Environmental Toxicology

Chronic Exposure of a Freshwater Mussel to Elevated pCO2: Effects on the Control of Biomineralization and Ion-Regulatory Responses

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

Abstract: Freshwater mussels may be exposed to elevations in mean partial pressure of carbon dioxide (pCO2) caused by both natural and anthropogenic factors. The goal of the present study was to assess the effects of a 28-d elevation in pCO2 at 15 000 and 50 000 µatm on processes associated with biomineralization, ion regulation, and cellular stress in adult Lampsilis siliquoidea (Barnes, 1823). In addition, the capacity for mussels to compensate for acid-base disturbances experienced after exposure to elevated pCO2 was assessed over a 14-d recovery period. Overall, exposure to 50 000 µatm pCO2 had more pronounced physiological consequences compared with 15 000 µatm pCO2. Over the first 7 d of exposure to 50 000 µatm pCO2, the mRNA abundance of chitin synthase (cs), calmodulin (cam), and calmodulin-like protein (calp) were significantly affected, suggesting that shell formation and integrity may be altered during pCO2 exposure. After the removal of the pCO2 treatment, mussels may compensate for the acid-base and ion disturbances experienced during pCO2 exposure, and transcript levels of some regulators of biomineralization (carbonic anhydrase [ca], cs, cam, calp) as well as ion regulation (Na+–K+–ATPase [nka]) were modulated. Effects of elevated pCO2 on heat shock protein 70 (hsp70) were limited in the present study. Overall, adult L. siliquoidea appeared to regulate factors associated with the control of biomineralization and ion regulation during and/or after the removal of pCO2 exposure. Environ Toxicol Chem 2018;37:538–550. © 2017 SETAC

Keywords: Chitin synthase; Na+–K+–ATPase; Calmodulin; Mollusk toxicology; Freshwater toxicology; Benthic macroinvertebrates

INTRODUCTION

Freshwater ecosystems may experience an increase in the mean partial pressure of carbon dioxide (pCO2) for a variety of reasons, including climate change [1], deforestation [2], agricultural and urban activities [3], and invasive species management [4]. Natural variations in the pCO2 of freshwater systems also occur at a local and global scale, with mean CO2 levels that can range from 647 to 38 000 µatm globally and that fluctuate seasonally [5]. As a result of the natural and anthropogenic sources of change in freshwater pCO2, it becomes important to understand the potential consequences for freshwater biota; however, at present our understanding of these consequences is limited [6]. Research into the impacts of elevations in aquatic pCO2 has largely focused on marine systems because of the well-characterized acidification of oceanic systems with increasing atmospheric CO2 [7]. For marine biota, physiological and behavioral alterations have been observed in a range of organisms [8–10]. Importantly, changes in seawater chemistry and carbonate saturation have resulted in reduced calcification and growth rates in marine calcifying organisms such as bivalves [9]. Therefore, similar negative consequences might be expected for freshwater bivalves if environmental pCO2 should rise.

In freshwater systems, unionid mussels provide a number of important ecological functions such as water filtration, generation of nutrient-rich areas, and food sources for other animals [11]; nevertheless, anthropogenic alterations to their habitats have resulted in their decline, making them one of most imperiled taxa in North America [12]. Moreover, because of their limited mobility, freshwater mussels may be acutely sensitive to environmental disturbances such as elevations in pCO2. In addition to other potential natural and anthropogenic sources [6], populations of freshwater mussels in the upper Midwest...
of the USA may be at risk of future exposures to elevated pCO2 because zones of elevated CO2 are being considered as a potential control mechanism to deter the movements of bigheaded carp (i.e., silver carp Hypophthalmichthys molitrix and bighead carp H. nobilis) [13]. This barrier would consist of artificially elevating pCO2 up to 100 times greater than ambient conditions in localized areas (Cupp et al. [14] and Donaldson et al. [15]), where the threat of carp invasion is high. The potential exposure to these extreme levels of CO2 provides an opportunity to study the responses of freshwater mussels to elevations in pCO2, an area that has until recently been poorly investigated [16–20], providing a framework for understanding the physiological and ecological impacts of natural and anthropogenic increases in pCO2 on threatened freshwater mussel populations.

The exposure of mussels to elevations in pCO2 results in the acidification of intracellular and extracellular fluids, which can lead to a host of physiological and ecological consequences. In unionoid mussels, hemolymph has a limited buffering capacity, no respiratory pigments, and a low osmotic concentration, and mussels rely on the bicarbonate-carbonate buffer system to at least, in part, respond to acidosis [21]. In response to acidosis, hemolymph HCO3\(^-\) (as well as Ca\(^{2+}\)) levels increase as CaCO3 is released from the shell [22]. The same bicarbonate buffering system is necessary for CaCO3 crystal formation for shell deposition through the reverse reaction, and this process is dependent on the maintenance of extrapallial fluid (fluid in contact with the inner shell surface) pH by the removal of H\(^+\) [23, 24]. Thus, formation of the shell can be compromised in high pCO2 environments as a result of alterations in the speciation of the carbonate system of the hemolymph and extrapallial fluid, and because of the utilization of CaCO3 stores from the shell (i.e., source of HCO3\(^-\)) for acid-base regulation, as has been shown in marine bivalves (Melzner et al. [25] and Michaelidis et al. [26]). Overall, the link between acid-base regulation and calcification of the shell may result in negative consequences for freshwater mussel shell integrity and growth in high pCO2 environments, which may lead to ultimate decreases in survival and further population declines.

