NRC Research Press

ARTICLE

Physiological effects of short- and long-term exposure to elevated carbon dioxide on a freshwater mussel, *Fusconaia flava*

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

Abstract: Zones of elevated carbon dioxide (CO_2) have the potential to deter the movement of fishes. Should CO_2 be used as a barrier, non-target organisms, such as freshwater mussels, have the potential to be impacted. In this study, the physiological responses of adult *Fusconaia flava* exposed to elevated partial pressures of CO_2 (pCO_2) over both short-term (6 h exposure with 6 h recovery) and long-term (4-, 8-, and 32-day exposure) periods were measured. A 6 h exposure to either ~15 000 μatm (1 atm = 101.325 kPa) or ~200 000 μatm caused an elevation in hemolymph Ca^{2+}. Exposure to ~200 000 μatm resulted in a decrease in hemolymph Cl^-, and exposure to ~15 000 μatm caused an increase in hemolymph Na^+, whereas ~200 000 μatm caused a decrease. Exposure to elevated pCO_2 for long-term periods caused a decrease in hemolymph Mg^{2+} and an initial increase in hemolymph Ca^{2+}. Body condition and hemolymph glucose were not significantly influenced by elevated pCO_2 for both experiments. This study shows that elevated pCO_2 had limited impacts on the physiological responses of adult freshwater mussels.

Résumé : Les zones de concentrations élevées de dioxyde de carbone (CO_2) pourraient faire entrave aux déplacements de poissons. L'utilisation éventuelle du CO_2 comme barrière pourrait avoir un impact sur des organismes non ciblés, comme les moules d'eau douce. Nous avons mesuré les réactions physiologiques de fusconaias jaunes (*Fusconaia flava*) adultes exposées à des pressions partielles de CO_2 (pCO_2) élevées pendant de courtes (exposition de 6 heures avec récupération de 6 heures) et longues durées (expositions de 4, 8 et 32 jours). Une exposition de 6 heures à des pCO_2 de ~15 000 μatm (1 atm = 101.325 kPa) ou ~200 000 μatm entraînait une hausse du Ca^{2+} hémolymphatique. Une exposition à ~200 000 μatm se traduisait par une baisse du Cl^- hémolymphatique et une exposition à ~15 000 μatm entraînait une hausse du Na^+ hémolymphatique, alors qu'une exposition à ~200 000 μatm entraînait sa baisse. L'exposition à de fortes pCO_2 pendant de longues périodes causait une baisse du Mg^{2+} hémolymphatique et une baisse initiale du Ca^{2+} hémolymphatique. Dans les deux expériences, des pCO_2 élevées n'ont pas eu d'influence significative sur l'embonpoint et le glucose hémolymphatique. L'étude démontre que de fortes pCO_2 ont des effets limités sur les réactions physiologiques des moules d'eau douce adultes. [Traduit par la Rédaction]

Introduction

The introduction of non-native species can have long-lasting impacts on resident populations and "invaded" environments (Pimentel et al. 2005; Ricciardi et al. 2013). Many invaders have been concentrated in the Great Lakes and Mississippi River basin. Since 1840, more than 182 species have been introduced into this region, which is the highest introduction rate recorded for a freshwater system (Ricciardi 2006). Recently, two species of invasive fish, silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*) (hereinafter collectively referred to as bigheaded carps), have experienced dramatic increases in population size coupled with rapid range expansion throughout the Mississippi basin and are spreading towards the Great Lakes basin, causing substantial concern. Bigheaded carps have the potential to negatively affect the structure of the aquatic food web and transform aquatic invaded habitats (Sampson et al. 2009). The leading edge of bigheaded carps is about 75 km away from the Chicago Area Waterway System, an artificial, anthropogenic connection between the Mississippi River and Laurentian Great Lakes basin (Patel et al. 2011). One pillar of the current management strategy to impede the movement of bigheaded carps from their current range into the Great Lakes is a trio of electrified barriers in the Chicago Area Waterway System. While it is currently believed that no bigheaded carps have traversed this barrier, no nonphysical barrier is 100% effective at stopping all fish movement (Noatch and Suski 2012), suggesting a need to develop additional, redundant technologies to contain bigheaded carps in the Mississippi basin.

Carbon dioxide gas (CO_2) added to water has the potential to be an effective tool to deter fish movements (Kates et al. 2012), which may be a promising option to supplement the current electric barrier system for deterring the spread of bigheaded carps. The concept behind a CO_2 barrier is that fish should avoid zones of elevated CO_2 once a target CO_2 level has been reached, thereby acting as a nonphysical barrier to influence movement (Noatch and Suski 2012). For example, Dennis et al. (2015*b*) used laboratory studies to show that several species of juvenile fishes, including bigheaded carps, would actively avoid zones of CO_2 once partial pressures had been elevated to approximately ~200 000 μatm (1 atm = 101.325 kPa). There are a number of potential methods by which CO_2 gas could be deployed in a real-world setting to act as a fish barrier, including both short-term (temporary) or long-term applications that may be installed at strategic movement locations frequented by bigheaded carps, such as in natural backwater habitats or at navigational structures (United States Army Corps of Engineers 2014). Should CO_2 barriers be deployed at a target area, it is likely that CO_2 gas would spread beyond the point of application, with CO_2 levels falling owing to dilution as distance from the application point increases. Despite the promise of CO_2

Received 19 February 2016. Accepted 21 March 2016.

K.D. Hannan, J.D. Jeffrey, C.T. Hasler, and C.D. Suski. Department of Natural Resources and Environmental Science, University of Illinois at Urbana–Champaign, 1102 South Goodwin Avenue, Urbana, IL 61801, USA.

Corresponding author: Kelly D. Hannan (email: Kelly.hannan3@gmail.com).

as a novel barrier technology, many challenges exist prior to its deployment, including assessing the potential impacts of elevated partial pressures of CO_2 (pCO_2) on nontarget organisms.

One group of nontarget organisms that may be affected by the deployment of a CO_2 barrier is freshwater mussels, which can be found in a variety of aquatic habitats and are key components of freshwater ecosystems. Freshwater mussels serve many crucial ecological functions, influence many ecosystem processes in freshwater systems, and are often used as an indicator of ecosystem health (Williams et al. 1993). The volume of water filtered by mussels can equal or exceed daily stream discharge if there is a dense bed of unionids, and they can remove harmful particles from the environment (Vaughn and Hakenkamp 2001). Mussels influence phytoplankton abundance, water clarity, and nitrogen availability and can create nutrient productivity hotspots (Howard and Cuffey 2006). Freshwater mussels also provide links to higher trophic levels as food for fish, mammals, and birds (Williams et al. 1993). North American freshwater ecosystems contain the highest diversity of freshwater mussels in the world — 297 species, which is one-third of the entire freshwater fauna (Williams et al. 1993; Ricciardi et al. 1998). Unfortunately, even though North America has the highest diversity of freshwater mussels, more than half of these mussels — 213 species (71%) — are listed as endangered, threatened, or of special concern (Williams et al. 1993), and 7% are presumed extinct or functionally extinct (i.e., cannot reproduce), indicating their fragile nature and vulnerability to environmental perturbation. Importantly, there is a paucity of studies on the effects of elevated pCO_2 on mussels in fresh water, making it difficult to predict how these organisms might respond to exposure to elevated pCO_2.

