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The response of two species of unionid mussels to extended exposure to elevated carbon dioxide

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ABSTRACT

Changes in environmental conditions can act as stressors, with potential consequences for the health and fitness of organisms. Rising levels of carbon dioxide (CO_2) is one potential environmental stressor that is occurring more frequently in the environment and can be a stressor for aquatic organisms. In this study, the physiological responses of two species of unionid mussel, *Lampsilis siliquoidea* and *Amblema plicata*, were assessed in response to exposure to two levels of elevated partial pressure of CO_2 (pCO_2) (~20,000 and ~55,000 μatm) over a 28 d period, followed by a subsequent 14 d recovery period. Observations were consistent with responses associated with respiratory acidosis, as demonstrated by changes in hemolymph HCO_3^-, Ca^{2+}, Cl^-, and Na^{2+}. Both species exposed to elevated pCO_2 had elevated hemolymph HCO_3^- during the pCO_2 treatment period compared to control mussels, but recovered once pCO_2 was removed. Similarly, both species had elevated hemolymph Na^+ during exposure to elevated pCO_2, and this returned to control levels for *A. plicata* but remained elevated for *L. siliquoidea* once the pCO_2 stimuli was removed. Changes in hemolymph Ca^{2+} and Cl^- in response to elevated pCO_2 were also observed, but these changes were species-specific. Additional physiological responses to elevated pCO_2 (e.g., changes in hemolymph glucose and Mg^{2+}) were consistent with a stress response in both species. This study demonstrates the importance of considering inter-specific differences in the response of organisms to stress, and also that responses to elevated pCO_2 may be transient and can recover once the stress is removed.

1. Introduction

Changes to the environment can induce physiological responses in organisms, which can have ultimate effects on overall health and fitness (Wikelski and Cooke, 2006). While understanding environmental change and its consequences for overall fitness and survival is critical for predicting future population changes, it is also important to define the sub-lethal impacts of stressors on animals, as sub-lethal physiological changes can provide valuable insight into how organismal physiology predicts population-level effects (Madliger and Love, 2014). With a finite level of resources available to an organism, increasing maintenance costs to respond to an environmental stressor can reduce the potential allocation of these resources to growth and reproduction (Maltby, 1999), thereby providing a link to potential consequences for a population following perturbations (Fefferman and Romero, 2013). Defining physiological endpoints that relate to population-level consequences has become a critical management and conservation tool with increasing human population causing major alterations to the biotic and abiotic environment at an accelerated rate (Bijlsma and Loeschcke, 2005).

One environmental change that has the potential to impact populations, and is occurring more frequently in both the terrestrial and aquatic environments, is an increase in carbon dioxide (CO_2) (Manabe and Wetherald, 1980). Carbon dioxide has increased due to a number of potential sources, both natural (i.e., daily and seasonal fluctuations in CO_2) (Maberly, 1996) and anthropogenic (e.g., climate change, and more recently CO_2 fish barriers) (Hasler et al., 2016; Noatch and Suski, 2012). Due to its effectiveness at deterring fish movement, the use of CO_2 has been considered as a non-physical barrier to prevent the movement of fishes (Donaldson et al., 2016; Noatch and Suski, 2012). In the aquatic environment, these natural and anthropogenic increases in CO_2 raise the partial pressure of CO_2 (pCO_2) in the water, which can cause a number of negative consequences for aquatic organisms (Heuer and Grosell, 2014). Studies that quantify the consequences of elevated aquatic pCO_2 have mainly focused on marine systems, which tend to parallel atmospheric levels of CO_2. However, freshwater systems, unlike marine systems, are highly variable in terms of the levels of pCO_2 that occur (Hasler et al., 2016). These levels can vary from below 100 μatm to over 4000 μatm depending on the substrate, productivity of the surrounding terrestrial environment, precipitation, aquatic respiration, and other factors (reviewed in Hasler et al., 2016). Furthermore, in a review of ~7000 global streams and rivers, the average median values for pCO_2 was ~3100 μatm (Raymond et al., 2013). In another review of 47 large rivers found globally, mean pCO_2 varied

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from 679 ± 543 μatm to 35,617 ± 46,757 μatm, and specifically, the means of rivers in the USA ranged from 679 ± 543 μatm to 9475 ± 993 μatm (Cole and Caraco, 2001). In addition, unlike marine systems, there is currently no clear consensus on how pCO_2 may change in freshwater systems as a result of environmental change, thus increasing the uncertainty of how freshwater biota will be affected by future changes to climate (Hasler et al., 2016).

Due to links between climate change and elevated pCO_2 in the ocean (Kurihara, 2008), the response of marine invertebrates to elevated pCO_2 have been well studied, and are highly variable (Kroeker et al., 2014; Pörtner et al., 2005). The varied responses in marine invertebrates, coupled with the fluctuations/lack of predictions for future changes in freshwater pCO_2, make the impacts of elevated pCO_2 on freshwater mussels difficult to predict. Elevated pCO_2 causes acidosis in the tissues and body fluids of aquatic animals (Pörtner et al., 2004), and marine invertebrates display a suite of physiological disturbances in response to acidosis, including increases and decreases in metabolic rate, reduced protein synthesis, altered ion exchange rates, reduced calcification, and reduced growth (Bibby et al., 2008; Dissanayake et al., 2010; Michaelidis et al., 2005). However, there are mechanisms to counteract the acidosis experienced by aquatic invertebrates, such as the accumulation of bicarbonate (HCO_3^-), excretion of hydrogen ions, and other regulatory processes (Pörtner et al., 2004). One mechanism by which mussels may increase hemolymph HCO_3^- when exposed to elevated pCO_2 is by utilizing $CaCO_3$ released from the shell into their hemolymph. This release of $CaCO_3$ also results in an increase in Ca^{2+} in the hemolymph (Michaelidis et al., 2005; Bibby et al., 2008). Another strategy to increase HCO_3^- in the hemolymph is through regulation of the Cl^-/HCO_3^- exchanger, which may result in changes in hemolymph Cl^- levels (Byrne and Dietz, 1997). An additional mechanism to reduce acidosis of internal fluids is through the active transport and removal of H^+ across cell and epithelial membranes (Gazeau et al., 2013). An increase in the activity of the Na^+/H^+ exchanger to excrete H^+ is a common strategy used by aquatic animals experiencing acidosis, which can result in an increase in hemolymph Na^+ (Byrne and Dietz, 1997; Lannig et al., 2010). Even though regulating acid-base parameters may not be detrimental in the short-term, chronically altering these mechanisms is expected to affect long-term growth (Michaelidis et al., 2005; Kurihara, 2008), calcification (Gazeau et al., 2013), and immune function (Bibby et al., 2008; Beesley et al., 2008), which may translate to negative consequences at the population- and species-levels (Fefferman and Romero, 2013; Pörtner et al., 2004).