As a result of the intricate link between the transport of substrates for CaCO3 crystal formation and acid-base and ion-regulatory processes in mussels, exposure to elevated pCO2 may have consequences for the biological control of calcification and ion homeostasis. Bio-mineralization related to shell formation is a highly controlled and energetically expensive process [23]. The formation of the shell is controlled by a suite of regulators including chitin synthase (CS), carbonic anhydrase (CA), calmodulin (CaM), and CaM-like protein, and these regulators have been shown to be modulated by elevated pCO2 exposure in marine and/or freshwater bivalves [19, 27–29]. In addition, acid-base regulation during pCO2 exposure resulted in ionic disturbances (e.g., changes in hemolymph Ca\(^{2+}\), Na\(^+\), Cl\(^-\), and HCO3\(^-\) levels) in several freshwater mussel species [16–18], presumably caused by the role of the Na\(^+\)/H\(^+\) exchanger and the Cl\(^-\)/HCO3\(^-\) anion exchanger in H\(^+\) excretion and HCO3\(^-\) retention, respectively. As an important regulator of secondary active ion transport, Na\(^+\)-K\(^+\)-adenosine triphosphatase (NKA) provides the driving force necessary for these acid-base and ion-regulatory responses, and may thus be sensitive to changes in environmental pCO2. In addition to biomineralization and acid-base responses, regulators of cellular stress such as heat shock protein 70 (HSP70) have been suggested as biomarkers of stress in response to changes in freshwater [19] and marine [30] pCO2. Overall, these physiological responses to elevated pCO2 are energetically demanding and, as a result, freshwater mussels may experience reduced survival and stress coping because of increased environmental pCO2.

Thus far, studies suggest that exposure to elevated pCO2 results in changes in hemolymph ion concentrations in adult freshwater mussels, likely caused by acid-base regulatory responses [16–18], and mussels may recover, at least in part, once the CO2 stressor is removed [16]. Furthermore, biological control of shell formation in adult freshwater mussels (i.e., cs mRNA) [19] and shell growth in juvenile mussels [20] were affected by long-term pCO2 exposure (>28 d), and normal shell growth may resume post-CO2 exposure [20]. Overall, however, the mechanisms underlying the observed physiological responses to exposure to elevations in pCO2 remain poorly understood. With the possibility that freshwater pCO2 may rise in the future because of natural or anthropogenic factors, an increased understanding of the physiological mechanisms utilized by mussels to respond to elevated pCO2 will help to define how mussel individuals and populations may be impacted in the future [6]. Consequently, the goals of the present study were to quantify, at a molecular level: 1) effects of a sustained increase in pCO2 on freshwater mussels, and 2) potential for mussels to recover from disturbances following the removal of the CO2 stressor by assessing regulators of biomineralization, ion-regulatory responses, and cellular-stress responses. To accomplish these goals, adult Lampsilis siliquoidea (Barnes, 1823) were exposed to either 15 000 or 50 000 µatm pCO2 for up to 28 d, representing scenarios in which mussels may be exposed to elevated pCO2 at a location close to the deployment of a CO2 barrier (50 000 µatm), or downstream of the barrier application area (15 000 µatm) where water pCO2 would have dissipated to some extent. Because of their limited mobility, it is possible that mussels could experience elevations in pCO2 for an extended period similar to that used in the present study; however, if mussels were relocated away from areas of CO2 infusion for conservation purposes, or if exposures were intermittent [18], mussels may also be provided with the opportunity to recover from pCO2 exposure. Thus, a recovery period of up to 14 d was evaluated after the period of pCO2 exposure. Gene expression patterns of cs, ca, cam, calp, nka, and hsp70 were assessed in the gill and/or mantle of mussels, representing candidates for the control of biomineralization, ion-regulatory responses, and the stress response. The approach used in the present study provides an opportunity to assess, at the organismal level, how mussels respond to elevations in pCO2, providing a framework for a greater understanding of the mechanisms underlying potential population level responses of freshwater mussels in high pCO2 environments.
MATERIALS AND METHODS

