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RESEARCH ARTICLE | *Fluid and Electrolyte Homeostasis*

Hot and bothered: effects of elevated P_{CO_2} and temperature on juvenile freshwater mussels

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Jeffrey JD, Hannan KD, Hasler CT, Suski CD. Hot and bothered: effects of elevated P_{CO_2} and temperature on juvenile freshwater mussels. *Am J Physiol Regul Integr Comp Physiol* 315: R115–R127, 2018. First published March 16, 2018; doi:10.1152/ajpregu.00238.2017.—Multiple environmental stressors may interact in complex ways to exceed or diminish the impacts of individual stressors. In the present study, the interactive effects of two ecologically relevant stressors [increased temperature and partial pressure of carbon dioxide (P_{CO_2})] were assessed for freshwater mussels, a group of organisms that are among the most sensitive and rapidly declining worldwide. The individual and combined effects of elevated temperature (22°C–34°C) and P_{CO_2} (~230, 58,000 μatm) on juvenile *Lampsilis siliquoidea* were quantified over a 5- or 14-day period, during which physiological and whole animal responses were measured. Exposure to elevated temperature induced a series of physiological responses, including an increase in oxygen consumption rates following 5 days of exposure at 31°C and an increase in carbonic anhydrase (*ca*) and heat shock protein 70 mRNA levels following 14 days of exposure at 28°C and 34°C, respectively. Treatment with elevated P_{CO_2} activated acid-base regulatory responses including increases in CA and Na^+-K^+-ATPase activity and a novel mechanism for acid-base regulation during P_{CO_2} exposure in freshwater mussels was proposed. Thermal and CO_2 stressors also interacted such that responses to the thermal stressor were diminished in mussels exposed to elevated P_{CO_2}, resulting in the greatest level of mortality. Additionally, larger mussels were more likely to survive treatment with elevated P_{CO_2} and/or temperature. Together, exposure to elevated P_{CO_2} may compromise the ability of juvenile freshwater mussels to respond to additional stressors, such as increased temperatures, highlighting the importance of considering not only the individual but also the interactive effects of multiple environmental stressors.

acidification; carbonic anhydrase; HSP70; multiple stressors; Na^+-K^+-ATPase

INTRODUCTION

With ongoing global change, increased attention has been paid to understanding the cumulative impacts of novel and extreme environmental challenges (i.e., stressors) on biota. Evaluating environmental impacts on ecosystems and organisms is particularly challenging because of the potential for multiple stressors to interact in complex and unexpected ways, and the need to understand the interactive and cumulative effects of multiple stressors has been cited as one of the most pressing questions in ecology and conservation (e.g., 75, 92). Multiple stressor effects are often considered to be additive; however, of particular interest are situations in which multiple stressors interact to create "ecological surprises" in the form of nonadditive effects (13). These nonadditive effects include synergism, in which stressor effects are amplified or exacerbated when acting together, and antagonism, in which effects resulting from multiple stressors are less than expected based on the individual stressor effects (24). Complex interactions between stressors are likely highly prevalent in natural environments (13, 50); however, mechanisms underlying responses to multiple stressors remain largely unexplored (32).

Although marine systems have gained increasing attention by researchers for the interactive effects of multiple stressors (13, 37), evidence for the net effects of multiple stressors on freshwater systems remains limited (50). Freshwater systems are particularly vulnerable to global change because of the integrative effects of local catchments and regional atmospheric processes (20, 65, 91). Two ecologically relevant stressors that have been well studied in marine systems, but may also occur together in freshwater systems, are increases in water temperature and carbon dioxide [measured as the partial pressure of CO_2 (P_{CO_2})] (19, 71). Although less well understood compared with marine systems, current and future freshwater P_{CO_2} may increase as a result of natural or anthropogenic factors (38). A global assessment of 47 rivers revealed a mean P_{CO_2} of 3,230 μatm for freshwater systems that ranged from 647 to 38,000 μatm (9). Freshwater P_{CO_2} can fluctuate daily and seasonally and can exceed atmospheric levels (i.e., supersaturation of CO_2) because of factors including terrestrial productivity, precipitation, and local geology (14, 25). River environments may therefore experience a wide range of P_{CO_2} over the course of a year, from less than 100 to more than 15,000 μatm, with higher levels occurring in warmer, drier periods (9). Additionally, anthropogenic increases in P_{CO_2} may result from deforestation (93), agricultural and urban activities (5), climate change (70), and invasive species management (64). Although the magnitude of the change is not known, freshwater P_{CO_2} is likely to increase with higher levels of atmospheric CO_2, greater terrestrial primary productivity, increased precipitation, and longer periods of dry conditions (38, 69, 70). Levels of freshwater CO_2 may also be intentionally elevated up to 100 times greater than ambient conditions in localized areas for the purpose of invasive species management (i.e., nonphysical barrier for the movement of invasive fish) (16, 18, 83); however, the form of such barriers has not yet been fully defined. Freshwater temperature can also vary because of natural and anthropogenic factors (44) and can reach highs of 30°C–40°C in some temperate regions, which are

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often at or near the thermal limits of resident species (23). Overall, freshwater temperatures are expected to rise over the next 100 years as global air temperatures continue to warm (62, 65a, 84), which could push organisms beyond their thermal limits. As increases in P_{CO_2} and temperature are likely to occur together, it becomes important to understand the potentially complex interactive effects of these stressors on organisms, particularly those already at risk from other environmental disturbances.

North American freshwater mussels are among the most sensitive and rapidly declining faunal groups worldwide (58, 85). Freshwater mussels are sessile, often long-lived, benthic invertebrates that have a complex life cycle involving a parasitic larval stage and can act as sentinel species providing important information about the health of an ecosystem (e.g., 30, 68). By providing a number of important functions in aquatic systems, such as linking the water column and benthic food webs, increasing habitat complexity, and generating habitats for other aquatic organisms, freshwater mussels are integral contributors to freshwater environments (86, 87). Declines in mussel populations have been attributed to anthropogenic factors, including habitat alterations, pollutants, sedimentation, and invasive species (80), and warming or increases in P_{CO_2} because of climate change may additionally put these organisms at risk (38, 40, 78). Because of the imperiled status of several freshwater mussel species, quantifying the responses of mussels to complex stressor environments becomes important, particularly for young life stages, when mussels may be even more sensitive to environmental challenges (11).

The tolerance and the physiological responses of freshwater mussels to elevated temperature and P_{CO_2} have been independently assessed; however, the interactive effects of these two stressors have not been defined. Thermal stressors can reduce survival (27, 67), elicit sublethal responses, including altered energy balance (28), increases in metabolic rate [e.g., oxygen consumption (MO_2)], and heart rate that decreases beyond a critical temperature (28, 66). Other sublethal responses, include decreased byssus production in juvenile mussels (a structure important for attachment and dispersal capabilities) (2), changes in burrowing (2) and valve gaping behaviors (26, 28), as well as elevations in heat shock proteins (HSPs) (68), which play an important role in mediating cellular damage. With respect to CO_2, exposure to elevated P_{CO_2} results in the acidification of internal fluids, necessitating modulation of acid-base regulatory responses (31). As the hemolymph of unionid mussels has a limited buffering capacity and a low osmotic concentration, mussels appear to utilize acid-base and ion-regulatory responses (34–36) as well as a bicarbonate-carbonate buffer system (61) to respond to acidosis. Changes in hemolymph ion concentrations have been observed in adult freshwater mussels exposed to elevated P_{CO_2} (34–36), presumably as a consequence of H^+ excretion via Na^+/H^+ exchangers (NHE) or vacuolar-type H^+-ATPase (v-type HA) (e.g., increase in hemolymph Na^+), and HCO_3^- retention by regulating HCO_3^-/Cl^- anion exchangers (e.g., reduction in hemolymph Cl^- and increase in HCO_3^-). Growth, integrity of the shell, and survival have also been compromised by exposure to elevated P_{CO_2} in juvenile freshwater mussels (88), which may result, at least in part, from a shift in the direction of the bicarbonate buffering system from deposition of $CaCO_3$ for biomineralization (47, 90) to utilizing shell $CaCO_3$ stores to provide HCO_3^- for buffering acidosis (42) [e.g., hemolymph Ca^{2+} levels increase as a result (34–36)]. Additionally, alterations in burrowing and gaping behaviors were observed in both juvenile (88) and adult (39) freshwater mussels in response to elevated P_{CO_2} exposure. Although the consequences of exposure to elevated temperature and P_{CO_2} have been assessed to some extent, the mechanisms underlying these responses are not well understood. Additionally, as wild freshwater mussel populations are likely to experience alterations in temperature and P_{CO_2} simultaneously, determining the interactive effects of these stressors will be important for predicting future responses to global changes of individuals and populations.