Although the negative consequences of elevated pCO_2 have been repeatedly observed in marine mussels (Michaelidis et al., 2005; Kurihara, 2008; Bibby et al., Beesley et al., 2008), a paucity of research has been done on the effects of elevated pCO_2 on freshwater mussels. Studies evaluating the consequences of emersion (air exposure) in freshwater mussels have observed physiological consequences similar to those experienced by marine mussels exposed to elevated pCO_2 (Byrne and McMahon, 1994; Heming et al., 1988); however, the responses of freshwater mussels to elevated pCO_2 in water may differ to those of emersion, as mussels have the capacity to open their valves and exchange ions with the water more freely during exposure to elevated pCO_2, unlike during emersion. Understanding the impacts of elevated pCO_2 on freshwater mussels becomes critical with the ever-increasing CO_2 in aquatic systems (either naturally or due to anthropogenic alterations), coupled with the lack of research on its consequences for already threatened freshwater mussels.

Freshwater mussels have their highest abundance in North American and are one of the most threatened taxa worldwide (Williams et al., 1993). Mussels are generally thought of as characterless animals, however, they have a variety of differences in morphologies (e.g., shell thickness, size, reproductive strategies, etc.) and behaviors (e.g., feeding, righting, movement, burrowing, gaping), especially in response to disturbances (Waller et al., 1999). This high degree of variability in mussel characteristics supports the use of multiple species when defining responses to stressors as this variability can describe how different species respond stressors. Additionally, with increasing threats to their survival, and more than half of freshwater mussel species listed as threatened or endangered (71%), it is critical that precautions be taken to define how environmental changes will affect growth and survival (Williams et al., 1993). The potential exposure of freshwater mussels to elevated pCO_2 may occur due to a number of natural and anthropogenic scenarios, and thus, it is important to understand the potential impacts of CO_2 exposure on this threatened taxon.

Based on this background, the goals of this study were to (1) quantify the physiological impacts of chronic exposure to elevated pCO_2 on two species of freshwater mussels from two different mussel tribes that have different life history strategies, shell thicknesses, and sensitivity to toxicity, and (2) define whether or not physiological disturbances associated with chronic exposure to elevated pCO_2 would recover once the exposure ended. To address these goals, fatmucket (*Lampsilis siliquoidea*) and threeridge (*Amblema plicata*) mussels were exposed to two different CO_2 levels for up to 28 d and then given up to 14 d to recover. It was predicted that changes in hemolymph ion levels will occur as a consequence of the acid-base disturbance experienced during exposure to elevations in pCO_2 similar to those of marine mussels described above.

2. Methods

2.1. Mussel collection and husbandry

Adult fatmucket (*L. siliquoidea*) mussels (65.3 ± 0.04 mm length (standard error), 34.6 ± 0.03 mm width, and 24.2 ± 0.02 mm depth) were delivered overnight from Missouri State University, Springfield, MO, in June 2015 to the Aquatic Research Facility at the University of Illinois, Urbana-Champaign, IL, USA. Adult threeridge mussels (*A. plicata*) (76.4 ± 0.06 mm length, 63.0 ± 0.06 mm width, and 39.8 ± 0.03 mm depth) were collected by benthic grab from the Mississippi River, near Cordova, IL, in July 2015. Following collection, mussels were transported to the Aquatic Research Facility at the University of Illinois, Champaign-Urbana, IL in coolers (travel time < 3 h). Upon arrival at the Aquatic Research Facility, both species of mussels were cleaned of epibionts, tagged for individual identification with Queen Marking Kit tags (The Bee Works, Orillia, ON, CA) (Neves and Moyer, 1988) or marked with a permanent marker. They were then placed in one of three holding tubs (1135.6 L) supplied with water from a 0.04 ha natural, earthen-bottom pond with ample vegetation, where they remained for at least one wk to recover from transport and hauling stressors and acclimate to lab conditions (Dietz et al., 1994; Dietz, 1974; Horohov et al., 1992). All tubs were equipped with a Teco 500 aquarium heater/chiller (TECO-US, Aquarium Specialty, Columbia, SC, USA) to prevent temperature fluctuations, and a low-pressure air blower (Sweetwater, SL24H Pentair, Apopka, FL, USA) for aeration. Fifty percent water changes using pond water were performed weekly to maintain water quality. Mussels were fed a commercial shellfish diet with multiple particle sizes consisting of *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 (Wang et al., 2007); mussels did not receive supplemental food 24 h prior to sampling. Dissolved oxygen (DO) and temperature were recorded daily in all three tubs with a portable meter (YSI 550 A, Yellow Springs Instruments, Irvine, CA, USA) and averaged 8.2 ± 0.02 mg L^{-1} (mean ± standard error, SE) and 21.6 ± 0.1 °C (respectively) throughout both the acclimation and experimental periods. Water pH and total alkalinity (TA) were also measured daily using a handheld meter (WTW pH 3310 meter, Germany) calibrated regularly (Moran, 2014) and a digital titration kit (Titrator model 16,900, cat. no. 2271900, Hach Company, Loveland, CO, USA), respectively, and averaged 8.425 ± 0.031 for pH (<100 μatm) and 1178.9 ± 29.9 μmol/kg for TA during the acclimation period.

2.2. Chronic CO_2 exposure

To quantify physiological disturbances due to chronic CO_2 exposure, mussels (*A. plicata*, $N = 120$; *L. siliquoidea*, $N = 132$) were removed from the holding tubs described above and separated randomly into one of three 128.7 L recirculating tank systems, as above. Each recirculating tank system was held at either ambient (<100 μatm, the lowest detectable limit of the CO_2 probe, see below; pH of 8.242 ± 0.028), ~20,000 μatm (17,181.90 ± 0.05 μatm; pH 7.261 ± 0.025), or ~55,000 μatm (52,644.70 ± 0.10 μatm; pH 6.857 ± 0.059) pCO_2 for 28 d. Air pressure throughout the experimental period was 1019.30 ± 0.33 hPa/mb and TA was 1189.6 ± 25.8 μmol/kg. A target of ~55,000 μatm was included because is the proposed target for a possible CO_2 barrier to invasive fish movement (Donaldson et al., 2016). The ~20,000 μatm level was targeted because previous work by Dennis et al. (2015) found that, for fish, extended holding at pCO_2 above 15,000 μatm began to induce negative physiological disturbances, and Heuer and Grosell (2014) indicated that the use of multiple CO_2 levels within a single study can help define mechanisms of CO_2 impacts. Values of pCO_2 were 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 pH rise above a target level during exposure (Reynaud et al., 2003; Riebesell et al., 2010). Temperature, pH and TA data collected from the tank systems were entered into CO2calc to calculate pCO_2 (Robbins et al., 2010). A modified infrared probe (Vaisala GMP220 and GMT221, St. Louis, MO, USA) was also used to verify pCO_2 (Johnson et al., 2010), along with the daily use of a CO_2 titration kit to determine the concentration of CO_2 (Hach Company, cat. no. 2272700).