Experimental animals

Adult L. siliquoidea (n = 138, length 65.5 ± 0.5 mm, mean ± standard error [SE]) were obtained from a captive propagation program at Missouri State University, Springfield, MO, and held at the Aquatic Research Facility at the University of Illinois, Urbana–Champaign, IL, as in Hannan et al. [16]. Mussels were measured for size (length, width, and depth) using digital calipers (Fisher Scientific) and tagged for identification using Queen Marking Kit tags (The Bee Works). Mussels were distributed evenly among 3 recirculating holding systems and were held for at least 1 wk before experimentation, at which time each holding system was assigned to a pCO2 treatment (i.e., one replicate per treatment, see section Chronic pCO2 exposure). Each holding system consisted of a 128.7-L tank (82.6 × 42.5 × 47.0 cm) for holding mussels that contained sediment (5 cm of sand; Old Castle all-purpose sand). Holding systems were supplied with water from a 0.04-ha natural earthen-bottom pond with ample vegetation that overflowed from the holding tank into a reservoir and was pumped back into the holding tank, creating a closed recirculating system. Each holding system was equipped with a Teco 500 aquarium heater/chiller (TECO-US; Aquarium Specialty) to maintain water temperatures and a low-pressure air blower (Sweetwater; SL24H Pentair) for aeration. Water changes of 50% were performed weekly to maintain water quality, and water quality was assessed daily. During the pre-exposure period, dissolved oxygen (7.84 ± 0.17 mg/L) and temperature (21.1 ± 0.1°C) were recorded with a portable meter (YSI 550A; Yellow Springs Instruments). Water pH (8.42 ± 0.03) and alkalinity (188 ± 3 mg/L CaCO3) were measured using a handheld meter (WTW; pH 3310 meter) calibrated regularly and a digital titration kit (Titrator model 16900, cat. no. 2271900; Hach), respectively. Concentrations of CO2 (5.5 ± 0.2 mg/L) were quantified using a CO2 titration kit (Hach; catalog no. 2272700). In addition to the naturally occurring sources that would be present in the pond water, mussels were fed a commercial shellfish diet every other day consisting of algae species ranging in particle sizes (Shellfish Diet 1800; Reed Mariculture). Mussel tanks were carefully monitored for the clearance of algae from the water, to ensure that food availability was not restricted. Mussels did not receive food 24 h before sampling.

Chronic pCO2 exposure

As part of the chronic pCO2 exposure, mussels were exposed to one of 3 treatments: control conditions (< 100 μatm pCO2), approximately 15 000 μatm pCO2, or approximately 50 000 μatm pCO2 for up to 28 d. Fish have been shown to avoid pCO2 of approximately 30 000 to 50 000 μatm (60–70 mg/L) [14,15]; thus levels of this magnitude or higher (to ensure 100% effectiveness) were considered for the highest concentrations used in the present study. The intermediate concentration of 15 000 μatm might be expected should freshwater rivers experience an approximately 1.5 to 15 times increase in current CO2 levels [5]. For the control group, pCO2 levels remained unaltered for the duration of the experiment (< 100 μatm, the lowest detectable limit of the CO2 probe). Target CO2 levels were maintained with a pH controller (PINPOINT; American Marine) that added compressed CO2 gas (commercial grade, 99.9% purity) into the system through an air stone if the pH rose above a target level [31]. Water pCO2 was monitored throughout the exposure period using a modified infrared probe (Vaisala GMP220 and GMT221; Table 1). The concentration of CO2, temperature, dissolved oxygen, pH, and alkalinity were also monitored daily as described earlier (Table 1). After 28 d of exposure to elevated pCO2, a set of mussels was also moved to control conditions (< 100 μatm) for up to 14 d to quantify recovery from pCO2 exposure.

Mussels were terminally sampled following 1, 4, 7, or 28 d of pCO2 exposure, or 7 or 14 d after their return to control conditions (n = 7–8 at each time point). Size (length, width, and depth) was determined using digital calipers. Mussels were sampled for mantle (consisted of a combination of mantle edge, center, and pallial regions) and gill, and tissues were placed in 1 mL of RNA later Stabilization Solution (Ambion, catalog no. AM7020; ThermoFisher) and stored overnight at 4°C before storage at –80°C until analysis.

Body condition index

Body condition index (BCI), a traditional metric used to quantify bivalve condition [17,32], calculated as BCI = Wdry tissue / Vshell cavity × 1000, where Wdry tissue (grams), was determined by drying the soft tissues in a pre-weighed container to a constant weight at 99°C for 24 h [32]. Shell cavity volume (Vshell cavity; mL) was calculated as the total displacement volume of the whole mussel, minus the displacement volume of the open shell after removal of the soft tissues.

RNA and first-strand complementary DNA synthesis

Total RNA was extracted from 20 to 50 mg of tissue using TRIzol Reagent (Invitrogen, catalog no. 15596018; ThermoFisher) according to the manufacturer’s protocol. Tissues were disrupted and homogenized with a BeadBug Microtube homogenizer (Denville Scientific). Extracted RNA was quantified using a NanoDrop One spectrophotometer (Fisher Scientific), and 1 μg of RNA was treated with deoxyribonuclease I (Amplification Grade, DNase; catalog no. 18068015, Invitrogen; ThermoFisher). To synthesize complementary DNA (cDNA), MultiScribe Reverse Transcriptase, RNase inhibitor, and random primers were used according to the manufacturer’s protocol (High-Capacity cDNA Reverse Transcription kit; Applied Biosystems, catalog no. 4374966; ThermoFisher).