The goal of the present study was to quantify the physiological responses of freshwater mussels to elevated pCO_2 exposure. Because of the potential for a CO_2 barrier to take different forms and the exposure of mussels to occur at different distances from the application site, mussels were exposed to elevated pCO_2 levels for both a short- and long-term period. To reflect exposure to a pulse of CO_2, adult Wabash pigtoe mussels (*Fusconaia flava*) were exposed to elevated pCO_2 (~15 000 and ~200 000 μatm) for 6 h and then returned to control water for an additional 6 h to assess recovery potential. To examine the impacts of an extended exposure to elevated pCO_2 that may be experienced by mussels downstream of a barrier, mussels were also exposed to 4, 8, and 32 days of elevated pCO_2 (~20 000 μatm). It was hypothesized that mussels would experience ionic and energetic disturbances in response to elevated pCO_2 exposure consistent with regulating acid–base disturbances. Exposure to elevated pCO_2 in marine bivalves (Michaelidis et al. 2005) and exposure of freshwater bivalves to strong acidification (i.e., sulfuric acid; Pynnönen 1994) and emersion (i.e., exposure to air; Byrne and McMahon 1994; Byrne et al. 1991) elicits a number of acid–base regulatory mechanisms, such as a decrease in hemolymph pH and an increase in HCO_3^-, and exposure to elevated pCO_2 was expected to invoke a similar response for freshwater mussels. Finally, as elevated pCO_2 may act as a stressor, it was predicted that glucose levels would increase and hemolymph Mg^{2+} would decrease (Fritts et al. 2015). Thus, mussels were sampled for hemolymph ions (Ca^{2+}, Na^+, Cl^-, Mg^{2+}) and glucose concentrations after short- and long-term pCO_2 exposure and assessed for body condition index (BCI) during the long-term exposure. Responses of freshwater mussels to short- and long-term exposure to elevated pCO_2 levels may differ, and therefore attaining a broad understanding of mussel responses to different levels and durations of pCO_2 exposure will help inform management and policy makers on the potential impacts of CO_2 barriers on native freshwater mussels.

Methods

Mussel collection and husbandry

Fusconaia flava were collected by benthic grab from Big Four Ditch, Paxton, Illinois, USA, in September 2014. Following collection, mussels were transported to the Aquatic Research Facility at the University of Illinois, Champaign–Urbana, Illinois, USA, in coolers (travel time <1 h). Upon arrival, mussels were cleaned of epibionts, tagged for individual identification with Queen Marking Kit tags (The Bee Works, Orillia, Ontario, Canada) (Neves and Moyer 1988), and divided among three separate 128.7 L recirculating tank systems supplied with 64.4 L of fresh water every 7 days from a 0.04 ha naturalized, earthen-bottom pond. Prior to commencement of experimental treatments, mussels remained in this recirculation system for at least 1 week to recover from transport and handling stressors and to acclimate to laboratory conditions (Horohov et al. 1992; Dietz et al. 1994). The recirculating system consisted of the three plastic holding containers, a UV sterilizer (TMC Vecton 8 W, 11 L·min^{-1} flowrate, Pentair, Apopka, Florida, USA), a Teco 500 aquarium heater–chiller (TECO-US, Aquarium Specialty, Columbia, South Carolina, USA), 5 cm of sand, and a low-pressure air blower (Sweetwater, SL24H, Pentair). Mussels were fed a commercial shellfish diet with multiple particle sizes: *Nannochloropsis* sp. 1–2 μm and a mixed diet of *Isochrysis*, *Pavlova*, *Thalassiosira*, and *Tertraselmis* spp. 5–12 μm (Instant Algae, Reed Mariculture Inc., Campbell, California, USA) every other day (Wang et al. 2007). Mussels did not receive supplemental food 48 h prior to sampling for hemolymph. Dissolved oxygen (DO) and temperature were recorded daily across all holding tanks with a portable meter (YSI 550A, Yellow Springs Instruments, Irvine, California, USA) and averaged 8.02 ± 0.6 mg·L^{-1} and 17.5 ± 0.2 °C, respectively (mean ± standard error, SE).

Short-term pCO_2 exposure

To quantify physiological disturbances due to short-term pCO_2 exposure, mussels (N = 48) were placed individually into eight 0.71 L plastic containers held within one of two raceways, and all experiments were run over 4 consecutive days. Running the experiments on 4 days made it possible to run controls alongside elevated pCO_2 treatments. Each raceway contained pond water (described above) that was pumped from a central basin into the eight containers, allowed to overflow from the containers into the raceway, and then drain back into the central basin, forming a closed, recirculating system (Vanglandeghem et al. 2010; Dennis et al. 2015*a*). Each individual container was supplied with an air stone to maintain DO levels. Mussels were introduced to individual containers 24 h prior to experimentation, after which water quality in the central basin was recorded. Temperature and DO were measured as stated above, and pH was measured using a handheld meter (WTW pH 3310 m, Cole Palmer, Vernon Hills, Illinois, USA) calibrated regularly throughout the study. Free CO_2 and total alkalinity levels were measured using digital titration kits (Hach Company, Loveland, Colorado, USA). Temperature and pH data were entered into CO2Calc to define pCO_2 (Robbins et al. 2010).

Following the 24 h introduction period, elevations in pCO_2 were achieved by the common technique of bubbling compressed CO_2 gas (commercial grade, 99.9% purity) into the central basin through an air stone (Summerfelt and Sharrer 2004; Clingerman et al. 2007), until one of two target dissolved pCO_2 levels was reached: ~200 000 μatm (188 114 ± 12 669 μatm) or ~15 000 μatm (14 772 ± 1685 μatm). Previous work has shown that a level of approximately ~200 000 μatm is a conservative pCO_2 that caused active avoidance in several species of juvenile fishes, including bigheaded carps, making this a potential target for nonphysical fish barriers (Dennis et al. 2015*b*). The second pCO_2 level of ~15 000 μatm was used because previous work by Dennis et al. (2015*b*) found that for fish, extended holding at pressures above ~15 000 μatm began to

induce negative physiological changes, thereby suggesting that this would be a conservative pCO_2 value that would permit extended holding of mussels without resulting in mortality. In addition, the lack of knowledge of the speed of CO_2 dissipation makes using different ranges of lower pCO_2 values important, and ~15 000 μatm is a possible pCO_2 value that would be expected downstream of a CO_2 barrier for mussels not residing immediately within a CO_2 addition zone. Because the addition of CO_2 to fresh water results in the formation of carbonic acid and thus a concomitant decrease in pH, values of pCO_2 within the central basin were maintained with a pH controller (PINPOINT, American Marine Inc., Connecticut, USA) that automatically added CO_2 to the basin should the pH rise above a target level during the 6 h exposure (Reynaud et al. 2003). Mussels were removed from their containers and sampled after either a 6 h exposure to one of the two pCO_2 levels (N = 8) or after a 6 h exposure to elevated pCO_2 followed by an additional 6 h recovery period in control conditions (~300 μatm; N = 8). A third group of control animals were handled in an identical manner as the CO_2-treated mussels but did not receive supplemental CO_2 additions and were held at control pCO_2 of ~300 μatm (273 ± 30 μatm) for the duration of the experiment.

Mussel sampling included measurements for length, width, depth, mass of the whole animal, and volume. Length, width, and depth were measured using a digital caliper (Traceable digital carbon fiber calipers, Fisher Scientific, Pittsburgh, Pennsylvania, USA), and whole animal mass (tissue + shell) was collected to the nearest 0.01 g using a waterproof balance (HL-300WP, A&D, Ann Arbor, Michigan, USA). Whole animal volume was collected by immersing the mussel in a graduated cylinder with a known volume of water and quantifying displacement, and shell cavity volume was generated by immersing just the shell in a graduated cylinder with a known water volume and subtracting from the volume of the whole animal. Hemolymph was extracted from the anterior adductor muscle with a 1 mL syringe and 26 G needle (Gustafson et al. 2005; Al-Subiai et al. 2009; McCartney et al. 2009) and centrifuged at 12 000 × gravity (g) for 2 min. Following centrifugation, the supernatant was removed, flash-frozen in liquid nitrogen, and stored at –80 °C until processing.