Mussels were sampled on day 1, 4, 7, or 28 of exposure to ~55,000 μatm ($N = 6$ for *A. plicata* and $N = 7$ for *L. siliquoidia*), ~20,000 μatm ($N = 6$ for *A. plicata* and $N = 7$ for *L. siliquoidia*) or control/ambient conditions (<100 μatm; $N = 8$ for both species). An additional set of mussels ($N = 50$ for *L. siliquoidiea* and $N = 41$ for *A. plicata*) was returned to control pCO_2 levels (<100 μatm, the lowest detectable limit of the probe) following the 28 d exposure period. Mussels that were previously held at ~55,000 μatm ($N = 6, 7$ for *A. plicata* and *L. siliquoidea*, respectively), ~20,000 μatm ($N = 6, 7$ for *A. plicata* and *L. siliquoidea*, respectively), or control pCO_2 conditions ($N = 8$ for both species) were sampled following an additional 7 or 14 d at ambient conditions to quantify recovery from CO_2 exposure. As it has been found that the CO_2 concentrations of freshwater systems have a very large range, including 175 fold below and 57 fold above atmospheric equilibrium (Cole et al., 1994), <100 μatm is a reasonable value control value, 20,000 μatm is a reasonable extreme high value, and 55,000 μatm is a value that mussels would only experience at a CO_2 barrier to fish movement or in the far future.

Mussel sampling included measurements for length, width, and depth of the whole animal using a digital caliper (traceable digital carbon fiber calipers, Fisher Scientific, Pittsburgh, PA, USA), and a waterproof balance (HL-300WP, A&D, Ann Arbor, MI, USA) for weight of the whole animal (tissue + shell) to the nearest 0.01 g. Volume of the whole animal was collected by immersing the mussel in a graduated cylinder with a known volume of water and quantifying the water displacement (Okumus and Stirling, 1998). Shell cavity volume was generated by immersing just the shell in a graduated cylinder with a known volume of water and subtracting from the volume of the whole animal. If possible, sex was determined for *L. siliquoidia* using both their external sexual dimorphism and by examination of the gills for glochidia, which are only found in female mussels (Trdan, 1981). Hemolymph was extracted from the anterior adductor muscle with a 1 mL syringe and 26 G needle (Gustafson et al., 2005b), and centrifuged at 12,000 × gravity (g) for 2 min (see (Gustafson et al., 2005a). Following centrifugation, the supernatant was removed, flash frozen in liquid nitrogen, and stored at −80 °C until processing.

2.3. Laboratory analyses

Hemolymph Cl^-, Mg^{2+} and Ca^{2+} concentrations were assayed in duplicate using commercially available kits (QuantiChrom assay kits Cl^-, cat. no. DICL-250; Mg^{2+}, cat. no. DIMG-250; Ca^{2+}, cat. no. DICA-500; BioAssay Systems, Hayward, CA, USA). Hemolymph Na^+ and HCO_3^- levels were measured by the Diagnostic Clinical Pathology Lab at the University of Illinois Urbana-Champaign, IL, USA using a Beckman chemistry analyzer (Beckman Coulter AU680, Beckman Coulter, Brea, CA, USA). Quality control testing for this analyzer was performed at least every 24 h, and the lab participates in a quality assurance program that performs surveys periodically throughout the year to ensure accuracy and precision of samples. Hemolymph 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). Samples were re-run if the intra-assay coefficients of variability were >10%. Additionally, inter-assay coefficients of variability were <10%.

2.4. Statistical analyses

A two-way analysis of variance (ANOVA) showed that there were no differences in length, width, depth, volume, and dry weight for both species of mussels across all treatment and time periods ($p > 0.400$). The effect of CO_2 exposure on hemolymph ion levels and glucose concentrations was assessed using a two-way analysis of variance, with pCO_2 (~55,000 μatm, ~20,000 μatm, and <100 μatm [control]), sampling day, and their interaction ($pCO_2 \times$ day) entered into each model as fixed effects. Volume, dry weight, and sex were initially included in the models as co-factors to quantify their potential influence on response variables, but were removed if they had no significant effect on the model (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).

For all statistical tests, analysis of fitted residuals using a Shapiro Wilk test (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 and then re-analyzed 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 ± SE where appropriate. All tests were performed using R (3.2.2 for Macintosh HD) (R Core Team, 2015), and differences were considered significant if α was <0.05.

3. Results

Following 1 d of exposure to elevated pCO_2, *L. siliquoidea* held at the highest level (~55,000 μatm) exhibited a 34% increase in hemolymph HCO_3^- compared to mussels held at control conditions, and HCO_3^- in mussels exposed to the highest pCO_2 remained significantly elevated for the entire length of the exposure period (i.e., 28 d; Table 1, Fig. 1A). Concentrations of HCO_3^- in the hemolymph of *A. plicata* held at both ~20,000 and ~55,000 μatm pCO_2 increased relative to controls throughout the treatment period, but this increase was statistically significant only at 4 d (57% increase for mussels held at ~55,000 μatm) and 28 d (45% increase for mussels held at ~20,000 μatm) of exposure (Table 2, Fig. 1B). For both *L. siliquoidea* and *A. plicata*, the elevated hemolymph HCO_3^- returned to control levels following 7 d at control conditions (<100 μatm pCO_2; i.e., recovery period) for (Tables 1, 2, Fig. 1).

Exposure of *L. siliquoidea* to elevated pCO_2 did not result in significant changes to hemolymph Ca^{2+} relative to mussels held at ambient

Table 1
Results of two-way analyses of variance (ANOVAs) examining the impact of chronic exposure to elevated pCO_2 on fatmucket mussels (*Lampsilis siliquoidea*) exposed to one of three different pCO_2 treatments (<100 [control], ~20,000, and ~55,000 μatm) for 1, 4, 7, or 28 d with an additional 7 or 14 d recovery period at control conditions (<100 μatm). Bolded lines indicate statistical significance across treatment groups within a measured variable.