Gene sequences

For the purpose of developing primers for quantitative real-time polymerase chain reaction (qPCR; see following), partial sequences were generated for ca, calp, cam, cs, hsp70, nka, glyceraldehyde 3-phosphate dehydrogenase (gapdh),
Responses of *Lampsilis siliquoidea* to chronically elevated pCO$_2$ treatments

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<th>Temperature, dissolved oxygen, pH, alkalinity, CO$_2$ concentration, and pCO$_2$</th>
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All PCR reactions were performed in the same way using an Eppendorf Mastercycler where reaction compositions (total vol 25 µL were 2 µL cDNA, 0.2 µM primer, and 5 µL Taq 5X Master Mix (Taq 5X Master Mix, catalog no. M0258L; New England BioLabs). Cycling conditions were 95 °C (30 s), 55 °C (30 s), and 68 °C (30 s) for 38 cycles and resulting products were run on 1.5% agarose gels with ethidium bromide and extracted using a QIAquick gel extraction kit (catalog no. 28704; QIAGEN). A PCR cloning kit (catalog no. 231122; QIAGEN) and Subcloning Efficiency DH5α Competent Cells (Invitrogen, catalog no. 18265017; Life Technologies) were used to clone the PCR products following the manufacturers’ protocols, with the exception that cloning reactions were scaled to 5 µL instead of 10 µL. Plasmids were extracted using a QIAPrep Spin Miniprep Kit (cat. no. 27104; QIAGEN) and sequenced by the Core DNA Sequencing Facility (University of Illinois at
The resulting partial sequences (Table 2) were sufficient to generate primers for qPCR (see section qPCR).

### qPCR

The relative abundance of ca, calp, cam, cs, hsp70, and nka mRNA was determined by qPCR. Primers were generated using Primer3plus for the target genes as well as the reference genes ef1-α, gapdh, and 18s (Table 3), and their specificity was verified by sequencing the product from each primer set. To optimize reaction compositions, standard curves were generated for each primer set using cDNA pooled from individuals across treatment groups (efficiencies were >0.92). Real-time PCR was carried out using PowerUp SYBR Green Master Mix (Applied Biosystems catalog no. A25778; ThermoFisher) and an ABI 7900HT Fast Real-Time PCR System (ThermoFisher) according to manufacturer's instructions for 10-μL reactions with a primer concentration of 0.5 μM. For all genes, cDNA was diluted 20-fold with the exception that a 20 000-fold dilution was used for 18s. Cycling conditions were 95 °C for 15 s and 60 °C for 60 s over 40 cycles.

### Table 2: Oligonucleotide primer sets used for gene cloning in Lampsilis siliquoidea

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<th>Gene</th>
<th>Primers (5′–3′)</th>
<th>Sequenced product size (bp)</th>
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<td>ca</td>
<td>Forward—TCA ATA TGC CGT CCT ACC GC</td>
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<td>Reverse—CCT GGC TCT GTG TAC GTG TT</td>
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<tr>
<td>calp</td>
<td>Forward—TGGCAGACCAACTAACAGAAAGA</td>
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<td>Reverse—TCACCTGCACTCATCTCCTCA</td>
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<tr>
<td>cam</td>
<td>Forward 1—TTG CTG AGT TCA AGG AGG CA</td>
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<td></td>
<td>Reverse 1—ACT CGT CAT CTG CAC GA</td>
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<tr>
<td></td>
<td>Forward 2—TGA CCA ACT GAC GGA AGA ACA</td>
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<td></td>
<td>Reverse 2—TGG CCA TCT CCA ATA TCT G</td>
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<tr>
<td>cs</td>
<td>Forward—TGT GCT ACA ATG TGG CAC GA</td>
<td>712</td>
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<td>Reverse—TAC CAC ACC ATG GCA CCT GA</td>
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<td>ef1-α</td>
<td>Forward—TCT CTG GAT GGC ATG AGAC</td>
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<td>Reverse—GAT GAC TCC AAG GGC GAC AG</td>
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<td>gapdh</td>
<td>Forward—TGG ATT TGG TCG TAT CGG GC</td>
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<td>hsp70</td>
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<td>Forward 2—GAT GTG GCC CCA CTG TCT C</td>
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<td>Reverse 2—TTG CTG AGA CGA CGA TTG TC</td>
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<td>nka</td>
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<td>Reverse—ACC ACC TCC AGC AGA TCT GT</td>
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<td>Reverse 2—CAC CAC CCA CCG AAT CAA GA</td>
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bp = base pair; ca = carbonic anhydrase; calp = calmodulin-like protein; cam = calmodulin; cs = chitin synthase; ef1-α = elongation factor 1-α; gapdh = glyceraldehyde 3-phosphate dehydrogenase; hsp70 = heat shock protein 70; nka = Na⁺-K⁺-adenosine triphosphatase.

### Table 3: Oligonucleotide primer sets used for quantitative real-time polymerase chain reaction in Lampsilis siliquoidea

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<tr>
<td>calp</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>Reverse—CCC GAC ATC CAA CTG CAA GA</td>
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</table>

*aQuantitative polymerase chain reaction.

*bSee Table 2 footnote for defined abbreviations.*
RESULTS

Mussel BCI did not differ across pCO2 treatments but did decline over the holding period (2-way ANOVA; Table 4). Mean BCI levels were significantly lower at 28 d of exposure and during the recovery period (49.6 ± 1.4, 48.4 ± 1.4, and 49.4 ± 2.1 for 28 d of exposure, and 7 and 14 d of post-exposure, respectively) relative to the first 7 d of the exposure period (72.1 ± 2.2, 68.0 ± 2.6, and 66.4 ± 2.6 for 1, 4, and 7 d, respectively), regardless of pCO2 treatment.

Mantle cs mRNA abundance was only significantly affected by treatment with 50,000 μatm pCO2 (Figure 1A; 2-way ANOVA, Table 4). After 7 d at 50,000 μatm pCO2, mantle cs mRNA levels were 2-fold lower than in control mussels and these levels increased above control after 7 d of recovery. Mantle cs mRNA levels were no longer different from control after 14 d of recovery in mussels previously exposed to 50,000 μatm pCO2.