Long-term pCO_2 exposure

To define the impacts of prolonged exposure to elevated pCO_2, mussels (N = 48) were first separated into one of two 128.7 L recirculating tank systems (identical to the ones described above). Throughout the long-term experiment, mussels were fed every other day, as stated above, which did not affect the pH of the recirculating tank systems. Mussels were then held at either control (~1000 μatm; 994 ± 62 μatm) or elevated (~20 000 μatm; 22 712 ± 2482 μatm) pCO_2 for up to 32 days (note that the difference in pCO_2 for the two control conditions in the short- and long-term experiments was due to natural fluctuations in the pCO_2 levels of the pond water; Hasler et al. 2016). The ~20 000 μatm pCO_2 level was targeted because it was unknown if mussels would survive extended holding at higher levels, as previous work by Dennis et al. (2015*b*) found that for fish, extended holding at pressures above ~15 000 μatm began to induce negative physiological disturbances related to pCO_2 exposure. Target pCO_2 levels were achieved by bubbling CO_2 gas into the water to a pH that corresponded to the target pCO_2 level, as described in the short-term experiment. Water quality was monitored similarly to the experiment described above. Mussels (N = 8) were sampled as described above on days 4, 8, or 32 of exposure to ~20 000 μatm pCO_2 or control conditions.

Laboratory analyses

Hemolymph Cl^-, Mg^{2+}, and Ca^{2+} concentrations were assayed in duplicate using commercially available kits (QuantiChrom assay kits: Cl^-, catalogue No. DICL-250; Mg^{2+}, catalogue No. DIMG-250; Ca^{2+}, catalogue No. DICA-500; BioAssay Systems, Hayward, California, USA). Hemolymph Na^+ levels were measured by the diagnostic clinical pathology lab at the University of Illinois, Urbana–Champaign, using a Beckman chemistry analyzer (Beckman Coulter AU680, Beckman Coulter, Brea, California, USA); quality control testing for this analyzer was performed at least every 24 h. Hemolymph glucose concentrations were assayed in duplicate according to the method of Bergmeyer (1974) using a 96-well microplate and a plate spectrophotometer (Molecular Devices, SpectraMax Plus 384, Sunnyvale, California, USA), and all inter- and intra-assay coefficients of variation were less than 10%.

Body condition index (BCI)

BCI, a traditional metric used to quantify bivalve condition (Widdows and Johnson 1988; Lundebye et al. 1997), was calculated as follows:

$$\text{BCI} = \frac{1000 \times \text{soft tissue dry mass}}{\text{shell cavity volume}}$$

Soft tissue dry mass (mg) was determined by removing mussel soft tissues and drying them at 99 °C for 24 h prior to weighing (Widdows et al. 2002).

Statistical analyses

The effect of pCO_2 exposure on hemolymph ion and glucose levels, as well as dry mass, wet mass, length, and BCI was assessed using two-way analysis of variance (ANOVA), with pCO_2 (elevated or control), sampling time, and their interaction (pCO_2 × sampling time) entered into each model as fixed effects. If at least one of the main effects in the ANOVA model was significant, or if the interaction term was significant, a Tukey–Kramer honestly significant difference (HSD) post hoc test was applied to separate means (Rohlf and Sokal 1995). When the interaction term was significant, the resulting multiple comparisons were examined, regardless of whether the main effects on their own were significant. In the absence of a significant interaction term, group differences were examined for significant individual main effects (i.e., pCO_2 or sampling time).

For all statistical analyses, a visual analysis of fitted residuals using a normal probability plot (Anscombe and Tukey 1963) was used to assess normality, while a Hartley's F_{max} test (Hartley 1950), combined with visual inspection of the distribution of fitted residuals, was used to assess homogeneity of variances. If either normality or homogeneity of variance assumptions were violated (Siegel and Castellan 1988), data were rank-transformed and then reanalyzed within the same parametric model described above, and the assumptions of both normality and equal variances were confirmed (Conover and Iman 1981; Iman et al. 1984; Potvin and Roff 1993). All data have been presented as means ± SE where appropriate, all tests were performed using JMP Pro 11 (SAS Institute Inc., Cary, North Carolina), and differences were considered significant at $\alpha < 0.05$.

Results

Short-term pCO_2 exposure

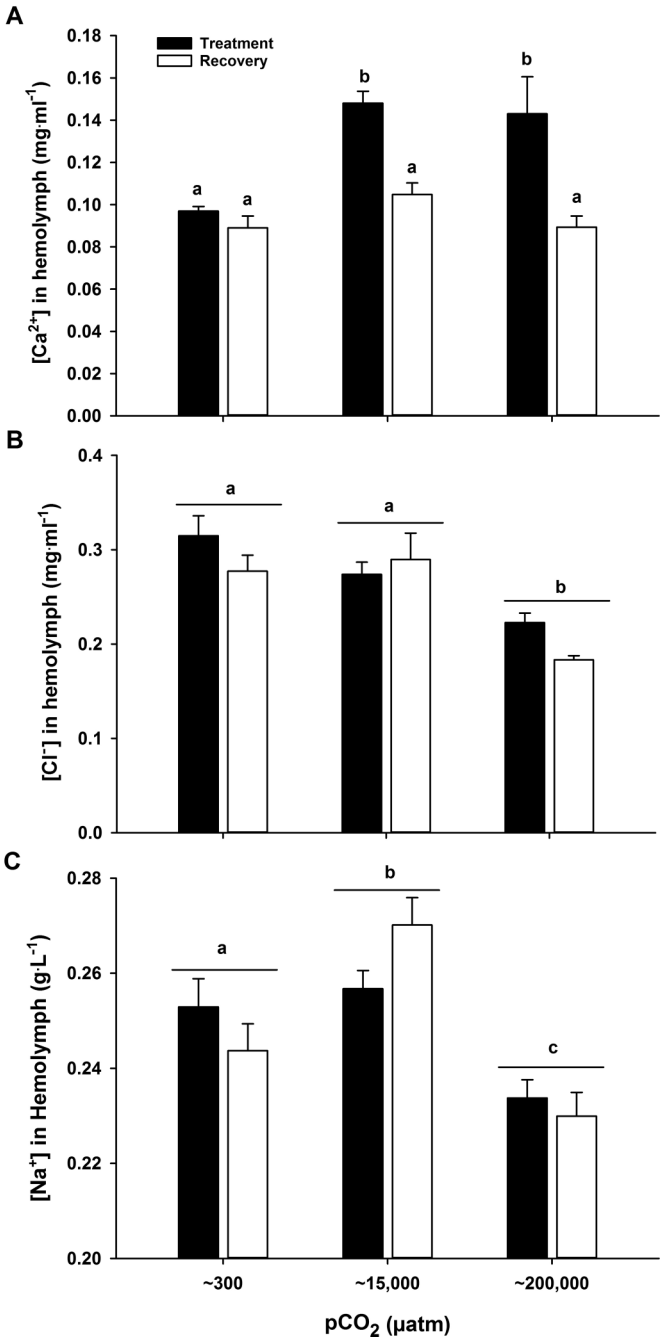
Following a 6 h exposure, mussels held at both elevated pCO_2 levels (~15 000 and ~200 000 μatm) exhibited nearly a 1.5-fold increase in hemolymph Ca^{2+} when compared with mussels exposed to ~300 μatm, and these elevated levels returned to baseline levels following exposure to control conditions for 6 h (Table 1; Fig. 1A). In contrast, the concentration of Cl^- in mussel hemolymph was only affected by exposure to a pCO_2 level of ~200 000 μatm and decreased by approximately 33% from control levels (Table 1; Fig. 1B). Owing to the lack of a significant interaction (Table 1), we cannot separate the effect of pCO_2 treatment and recovery on hemolymph Cl^-; however, hemolymph Cl^- levels did not appear to return to baseline levels following a 6 h recovery

Table 1. Results of two-way analyses of variance (ANOVA) examining the impact of a short-term exposure to elevated CO_2.