Based on this background, the goal of the present study was to quantify the interactive effects of elevated P_{CO_2} and temperature on juvenile freshwater mussels using an integrative approach. In this study, juvenile *Lampsilis siliquoidea* were exposed to either control (~230 μatm) or high (~58,000 μatm) P_{CO_2} for up to 14 days, in combination with one of five experimental temperatures ranging from 22°C to 34°C. These exposure conditions represent ecologically relevant and extreme conditions that freshwater mussels may experience in many parts of the United States. Within the native range of *L. siliquoidea* (15, 63), summer water temperatures in shallow waters of the Upper Mississippi River can exceed 30°C (27), streams in the southern United States can reach 35°C–40°C (78), and mean water temperatures are expected to increase in the future with global warming (62, 65a, 84). Freshwater P_{CO_2} levels may also increase in freshwater systems because of the variety of factors described above and may reach levels above 50,000 μatm in the upper Midwest at locations near proposed fish deterrent barriers where P_{CO_2} levels may potentially be elevated up to 100 times above ambient conditions (e.g., 16, 18). In the present study, survival was assessed over the 14-day exposure period, after which cellular-level responses were quantified, including regulators of acid-base status [carbonic anhydrase (CA), Na^+-K^+-ATPase (NKA), and v-type HA activity and/or mRNA level] and indicators of cellular stress (*hsp70* mRNA, antioxidant capacity, and oxidative damage). In addition, whole animal MO_2 was evaluated following a 5-day exposure at either 22°C or 31°C and either control or high P_{CO_2}. It was hypothesized that mussels exposed to elevated P_{CO_2} would be more sensitive to the additional thermal stressor, resulting in decreased survival because of the increased demand of regulating acid-base status, evidenced by elevated enzyme activity and mRNA levels.

MATERIALS AND METHODS

Experimental Animals

Juvenile *L. siliquoidea* ($n = 331$) of approximate age 16 mo were obtained from the Genoa National Fish Hatchery, Genoa, WI, and held at the Aquatic Research Facility at the University of Illinois, Urbana–Champaign, IL. Mussels were held for at least 1 wk before experimentation in a recirculating tank system with sediment (2 cm of sand; Old Castle all-purpose sand, Atlanta, GA) that was supplied with water from a 0.04-ha natural earthen-bottom pond with vegetation. The holding system was equipped with a Teco 500 aquarium heater/chiller (TECO-US, Aquarium Specialty, Columbia, SC) to maintain a water temperature of 22°C and a low-pressure air blower (Sweetwater, SL24H Pentair, Apopka, FL) for aeration. Fifty percent water changes were performed twice weekly, and water quality was

assessed daily. During this period, dissolved oxygen (8.17 ± 0.10 mg/l, means ± SE) and temperature (21.5°C ± 0.3°C) were measured with a YSI 550A portable meter (Yellow Springs Instruments, Irvine, CA), pH (8.33 ± 0.04) was measured using a WTW pH 3310 handheld meter (WTW) that was calibrated regularly, and alkalinity (217 ± 5 mg/l $CaCO_3$) was measured using a digital titration kit (Hach, Loveland, CO) (Table 1). Water P_{CO_2} (427 ± 179 μatm) was monitored using a modified infrared probe (Vaisala GMP220 and GMT221, St. Louis, MO) (53), and the concentration of CO_2 (9.0 ± 0.6 mg/l) was determined using a CO_2 titration kit (Hach). Ammonia levels were also measured during the preexposure and exposure periods using an Ammonia Nitrogen Kit (No. 3351–02, LaMotte, Chestertown, MD) and did not exceed the lowest detectable limit of 0.2 parts/million NH_3-N. Mussels were fed daily with a commercial shellfish diet with multiple particle sizes consisting mainly of *Nannochloropsis* sp. 1–2 μm, as well as a mixed diet of *Isochrysis*, *Pavlova*, *Thalassiosira*, and *Tertraselmis* spp. 5–12 μm (Instant Algae, Reed Mariculture, Campbell, CA). Mussel tanks were carefully monitored for the clearance of algae from the water to ensure that food availability was not restricted.

Experiment 1

Elevated carbon dioxide and temperature exposure. Juvenile mussels that displayed foot movement (55) (n = 303; length, 11.4 ± 0.1 mm, mean ± SE; length range, 7–16.2 mm) were moved to one of ten recirculating systems (Fig. 1*A*) 24 h before the onset of the experiment. Within each recirculating system mussels (n = 30–32) were divided evenly among three 283-ml containers (i.e., 10–11 mussels per container) with sediment (2 cm of sand; Old Castle all-purpose sand) that were supplied with pond water. Water was allowed to overflow into a 3.5-liter trough that drained into a reservoir tank below the setup. Water was then recirculated from the reservoir tank back into the individual mussel holding containers with a pump. Water was also passed through an aquarium heater/chiller (TECO-US, Aquarium Specialty) to maintain water temperature, and the reservoir was aerated with a low-pressure air blower (Sweetwater, SL24H Pentair). Mussel husbandry was carried out as for the preexposure setup, with mussels being fed daily and 25%–50% water changes occurring every other day.

Mussels were exposed to one of five temperatures (22°C, 25°C, 28°C, 31°C, or 34°C) and to either control P_{CO_2} (232 ± 31 μatm) or a high P_{CO_2} treatment of ~58,000 μatm (58,661 ± 576 μatm) for up to 14 days (Fig. 1*B*). The highest temperature of 34°C was chosen as it is the upper thermal tolerance for juvenile *L. siliquoidea* acclimated to 22°C (67). Temperature was increased by a rate of 0.5°C/h until the target temperatures were achieved, which was slower than the suggested maximum rate recommended in the Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels (3). Freshwater P_{CO_2} may rise because of a variety of natural and anthropogenic factors (see INTRODUCTION). Similar to targets used in the temperature portion of this study, a CO_2 level of 58,000 μatm was chosen as it is near the upper tolerance for juvenile mussels (88), making this pressure valuable for identifying mechanisms of tolerance relevant at lower partial pressures (45). In addition, studies of eutrophic environments have shown that freshwater P_{CO_2} can exceed 40,000 μatm (4), and levels are projected to exceed 50,000 μatm in areas adjacent to nonphysical fish barriers that use elevated CO_2 to deter the movement of fishes (16, 18). Target P_{CO_2} was maintained with the common method of using a pH controller (PINPOINT, American Marine, Ridgefield, CT) that added compressed CO_2 gas (commercial grade, 99.9% purity) into the system's reservoir through an air stone if the pH rose above a target level (74). Each recirculating system contained an additional 2-liter container that allowed for water chemistry to be monitored daily (Table 1), as described for the preexposure period.

Mussels were held for a maximum of 14 days at treatment conditions, and their survival was monitored over this period. Dead mussels, indicated by a persistent opening of the shell and no foot retraction, were counted and removed daily. After 14 days of exposure to treatment conditions, surviving mussels were measured for length, and their soft tissues were removed with forceps. Because of their small size, whole body soft tissues (i.e., no differentiation of internal tissues) from individual mussels were used for analysis of physiological variables (29, 55). Within a treatment group, individual mussels were randomly selected for analysis of mRNA levels, enzyme activity, or oxidative stress analysis. Soft tissues of individual mussels were stored in either RNAlater (Ambion, Fisher Scientific, Hanover Park, IL) or flash frozen in liquid nitrogen. Samples stored in RNAlater were stored overnight at 4°C before being stored at −20°C, and flash-frozen samples were stored at −80°C until analysis.

Quantitative real-time RT-PCR. Total RNA was extracted from the soft tissue of individual mussels that were stored in RNAlater (n = 7–8) using TRIzol reagent (Ambion, Fisher Scientific) according to the manufacturer's protocol. Tissues were disrupted and homogenized with a BeadBug Microtube homogenizer (Denville Scientific, Holliston, MA). Extracted RNA was quantified using a Qubit 3.0 Fluorometer (Fisher Scientific), its quality was assessed using a NanoDrop One spectrophotometer (Fisher Scientific), and 1 μg of RNA was treated with deoxyribonuclease I (Amplification Grade, DNase; Invitrogen, Fisher Scientific). To synthesize cDNA, MultiScribe Reverse Transcriptase, RNase inhibitor, and random primers were used according to the manufacturer's protocol (High-Capacity cDNA Reverse Transcription kit, Applied Biosystems, Fisher Scientific).