Mantle ca mRNA levels were significantly affected in mussels previously exposed to elevated pCO2, after mussels had been moved to recovery conditions (Figure 1B; 2-way ANOVA, Table 4). Mantle ca mRNA was significantly elevated above control levels by approximately 3-fold in mussels exposed to 50,000 μatm pCO2 after 7 d of recovery, and in mussels exposed to 15,000 μatm pCO2 after 14 d of recovery. Mantle ca mRNA levels decreased significantly over time in mussels held at control conditions (i.e., ~2-fold lower at 14 d of recovery compared with 1 and 7 d of holding). Levels of ca mRNA were also measured in the gill; however, these levels were significantly lower than those detected in the mantle (Figure 2A; Student’s t test: \( t = -9.499, df = 33, p < 0.001 \)) and were too low to make comparisons among treatments.

Overall, cam and calp were differentially expressed in the gill and mantle. Levels of cam mRNA were significantly higher in the gill compared with the mantle (Figure 2C; Student’s t test: \( t = -9.499, df = 33, p < 0.001 \)). Although mantle cam mRNA levels were too low to make comparisons among treatments, gill cam mRNA levels were significantly affected by pCO2 exposure (Figure 3A; 2-way ANOVA, Table 4). In mussels exposed to 50,000 μatm pCO2, gill cam mRNA levels were significantly lower than control mussels following 1 d of treatment and again during the recovery period. Levels of cam mRNA were not significantly affected by treatment with 15,000 μatm pCO2, with the exception that mRNA levels were significantly elevated by approximately 2-fold at 4 d of

### Statistical analysis

The effects of pCO2 exposure on BCI and the mRNA abundance of target genes were assessed using a 2-way analysis of variance (ANOVA) with pCO2 level, sampling time, and their interaction \((\text{pCO2} \times \text{sampling time})\) entered into the model as fixed effects. If at least one of the main effects or the interaction was significant, a Tukey–Kramer honest significant difference post hoc test was performed. Student’s t tests were used to assess tissue differences in the mRNA level of ca, calp, and cam between the gill and mantle.

For all statistical analyses, a visual analysis of fitted residuals, using a normal probability plot and/or a Shapiro–Wilk normality test, was used to assess normality. In addition, homogeneity of variances was assessed using a Levene’s test, as well as a visual inspection of the fitted residuals for all 2-way ANOVA statistical analyses and a Bartlett test for all Student’s t statistical tests. Normality was also assessed using a normal probability plot and/or a Shapiro–Wilk normality test, was used to assess normality. In addition, homogeneity of variances was assessed using a Levene’s test, as well as a visual inspection of the fitted residuals for all 2-way ANOVA statistical analyses.
treatment compared with the control and 50 000 μatm pCO2 treatment groups. Although calp mRNA levels were significantly higher in the mantle compared with the gill (Figure 2C), pCO2 treatment only had a significant effect on gill calp mRNA levels (Figure 3B,C; 2-way ANOVA, Table 4). In the gill, calp mRNA levels were significantly lower at 14 d of recovery compared with 1 d of treatment and at 7 and 14 d of recovery compared with 7 d of treatment in mussels previously exposed to 50 000 μatm pCO2. In addition, gill calp mRNA levels were significantly lower in mussels treated with 50 000 μatm pCO2 relative to the 15 000 μatm pCO2 group at 14 d of recovery. Exposure to 15 000 μatm pCO2 also significantly reduced gill calp mRNA levels by approximately 2-fold compared with the control group at 1 d of treatment but not at any other point in the treatment or recovery period. Similar to mantle ca, gill calp mRNA levels decreased with holding in the control group, with mRNA levels being lower at 14 d of recovery (i.e., 42 d of holding) compared with 7 d of holding at control conditions.

Gill and mantle nka mRNA levels were only affected during the recovery period in mussels previously exposed to 50 000 μatm pCO2 (Figure 4; 2-way ANOVA, Table 4). In the gill, nka mRNA levels decreased significantly by approximately 2-fold during the recovery period compared with 7 and 28 d of treatment with 50 000 μatm pCO2, and these levels were also significantly lower than the control group at 14 d of recovery. Similarly, mantle nka mRNA levels were significantly lower at 14 d of recovery in mussels previously exposed to 50 000 μatm pCO2 relative to the control group.

Levels of hsp70 mRNA were only significantly affected in gill and not mantle by treatment with elevated pCO2 (Figure 5; 2-way ANOVA, Table 4). Gill hsp70 mRNA levels were significantly lower in mussels exposed to 15 000 μatm pCO2 relative to the control group at 1 and 28 d of treatment as well as during the recovery period. Within the 15 000 μatm pCO2 treatment group, hsp70 mRNA levels marginally increased from 1 to 4 d of treatment but remained unaffected thereafter. Similarly, gill hsp70 mRNA levels increased in mussels exposed
to 50,000 μatm pCO₂ from 4 to 7 d of treatment and remained elevated until mussels were moved to recovery conditions, after which hsp70 mRNA levels decreased below those at the onset of the treatment (i.e., 1 d) and those of mussels held at control or 15,000 μatm pCO₂. Levels of gill hsp70 mRNA did increase marginally with holding in control mussels, where mRNA levels remained significantly elevated after 28 d of holding compared with 4 d of holding.