Measured variable	Main effect	SS	df	*F*	*p*
Dry mass (g)	Entire model	0.24	5	0.95	0.218
	pCO_2	0.08	2	0.18	0.837
	Time	0.01	1	0.05	0.831
	pCO_2 × time	0.15	2	0.34	0.711
	Error	9.44	42		
Wet mass (g)	Entire model	4.85	5	0.01	1.000
	pCO_2	1.07	2	0.01	0.995
	Time	1.14	1	0.01	0.921
	pCO_2 × time	2.64	2	0.01	0.988
	Error	4769.23	42		
Length (cm)	Entire model	18.60	5	0.08	0.955
	pCO_2	0.19	2	17.78	0.828
	Time	0.30	1	0.30	0.937
	pCO_2 × time	0.52	2	0.52	0.995
	Error	1993.06	42		
Ca^{2+} (mg·mL^{-1})	**Entire model**	**0.02**	**5**	**9.92**	**<0.001**
	pCO_2	**0.01**	**2**	**8.15**	**0.001**
	Time	**0.02**	**1**	**25.37**	**<0.001**
	pCO_2 × time	**0.01**	**2**	**3.98**	**0.026**
	Error	0.02	42		
Cl^- (mg·mL^{-1})	**Entire model**	**0.07**	**5**	**7.75**	**<0.001**
	pCO_2	**0.06**	**2**	**16.70**	**<0.001**
	Time	<0.01	1	0.17	0.678
	pCO_2 × time	0.01	2	2.60	0.087
	Error	0.08	42		
Na^+ (g·L^{-1})	**Entire model**	**0.006**	**5**	**8.410**	**<0.001**
	pCO_2	**0.006**	**2**	**19.370**	**<0.001**
	Time	<0.001	1	0.001	0.976
	pCO_2 × time	<0.001	2	2.460	0.104
	Error	0.004	33		
Mg^{2+} (mg·mL^{-1})	Entire model	<0.001	5	1.47	0.219
	pCO_2	<0.001	2	2.03	0.144
	Time	<0.001	1	1.86	0.180
	pCO_2 × time	<0.001	2	0.72	0.491
	Error	<0.001	42		
Glucose (μmol·L^{-1})	Entire model	719.25	5	0.71	0.618
	pCO_2	370.50	2	0.63	0.433
	Time	126.75	1	0.92	0.408
	pCO_2 × time	222.00	2	0.55	0.582
	Error	9212.00	42		

Note: *Fusconaia flava* were exposed to one of three different pCO_2 treatments (~300 μatm (control), ~15 000 μatm, or ~200 000 μatm; 1 atm = 101.325 kPa) for 6 h and following a 6 h recovery period at control pCO_2 (~300 μatm). Bold rows indicate statistical significance across treatment groups within a measured variable.

Fig. 1. Concentrations of Ca^{2+} (A), Cl^- (B), and Na^+ (C) in the hemolymph of *Fusconaia flava* exposed to three treatments of pCO_2 (~300 (control), ~15 000, and ~200 000 μatm; 1 atm = 101.325 kPa) for 6 h (solid bars) followed by a subsequent 6 h recovery in water at control pCO_2 (~300 μatm; open bars). Data are presented as means + SE (N = 8). Groups that do not share a letter are significantly different from one another (two-way ANOVA; see Table 1). Lines over multiple bars represent an overall significant effect of pCO_2 treatment but not sampling time.



period at control conditions (Table 1; Fig. 1B). Similarly, there was no significant interaction of pCO_2 treatment and sampling time on hemolymph Na^+, but there was a significant impact of pCO_2 treatment (Table 1). Overall, hemolymph Na^+ decreased relative to control levels following an exposure to ~200 000 μatm pCO_2, but hemolymph Na^+ increased relative to controls following exposure to a pCO_2 level of ~15 000 μatm (Table 1; Fig. 1C). No changes in hemolymph Mg^{2+} or glucose concentrations were observed when mussels were exposed to either pCO_2 treatment during the short-term experiment (Tables 1 and 2). Mussel size did not vary significantly across treatments or pCO_2 levels (Table 1).

Long-term pCO_2 exposure

Mussels held at ~20 000 μatm for 4 days exhibited no change in hemolymph Mg^{2+} concentration relative to animals held at control pCO_2 levels, but after 8 days of treatment, mussels exhibited a 40% decline in hemolymph Mg^{2+} relative to control mussels and a subsequent 70% decline from control levels after 32 days of treatment (Table 3; Fig. 2A). In contrast, hemolymph Ca^{2+} increased by approximately 1.5-fold after 4 and 8 days of elevated pCO_2 exposure relative to control animals, but Ca^{2+} concentrations returned to control levels by 32 days of treatment (Table 3; Fig. 2B). At 8 days of CO_2 treatment, hemolymph Na^+ was elevated by approximately 13% in mussels exposed to pCO_2 of ~20 000 μatm compared with mussels held at control conditions for the same length of time, and then these levels were no longer different from mussels held at control conditions at 32 days of treatment (Table 3; Fig. 2C).

Table 2. Concentrations of hemolymph Mg^{2+} and glucose.

Measured variable	Treatment	~300 μatm	~15 000 μatm	~200 000 μatm
Mg^{2+} (mg·mL^{-1})	CO_2 exposure	0.02±0.002	0.02±0.002	0.02±0.001
	Recovery	0.02±0.001	0.02±0.002	0.02±0.001
Glucose (μmol·L^{-1})	CO_2 exposure	106.0±17.0	104.3±22.3	97.9±7.7
	Recovery	161.7±60.2	80.0±10.0	84.0±14.1

Note: *Fusconaia flava* were exposed to one of three different elevated pCO_2 treatments (~300 μatm (control), ~15 000 μatm, or ~200 000 μatm; 1 atm = 101.325 kPa) and for a 6 h recovery period at control pCO_2 (~300 μatm). Data are presented as means ± SE (N = 8). No statistical differences were detected across treatments within a measured variable (two-way ANOVA; Table 1).

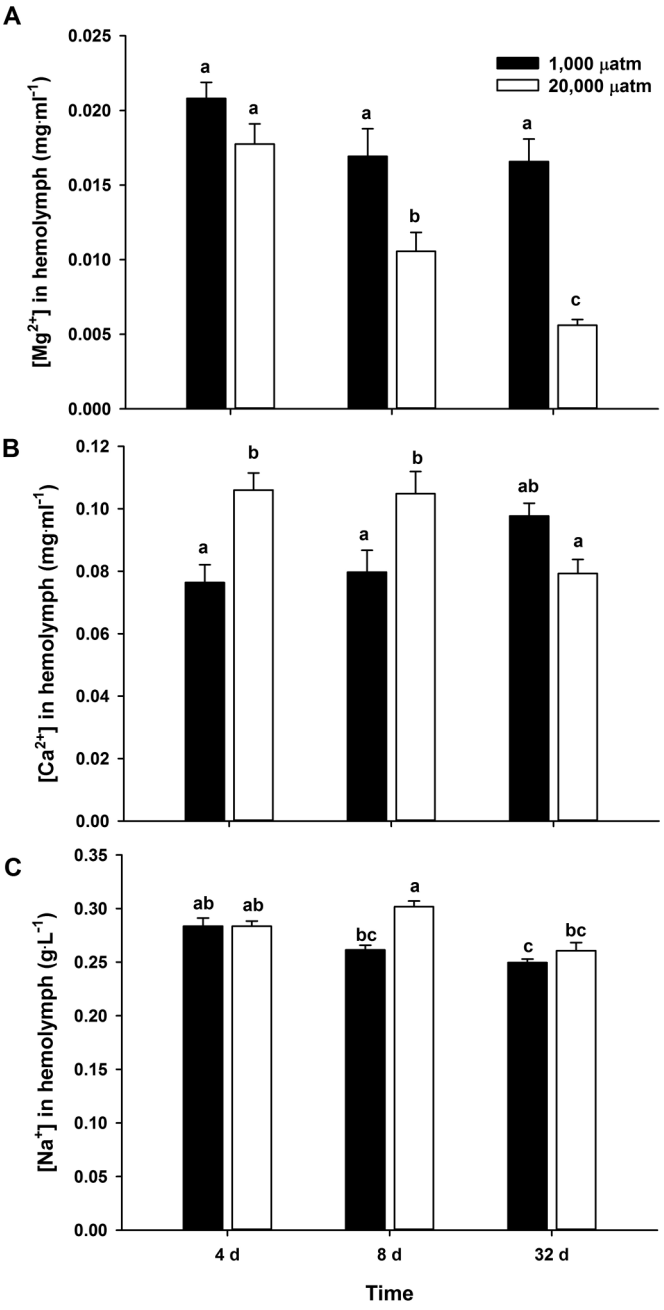
Table 3. Results of two-way analyses of variance (ANOVA) quantifying the impact of long-term exposure to elevated pCO_2.