Measured variable	Main effects	*SS*	*df*	*F*	*p*
HCO_3^-	Treatment	142.6	2	40.54	<**0.001**
(mmol L^{1-})	Day	434.0	5	49.36	<**0.001**
	Treatment × Day	121.9	10	6.93	<**0.001**
	Residuals	205.7	117		
Ca^{2+}	Treatment	0.0033	2	1.64	0.198
(mg mL^{-1})	Day	0.3253	5	65.44	<**0.001**
	Volume	0.009	1	9.03	**0.003**
	Treatment × Day	0.0461	10	4.64	<**0.001**
	Residuals	0.1203	121		
Cl^-	Treatment	26,085	2	25.06	<**0.001**
(mg mL^{-1})	Day	76,354	5	29.34	<**0.001**
	Treatment × Day	62,711	10	12.05	<**0.001**
	Residuals	63,504	122		
Na^+	Treatment	52,728	2	83.31	<**0.001**
(g L^{-1})	Day	64,696	5	40.89	<**0.001**
	Treatment × Day	47,948	10	15.15	<**0.001**
	Residuals	36,077	114		
Glucose	Treatment	19,196	2	8.36	<**0.001**
(μM)	Day	32,557	5	5.67	<**0.001**
	Treatment × Day	36,706	10	3.20	**0.001**
	Residuals	140,147	122		
Mg^{2+}	Treatment	3574	2	2.74	0.069
(mg mL^{-1})	Day	98,991	5	30.31	<**0.001**
	Volume	3789	1	5.80	<**0.001**
	Treatment × Day	43,261	10	6.62	<**0.001**
	Residuals	79,039	121		

pCO_2, even after 28 d (Fig. 1C; Table 1). However, following a return to control pCO_2 for 7 and/or 14 d, *L. siliquoidea* previously held at either ~20,000 μatm or ~55,000 μatm showed a 28% decrease in hemolymph Ca^{2+} relative to control mussels (Table 1, Fig. 1C). This reduction in hemolymph Ca^{2+} did not return to control levels even following 14 d at control conditions (Table 1, Fig. 1C). Additionally, hemolymph Ca^{2+} was elevated in control mussels at day 28 relative to day 1 of holding ($p < 0.001$). The concentration of Ca^{2+} in the hemolymph of *A. plicata* was elevated by at least 28% throughout the treatment period at both ~20,000 and ~55,000 μatm compared to control conditions, with the exception of day 4 (Table 2, Fig. 1D). Following a subsequent reduction in pCO_2 to control levels for 7 and 14 d, hemolymph Ca^{2+} levels for *A. plicata* from both elevated pCO_2 treatments returned to control levels (Table 2, Fig. 1D).

The concentration of Cl^- in *L. siliquoidea* hemolymph initially fell by 37% in animals held at ~55,000 μatm pCO_2 relative to control mussels at day 1 of exposure, but this disturbance returned to control levels by 4 d of exposure. However, by 28 d of exposure, hemolymph Cl^- increased by 28% relative to control animals for both the mussels held at ~20,000 and ~55,000 μatm pCO_2, and this elevation in Cl^- persisted even after 14 d of recovery at ambient pCO_2 (Table 1, Fig. 1E). The concentration of Cl^- in the hemolymph of *A. plicata* exposed to elevated pCO_2 was only different from control mussels at day 28 of exposure to the highest level of pCO_2 (28% elevated at ~55,000 μatm; Table 2, Fig. 1F).

We observed an initial decrease of Na^+ in the hemolymph of *L. siliquoidea* after only 1 d exposure to ~55,000 μatm pCO_2 relative to control mussels. After 4 d of exposure to elevated pCO_2, however, *L. siliquoidea* from both the ~20,000 μatm and ~55,000 μatm treatments displayed an elevation of hemolymph Na^+ of approximately 13% relative to control mussels. Hemolymph Na^+ levels remained elevated in *L. siliquoidea* throughout the remainder of the experimental period, including the 14 d recovery period at control pCO_2 levels (Table 1, Fig. 2A). As with Ca^{2+}, the concentration of Na^+ in the hemolymph of the control *L. siliquoidea* was different at 28 d of holding compared to day 1, with Na^+ showing a decrease in control mussels on day 28 ($p < 0.001$). *A. plicata* held at both levels of elevated pCO_2 (~20,000 and ~55,000 μatm) had at least an 11% higher hemolymph Na^+ concentration compared to control mussels during the exposure period. Hemolymph Na^+ decreased to control levels once *A. plicata* were moved to control conditions (<100 μatm pCO_2) for 7 and 14 d, with the exception that hemolymph Na^+ levels first returned to control levels at 28 d of exposure to ~55,000 μatm pCO_2 (Table 2, Fig. 2B).

The concentration of glucose in the hemolymph of *L. siliquoidea* increased two-fold relative to control mussels following 4 and 7 d of exposure to ~55,000 μatm pCO_2 (Table 1, Fig. 3A). The elevation in glucose returned to control levels by 28 d of exposure, and remained at control levels during the 14 d recovery period at <100 μatm pCO_2 (Table 1, Fig. 3A). *A. plicata* held at the highest level of pCO_2 (~55,000 μatm) experienced a treatment wide elevation in hemolymph glucose relative to mussels held at control conditions throughout the experiment (i.e., only a significant effect of pCO_2 treatment; Table 2, Fig. 3B). Concentrations of Mg^{2+} in the hemolymph of *L. siliquoidea* were reduced by exposure to both levels of pCO_2 (~20,000 and ~55,000 μatm) compared to control mussels at 28 d of exposure (Table 1, Fig. 4A). However, once the CO_2 stressor was removed, hemolymph Mg^{2+} was no longer different from control mussels in those *L. siliquoidea* previously exposed to elevated pCO_2 (Table 1, Fig. 4A). Concentrations of Mg^{2+} in the hemolymph of *A. plicata* were 38% higher relative to control mussels only on day 1 of holding at the highest level of pCO_2 (~55,000 μatm) (Table 2, Fig. 4B).

4. Discussion

Following exposure to elevations in pCO_2, both *L. siliquoidea* and *A. plicata* showed numerous indicators of physiological disturbances related to acid-base disturbances. As environmental pCO_2 increases, CO_2 enters an organism's fluids by diffusion until a new steady-state gradient is achieved (Seibel and Walsh, 2003). This increase in pCO_2 levels (hypercapnia) results in a simultaneous rise in H^+, which causes a reduction in pH (i.e., acidosis) of internal body fluids (Wicks and Roberts, 2012). In response to this reduction in pH, buffering of fluids is achieved primarily by increasing HCO_3^- levels (Lindinger et al., 1984). The results of the present study support this mechanism, and show that exposure to the highest level of pCO_2 (~55,000 μatm) caused an elevation in hemolymph HCO_3^- relative to control mussels in both species. As previously stated, one strategy to increase HCO_3^- in the hemolymph is to obtain $CaCO_3$ from the shell. The release of $CaCO_3$ from the shell when exposed to elevated pCO_2 and reduced pH results in the production of HCO_3^- and Ca^{2+} in equimolar amounts (Bibby et al., 2008; Michaelidis et al., 2005). Michaelidis et al. (2005), as well as Bibby et al. (2008), both observed elevations in Ca^{2+} in the hemolymph of marine invertebrates exposed to elevated pCO_2. Similar elevations in Ca^{2+} were also observed in this study along with an increase in hemolymph HCO_3^- in *A. plicata*. However, these elevations did not occur in tandem, suggesting that there may be another strategy for increasing HCO_3^- of the hemolymph besides the release of $CaCO_3$ from the shell. Another strategy to increase HCO_3^- in the hemolymph is by altering the activity of the Cl^-/HCO_3^- exchanger. Mussels actively uptake Cl^- ions by exchanging Cl^- for HCO_3^- (Byrne and Dietz, 1997), and mussels may down-regulate activity of this exchanger in an effort to retain HCO_3^- at the cost of Cl^- uptake. This down-regulation of the Cl^-/HCO_3^- exchanger would result in a decrease in hemolymph Cl^-, which was observed initially in *L. siliquoidea* in the present study. *Fusconaia flava* mussels held for short- and long-term exposures to elevated CO_2 also demonstrated an acute decrease in Cl^-, suggesting that, for some species of mussels, this decrease in activity of the Cl^-/HCO_3^- exchanger may be a short-term response (Hannan et al., 2016). Increases in the activity of the Na^+/H^+ exchanger to excrete H^+ is an additional strategy to reduce acidosis, and can result in an increase in hemolymph Na^+ (Byrne and Dietz, 1997; Lannig et al., 2010). An increase in