The relative abundance of *hsp70*, *ca*, and *nka* mRNA was determined by quantitative real-time RT-PCR. Primers for the target genes, as well as the reference genes *ef1-α*, *gapdh*, and *18s*, were previously

Table 1. *Water chemistry variables for control and high P_{CO_2} treatments at either 22°C, 25°C, 28°C, 31°C, or 34°C assessed daily over the 14-day exposure period*

Treatment		Water Chemistry Variable					
CO_2	Temperature, °C	Temperature, °C	dO_2, mg/l	pH	P_{CO_2}, μatm	$[CO_2]$, mg/l	Alkalinity, mg/l $CaCO_3$
Control	22	21.5 ± 0.1	8.24 ± 0.04	8.314 ± 0.168	127 ± 52	1.5 ± 0.2	255 ± 8
	25	24.2 ± 0.2	8.03 ± 0.03	8.567 ± 0.015	358 ± 82	1.6 ± 0.2	246 ± 4
	28	28.0 ± 0.2	7.65 ± 0.07	8.552 ± 0.022	269 ± 88	0.5 ± 0.1	209 ± 9
	31	30.0 ± 0.2	7.07 ± 0.04	8.438 ± 0.106	221 ± 54	0.9 ± 0.1	255 ± 6
	34	33.2 ± 0.3	6.95 ± 0.06	8.559 ± 0.027	181 ± 56	0.4 ± 0.1	227 ± 9
High	22	22.3 ± 0.2	7.63 ± 0.05	6.860 ± 0.156	60241 ± 1187	72.4 ± 2.6	278 ± 4
	25	24.7 ± 0.1	7.24 ± 0.04	6.684 ± 0.010	60837 ± 1377	70.4 ± 2.4	277 ± 6
	28	27.7 ± 0.2	7.07 ± 0.03	6.700 ± 0.016	59423 ± 1168	64.2 ± 3.0	272 ± 6
	31	30.3 ± 0.2	6.64 ± 0.02	6.655 ± 0.025	56187 ± 1222	58.0 ± 2.5	233 ± 11
	34	33.5 ± 0.2	6.27 ± 0.03	6.669 ± 0.025	56071 ± 589	52.5 ± 2.3	262 ± 4

Data are presented as means ± SE. dO_2, dissolved oxygen.

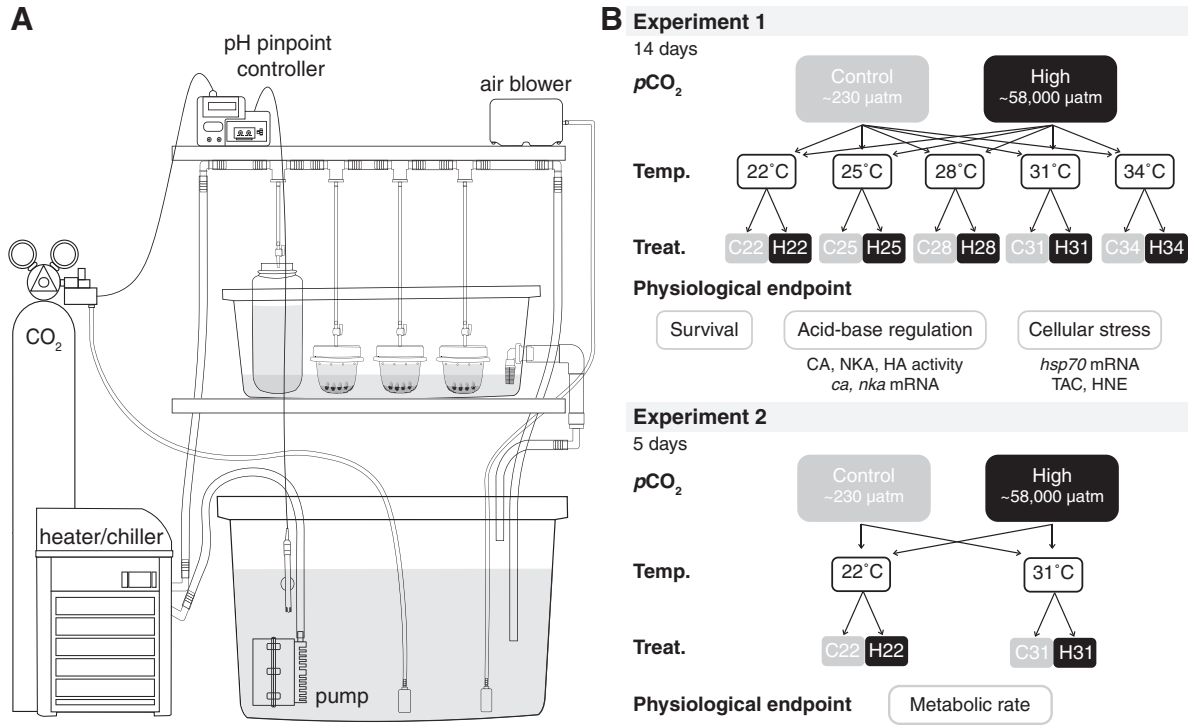

Fig. 1. *A*: schematic of the mussel holding system. *B*: experimental design for the exposure of juvenile *Lampsilis siliquoidea* to elevated temperature and P_{CO_2}. Mussels were held in recirculating systems that consisted of 283-ml containers with sediment that held the mussels and a 2-liter container for water chemistry monitoring. Water was allowed to overflow into a 3.5-liter container that drained into a reservoir. Water was then recirculated through a heater/chiller to the individual containers with a pump. The reservoir was aerated with a low-pressure air blower, and P_{CO_2} levels were elevated and maintained using a pH pinpoint controller. For *experiment 1*, mussels were exposed to either control (~230 μatm) or high (~58,000 μatm) P_{CO_2} as well as either 22°C, 25°C, 28°C, 31°C, or 34°C for 14 days. Survival was assessed daily over the 14-day period. Regulators of acid-base status were assessed following the 14-day exposure, including carbonic anhydrase (CA) activity and mRNA, Na^+-K^+-ATPase (NKA) activity and mRNA, and vacuolar-type H^+-ATPase (v-type HA) activity. Indicators of cellular stress were also assessed, including heat shock protein 70 (*hsp70*) mRNA, and total antioxidant capacity (TAC) and oxidative damage [hydroxynonenal (HNE)]. For *experiment 2*, oxygen consumption (MO_2) was assessed following 5 days of exposure to either control or high P_{CO_2} and either 22°C or 31°C.

used in Jeffrey et al. (51). To optimize reaction compositions, standard curves were generated for each primer set using cDNA pooled from individuals across treatment groups (efficiencies were >0.90). PowerUp SYBR Green Master Mix (Applied Biosystems, Fisher Scientific) and an ABI 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY) were used to carry out quantitative real-time RT-PCR according to manufacturer's instructions for 10-μl reactions. Primer concentrations were 0.5 μM, and cDNA was diluted 20-fold for *hsp70*, *nka*, *ef1-α*, and *gapdh*, 10-fold for *ca*, and 10,000-fold for *18s*. Cycling conditions were as follows: 95°C for 15 s and 60°C for 60 s over 40 cycles. The NORMA-gene approach of Heckmann et al. (41) was used to normalize threshold cycle (C_t) values that were then expressed relative to the control P_{CO_2} mean for mussels held at 22°C using the $-\Delta C_t$ method, where Δ indicates change (57).

Enzymatic analyses. Flash frozen whole body soft tissues of individual mussels (n = 5–8) were homogenized in eight volumes (wt/vol) of SEID buffer (250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, 0.1% Na deoxycholic acid; pH 7.3) using a Kimble, Kontes motorized pestle (Fisher Scientific). Samples were centrifuged at 5,000 *g* at 4°C for 3 min, and the supernatants were kept on ice throughout the analysis (29). The activity of NKA and v-type HA was measured according to McCormick (60) and Lin and Randall (56), respectively. These methods were used to calculate the difference in the amount of adenosine diphosphate (ADP) produced in samples assayed at control conditions and when in the presence of an inhibitor. Ouabain (0.5 mM; Sigma-Aldrich, St. Louis, MO) and *N*-ethylmaleimide (NEM; 1 mM; Sigma-Aldrich) with sodium azide (NaN_3; 5 mM; Sigma-Aldrich) were used to inhibit NKA and v-type HA, respectively. Additionally, bafilomycin A1 (10 μM; Research Products International, Fisher Scientific) was used to inhibit v-type HA and produced similar results to NEM. Samples (10 μl) were run in duplicate in a 96-well microplate across five working solutions. Each reaction contained 2 mM phosphoenolpyruvate (Gold Biotechnology, St. Louis, MO), 0.5 mM ATP (Sigma-Aldrich), 0.16 mM nicotinamide adenine dinucleotide (NADH; Sigma-Aldrich), 2.86 U/ml lactate dehydrogenase (Sigma-Aldrich), 3.75 U/ml pyruvate kinase (Sigma-Aldrich), 50 mM imidazole (Sigma-Aldrich), 47.25 mM NaCl, 2.625 mM $MgCl_2 \times 6H_2O$, and 10.5 mM KCl. *Solution A* contained no inhibitors, *solution B* was supplemented with ouabain, *solution C* contained ouabain and NaN_3, *solution D* was identical to *solution C* but also contained NEM, and *solution E* contained ouabain and bafilomycin. Plates were read at 340 nM at 20-s intervals for 20 min using a microplate spectrophotometer (Molecular Devices, SpectraMax Plus 384, Sunnyvale, CA). The average slope (mOD/10 μl/min) of each sample with and without inhibitors was determined. Standard curves for NADH (0–5 mM) and ADP (0–4 mM) were determined to verify the quality of the reagents. Protein concentrations were measured for each sample using a Bradford assay (Sigma Aldrich), and enzyme activity was expressed as μmol ADP/mg protein/h.

