_2$ and the species of mussels were not the same in our study and those of Hannan et al. (2016a, b), these data suggest that...
fluctuating exposures to elevated pCO₂ have a different effect on the Mg²⁺ 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 pCO₂ 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 pCO₂, 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 pCO₂ at 55 000 µatm over a 28 day period (Hannan et al. 2016b), suggesting that fluctuating and long-term exposure to pCO₂ may have similar effects on haemolymph glucose in this species. Taken together, changes in haemolymph Mg²⁺ and glucose concentrations suggest that all three species of mussel experienced physiological stress during exposure to fluctuating pCO₂; however, desensitization, acclimation or recovery might have occurred over extended exposure to the intermittent CO₂ 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 CO₂ concentration used in the present study did not permit recovery between pulses of high pCO₂, evidenced by the fact that mussels sampled before and after the CO₂ exposure were not statistically different from each other. Additionally, the responses of mussels to intermittent pCO₂ exposure (i.e. elevations of Ca²⁺ and Na⁺ and reduction in Mg²⁺) were similar to those observed in unionid mussels exposed to a chronically elevated pCO₂ (Hannan et al., 2016a, b), suggesting that mussels react to the intermittent and chronic CO₂ 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 Mg²⁺ returned to control values by the end of the intermittent CO₂ exposure, whereas in previous studies using either chronic exposure to elevated CO₂ (Hannan et al., 2016a, b) or elevated temperature (Fritts et al., 2015b), Mg²⁺ remained reduced throughout the exposure period. In addition, haemolymph Ca²⁺ (P. grandis and L. cardium) and Na⁺ (all three mussel species) remained elevated for the intermittent exposure to 55 000 µatm pCO₂, whereas these ions returned to control values by 32 days of chronic exposure to ~20 000 µatm pCO₂ for F. flava (Hannan et al., 2016a). This sustained increase in

![Figure 4: Concentrations of glucose in the haemolymph of Pyganodon grandis (n = 9-14; A), Amblema plicata (n = 12-14; B) and Lampsis cardium mussels (n = 13-14; C) exposed to two treatments of pCO₂ (~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 pCO₂ treatment and sampling day; *significant effect of pCO₂ treatment between mussels exposed to fluctuating ~55 000 µatm and those exposed to ~1000 µatm (two-way ANOVA; see Table 3).](https://academic.oup.com/conphys/article-abstract/4/1/cow066/2753367/10?c=1&printable=yes)
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 mussel 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 the freshwater mussel, *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 flavia*. 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 pCO2? Ecol Lett 19: 98–108.

Hemelraad J, Holwerda WA, Wijmijn 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, Noath 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.

Noath 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 CO2 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, Stierl 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 CO2 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 (Driendena 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 CO2 on shallow water marine benthos. *J Geophys Res-Oceans* 110: 1–7.

Siegel S, Castellan JN (1988) *Nonparametric Statistics for the Behavioral Sciences*. McGraw-Hill 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 (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, Hardey 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.