Measured variable	Main effect	SS	df	F	p
Dry mass (g)	Entire model	0.28	5	0.30	0.911
	pCO_2	0.01	1	0.07	0.797
	Time	0.18	2	0.46	0.633
	pCO_2 × time	0.10	2	0.25	0.78
	Error	7.99	42		
Wet mass (g)	Entire model	4.27	5	0.006	1.000
	pCO_2	0.08	1	0.001	0.981
	Time	3.31	2	0.011	0.989
	pCO_2 × time	0.87	2	0.003	0.997
	Error	6110.67	42		
Length (cm)	Entire model	15.56	5	0.06	0.998
	pCO_2	2.76	1	0.05	0.820
	Time	0.60	2	0.01	0.994
	pCO_2 × time	12.20	2	0.12	0.891
	Error	2206.87	42		
Ca^{2+} (mg·mL^{-1})	**Entire model**	**0.008**	**5**	**5.66**	**<0.001**
	pCO_2	**0.002**	**1**	**6.62**	**0.014**
	Time	<0.001	2	0.23	0.798
	pCO_2 × time	**0.005**	**2**	**10.61**	**<0.001**
	Error	0.020	42		
Cl^- (mg·mL^{-1})	**Entire model**	**0.02**	**5**	**6.10**	**<0.001**
	pCO_2	<0.001	1	1.17	0.285
	Time	**0.01**	**2**	**9.52**	**<0.001**
	pCO_2 × time	**0.01**	**2**	**5.14**	**<0.001**
	Error	0.04	42		
Na^+ (g·L^{-1})	**Entire model**	**0.013**	**5**	**13.38**	**<0.001**
	pCO_2	**0.002**	**1**	**14.28**	**<0.001**
	Time	**0.007**	**2**	**16.18**	**<0.001**
	pCO_2 × time	**0.003**	**2**	**7.53**	**0.002**
	Error	0.020	40		
Mg^{2+} (mg·mL^{-1})	**Entire model**	**0.001**	**5**	**22.99**	**<0.001**
	pCO_2	**<0.001**	**1**	**48.65**	**<0.001**
	Time	**<0.001**	**2**	**26.99**	**<0.001**
	pCO_2 × time	**<0.001**	**2**	**6.17**	**0.005**
	Error	<0.001	42		
Glucose (μmol·L^{-1})	**Entire model**	**2773.00**	**5**	**3.62**	**0.008**
	pCO_2	363.00	1	2.37	0.131
	Time	**1848.88**	**2**	**6.03**	**0.005**
	pCO_2 × time	561.13	2	1.83	0.173
	Error	9212.00	42		
Body condition index (g·mL^{-1})	Entire model	1407.17	5	0.61	0.692
	pCO_2	571.60	1	1.24	0.272
	Time	76.24	2	0.08	0.921
	pCO_2 × time	751.60	2	0.82	0.446
	Error	20755.76	42		

Note: *Fusconaia flava* were exposed to either control pCO_2 (~1000 μatm; 1 atm = 101.325 kPa) or ~20 000 μatm for 4, 8, or 32 days. Bold rows indicate statistical significance across treatment groups within a measured variable.

However, Na^+ decreased in control mussels over time (Fig. 2C). Hemolymph Cl^- and glucose moderately decreased over time in mussels held at both control and elevated pCO_2 conditions; however, these levels did not differ significantly between treatment groups, although there was a significant effect of time (Tables 3 and 4). Body condition index did not vary significantly across treatments (Tables 3 and 4), and mussel size did not vary across time or pCO_2 treatment (Table 3).

Fig. 2. Concentration of Mg^{2+} (A), Ca^{2+} (B), and Na^+ (C) in the hemolymph in *Fusconaia flava* held at either control (~1000 μatm (1 atm = 101.325 kPa); solid bars) or ~20 000 μatm pCO_2 (open bars) for 4, 8, or 32 days. Data are presented as means + SE (N = 8). Groups that do not share a letter are significantly different from one another (two-way ANOVA; see Table 3).



Discussion

Acute exposure to elevated pCO_2 caused a number of physiological disturbances in the freshwater unionid mussel *F. flava*. As the level of environmental pCO_2 increases, CO_2 enters the animal via diffusion until a new steady-state gradient is reached and is sufficient to allow CO_2 excretion (Seibel and Walsh 2003). A rise in pCO_2 of the hemolymph results in a concomitant increase in H^+

Table 4. Levels of hemolymph Cl^-, glucose, and body condition index (BCI) of *Fusconaia flava* exposed to either control (~1000 μatm; 1 atm = 101.325 kPa) or ~20 000 μatm pCO_2 for 4, 8, or 32 days.

Measured variable	CO_2	Day 4	Day 8	Day 32
Cl^- (mg·mL^{-1})	~1 000 μatm	0.25±0.01a	0.23±0.01abc	0.20±0.01c
	~20 000 μatm	0.23±0.01ab	0.21±0.01bc	0.22±0.01abc
Glucose (μmol·L^{-1})	~1 000 μatm	92.38±10.48ab	194.88±35.05a	95.87±29.55b
	~20 000 μatm	219.41±48.38ab	179.20±37.42a	86.94±15.13b
BCI (g·mL^{-1})	~1 000 μatm	99.73±4.70	102.15±5.51	90.03±8.45
	~20 000 μatm	102.89±5.18	101.74±5.29	107.99±12.92

Note: Data are presented as means ± SE (N = 8). For Cl^-, there was a significant interaction term, and groups that do not share a letter are significantly different from one another; for glucose, there was only a significant effect of sampling time, which are represented by "days" that do not share a letter (two-way ANOVA; see Table 3).