**DISCUSSION**

Several factors play a role in the biological control of shell formation in bivalves and have been shown to be sensitive to changing environmental conditions. Chitin synthase is a key enzyme in the synthesis of chitin, an insoluble polysaccharide that forms the structural framework of mollusk shells, and is important for coordination of mineralization processes and shell formation [35]. In the present study, a significant decrease in mantle cs mRNA was detected in *L. siliquoidea* after 7 d of exposure to 50,000 μatm pCO₂ compared with mussels held at control conditions. A similar decrease in *cs* mRNA was detected in the freshwater mussel *Fusconaia flava* exposed to 20,000 μatm pCO₂ for up to 32 d [19] and in the marine pearl oyster *P. fucata* after 72 h of exposure to approximately 900 to 2000 μatm pCO₂ [27]. Although not significant in the present study, exposure to 15,000 μatm pCO₂ also appeared to decrease cs mRNA levels in *L. siliquoidea* at 7 d of exposure. These decreases in the abundance of mantle cs mRNA may result from a decrease in the investment in shell formation. Shell formation is an energetically expensive process [36]; thus, investments in shell growth may be limited in situations where acid-base regulation is a priority, such as during exposure to elevated pCO₂. The mollusk shell is an important external structure that provides protection against predators and is used for mobility and feeding; consequently, inhibition of shell formation caused by elevations in pCO₂ could have consequences for mussel health and survival. Interestingly, mantle cs mRNA levels increased above those of control mussels after removal of the 50,000 μatm pCO₂ treatment. This post-exposure increase in
mantle cs mRNA likely provided a compensatory mechanism for alterations in shell formation that may have occurred during pCO2 exposure. A subsequent fall in mantle cs mRNA after 14 d of recovery further suggests that once the pCO2 stressor was removed, normal shell formation may resume—as was observed in juvenile L. siliquoidea after 16 d post-exposure to elevated pCO2 [20].

A similar post-recovery increase was observed for mantle ca mRNA abundance; however, the ca isoform assessed in the present study was not highly abundant in the gill [41]. Although the classification and modulatory functions of mollusk CA members are increasing, they are still relatively poorly understood. In the present study, sequencing of ca was based on the α-ca (predominant CA group for metazoans) of the freshwater pearl mussel H. cumingii (HcCA), which, as in the present study, was more highly expressed in the mantle compared with the gill [42]. This pattern of expression implies that the isoform of CA investigated in the present study likely plays a specific role in regulating shell formation. In the mantle, CA provides a source of HCO3– for CaCO3 formation by catalyzing the hydration of CO2 to HCO3–, hence increasing the driving force toward biomineralization. In the present study, no effect of pCO2 exposure on mantle ca mRNA was detected until the post-exposure period, where ca mRNA levels were elevated above those of control mussels in L. siliquoidea previously exposed to elevated pCO2. Although, the effects of elevated pCO2 on ca mRNA have not been previously investigated in freshwater mussels, mantle ca mRNA levels were sensitive to external environmental conditions in H. cumingii, effects that coincided with changes in shell growth and pH homeostasis [42]. Accordingly, in the present study, the post-exposure increase in mantle ca mRNA, similar to cs, may represent a compensatory response to increase biomineralization processes after exposure to elevated pCO2. Further assessments of other potential CA isoforms, and their responses to freshwater acidification, will provide a more complete understanding of the role of CAs in regulating responses to increased pCO2.

Calcium metabolism is another important regulator of biomineralization and includes the sequential processes of Ca2+ absorption, accumulation, transportation, and incorporation into the shell. Both CaM and CaM-like protein are considered important regulators of Ca2+ metabolism in marine bivalves [43–47]. Although calcium metabolism likely differs between freshwater and marine bivalves owing to the differences in environmental Ca2+ concentrations, the roles of CaM and CaM-like protein as calcium regulators have been studied to some extent in freshwater pearl mussels H. schlegeli [48] and H. cumingii [49]. In the present study, cam and calp mRNA abundance differed between the gill and mantle of L. siliquoidea, with cam mRNA being more abundant in the gill and calp mRNA levels being higher in the mantle. Similar transcript expression patterns of cam and calp in the gill and mantle have been found in both freshwater [48,49] and marine bivalves [43,44]. In the present study, cam and calp mRNA levels were only significantly affected by pCO2 exposure in the gill. In bivalves, the gill is the primary location for Ca2+ uptake from the external environment [50,51]. After 24 h of exposure, gill cam and calp mRNA concentrations, the roles of CaM and CaM-like protein as calcium regulators have been studied to some extent in freshwater pearl mussels H. schlegeli [48] and H. cumingii [49]. In the present study, cam and calp mRNA abundance differed between the gill and mantle of L. siliquoidea, with cam mRNA being more abundant in the gill and calp mRNA levels being higher in the mantle. Similar transcript expression patterns of cam and calp in the gill and mantle have been found in both freshwater [48,49] and marine bivalves [43,44]. In the present study, cam and calp mRNA levels were only significantly affected by pCO2 exposure in the gill. In bivalves, the gill is the primary location for Ca2+ uptake from the external environment [50,51]. 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nevertheless, *calp* mRNA in the mantle did not appear to be sensitive to changes in environmental *p*CO$_2$. Expression levels of CaM-like protein are location-specific within the mantle in other bivalve species (e.g., highest in the mantle edge compared with other regions in *H. cumingii* [49]). In the present study, a combination of mantle areas was used for mRNA analysis, which may have limited the ability to detect location-specific effects of *p*CO$_2$ on *calp* mRNA. Together, these results suggest that in *L. siliquoidea* a decrease in the uptake of environmental Ca$^{2+}$ is after exposure to elevated *p*CO$_2$ may be mediated by *calp* in the gill; nonetheless, deposition of Ca$^{2+}$ from the mantle via a *calp*-dependent mechanism may not be affected.