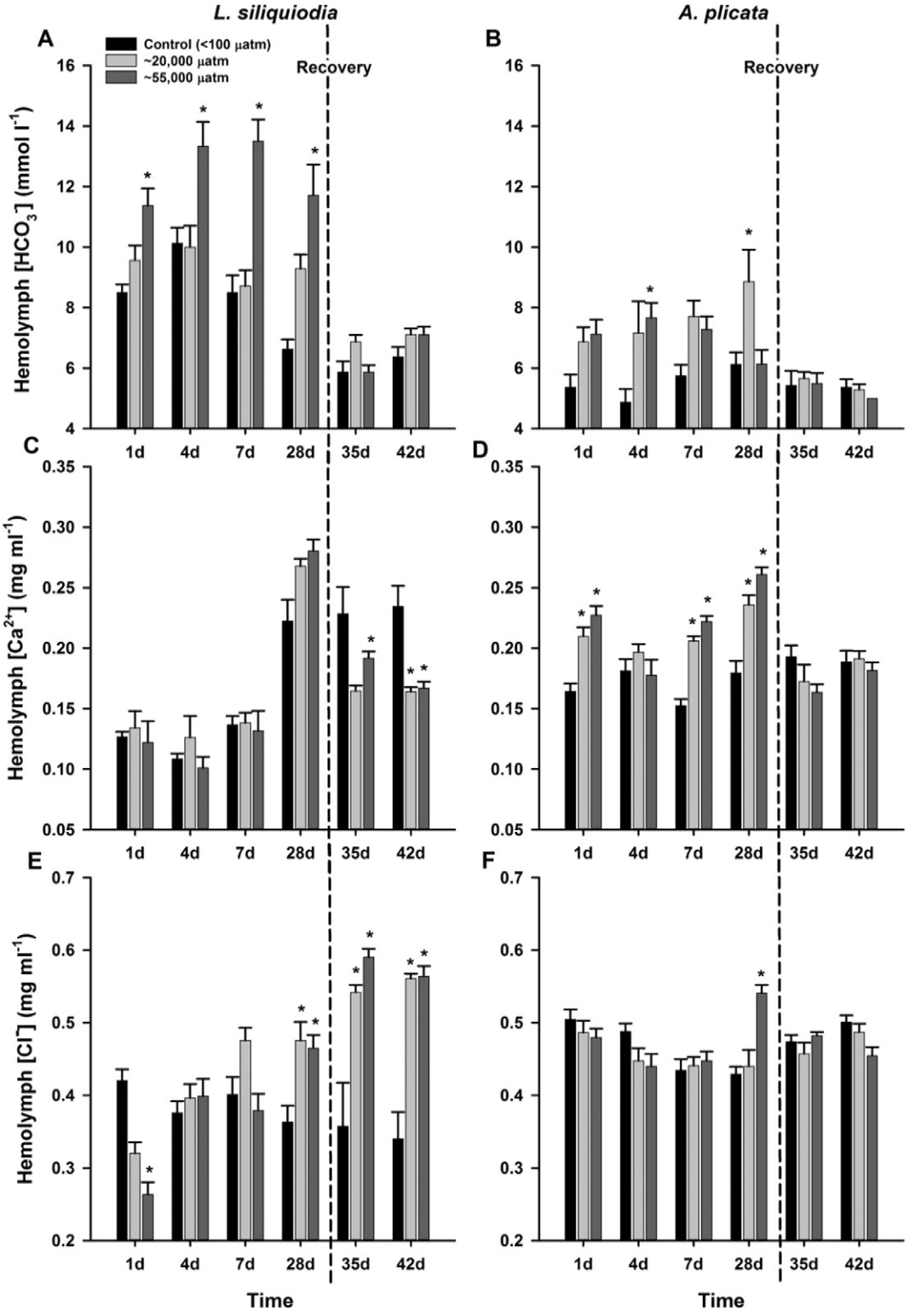

Fig. 1. Concentrations of HCO_3^- (A and B), Ca^{2+} (C and D), and Cl^- (E and F) in the hemolymph of fatmucket (*Lampsilis siliquoidea*) and threeridge (*Amblema plicata*) mussels exposed to three levels of pCO_2, <100 (control), ~20,000, and ~55,000 µatm for 1, 4, 7, or 28 d. The dashed line represents the onset of the recovery treatment, where all CO_2 levels were returned to control conditions (<100 µatm) for an additional 7 or 14 d. Data are presented as means ± SE ($N = 8–10$). An asterisk (*) represents groups that were significantly different from the control treatment within a time point (two-way ANOVA; see Tables 1, 2).

hemolymph Na^+ was observed for both species held at both elevations in pCO_2 during the exposure period. Together, the results of this study show that in both species of mussel exposed to elevated pCO_2, changes hemolymph ion levels are likely occurring in an effort to reduce acidosis of their hemolymph.

In addition to showing indications of regulating acid-base status in response to pCO_2 exposure, both species of mussels also displayed signs of physiological stress when exposed to the highest pCO_2. In bivalves, glucose fuels aerobic processes and is their primary energy store (de Zwaan and Wijsman, 1976). Additionally, the mobilization of

Table 2
Results of two-way analyses of variance (ANOVAs) examining the impact of chronic exposure to elevated pCO_2 on threeridge mussels (*Amblema plicata*) exposed to one of three different pCO_2 treatments (<100 [control], ~20,000, and ~55,000 μatm) for 1, 4, 7, or 28 d with an additional 7 or 14 d recovery period at control conditions (<100 μatm). Bolded lines indicate statistical significance across treatment groups within a measured variable.