CA activity was measured in the same homogenate as described above according to Henry (43). Homogenate (50 μl) was added to 5 ml of reaction buffer (225 mM mannitol; 75 mM sucrose; 10 mM Tris base; pH brought to 7.4 with orthophosphoric acid) held within a chamber that was kept at 4°C throughout the analysis. Using a pH electrode (GK2401C Combined pH Electrode, Hach; VWR, Rednor, PA) connected to a pH/ISE isoPod (eDAQ, Colorado Springs, CO), the change in pH over a 0.25 pH unit range was measured following

the addition of 200 μl of ice-cold CO_2-saturated H_2O using a 500-ml Hamilton syringe (VWR) in the presence (catalyzed; V_{cat}) and absence (uncatalyzed; V_u) of the sample homogenate. In addition, the buffer capacity (β; pH units/μmol H^+) of the reaction buffer was determined as the change in pH following the addition of 50 μl of 0.1 M HCl to 5 ml of reaction buffer. The activity of CA (μmol H^+/min/mg protein) was calculated as

$$\text{CA activity} = \frac{\left(\frac{V_{cat}}{\beta} - \frac{V_u}{\beta}\right) \times 60\ \text{sec}}{\text{Volume}_{lysate} \times [\text{protein}]}$$

Oxidative stress. Flash-frozen whole body soft tissues of individual mussels ($n = 4–6$) were homogenized in eight volumes (wt/vol) of 1X PBS buffer (from 10× PBS Buffer, Fisher Scientific) supplemented with protease inhibitors [50 μg/l aprotinin and 40 μM of phenylmethylsulfonyl fluoride; Thermofisher, Fisher Scientific] (59) using a Kimble, Kontes motorized pestle (Fisher Scientific). Samples were centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatant was removed and held on ice.

Oxidative stress was quantified by assessing an oxidative marker of lipid peroxidation, 4-hydroxynonenal (HNE), as well as total antioxidant capacity (TAC). Commercially available kits, HNE OxiSelect HNE-His adduct ELISA Kit and OxiSelect Total Antioxidant Capacity Assay Kit (Cell Biolabs, Fisher Scientific), were used following the manufacturer's protocol. TAC was expressed in Cu-reducing equivalents against a uric acid standard, where 1 mM of uric acid is equivalent to 2189 μM Cu^{2+}. Samples were run in duplicate and normalized to their protein concentrations that were determined using a Bradford assay (Sigma Aldrich).

Experiment 2

Metabolic rate. In a second experiment using a different group of mussels, MO_2 was obtained from four of the above treatments (22°C: control CO_2, 22°C: high CO_2, 31°C: control CO_2, and 31°C: high CO_2), following 5 days of exposure (Fig. 1*B*). Mussels were held in the same recirculating systems, within an additional 283-ml container ($n = 10$ for each treatment group) and treated as in *Experiment 1*. Mussels were not fed the day before MO_2 measurements. Oxygen consumption data were collected for seven mussels from each treatment group using computerized intermittent-flow respirometry (79). The system consisted of four glass chambers (~20 mm length, ~12 mm width) each connected to one peristaltic pump for flushing ambient water into the chamber. Each glass chamber was also equipped with a 5-mm stir bar to facilitate recirculation during the measurement period. The total volume of the setup including the glass chamber and all associated tubing was ~1.6 ml. Oxygen consumption in each individual chamber was quantified with twelve 30-min cycles consisting of 20-min measurement, 9-min flush, and 1-min wait period before commencing the next cycle. For each mussel, MO_2 (in mg $O_2 \cdot kg^{-1} \cdot h^{-1}$) was calculated as $MO_2 = \alpha \cdot V_{resp} \cdot \beta \cdot M_b^{-1}$, where V_{resp} was the volume of each chamber minus the volume of the mussel, β was the oxygen solubility (adjusted daily for the barometric pressure and temperature), and M_b was the weight of the whole animal (shell + tissues; g). During each trial the coefficient of determination (r^2) for all slope measurements was >0.95 and MO_2 was defined as the average of the 8 lowest of the 12 measurements for each animal (76).

Calibration of the fiber optic oxygen probes with oxygen-free and fully saturated water occurred regularly throughout the experiment, background O_2 data was collected and accounted for during each trial (79), and all tubing and chambers were rinsed with dilute bleach between trials to minimize bacterial growth and reduce background MO_2. Data were recorded with AutoResp software [version 1.4, Loligo, Tjele, Denmark (79)].

Statistical Analysis

A Cox proportional hazard regression model was used to assess survival in juvenile *L. siliquoidea* over the 14-day period of exposure to elevated temperature and P_{CO_2} using the "survival" package (82) in R. This model is an appropriate method for assessing time to event data, as it allows for the inclusion of censored data (12, e.g., 54). Temperature and P_{CO_2} were included in the model as fixed effects, and mussel length was included as a covariate. The interaction between P_{CO_2} and temperature was not significant and was thus removed and analyses were rerun (21). Additionally, a logistic regression was used to assess differences in mortality at the end of the 14-day exposure period, with P_{CO_2}, temperature, and the interaction of P_{CO_2} and temperature as fixed effects and mussel length as a covariate. If either mean or interaction effects were significant, Tukey-Kramer honestly significant difference post hoc analyses were performed using the "multcomView" and "lsmeans" packages in R. To verify that mussel size was not different across experimental treatments, a two-way analysis of variance (ANOVA) was used with mussel length as the response variable and P_{CO_2}, temperature, and their interaction (P_{CO_2} × temperature) as fixed effects.

The effects of elevated P_{CO_2} and temperature exposure on the mRNA abundance of target genes, enzyme activity, and oxidative stress markers were first assessed using a two-way analysis of covariance (ANCOVA) with temperature, CO_2 level, and their interaction (P_{CO_2} × temperature) entered into the model as fixed effects, and mussel length as a covariate. If mussel length was not significant in the model, it was removed and a two-way ANOVA was run with the fixed effects indicated above. For the statistical analysis of MO_2, length was not included in the model (i.e., analyzed using a two-way ANOVA) because these data already include an aspect of mussel size (e.g., M_b, mussel weight) in their calculation. If the interaction term was significant, a Tukey-Kramer honestly significant difference post hoc test was used, using the "multcomp" package (46) in R. Similarly, if the interaction term was not significant, a Tukey post hoc test was used to assess significant main effects. Additionally, if length as a covariate was significant in the above two-way ANCOVA models, a linear regression was performed to determine how length affected the variable of interest.

Assumptions of normality and homogeneity of variance were assessed for all models. A visual analysis of fitted residuals using a normal probability plot (1) and a Shapiro-Wilk normality test were used to assess normality. The homogeneity of variances was assessed using a Levene's test as well as a visual inspection of fitted residuals. If either the assumption of normality or the homogeneity of variance was violated (i.e., if either the tests or visual inspections described above failed), data were transformed with a rank or square root transformation and run with the same parametric model provided that the assumptions were met (10, 48, 72). Statistical analyses were performed using R v3.3.2 (81) and the level of significance (α) was 0.05. Data are presented as means ± SE.

RESULTS

After 14 days of exposure, whole body *ca* mRNA levels and CA activity were both significantly affected by high P_{CO_2} exposure, but only *ca* mRNA levels were affected by elevated temperature (Table 2). In neither case was the interaction of P_{CO_2} and temperature significant for *ca* mRNA or CA activity (Table 2). Overall and independent of holding temperature, exposure to high P_{CO_2} resulted in a significant reduction in *ca* mRNA levels and an elevation in CA activity compared with mussels held at control P_{CO_2}. The differences in *ca* mRNA and CA activity between CO_2-treated and control mussels appeared most pronounced at higher temperatures (i.e., >28°C) (Fig. 2); however, this was not significant (Table 2). Whole body *ca*

Table 2. *Results of two-way ANOVA or ANCOVA for effects of elevated P_{CO_2} and temperature exposure on juvenile Lampsilis siliquoidea*