ions and thus a reduction in pH (acidosis) of the internal body fluids (Wicks and Roberts 2012). The buffering of fluids is primarily achieved by increasing HCO_3^- concentrations through the dissolution of calcium carbonate ($CaCO_3$) exoskeletons in calcified animals (Lindinger et al. 1984), as well as mechanisms that actively transport acid–base relevant ions across cell and epithelial membranes to remove H^+ and accumulate HCO_3^- (reviewed by Gazeau et al. 2013). As a result of acidosis, marine calcified organisms have displayed a suite of physiological disturbances similar to those reported in the current study, including an increase in hemolymph Ca^{2+} and Na^+, as well as a decrease in hemolymph Cl^- (Michaelidis et al. 2005; Dissanayake et al. 2010). Although not directly measured, the results from the present study suggest that *F. flava* likely experienced a reduction in internal pH due to an influx of environmental CO_2 via diffusion, similar to what has been observed with marine mussels (Gazeau et al. 2013; Heuer and Grosell 2014). Mussels appeared to elicit a range of processes to buffer acidosis resulting from elevated pCO_2, including excreting H^+ and increasing hemolymph HCO_3^- (Bibby et al. 2008). The production of HCO_3^- from the dissolution of $CaCO_3$ would result in equimolar production of Ca^{2+} (Heming et al. 1988; Michaelidis et al. 2005; Bibby et al. 2008) and is likely the source of the increased hemolymph Ca^{2+} observed in the present study. An additional strategy to buffer acidosis is the down-regulation of Cl^-/HCO_3^- exchangers to retain HCO_3^-, resulting in a concomitant decrease in Cl^- uptake (Byrne and Dietz 1997), which is likely the mechanism for the net decrease in Cl^- observed in mussels exposed to pCO_2 of ~200 000 μatm (Byrne and Dietz 1997). Finally, an increase in the activity of Na^+/H^+ exchangers to excrete H^+ is a common strategy used by aquatic animals experiencing acidosis, which can result in an increase in hemolymph Na^+ (Byrne and Dietz 1997; Lannig et al. 2010), and provides a likely mechanism for the observed increase in hemolymph Na^+ in the ~15 000 μatm pCO_2 treatment. In response to ~200 000 μatm pCO_2, there may be a shift from less to more ATP-efficient ion transporters, resulting in a reduction of the electroneutral exchange of Na^+ transporters, which involves an active ion pump, resulting in a decrease in Na^+ in the hemolymph (Bibby et al. 2008; Dissanayake et al. 2010; Lannig et al. 2010). In freshwater bivalves, there have been conflicting reports of Na^+ and Cl^- responses to acidosis experienced due to emersion. In some cases, no change in hemolymph Na^+ and Cl^- levels were detected, suggesting the importance of maintaining these ions within strict limits to maintain stability (Byrne and McMahon 1994). However, in the present study, the observed responses more similarly mirrored those of marine bivalves, suggesting that emersion and exposure to water with elevated pCO_2 result in different physiological responses. Interestingly, following a return to control pCO_2 for 6 h after an exposure to both ~15 000 and ~200 000 μatm pCO_2, hemolymph Ca^{2+} returned to control levels, suggesting that once the CO_2 stimulus is removed, mussels may be able to recover from the stressor. In summary, results from the short-term experiment indicate that freshwater mussels exposed to elevated pCO_2 experienced a suite of physiological disturbances intended to regulate acid–base balance, and there was some evidence for recovery of hemolymph Ca^{2+} levels once pCO_2 was returned to control conditions.

Mussels exposed to elevated pCO_2 for an extended period of time demonstrated changes to hemolymph ion levels relative to control, but some disturbances had returned to control levels after 32 days of exposure. More specifically, long-term exposure to increased pCO_2 at ~20 000 μatm caused a decrease in Mg^{2+} compared with control animals, along with higher Na^+ and Ca^{2+} compared with controls, with Na^+ and Ca^{2+} disturbances returning to control levels by day 32. Similar to the results from the present study, a decrease in hemolymph Mg^{2+} has previously been observed in response to other stressors in mussels, such as elevated temperature (Fritts et al. 2015) and exposure to heavy metals (Hemelraad et al. 1990), and may represent an indicator of stress. Interestingly, there is a limited amount of information on the role of Mg^{2+} in freshwater mussels despite its use as a bioindicator (Dietz et al. 1994), but Mg^{2+} is thought to play a role in stabilizing cell membranes (Shumway 1977). Increases in hemolymph Ca^{2+} and elevated Na^+ compared with control mussels in response to elevated pCO_2 were similar to the responses in the short-term study and likely were caused by the similar mechanisms related to acid–base regulation to increase HCO_3^- and excrete H^+, respectively. However, it is important to note that both hemolymph Na^+ and Cl^- decreased in mussels held at control conditions over the holding period, making it difficult to parse out the effects of acid–base regulation and holding. Thus, the Na^+ concentration of mussel hemolymph did not increase throughout the treatment period in response to elevated pCO_2; rather, mussels held in control conditions had decreased Na^+ throughout time and compared with mussels held at ~20 000 μatm on day 8. Even with the impact of holding on control mussels, at 8 days of exposure, mussels held at ~20 000 μatm pCO_2 exhibited hemolymph Na^+ levels that were significantly higher than mussels held at control conditions for the same length of time, suggesting that the effect of pCO_2 exposure may have superseded the impact of holding at this time point. When exposed to pCO_2 levels of ~20 000 μatm over an extended period, hemolymph Ca^{2+} levels returned to control levels by 32 days, suggesting that mussels may have been able to correct acid–base disturbances during extended exposures. Together the results of the long-term study suggest that although mussels showed signs of stress, as demonstrated by the drop in Mg^{2+}, they were still attempting to achieve acid–base balance using similar mechanisms as during short-term exposure to elevated pCO_2.

Interestingly, two important parameters measured in the current study did not change following the different pCO_2 treatments. More specifically, despite the magnitude of pCO_2 exposure and period of exposure, glucose concentrations in hemolymph remained unchanged from control levels for both the short- and long-term experiments. Additionally, BCI was unaffected by long-

term exposure to elevated pCO_2. In both freshwater and marine ecosystems, mussels have been used as an indicator of pollution, heavy metal contamination, and overall ecosystem health and recovery (Simmons and Reed 1973; Viarengo and Canesi 1991; Hellou and Law 2003). Mussels are filter feeders and presumably one of the first species to show negative effects of a changing or unhealthy ecosystem (Viarengo and Canesi 1991; Van Hassel and Farris 2006), and BCI, as well as other parameters that assess growth and shell condition, are used as indicators of overall health of the ecosystem (Roper and Hickey 1994; Damiens et al. 2007). The BCI is a measure of soft tissue mass compared with the inner shell volume, and a low BCI score has been linked to physiological stress and thus used as a proxy for ecosystem health (Bayne et al. 1979; Widdows and Johnson 1988; Damiens et al. 2007). Similarly, glucose is the primary energy store in bivalves and fuels aerobic processes (de Zwaan and Wijsman 1976). Importantly, glucose used as part of the stress response comes at a cost to nonvital functions such as growth, reproduction, and movement, and changes in hemolymph glucose are used as a traditional stress indicator for aquatic invertebrates (Patterson et al. 1999; Fritts et al. 2015). While a lack of a change in body condition in the current study could be due to the relatively short duration of the study compared with other more long-term studies (i.e., a noticeable change in BCI may take longer than 32 days to manifest; Van Hassel and Farris 2006), the lack of a change in the glucose response relative to control animals would suggest that mussels are not showing activation of the stress response in a traditional way following pCO_2 exposure. Taken together, a lack of change in hemolymph glucose and BCI may indicate that within the timeframe and pCO_2 exposure of this study, overall mussel stress and body condition may not have been impacted.

In the present study, although differences between the control conditions and the intermediate pCO_2 were present, some overarching similarities between experiments emerged. The different control pCO_2 values of the short-term (~300 μatm) and long-term (~1000 μatm) experiments were artifacts of using ponds as the water source that have natural fluctuations in pCO_2 (Maberly 1996). As freshwater systems can be quite variable in their pCO_2, these control levels are still within the range of what a mussel may naturally experience because of daily or seasonal variation (Maberly 1996). The differences in the targeted "intermediate" pCO_2 (~15 000 and ~20 000 μatm) between experiments also make comparisons between the short- and long-term studies difficult; however, in the context of a CO_2 barrier, they both provide an example of what mussels may experience downstream of a barrier injection site. Even with these differences in pCO_2 between experiments, similar physiological responses (e.g., hemolymph Ca^{2+}) were observed. Although these differences in control and treatment pCO_2 made comparisons between studies difficult, the present study provides useful information about the effects of different durations and levels of CO_2 exposure on a freshwater mussel that may reflect their exposure to a CO_2 barrier.