Measured variable	Main Effects	*SS*	*df*	*F*	*p*
HCO_3^-	Treatment	21,128	2	12.79	<**0.001**
(mmol L^{-1})	Day	33,780	5	8.18	<**0.001**
	Treatment × Day	23,148	10	2.80	**0.004**
	Residuals	91,698	111		
Ca^{2+}	Treatment	0.026	2	28.12	<**0.001**
(mg mL^{-1})	Day	0.027	5	11.89	<**0.001**
	Treatment × Day	0.043	10	9.28	<**0.001**
	Residuals	0.051	111		
Cl^-	Treatment	0.004	2	1.73	0.182
(mg mL^{-1})	Day	0.033	5	5.07	<**0.001**
	Treatment × Day	0.073	10	5.53	<**0.001**
	Residuals	0.146	111		
Na^+	Treatment	90.8	2	42.13	<**0.001**
(g L^{-1})	Day	331.8	5	61.57	<**0.001**
	Treatment × Day	76.2	10	7.07	<**0.001**
	Residuals	119.6	111		
Glucose	Treatment	7740	2	9.96	<**0.001**
(μM)	Day	6606	5	3.40	**0.007**
	Treatment × Day	2534	10	0.65	0.766
	Residuals	41,169	106		
Mg^{2+}	Treatment	0.00002	2	0.19	0.826
(mg mL^{-1})	Day	0.003	5	13.50	<**0.001**
	Treatment × Day	0.002	10	3.84	<**0.001**
	Residuals	0.005	111		

glucose, a step in the stress response, comes as a cost to non-vital functions such as, growth, reproduction, and movement, and changes in hemolymph glucose is used as a traditional indicator of stress for aquatic invertebrates (Fritts et al., 2015; Patterson et al., 1999). Thus, an elevation of glucose in both species of mussels provides evidence of stress in mussels exposed to the highest pCO_2 (~55,000 μatm). The lack of glucose response at ~20,000 μatm was also observed in *F. flava* (Hannan et al., 2016) and suggests that this high level of CO_2 exposure may be below the threshold of stress for the freshwater mussels studied to date. Additionally, at ~55,000 μatm glucose was elevated throughout the experiment and recovery period for *A. plicata* but only on days 4 and 7 of CO_2 treatment for *L. siliquoidea*, suggesting that glucose may not be the ideal indicator of chronic stress in *L. siliquoidea* in response to CO_2 exposures. Another possible measure of stress in freshwater mussels is the concentration of Mg^{2+} in hemolymph. A decrease in hemolymph Mg^{2+} has been reported in response to a range of stressors in mussels, including elevated temperature (Fritts et al., 2015), exposure to heavy metals (Hemelraad et al., 1990), and, recently, in response to long-term exposure to elevated pCO_2 in *F. flava* (Hannan et al., 2016). At present, there is a lack of information on the role of Mg^{2+} in freshwater mussels despite its use as a bioindicator (Dietz et al., 1994). In the current study, a decrease in hemolymph Mg^{2+} was observed in *L. siliquoidea* exposed to both elevated levels of CO_2 by the end of the exposure period. Interestingly, this indication of stress at 28 d via a decrease in hemolymph Mg^{2+} was in contrary to the hemolymph glucose levels that return to control levels at 28 d in *L. siliquoidea*. Taken together, the elevation of glucose in both species, as well as decrease in Mg^{2+} in *L. siliquoidea*, suggests that both mussel species were experiencing physiological stress when exposed to elevated pCO_2.

Although there is evidence of changes in ion concentrations of the hemolymph and stress in both mussel species, there is also evidence of recovery back to control conditions once the elevated CO_2 stimulus (i.e., the stressor) was removed. For example, both species of mussel showed signs of recovery of hemolymph HCO_3^- to control levels when the CO_2 treatment was removed. *A. plicata* exhibited an increase in hemolymph Ca^{2+} during the exposure period at both levels of elevated pCO_2, which subsequently decreased to control levels once the stressor was removed. *L. siliquoidea* showed no effect of CO_2 treatment on hemolymph Ca^{2+} levels during the exposure period, but following a return to control conditions of mussels previously held at elevated pCO_2, hemolymph Ca^{2+} fell below those of mussels held for the same duration at control conditions. It should be noted that, beginning at 28 d of holding, hemolymph Ca^{2+} rose in control *L. siliquoidea*, possibly due to holding or confinement. Although it can be difficult to tease apart the effects of holding and pCO_2 treatment, by comparing mussels within the same time point, the effect of CO_2 was still evident in *L. siliquoidea* during the recovery period. Hemolymph Na^+ levels returned to control levels in *A. plicata* after the stressor was removed, but remained elevated during the recovery period for the *L. siliquoidea*. Again, holding may have

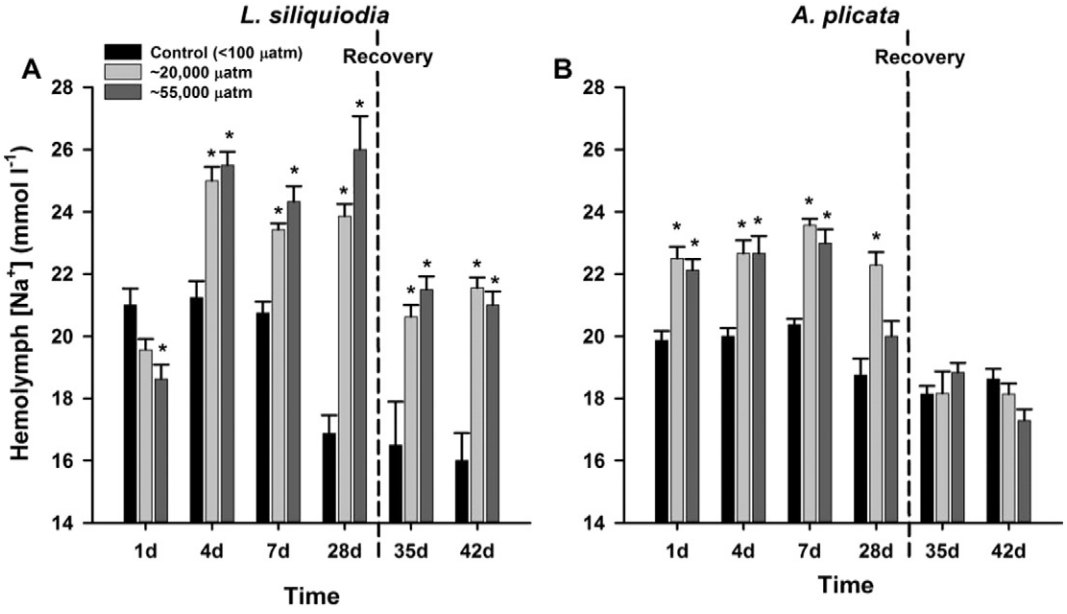

Fig. 2. Concentrations of Na^+ in the hemolymph of (A) fatmucket (*Lampsilis siliquoidea*) and (B) threeridge (*Amblema plicata*) mussels exposed to three levels of pCO_2, <100 (control), ~20,000, and ~55,000 μatm for 1, 4, 7, or 28 d. The dashed line represents recovery where all CO_2 levels were returned to control conditions (<100 μatm). Data are presented as means ± SE ($N = 8$–10). An asterisk (*) represents groups that are significantly different from the control treatment within a time point (two-way ANOVA; see Tables 1, 2).