Variable	Main Effects	Df	SS	*F* Value	*P* Value
Length	P_{CO_2}	1	0.2	0.066	0.797
	Temp.	4	8.0	0.705	0.589
	P_{CO_2} × Temp.	4	7.8	0.687	0.601
ca mRNA	P_{CO_2}	1	5964	16.445	**<0.001**
	Temp.	4	4415	3.043	**0.023**
	P_{CO_2} × Temp.	4	2262	1.559	0.196
CA activity	P_{CO_2}	1	267.0	11.591	**0.001**
	Temp.	4	97.2	1.055	0.388
	P_{CO_2} × Temp.	4	70.7	0.768	0.551
nka mRNA	Length	1	1717	4.661	**0.035**
	P_{CO_2}	1	921	2.501	0.119
	Temp.	4	6241	4.235	**0.004**
	P_{CO_2} × Temp.	4	4847	3.290	**0.016**
NKA activity	Length	1	0.027	6.587	**0.013**
	P_{CO_2}	1	0.034	8.403	**0.005**
	Temp.	4	0.011	0.685	0.606
	P_{CO_2} × Temp.	4	0.007	0.440	0.779
HA activity	Length	1	1643	6.293	**0.016**
	P_{CO_2}	1	73	0.279	0.600
	Temp.	4	1679	1.607	0.187
	P_{CO_2} × Temp.	4	1804	1.727	0.159
hsp70 mRNA	P_{CO_2}	1	0.026	0.475	0.493
	Temp.	4	1.333	6.012	**<0.001**
	P_{CO_2} × Temp.	4	0.601	2.709	**0.038**
TAC	P_{CO_2}	1	99	0.069	0.794
	Temp.	4	2499	0.435	0.783
	P_{CO_2} × Temp.	4	8352	1.452	0.232
HNE	Length	1	19.33	11.262	**0.002**
	P_{CO_2}	1	0.03	0.016	0.900
	Temp.	4	18.50	2.695	**0.042**
	P_{CO_2} × Temp.	4	5.22	0.760	0.557
MO_2	P_{CO_2}	1	151.9	6.458	**0.018**
	Temp.	1	944.5	40.153	**<0.001**
	P_{CO_2} × Temp.	1	0.1	0.005	0.942

ANCOVA, analysis of covariance; Df, degrees of freedom; SS, sum of squares; *ca*, carbonic anhydrase; HA, H^+-ATPase; HNE, 4-hydroxynonenal; *hsp70*, heat shock protein 70; MO_2, oxygen consumption; *nka*, Na^+-K^+-ATPase; TAC, total antioxidant capacity; DF, degrees of freedom; Temp., temperature. Significant *P* values are represented as bolded text.

mRNA levels were also significantly elevated in mussels held at 28°C compared with 25°C, regardless of P_{CO_2} treatment (Fig. 2*A*). Although not significant (Table 2), the increase in *ca* mRNA with temperature appeared to occur only in mussels exposed to control P_{CO_2} and not high P_{CO_2} (Fig. 2*A*).

Elevated temperature and P_{CO_2} significantly interacted to affect *nka* mRNA levels in mussels following 14 days of exposure (Table 2). Only mussels held at high P_{CO_2} showed a reduction in *nka* mRNA with an increase in temperature, where mRNA levels were significantly reduced in mussels held at 28°C and 34°C compared with 22°C, and these levels fell below those of mussels held at control P_{CO_2} at the highest holding temperature (34°C) (Fig. 3*A*). Although no interactive effect of elevated temperature and P_{CO_2} on NKA activity was found (Table 2), exposure to high P_{CO_2} resulted in an overall significant increase in NKA activity compared with mussels held at control P_{CO_2} (Fig. 3*B*). Additionally, mussel length was negatively correlated with NKA activity ($F_{1,62} = 6.197$, $P = 0.015$, $R^2 = 0.076$) and *nka* mRNA ($F_{1,75} = 3.545$, $P = 0.064$, $R^2 = 0.032$) levels (Table 2). Whole body v-type HA activity was not affected by exposure to either elevated temperature or high P_{CO_2} (Table 2), but was positively correlated with mussel length ($F_{1,58} = 5.829$, $P = 0.019$, $R^2 = 0.076$; Table 2).

Exposure to elevated P_{CO_2} and temperature significantly interacted to affect *hsp70* mRNA levels, but indicators of oxidative stress were relatively unaffected by either elevated temperature or P_{CO_2} following 14 days of exposure (Table 2). Whole body *hsp70* mRNA levels increased by about twofold at 34°C compared with all other holding temperatures in mussels held at control P_{CO_2}, and this heat-induced increase in *hsp70* mRNA was not observed in mussels also exposed to high P_{CO_2} (Fig. 4*A*). Measurements of oxidative stress (i.e., TAC and HNE) were largely unaffected by exposure to either high P_{CO_2} or elevated temperature (Table 2), with the exception that HNE adducts were elevated in mussels exposed to 34°C compared with 28°C, independent of P_{CO_2} exposure (Fig. 4, *B* and *C*). Additionally, mussel length was positively correlated with HNE ($F_{1,55} = 10.35$, $P = 0.002$, $R^2 = 0.143$; Table 2).

After a 5-day exposure, both elevated P_{CO_2} and temperature (31°C) significantly, but independently, affected mussel MO_2

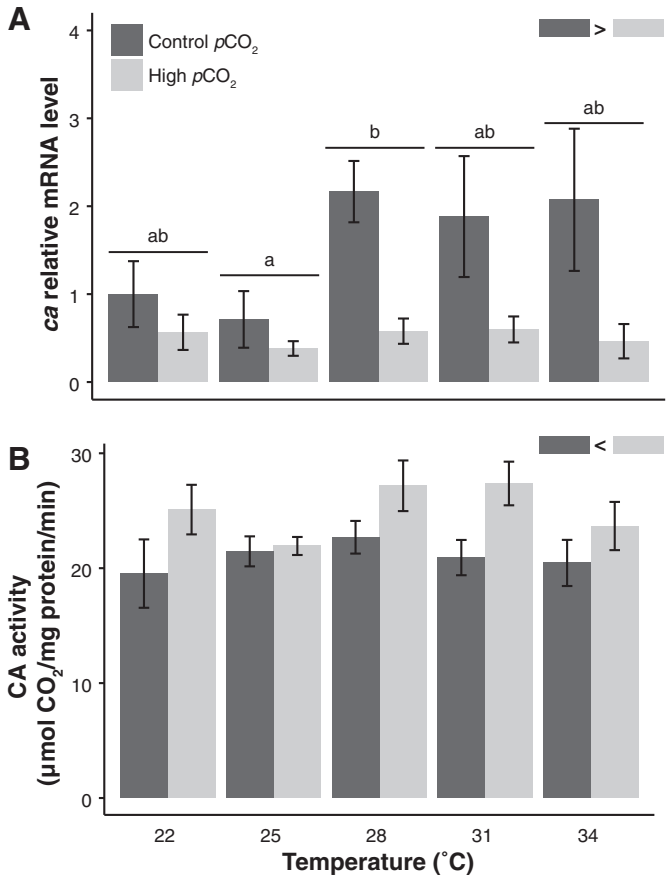

Fig. 2. *A*: carbonic anhydrase (*ca*) relative mRNA level. *B*: CA activity of juvenile *Lampsilis siliquoidea* exposed to elevated P_{CO_2} and temperature. Mussels were exposed to either control (~230 μatm) or high (~58,000 μatm) P_{CO_2}, and either 22°C, 25°C, 28°C, 31°C, or 34°C for 14 days. Values are presented as means ± SE (n = 5–8). Levels of mRNA were measured by quantitative real-time RT-PCR (qPCR), and data were expressed relative to the control P_{CO_2} group held at 22°C. The interaction of P_{CO_2} and temperature was not significant; however, both temperature and/or P_{CO_2} had significant independent effects (two-way ANOVA; see Table 2 for details). A significant effect of temperature is represented by groups that do not share a letter (i.e., letter above a line to indicate a mean effect of temperature). A significant effect of P_{CO_2} is represented by the *inset* box to indicate the mean effect of CO_2 treatment.