The main driver of secondary active ion transport, Na$^+$–K$^+$-ATPase also provides the motive force that is necessary for Na$^+$ uptake. In the present study, *nka* mRNA was not significantly affected by *p*CO$_2$ exposure until the post-exposure period. Gill *nka* mRNA decreased by approximately 2-fold during the recovery period in mussels previously exposed to 50 000 μatm *p*CO$_2$ and a similar decrease was observed in the mantle following 14 d of recovery. Ion-regulatory changes were previously assessed in freshwater mussels in response to similarly high levels (20 000–55 000 μatm) of sustained [16] and intermittent [18] *p*CO$_2$ exposure. In these studies, mussels showed a significant increase in hemolymph Na$^+$ levels in response to elevated *p*CO$_2$ that was thought to have resulted from an increase in H$^+$ excretion via the Na$^+$/H$^+$ exchanger, thus also increasing Na$^+$ uptake [54]. Hemolymph Na$^+$ levels subsequently decreased post-exposure in *L. siliquoidea* and *Amblema plicata* previously exposed to elevated *p*CO$_2$ [16]. The decrease in *nka* mRNA levels in *L. siliquoidea* during the recovery period in the present study may represent a decrease in Na$^+$ uptake during this time, allowing for Na$^+$ levels to decrease back to baseline (i.e., through passive loss to the freshwater environment). Interestingly, *nka* mRNA levels remained low after 14 d at control conditions, suggesting that an extended period post-*CO$_2$* exposure may be required for recovery of ionic disturbances experienced during *p*CO$_2$ exposure. Assessments of NKA activity are required to determine whether changes in *nka* transcript abundance reflect activity level changes of this enzyme; nevertheless, a comparable decrease in whole-body Na$^+$ during copper exposure was thought to result from a decrease in NKA activity in juvenile *L. siliquoidea* [55]. These results suggest that *L. siliquoidea* may regulate *nka* mRNA after exposure to elevated *p*CO$_2$ to clear a Na$^+$ load acquired during *p*CO$_2$ exposure.

Levels of *hsp70* mRNA have been previously shown to be sensitive to changes in environmental condition in bivalves [56]. In a previous study, *hsp70* mRNA levels were significantly increased by a 32-d exposure to 20 000 μatm *p*CO$_2$ in the gill but not the mantle of the freshwater mussel *F. flava* [19]. Mantle *hsp70* mRNA levels were similarly unaltered by *p*CO$_2$ exposure in *L. siliquoidea* in the present study. Within mussels exposed to 50 000 μatm *p*CO$_2$, gill *hsp70* mRNA levels increased significantly by approximately 1.5-fold from 4 to 7 d of exposure, and remained elevated until 28 d of exposure that may represent a cellular response to the *CO$_2$* stressor. Levels of gill *hsp70* mRNA then decreased by approximately 2-fold during the post-exposure period in mussels previously exposed to 50 000 μatm, which may have been caused by increased mRNA turnover or decreased mRNA production, although the consequences for HSP70 protein levels are unclear and warrant further investigation. The gill *hsp70* mRNA levels were also lower than those of control mussels, which may, in part, have been the result of a marginal but significant increase in *hsp70* mRNA levels in the control group over the holding period. Overall, the effect of *p*CO$_2$ exposure on *hsp70* mRNA levels was minimal in *L. siliquoidea* compared with *F. flava*, and may point to species-specific regulation of cellular components of the stress response, although a broader investigation of genes related to cellular stress is needed.

In many cases, the impacts of elevated *p*CO$_2$ exposure on the variables assessed in the present study were not evident until the post-exposure period. This delay in the assessed responses to elevated *p*CO$_2$ exposure may reflect the ability of *L. siliquoidea* to regulate during *p*CO$_2$ exposure (e.g., acid-base status), and utilize compensatory mechanisms after exposure to maintain a state of homeostasis. Alternatively, the changes that were observed in control mussels over the 42 d of holding (i.e., increase in gill *hsp70* mRNA, decrease in mantle *ca* and gill *calp* mRNA, and decline in BCI) suggest that long-term holding may also have contributed to the responses observed during the recovery period. Because of the decrease in BCI observed over the holding period, the amount of food supplied to mussels may have been insufficient; thus, in future studies, additional feeding may be necessary for these holding conditions (e.g., every day rather than every other day). However, the significant differences between mussels previously exposed to elevated *p*CO$_2$ and control mussels during the recovery period suggest that exposure to elevated *p*CO$_2$ likely still played a role in regulation of biomineralization and ion-regulatory responses in *L. siliquoidea*. Long-term holding of animals in the laboratory can be difficult; nonetheless, manipulation of the animals during the holding period was limited, mussels were provided with sediment in which to bury, and no mortality occurred. Consequently, the results of the present study are valuable and point to mechanisms that *L. siliquoidea* may utilize to regulate against acid-base and ionic disturbances experienced after exposure to elevated *p*CO$_2$.