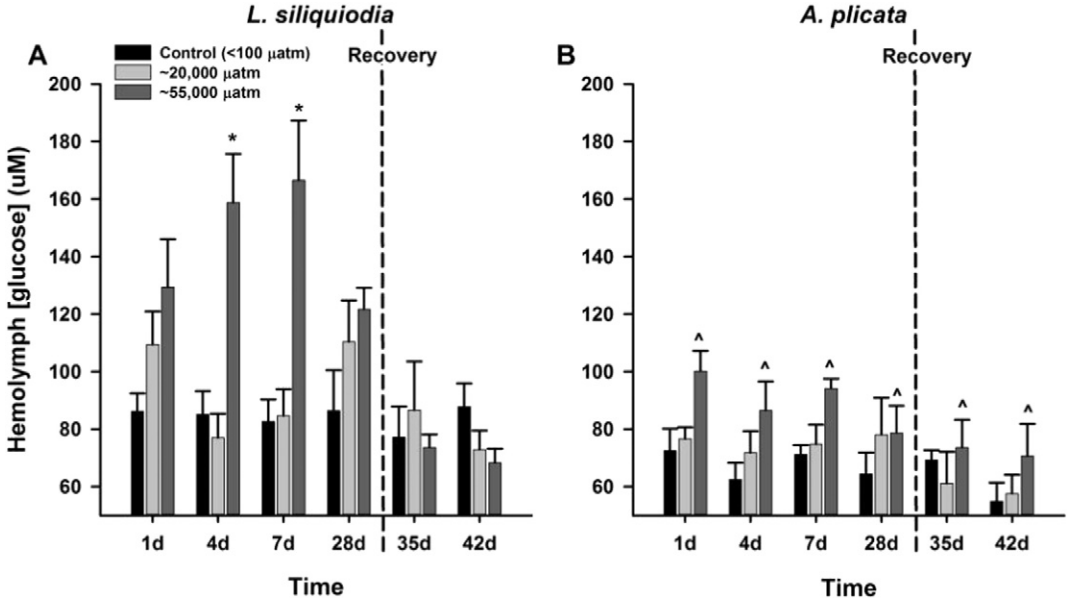

Fig. 3. Concentrations of glucose in the hemolymph of (A) fatmucket (*Lampsilis siliquoidea*) and (B) threeridge (*Amblema plicata*) mussels exposed to three levels of pCO_2, <100 (control), ~20,000, and ~55,000 µatm for 1, 4, 7, or 28 d. The dashed line represents recovery where all CO_2 levels were returned to control conditions (<100 µatm). Data are presented as means ± SE ($N = 8–10$). For panel A, an asterisk (*) represents groups that are significantly different from the control treatment within a time point (two-way ANOVA; see Table 1). For panel B, there was no significant interaction between pCO_2 treatment and sampling day; a caret (^) represents a significant effect of pCO_2 treatment between mussels exposed to ~55,000 and those exposed to <100 µatm (control) (two-way ANOVA; see Table 2).

influenced hemolymph Na^+ levels in *L. siliquoidea* beginning at 28 d of holding; however, by comparing mussels within the same time point, the effect of CO_2 was still evident in *L. siliquoidea* during entire experimental period. The persistent effect of elevated pCO_2 suggests that 14 d was not a long enough recovery period for *L. siliquoidea* in terms of hemolymph Na^+. The concentration of Cl^- in the hemolymph of *A. plicata* also returned to control levels following the removal of the elevated CO_2 stimuli. Conversely, for *L. siliquoidea* that showed an initial decrease in hemolymph Cl^- at 1 d of exposure to elevated pCO_2, hemolymph Cl^- levels rose above control levels at 28 d of exposure and these high levels persisted following a return to control conditions for 14 d. The initial decrease and subsequent increase in hemolymph Cl^- suggests that *L. siliquoidea* may initially down-regulate the exchange of HCO_3^- and Cl^- through the Cl^-/HCO_3^- exchanger, a process that is resumed following long-term exposure to elevated pCO_2, and may help to explain the concurrent decrease in hemolymph HCO_3^- during the recovery period. There was ample evidence of recovery for *A. plicata* as most measured factors returned to control levels once the CO_2 stressor was removed. However, this was not the case for *L. siliquoidea*, as they showed changes in hemolymph Ca^{2+} and Cl^- that occurred almost exclusively during the recovery period, and hemolymph Na^+ remained elevated after the stressor was removed. The concentration of glucose in *L. siliquoidea* was able to recover back to control conditions during recovery; however, *A. plicata* showed a treatment effect and was elevated

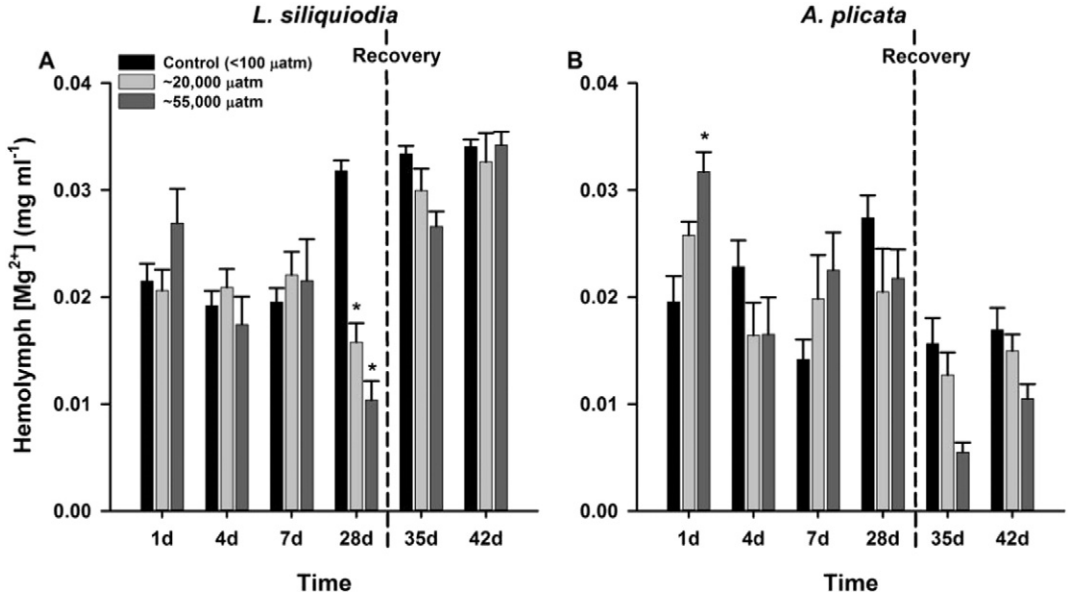

Fig. 4. Concentrations of Mg^{2+} in the hemolymph of (A) fatmucket (*Lampsilis siliquoidea*) and (B) threeridge (*Amblema plicata*) mussels exposed to three levels of pCO_2, <100 (control), ~20,000, and ~55,000 µatm for 1, 4, 7, or 28 d. The dashed line represents recovery where all CO_2 levels were returned to control conditions (<100 µatm). Data are presented as means ± SE ($N = 8–10$). An asterisk (*) represents groups that are significantly different from the control treatment within a time point (two-way ANOVA; see Tables 1,2).

throughout the experiment. The concentration of Mg^{2+} for both species of mussel was able to recover back to control conditions once the stress was removed. Taken together, these results of the present study suggest that, if freshwater mussels are returned to ambient conditions (i.e., on their own, through management practices, or a CO_2 barrier is moved), there is potential to recover from exposure to elevated pCO_2; however, there are species-specific differences in the extent of and the time required for recovery from exposure to elevated pCO_2.