Results from this study represent some of the first data on the physiological impacts of elevated pCO_2 on freshwater mussels. Other studies have shown that pCO_2 in freshwater environments demonstrates daily and seasonal variation (Maberly 1996) and also changes across landscapes, with low-order streams experiencing a wide variation in pCO_2 values dependent on forest cover, precipitation, and surface area (in particular freshwater environments, mean pCO_2 can be nearly 20 times atmospheric levels; Butman and Raymond 2011; Kokic et al. 2015). Similarly, global mean pCO_2 for lakes is ~1000 μatm (Cole et al. 1994), with variation in pCO_2 driven by various factors, including surface area, biotic processes, terrestrial primary productivity, and atmospheric pCO_2 (reviewed by Hasler et al. 2016). In addition, while future levels of pCO_2 in fresh water are difficult to predict, some freshwater systems are predicted to have heightened pCO_2 due to climate change (Phillips et al. 2015; Pilcher et al. 2015; Hasler et al. 2016). Should freshwater mussels be exposed to elevated pCO_2, either from natural processes or through the deployment of a CO_2 barrier, they would be expected to respond to acidosis by adjusting hemolymph ion levels to excrete H^+ and retain HCO_3^- in the short term. Should CO_2 stressors be extended or removed, mussels would likely recover from or correct disturbances induced by elevated pCO_2 with little evidence for negative impacts to overall stress and health (e.g., BCI and glucose). Future research should focus on studying other age classes of freshwater mussels, other species, as well as the measurement of other parameters such as growth and reproductive success, to provide a more complete picture of the overall impacts of elevated pCO_2 on nontarget freshwater mussels. The present study provides evidence that a CO_2 barrier used to prevent the movement and spread of invasive fish species may have limited physiological impacts on nontarget freshwater mussel species.

Acknowledgements

This work was supported by the Illinois Department of Natural Resources and the United States Geological Survey through funds provided by the United States Environmental Protection Agency's Great Lakes Restoration Initiative. Jeremy Tiemann, Kevin Cummings, John Tix, Cody Sullivan, Eric Schneider, and Josh Sherwood provided valuable help collecting mussels. We thank Adam Wright for providing valuable help with mussel husbandry and laboratory assistance.

References

Al-Subiai, S.N., Jha, A.N., and Moody, A.J. 2009. Contamination of bivalve haemolymph samples by adductor muscle components: implications for biomarker studies. Ecotoxicology, **18**(3): 334–342. doi:10.1007/s10646-008-0287-9. PMID:19083092.

Anscombe, F.J., and Tukey, J.W. 1963. The examination and analysis of residuals. Technometrics, **5**(2): 141–160. doi:10.1080/00401706.1963.10490071.

Bayne, B.L., Moore, M.N., Widdows, J., Livingstone, D.R., Salkeld, P., Crisp, D.J., Morris, R.J., Gray, J.S., Holden, A.V., Newell, R.C., and McIntyre, A.D. 1979. Measurement of the responses of individuals to environmental stress and pollution: Studies with bivalve molluscs. Philos. Trans. R. Soc. B Biol. Sci. **286**: 563–581. doi:10.1098/rstb.1979.0046.

Bergmeyer, H. 1974. Methods of enzymatic analysis. 2nd ed. Verlag Chemie International, Deerfield Beach, Fla.

Bibby, R., Widdicombe, S., Parry, H., Spicer, J., and Pipe, R. 2008. Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. Aquat. Biol. **2**(1): 67–74. doi:10.3354/ab00037.

Butman, D., and Raymond, P.A. 2011. Significant efflux of carbon dioxide from streams and rivers in the United States. Nat. Geosci. **4**(12): 839–842. doi:10.1038/ngeo1294.

Byrne, R., and Dietz, T. 1997. Ion transport and acid-base balance in freshwater bivalves. J. Exp. Biol. **200**: 457–465. doi:10.2174/138920209787847032. PMID:9318114.

Byrne, R.A., and McMahon, R.F. 1994. Behavioral and physiological responses to emersion in freshwater bivalves. Am. Zool. **34**: 194–204. doi:10.1093/icb/34.2.194.

Byrne, R.A., Shipman, B.N., Smatresk, N.J., Dietz, T.H., and McMahon, R.F. 1991. Acid–base balance during emergence in the freshwater bivalve *Corbicula fluminea*. Physiol. Zool. **64**: 748–766. doi:10.1086/physzool.64.3.30158205.

Clingerman, J., Bebak, J., Mazik, P.M., and Summerfelt, S.T. 2007. Use of avoidance response by rainbow trout to carbon dioxide for fish self-transfer between tanks. Aquacult. Eng. **37**(3): 234–251. doi:10.1016/j.aquaeng.2007.07.001.

Cole, J.J., Caraco, N.F., Kling, G.W., and Kratz, T.K. 1994. Carbon dioxide supersaturation in the surface waters of lakes. Science, **265**: 1568–1570. doi:10.1126/science.265.5178.1568. PMID:17801536.

Conover, W.J., and Iman, R.L. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. American Statistics, **35**(3): 131–132. doi:10.2307/2683977.

Damiens, G., Gnassia-Barelli, M., Loquès, F., Roméo, M., and Salbert, V. 2007. Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere, **66**(3): 574–583. doi:10.1016/j.chemosphere.2006.05.032. PMID:16828146.

de Zwaan, A., and Wijsman, T.C.M. 1976. Anaerobic metabolism in bivalvia (Mollusca) characteristics of anaerobic metabolism. Comp. Biochem. Physiol. Part B Comp. Biochem. **54**(3): 313–323. doi:10.1016/0305-0491(76)90247-9.

Dennis, C.E., III, Kates, D.F., Noatch, M.R., and Suski, C.D. 2015*a*. Molecular responses of fishes to elevated carbon dioxide. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. **187**: 224–231. doi:10.1016/j.cbpa.2014.05.013.

Dennis, C.E., III, Adhikari, S., and Suski, C.D. 2015*b*. Molecular and behavioral responses of early-life stage fishes to elevated carbon dioxide. Biol. Invasions. **17**(11): 3133–3151. doi:10.1007/s10530-015-0941-0.

Dietz, T.H., Lessard, D., Silverman, H., and Lynn, J.W. 1994. Osmoregulation in *Dreissena polymorpha*: the importance of Na, Cl, K, and particularly Mg. Biol. Bull. **187**(1): 76–83. doi:10.2307/1542167.

Dissanayake, A., Clough, R., Spicer, J.I., and Jones, M.B. 2010. Effects of hypercapnia on acid–base balance and osmo-/iono-regulation in prawns (Decapoda: Palaemonidae). Aquat. Biol. **11**(1): 27–36. doi:10.3354/ab00285.

Fritts, A.K., Peterson, J.T., Hazelton, P.D., and Bringolf, R.B. 2015. Evaluation of methods for assessing physiological biomarkers of stress in freshwater mussels. Can. J. Fish. Aquat. Sci. **72**(10): 1450–1459. doi:10.1139/cjfas-2014-0564.

Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J., O'Connor, W.A., Martin, S., Pörtner, H., and Ross, P.M. 2013. Impacts of ocean acidification on marine shelled molluscs. Mar. Biol. **160**(8): 2207–2245. doi:10.1007/s00227-013-2219-3.

Gustafson, L.L., Stoskopf, M.K., Bogan, A.E., Showers, W., Kwak, T.J., Hanlon, S., and Levine, J.F. 2005. Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). Dis. Aquat. Org. **65**(2): 159–165. doi:10.3354/dao065159. PMID:16060269.

Hartley, H.O. 1950. The maximum *F*-ratio as a short-cut test for heterogeneity of variance. Biometrika, **37**(3–4): 308–312. doi:10.1093/biomet/37.3-4.308. PMID: 14801057.

Hasler, C.T., Butman, D., Jeffrey, J.D., and Suski, C.D. 2016. Freshwater biota and rising pCO_2? Ecol. Lett. **19**(1): 98–108. doi:10.1111/ele.12549. PMID:26610406.

Hellou, J., and Law, R.J. 2003. Stress on stress response of wild mussels, *Mytilus edulis* and *Mytilus trossulus*, as an indicator of ecosystem health. Environ. Pollut. **126**(3): 407–416. doi:10.1016/S0269-7491(03)00231-8. PMID:12963304.

Hemelraad, J., Holwerda, D.A., Wijnne, H.J., and Zandee, D.I. 1990. Effects of cadmium in freshwater clams. interaction with essential elements in *Anodonta cygnea*. Arch. Environ. Contam. Toxicol. **19**(5): 686–690. doi:10.1007/BF01183985. PMID:21318499.