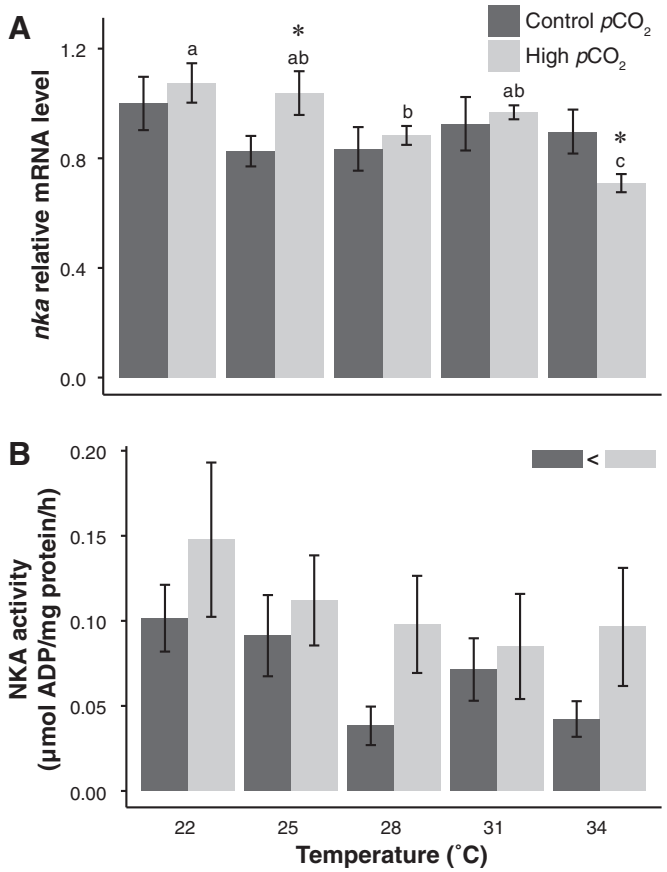

Fig. 3. *A*: Na^+-K^+-ATPase (*nka*) relative mRNA level. *B*: NKA activity of juvenile *Lampsilis siliquoidea* exposed to elevated P_{CO_2} and temperature. Mussels were exposed to either control (~230 μatm) or high (~58,000 μatm) P_{CO_2}, and either 22°C, 25°C, 28°C, 31°C, or 34°C for 14 days. Values are presented as means ± SE (n = 5–8). Levels of mRNA were measured by quantitative real-time RT-PCR (qPCR), and data were expressed relative to the control P_{CO_2} group held at 22°C. For *A*, within the high P_{CO_2} treatment group, groups that do not share a letter are significantly different from one another; *significant difference between P_{CO_2} treatment groups within a temperature treatment group [two-way analysis of covariance (ANCOVA); see Table 2 for details]. For *B*, there was only a significant independent effect of P_{CO_2} on NKA activity, which is represented by the *inset* box to indicate the mean effect of CO_2 treatment (two-way ANCOVA; see Table 2 for details).

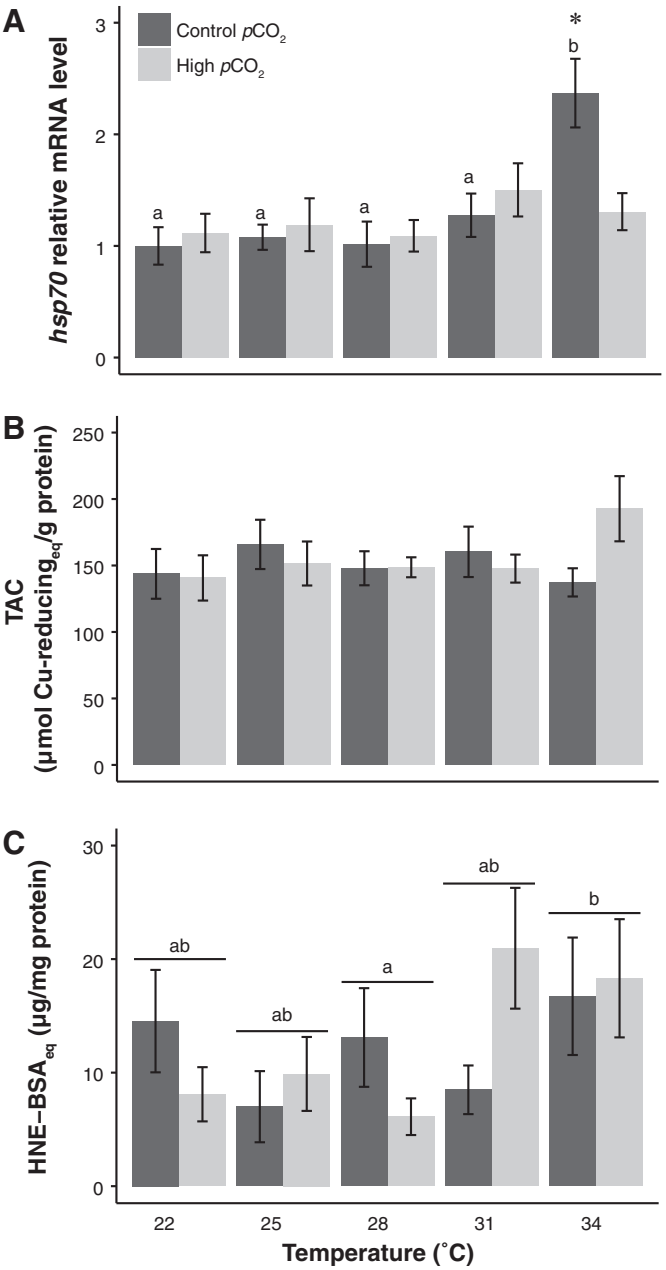

Fig. 4. Heat shock protein 70 (*hsp70*) relative mRNA level (*A*) total antioxidant capacity (TAC) (*B*), and hydroxynonenal (HNE) (*C*) of juvenile *Lampsilis siliquoidea* exposed to elevated P_{CO_2} and temperature. Mussels were exposed to either control (~230 μatm) or high (~58,000 μatm) P_{CO_2}, and either 22°C, 25°C, 28°C, 31°C, or 34°C for 14 days. Values are presented as means ± SE. Levels of mRNA (n = 6–8) were measured by quantitative real-time RT-PCR (qPCR), and data were expressed relative to the control P_{CO_2} group held at 22°C. TAC and HNE (n = 4–6) were assessed as indicators of oxidative stress. For *hps70*, within the control P_{CO_2} treatment group, groups that do not share a letter are significantly different from one another; *significant difference between P_{CO_2} treatment groups within a temperature treatment group (two-way ANOVA; see Table 2 for details). For HNE, there was only a significant independent effect of temperature, which is represented by groups that do not share a letter (i.e., letter above a line to indicate a mean effect of temperature) [two-way analysis of covariance (ANCOVA); see Table 2 for details]. No significant effect of elevated temperature or P_{CO_2} was detected for TAC (two-way ANOVA; see Table 2 for details).

(Table 2). Although the interaction between P_{CO_2} and temperature was not significant (Table 2), the increase in MO_2 that was observed in mussels held at 31°C appeared to be attenuated in mussels also exposed to high P_{CO_2} (Fig. 5).

Over the 14-day exposure period, mortality was highest in mussels exposed to both high P_{CO_2} and 34°C, where nearly 50% of mussels did not survive (Fig. 6, *A* and *B*). With respect to the Cox proportional hazards model, there was no significant interactive effect of elevated temperature and P_{CO_2} on survival; however, each factor had an individual and significant effect on survival (Table 3). Mussel length did not differ significantly across treatments (Table 2), but larger mussels within each treatment were more likely to survive longer (Table 3). More specifically, for each increase in unit length (mm), the likelihood of death decreased by a factor of 0.36 or 64% (Table 3, Fig. 6*C*). Exposure to elevated P_{CO_2} increased the hazard by a factor of 2.45, indicating that mussels were more likely to die earlier when exposed to high P_{CO_2}, independent of temperature (Table 3). Additionally, exposure to elevated temperatures

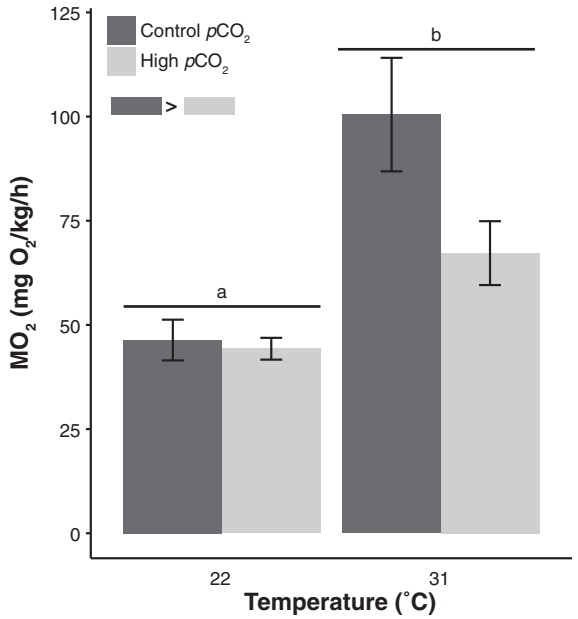

Fig. 5. Oxygen consumption (MO_2) of juvenile *Lampsilis siliquoidea* exposed to elevated P_{CO_2} and temperature. Mussels were exposed to either control (~230 μatm) or high (~58,000 μatm) P_{CO_2} and either 22°C or 31°C for 5 days. Values are presented as means ± SE ($n = 7$). The interaction of P_{CO_2} and temperature was not significant; however, both temperature and P_{CO_2} had significant independent effects (two-way ANOVA; see Table 2 for details). A significant effect of temperature is represented by groups that do not share a letter (i.e., letter above a line to indicate a mean effect of temperature). A significant effect of P_{CO_2} is represented by the *inset* box to indicate the mean effect of CO_2 treatment.

increased the probability of mussel death occurring earlier, and this was highest in the group exposed to 34°C (Table 3). Further analysis of the total mortality of mussels following the 14-day exposure period also found no significant interactive effect of P_{CO_2} and temperature; however, temperature and P_{CO_2} had significant individual mean effects (logistic regression; $P < 0.001$ for length and temperature, $P = 0.015$ for P_{CO_2}, $P = 0.690$ for P_{CO_2} × temperature). At the end of the 14-day exposure period, mortality was highest in mussels exposed to temperatures at or above 28°C and in mussels exposed to high P_{CO_2} (Fig. 6*C*).