Overall, the data from the present study provide a mechanistic understanding of the potential consequences for biomineralization and ion-regulatory processes of freshwater mussels to elevations in *p*CO$_2$. No mortality occurred over the course of the experimental period in the present study, and similar levels of *p*CO$_2$(42 mg/L) caused low mortality in juvenile *L. siliquoidea* [20], indicating that *L. siliquoidea* were reasonably tolerant to elevations in *p*CO$_2$. However, although survival was not significantly impacted by exposure to 28 d of elevated *p*CO$_2$, sublethal impacts were evident—particularly when mussels were exposed to 50 000 μatm *p*CO$_2$ (Figure 6). During *p*CO$_2$ exposure, mussels may reduce chitin synthesis through a decrease in cs mRNA in the mantle that may have negative consequences for shell integrity and structure, and mussels may potentially regulate other aspects of biomineralization via the uptake of Ca$^{2+}$ in the gill by adjustments in *ca* and *calp* mRNA (Figure 6). These decreases in factors associated with biomineralization also coincided with increases in hemolymph HCO$_3^-$ and Na$^+$, which
FIGURE 6: Summary of the regulatory responses during and after exposure to elevated partial pressures of carbon dioxide ($p$CO$_2$) in adult Lampsilis siliquoidea. Results for mussels exposed to $\sim$ 50 000 μatm $p$CO$_2$ for a period of 1, 4, 7, and 28 d, and then for an additional 2 wk at control conditions are displayed. Factors involved in biomineralization (i.e., calmodulin [cam], calmodulin-like protein [calp], chitin synthase [cs], and carbonic anhydrase [ca]) were regulated during the first 7 d of $p$CO$_2$ exposure and during the post-exposure period. Ion-regulatory responses (i.e., Na$^+$/H$^+$ exchanger [nka]) were also regulated during the post-exposure period. The compensatory reactions during the post-exposure period are likely in answer to the acid-base and ion disturbances experienced during the $p$CO$_2$ exposure period. Increases and decreases in acid-base and ion-regulatory responses (i.e., hemolymph HCO$_3^-$, Cl$^-$, Ca$^{2+}$, and Na$^+$) are represented by solid and dashed lines, respectively [16]. Note that the absence of a line for acid-base and ion-regulatory reactions indicates that hemolymph ion levels of mussels held at high $p$CO$_2$ conditions were not different from mussels held at control conditions [16].

are thought to occur in response to the release of CaCO$_3$ stores from the shell and increases in H$^+$ excretion via the Na$^+$/H$^+$ exchanger, respectively [16]. Thus, periods of increased $p$CO$_2$ exposure may result in a shift from an investment in shell growth to acid-base regulation caused by the intricately linked processes of calcification and acid-base regulation (i.e., carbonate buffering system). In the short term, these responses are likely to be beneficial; moreover, a decrease in shell integrity may have long-term consequences for survival in mussels because of the important protective properties of the shell. Intriguingly, freshwater mussels exhibited resilience in the compensatory responses observed during the post-exposure period. Increases in mantle cs and ca transcript abundance as well as decreases in nka mRNA after $p$CO$_2$ exposure may represent re-investment in biomineralization processes as well as ion regulation, respectively (Figure 6). Because many of these post-exposure responses continued to be affected even after 14 d of recovery, a longer post-exposure period (e.g., equal to or greater than the exposure period) may be necessary for full recovery from $p$CO$_2$ exposure in adult L. siliquoidea. Furthermore, if mussel nutrition was compromised during the exposure/post-exposure periods, this may have additionally decreased the ability of mussels to recover from $p$CO$_2$ exposure. The results from the present study have the potential to provide a foundation for the physiological responses of freshwater mussels to future increases in freshwater $p$CO$_2$ caused by both natural and anthropogenic factors. As previously indicated, the responses of freshwater bivalves to elevations in $p$CO$_2$ have been largely understudied compared with the work that has been carried out on marine bivalves in the context of ocean acidification. Although the levels of CO$_2$ in the present study exceed those typically used in ocean acidification studies (i.e., $\sim$ 15 000–50 000 μatm vs $\sim$ 800–4000 μatm for marine studies [24,27,28,30]), these levels may not be improbable for some freshwater systems [5], particularly in the case of invasive species management [14,15]. Further studies into the carry-over effects of elevated $p$CO$_2$ exposure—as well as consequences for reproduction and the role that reproductive state and sex of the mussel may play in mediating responses—would provide additional information on population level consequences for freshwater mussels exposed to high $p$CO$_2$.

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Disclaimer—The present study is solely the responsibility of the authors and does not necessarily represent the official views of the US Geological Survey.

Data Availability—DNA sequences have been submitted to the GenBank, with accession numbers KY978468–76. Body condition index and mRNA data are available on request from the corresponding author (jjjeffrey@illinois.edu).
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