The above-described species-specific differences in the responses of freshwater mussels to elevated pCO_2 may have resulted from variations in their physiology and behavior. *L. siliquoidea* belong to the tribe Lampsilini (Campbell et al., 2005), which has a wide range of relative shell masses, but generally tend to be thin shelled relative to other tribes (Haag and Rypel, 2011; Haag, 2012). In contrast, *A. plicata* belong to the tribe Ambleminae (Campbell et al., 2005) that are generally longer-lived and tend to have thicker shells relative to other tribes (Haag and Rypel, 2011). We hypothesize that shell thickness may play a role in how mussels mediate their responses to elevated pCO_2. For instance, due to the thickness of their shells, thinner shelled mussels (e.g., *L. siliquoidea*) may be able to rely less on shell $CaCO_3$ stores to buffer acidosis than thicker shelled unionids (e.g., *A. plicata*) during long-term exposure to elevated pCO_2. Indeed, hemolymph Ca^{2+} and HCO_3^- were elevated in the thicker shelled *A. plicata* during CO_2 exposure; whereas hemolymph Cl^- was relatively unaffected suggesting that these mussels may rely primarily on shell $CaCO_3$ stores to regulated acid-base status rather than down-regulation of the Cl^-/HCO_3^- exchanger. Conversely, the thinner shelled *L. siliquoidea* may rely on additional mechanisms to increase hemolymph HCO_3^-, such as regulation of the Cl^-/HCO_3^- exchanger as evidenced by changes in hemolymph HCO_3^- and Cl^-. Although not measured in the present study, shell composition and strength of marine mussels can be affected by elevated pCO_2 (Kurihara, 2008), and presumably will affect thinner shelled unionids more than thicker shelled species. Additionally, Lampsilini mussels have a unique reproductive strategy (i.e., using a lure to attract fish hosts), and as a result, spend more time with their valves open than other unionid mussels (Zanatta and Murphy, 2006), which may increase their sensitivity to changes in the external environment (e.g., elevations in pCO_2). The tendency for Lampsilini mussels to remain open for longer periods of time may have contributed to the lack of an observed change in the concentration of hemolymph Ca^{2+} in *L. siliquoidea* during the exposure period (i.e., Ca^{2+} from shell $CaCO_3$ stores may have been more easily lost to the environment if the mussels were open). The present study supports the idea that *L. siliquoidea* may be more sensitive to environmental stressors compared to *A. plicata*, as demonstrated by the both a robust glucose response and decreased hemolymph Mg^{2+} during exposure to elevated pCO_2. During the recovery period, *A. plicata* were able to recover within a 14 d period, whereas *L. siliquoidea* were not. Furthermore, *L. siliquoidea* showed changes from control conditions almost exclusively in the recovery period, suggesting their response to elevated pCO_2 may be delayed. This delayed response in *L. siliquoidea*, highlights the importance of also considering post-stressor periods to provide a more complete and comprehensive picture of responses to chronic elevations in pCO_2. This study was focused on observing the response to chronic CO_2 exposure and a recovery period; however, in nature, many stressors are intermittent or fluctuating, and are not applied on a continual or chronic basis (e.g., pollution, temperature, or noise). Responses to intermittent exposures may result in different effects on organismal health and fitness relative to chronic, sustained exposures (Handy, 1994). For example, following an initial exposure to a stressor, subsequent exposure to the same challenge can result in responses that are similar, exacerbated [i.e., carry over effects (O'Connor et al., 2014)], or attenuated compared to short-term or chronic time periods (Reinert et al., 2002). The differences in the responses to the treatment and recovery period between the two studied mussel species suggest that further studies on intermittent exposures to this CO_2 stressor would be valuable. Taken together, the results from the present study suggest that *L. siliquoidea* and *A. plicata* respond to elevated pCO_2 in a species-specific manner, and may rely on different mechanisms to deal with the acid-base disturbances associated with elevations in pCO_2. In addition, although a two week period appears to be long enough for *A. plicata* to recover from exposure to elevated pCO_2, this period was not sufficient for the recovery of *L. siliquoidea*. The potential differences in the gaping behaviors of mussels from different tribes (i.e., time that they remain open vs. closed) may be an important factor to consider when interpreting physiological responses to environmental stressors, and constitutes and interesting avenue to explore in future studies.

The results of this study help define the responses of freshwater mussels to chronic exposures of elevated pCO_2 and subsequent recovery. Should unionid mussels be exposed elevated CO_2 levels, either through natural (diel and seasonal variation) (Maberly, 1996) or anthropogenic sources (elevated CO_2 from climate change or a non-physical fish barrier) (Hasler et al., 2016; Noatch and Suski, 2012), they would likely experience stress and respond to the resulting acidosis by increasing/retaining HCO_3^-, and excreting H^+. This data suggests that if mussels are exposed to elevated pCO_2 for an extended period of time, it has the potential to redirect energy from growth and reproduction to dealing with stress and maintaining homeostasis, which can translate to population-level consequences through stress and/or energy depletion (i.e., increased mobilization of glucose) (Fefferman and Romero, 2013). However, if CO_2 stressors are removed, or if mussels are moved out of the range of elevated pCO_2, physiological changes are likely to be transient for most metrics monitored in this study (e.g., for *A. plicata* mussels, and potentially for *L. siliquoidea* over a recovery period > 14 d). The species-specific differences observed in response to elevated pCO_2 further encourages the use of multiple species when examining responses to environmental stressors, as variations in behavior and physiology may play a role in determining how different species and populations experience their environment. *L. siliquoidea* seemed to be the more sensitive to elevated pCO_2 exposure, possibly due to both their morphology (e.g., thin shells (Campbell et al., 2005; Haag and Rypel, 2011) and behavior (e.g., tendency to spend more time open) (Zanatta and Murphy, 2006); whereas the *A. plicata* may be better equipped to deal with harsh environmental conditions. It is therefore important to consider that elevations in pCO_2 may have tribe-specific, and species-specific effects (i.e., what is stressful for one mussel species may not be for another), that may have population- and community-level impacts.

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