DISCUSSION

High P_{CO_2} Exposure Effects

Regardless of temperature exposure, mechanisms of acid-base regulation became evident for juvenile *L. siliquoidea* exposed to high P_{CO_2} over a 14-day period, and a proposed model for responding to this challenge is presented in Fig. 7. Exposure to a high P_{CO_2} environment results in acidification of internal fluids, leading to an increase in acid-base regulatory processes. The hydrolysis of CO_2 to H^+ and HCO_3^- is made possible by CA, allowing for H^+ to be excreted via either v-type HA or NHE along the apical membrane of ionocytes (31). Correspondingly, CA activity was elevated in juvenile *L. siliquoidea* during exposure to high P_{CO_2} and is likely related to the need to excrete accumulated H^+. Additionally, NKA activity was elevated in CO_2-exposed mussels, suggesting that mussels maintained the gradient necessary for Na^+ uptake allowing for H^+ excretion, likely via NHE because v-type HA activity was unaffected. This mechanism of H^+ excretion via NHE facilitated by NKA was further supported by an observed increase in hemolymph Na^+ levels in adult *L. siliquoidea* during a 28-day exposure to ~55,000 μatm P_{CO_2} (36). Additionally, though not assessed in the present study, a decrease in the activity of the HCO_3^-/Cl^- anion exchanger has been proposed to play a role in the retention of HCO_3^- during periods of acidosis (7, 34–36), and thus was included in the model. Overall, the results from the present study, including increases in CA and NKA activity, as well as the lack of a change in v-type HA activity, provide further insight into the potential mechanisms underlying the acid-base regulatory responses of freshwater mussels to high P_{CO_2} exposure.

Mussels exposed to elevated P_{CO_2} also exhibited a lower survival rate over the 14-day exposure period. Regardless of exposure temperature, mussel survival decreased by a factor of 2.45 for individuals exposed to high P_{CO_2} (~58,000 μatm). Although mussels likely attempted to regulate acid-base status during P_{CO_2} exposure using the mechanisms described above (Fig. 7), freshwater mussel hemolymph has a limited buffering capacity, no respiratory pigments, and low osmotic concentration (61). Thus, freshwater mussels rely, at least in part, on the bicarbonate-carbonate buffering system to respond to acidosis, where HCO_3^- (as well as Ca^{2+}) is released from $CaCO_3$ stores of the shell (42). Because the reverse reaction is also necessary for shell mineralization (i.e., $CaCO_3$ deposition) (47, 90), acid-base regulation during P_{CO_2} exposure can come at a cost of shell growth and maintenance (51, 52). Waller et al. (88) also found decreased survival of juvenile *L. siliquoidea* exposed to elevations in P_{CO_2} over a 28-day period, which occurred in concert with decreased shell growth and increased shell damage. Because of the important protective properties of the shell, although juvenile *L. siliquoidea* may modulate acid-base regulatory processes during exposure to elevated P_{CO_2}, negative impacts on shell growth and maintenance may increase mortality, particularly for smaller mussels.

Elevated Temperature Exposure Effects

As ectotherms, performance is expected to be mediated by environmental temperature in mussels (78), and exposure to elevated temperatures up to 34°C resulted in an increase in the physiological responses of juvenile *L. siliquoidea*. Mussels exposed to a 14-day increase in temperature exhibited an elevation in *ca* mRNA by approximately twofold. Although the modulatory function of mollusc CA members remains poorly understood, the *ca* isoform investigated in this study was highly expressed in the mantle tissue of adult *L. siliquoidea* (51), and in the freshwater mussel *Hyriopsis cumingii* (73) compared with other tissues, suggesting an important role for this isoform in biomineralization. An increase in *ca* mRNA with elevated temperature may represent a greater driving force toward biomineralization and growth at higher environmental temperatures, and previous studies have found increased growth with increased environmental temperature (e.g., 8, 17). Additionally, whole body mRNA levels of *hsp70* were elevated by more than twofold in mussels exposed to 34°C compared with all other experimental temperatures, suggesting an increase in the cellular stress response (22, 77). After a 5-day exposure to 31°C, mussels exhibited an increase in MO_2 representing an increased energy demand, potentially neces-

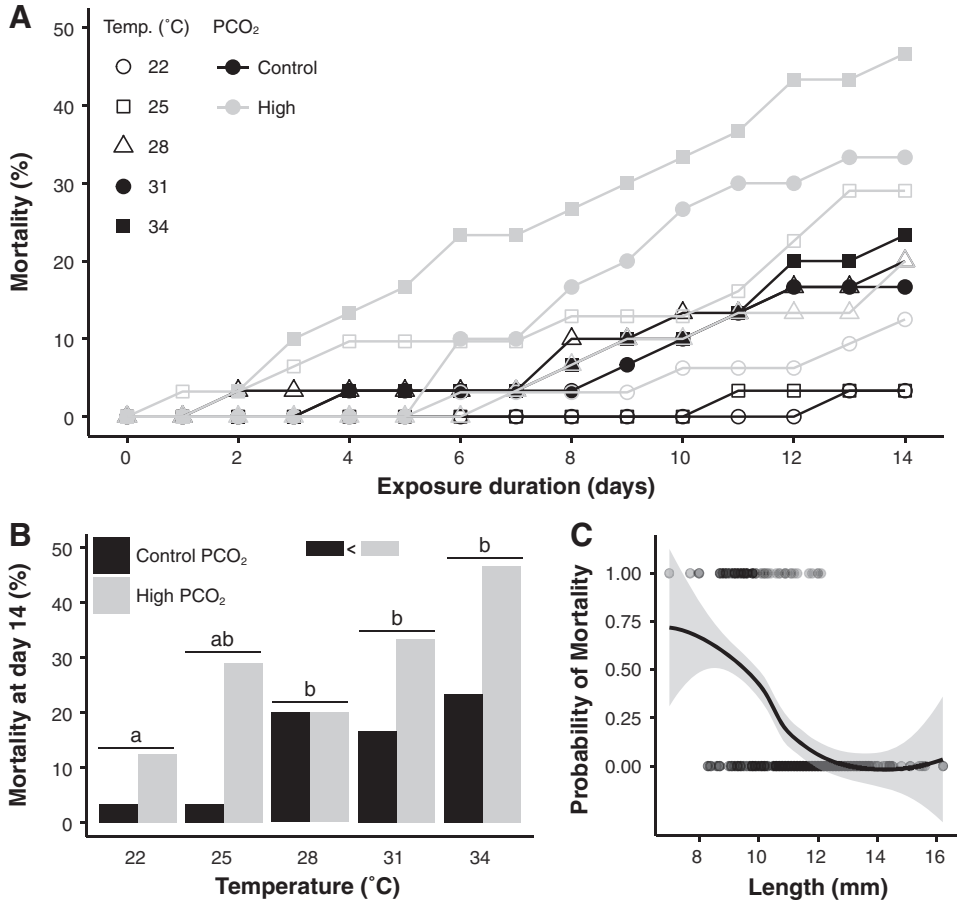

Fig. 6. *A*: cumulative mortality over the exposure period. *B*: total mortality at 14 days of exposure. *C*: effect of length on the probability of mortality of juvenile *Lampsilis siliquoidea* exposed to elevated P_{CO_2} and temperature. Mussels (n = 30–32) were exposed to either control (~230 μatm) or high (~58,000 μatm) P_{CO_2}, and either 22°C, 25°C, 28°C, 31°C, or 34°C for 14 days. For *B*, the interaction of P_{CO_2} and temperature was not significant; however, both temperature and/or P_{CO_2} had significant independent effects (logistic regression; $P < 0.001$ for length and temperature, $P = 0.015$ for P_{CO_2}, $P = 0.690$ for P_{CO_2} × temperature). A significant effect of temperature is represented by groups that do not share a letter (i.e., letter above a line to indicate a mean effect of temperature). A significant effect of P_{CO_2} is represented by the *inset* box to indicate the mean effect of CO_2 treatment. For *C*, the black regression line was fit using ggplot2 (89) in R (method = loess; local polynomial regression fitting), and the shaded area around the regression line indicates a 95% confidence band. Each slightly transparent point represents an individual mussel; thus, darker points indicate multiple individuals.

sary to support an increase in physiological and behavioral modifications induced by thermal stress. Oxygen consumptions rates were not assessed in *L. siliquoidea* following the longer 14-day exposure period in the present study; however, lampsiline mussels (e.g., *Lampsilis cardium*) are considered thermally sensitive species that are unable to sustain high metabolic rates, resulting in a decrease in clearance rates and MO_2 and a shift to anaerobic respiration (78). This potential decrease in metabolic rate following a longer term exposure to elevated temperatures could result in decreased energy allocation to growth in juvenile mussels, ultimately leading to mussel death (28). In fact, survival decreased over the 14-day period with exposure to elevated temperatures, and this was most severe for mussels exposed to the highest temperature of 34°C, the upper thermal tolerance for juvenile *L. siliquoidea* (67). Overall, the physiological responses exhibited by juvenile *L. siliquoidea* are consistent with responses to exposure to a thermal stressor; however, long-term maintenance of these responses may not be possible, resulting in increased mortality.

Interactive Effects of Elevated Temperature and P_{CO_2} Exposure

Overall, results from the present study suggest a depression of thermally induced stress responses in juvenile *L. siliquoidea* held at high P_{CO_2}. For instance, mussels only exposed to an elevation in temperature exhibited an increase in *hsp70* and *ca* mRNA as well as an increase in MO_2, and these responses were either diminished or completely suppressed when mussels were held at high P_{CO_2}. The suppression of a heat-induced increase in *hsp70* mRNA in juvenile *L. siliquoidea* exposed to high P_{CO_2} may suggest that exposure to elevated P_{CO_2} resulted in a decrease in investment in the cellular stress response, potentially making mussels more susceptible to the negative consequences of additional stressors (e.g., temperature). Additionally, during periods of exposure to high P_{CO_2}, investment in shell formation may decrease in favor of acid-base regulation, resulting in a suppression of the increase in mRNA with increasing temperature of a *ca* isoform likely to be involved in biomineralization. Furthermore, attenuation of the thermally induced increase in MO_2 in mussels also exposed to high P_{CO_2} suggests that juvenile *L. siliquoidea* may have a decreased capacity to respond to the additional thermal stressor. Finally,

Table 3. *Cox proportional hazards regression model for the risk of mortality with increased temperature, P_{CO_2}, and length in juvenile Lampsilis siliquoidea*

Factor	Hazard Ratio	95% CI	z Value	*P* Value
Length	0.364	0.292–0.453	−9.043	**<0.001**
High P_{CO_2}[a]	2.450	1.431–4.195	3.265	**0.001**
25°C[b]	4.430	1.495–13.126	2.686	**0.007**
28°C[b]	4.008	1.408–11.411	2.601	**0.009**
31°C[b]	7.589	2.702–21.311	3.847	**<0.001**
34°C[b]	13.465	4.940–36.701	5.082	**<0.001**

CI, confidence interval. [a]Expressed relative to control P_{CO_2}. [b]Expressed relative to 22°C. Significant *P* values are represented as bolded text.

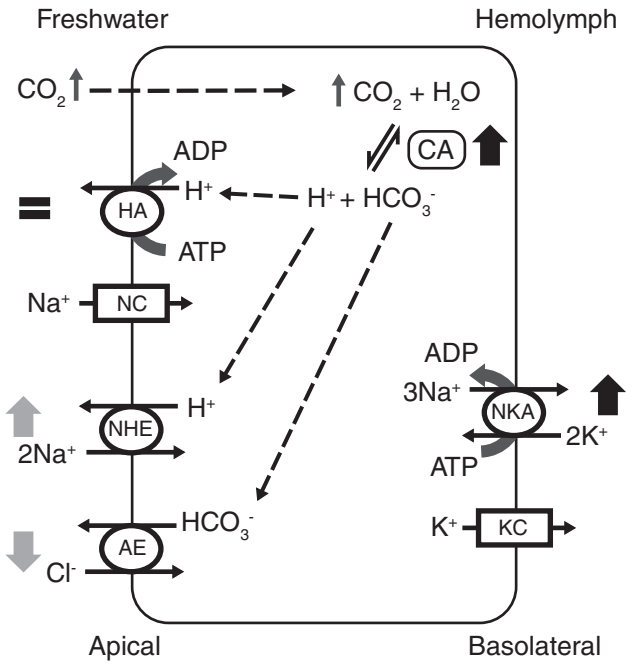

Fig. 7. Model for acid-base regulatory mechanism of *Lampsilis siliquoidea* in response to exposure to elevated P_{CO_2}. Along the apical membrane are vacuolar-type H^+-ATPase (v-type HA) paired with an Na^+ channel (NC), Na^+/H^+ exchanger (NHE), and Cl^-/HCO_3^- anion exchanger (AE). Along the basolateral membrane is Na^+-K^+-ATPase (NKA), paired with a K^+ channel (KC). Carbonic anhydrase (CA) catalyzes the hydrolysis of CO_2 to H^+ and HCO_3^-. Thin black arrows with solid and hatched lines represent directional movements of ions. Thin dark gray arrows represent an increase in CO_2. Thick black arrows and equal signs represent changes in activity of CA, NKA, and HA, and thus proposed mechanisms of acid-base regulation based on results from the present study. Thick light gray arrows represent hypothesized mechanisms of acid-base regulation based on the results of the present study and from Hannan et al. (34–36). Additional putative transporters/proteins not examined in the present study but that are thought to play an important role in acid-base regulation include NC, NHE, Cl^-/HCO_3^- AE, and KC (see text for details).

nka mRNA levels were reduced with exposure to elevated temperature, but only in mussels exposed to high P_{CO_2}. Together, the results of the present study suggest that exposure to high P_{CO_2} may compromise the capacity of juvenile *L. siliquoidea* to sufficiently respond to an additional stressor such as an increase in temperature, which may have resulted in the lowest survival being observed in mussels held at both elevated P_{CO_2} and temperature.

Differential Regulation of mRNA and Activity of CA and NKA

Generally, there was no consistent relationship between the mRNA and activity levels of CA and NKA. The differences in observed mRNA abundance and activity level of CA and NKA following 14 days of exposure to elevated temperature and P_{CO_2} may have resulted from posttranscriptional or posttranslational modifications. Alternatively, for CA, increases in additional isoforms of *ca* that were not measured in this study may have contributed to the overall observed increase in CA activity with elevated P_{CO_2} exposure (i.e., cytoplasmic *ca* isoforms that play a greater role in acid-base regulation), since total CA activity was assessed. For NKA, the abundance of *nka* mRNA may have been modulated by P_{CO_2} earlier in the exposure period, resulting in the observed elevation in NKA activity after 2 wk of exposure, which would not have been captured by the experimental design of the present study. Overall, an assessment of *ca* and *nka* mRNA abundance over the exposure period, as well as the investigation of additional *ca* isoforms, may help to elucidate the changes in activity that were observed in the present study in response to elevated P_{CO_2} exposure.

Mussel Size Matters

An important finding of the present study was the impact of mussel length on survival during exposure to elevated temperature and P_{CO_2}. Mussel length played a role in determining mussel survival over the 14-day exposure period, and no mortality occurred for mussels with a length greater than 12.1 mm. Previous studies suggest that mussel length positively correlates with overwintering success and survival in harsh conditions (6, 17, 33). Additionally, in some cases, physiological variables were related to mussel length and may point to a physiological basis for the potential differences in survival of juvenile mussels exposed to thermal and CO_2 stressors. Mussels in early life stages are likely to be more sensitive to changing environmental conditions compared with adult mussels (11), and the results of the present study suggest that, even within a life stage, larger individuals may do better in challenging conditions. Therefore, faster-growing mussels that invest more in early life growth may be more resilient to exposures to elevation in P_{CO_2} and temperature, and this could ultimately lead to population level impacts should conditions persist. The present study highlights the importance of considering multiple life stages when assessing environmental stressor effects, as well as size differences within these stages, to better understand the sensitivity of organisms to environmental stressors.

Perspectives and Significance

Mussels are important components of freshwater ecosystems, providing nutrient-rich areas, water filtration, and food sources for other animals. With the extensive declines to North American mussel populations because of habitat alterations, freshwater mussels are an imperiled taxon. The current study showed that increases in temperature and P_{CO_2}, two ecologically relevant stressors, resulted in physiological responses in mussels that may have eventually led to their increased mortality. In particular, thermal stress responses were attenuated in mussels also exposed to elevated P_{CO_2}, potentially contributing to the highest mortality in mussels held near their thermal limit, indicating the likelihood of synergistic responses to these stressors. Additionally, mussel length was positively associated with survival over the 14-day exposure period, suggesting that younger or slower-growing mussels may be more sensitive to environmental increases in both temperature and P_{CO_2}. Wild populations of mussels are likely to encounter multiple stressors concurrently, and the present study highlighted the importance of considering how multiple stressors might interact in complex ways. In particular, juvenile mussels exposed to elevations in P_{CO_2} because of natural or anthropogenic factors may be less equipped to respond to additional thermal stressors, emphasizing the importance of considering the interactive effects of elevated P_{CO_2} with other environmental stressors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.D.J., K.D.H., C.T.H., and C.D.S. conceived and designed research; J.D.J. and K.D.H. performed experiments; J.D.J. analyzed data; J.D.J., K.D.H., C.T.H., and C.D.S. interpreted results of experiments; J.D.J. prepared figures; J.D.J. drafted manuscript; J.D.J., K.D.H., C.T.H., and C.D.S. edited and revised manuscript; J.D.J., K.D.H., C.T.H., and C.D.S. approved final version of manuscript.

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