Heming, T.A., Vinogradov, G.A., Klerman, A.K., and Komov, V.T. 1988. Acid–base regulation in the freshwater pearl mussels *Margaritifera magaritifera*: effects of emersion and low water pH. J. Exp. Biol. **137**: 501–511.

Heuer, R.M., and Grosell, M. 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am. J. Physiol. **307**(9): R1061–R1084. doi:10.1152/ajpregu.00064.2014.

Horohov, J., Silverman, H., Lynn, J.W., and Dietz, T.H. 1992. Ion transport in the freshwater zebra mussel, *Dreissena polymorpha*. Biol. Bull. **183**(2): 297–303. doi:10.2307/1542216.

Howard, J.K., and Cuffey, K.M. 2006. The functional role of native freshwater mussels in the fluvial benthic environment. Freshw. Biol. **51**: 460–471. doi:10.1111/j.1365-2427.2005.01507.x.

Iman, R.L., Hora, S.C., and Conover, W.J. 1984. Comparison of asymptotically distribution-free procedures for the analysis of complete blocks. J. Am. Stat. Assoc. **79**(387): 674–685. doi:10.1080/01621459.1984.10478096.

Kates, D., Dennis, C.E., III, Noatch, M.R., and Suski, C.D. 2012. Responses of native and invasive fishes to carbon dioxide: Potential for a nonphysical barrier to fish dispersal. Can. J. Fish. Aquat. Sci. **69**(11): 1748–1759. doi:10.1139/f2012-102.

Kokic, J., Wallin, M.B., Chmiel, H.E., Denfeld, B.A., and Sobek, S. 2015. Carbon dioxide evasion from headwater systems strongly contributes to the total export of carbon from a small boreal lake catchment. J. Geophys. Res. Biogeosci. **120**(1): 13–28. doi:10.1002/2014JG002706.

Lannig, G., Eilers, S., Pörtner, H.O., Sokolova, I.M., and Bock, C. 2010. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas* — changes in metabolic pathways and thermal response. Mar. Drugs, **8**(8): 2318–2339. doi:10.3390/md8082318. PMID:20948910.

Lindinger, M.I., Lauren, D.J., and McDonald, D.G. 1984. Acid–base balance in the sea mussel, *Mytilus edulis*. III: Effects of environmental hypercapnia on intra- and extracellular acid–base balance. Mar. Biol. Lett. **5**: 371–381.

Lundebye, A., Langston, W.J., and Depledge, M.H. 1997. Stress proteins and condition index as biomarkers of tributyltin exposure and effect in mussels. Ecotoxicology, **6**(3): 127–136. doi:10.1023/A:1018662324296.

Maberly, S.C. 1996. Diel, episodic and seasonal changes in pH and concentrations of inorganic carbon in a productive lake. Freshw. Biol. **35**: 579–598. doi:10.1111/j.1365-2427.1996.tb01770.x.

McCartney, M.A., Sommer, K., and Wilbur, A.E. 2009. Field evaluation of mortality from hemolymph extraction as a source of DNA, and application to PCR-RFLP identification of threatened freshwater mussel species. J. Shellfish Res. **28**(2): 345–354. doi:10.2983/035.028.0217.

Michaelidis, B., Ouzounis, C., Paleras, A., and Pörtner, H.O. 2005. Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. **293**(2): 109–118. doi:10.3354/meps293109.

Neves, R.J., and Moyer, S.N. 1988. Evaluation of techniques for age determination of freshwater mussels (Unionidae). Am. Malacol. Bull. **6**(2): 179–188.

Noatch, M.R., and Suski, C.D. 2012. Non-physical barriers to deter fish movements. Environ. Rev. **20**(1): 71–82. doi:10.1139/a2012-001.

Patel, A.G., Glassner-Shwayder, K., and Eder, T. 2011. Halting the invasion: maintaining the health of the Great Lakes and Mississippi River basins by preventing further exchange of aquatic invasive species. Environ. Pract. **12**(4): 342–356. doi:10.1017/S1466046610000499.

Patterson, M.A., Parker, B.C., and Neves, R.J. 1999. Glycogen concentration in the mantle tissue of freshwater mussels (Bivalvia: Unionidae) during starvation and controlled feeding. Am. Malacol. Bull. **15**: 47–50.

Phillips, J.C., McKinley, G.A., Bennington, V., Bootsma, H.A., Pilcher, D.J., Sterner, R.W., and Urban, N.R. 2015. The potential for CO_2-induced acidification in freshwater: a Great Lakes case study. Oceanography, **28**(2): 136–145. doi:10.5670/oceanog.2015.37.

Pilcher, D.J., McKinley, G.A., Bootsma, H.A., and Bennington, V. 2015. Physical and biogeochemical mechanisms of internal carbon cycling in Lake Michigan. J. Geophys. Res. Oceans, **120**(3): 2112–2128. doi:10.1002/2014JC010594.

Pimentel, D., Zuniga, R., and Morrison, D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol. Econ. **52**(3): 273–288. doi:10.1016/j.ecolecon.2004.10.002.

Potvin, C., and Roff, D.A. 1993. Distribution-free and robust statistical methods: Viable alternatives to parametric statistics. Ecology, **74**: 1617–1628. doi:10.2307/1939920.

Pynnönen, K. 1994. Hemolymph gases, acid-base status, and electrolyte concentration in the freshwater clams *Anodonta anatina* and *Unio tumidus* during exposure to and recovery from acidic conditions. Physiol. Zool. **67**: 1544–1559. doi:10.1086/physzool.67.6.30163911.

Reynaud, S., Leclercq, N., Romaine-Lioud, S., Ferrier-Pagés, C., Jaubert, J., and Gattuso, J. 2003. Interacting effects of CO_2 partial pressure and temperature on photosynthesis and calcification in a scleractinian coral. Glob. Change Biol. **9**: 1660–1668. doi:10.1046/j.1365-2486.2003.00678.x.

Ricciardi, A. 2006. Patterns of invasion in the Laurentian Great Lakes in relation to changes in vector activity. Divers. Distrib. **12**: 425–433. doi:10.1111/j.1366-9516.2006.00262.x.

Ricciardi, A., Neves, R.J., and Rasmussen, J.B. 1998. Impending extinctions of North American freshwater mussels (Unionida) following zebra mussel (*Dressena polymorpha*) invasion. J. Anim. Ecol. **67**(4): 613–619. doi:10.1046/j.1365-2656.1998.00220.x.

Ricciardi, A., Hoopes, M.F., Marchetti, M.P., and Lockwood, J.L. 2013. Progress toward understanding the ecological impacts of nonnative species. Ecol. Monogr. **83**(3): 263–282. doi:10.1890/13-0183.1.

Robbins, L.L., Hansen, M.E., Kleypas, J.A., and Meylan, S.C. 2010. CO2calc: a user-friendly seawater carbon calculator for Windows, Mac OS X, and iOS(iPhone). Florida Shelf Ecosystems Response to Climate Change Project Open-File Report 1280. US Geological Survey, Reston, Va.

Rohlf, F.J., and Sokal, R.R. 1995. Statistical tables. W.H. Freeman and Company, New York.

Roper, D.S., and Hickey, C.W. 1994. Population structure, shell morphology, age and condition of the freshwater mussel *Hyridella menziesi* (Unionacea: Hyriidae) from seven lake and river sites in the Waikato River system. Hydrobiologia, **284**: 205–217. doi:10.1007/BF00006690.

Sampson, S.J., Chick, J.H., and Pegg, M.A. 2009. Diet overlap among two Asian carp and three native fishes in backwater lakes on the Illinois and Mississippi Rivers. Biol. Invasions, **11**(3): 483–496. doi:10.1007/s10530-008-9265-7.

Seibel, B.A., and Walsh, P.J. 2003. Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. J. Exp. Biol. **206**: 641–650. doi:10.1242/jeb.00141. PMID:12517981.

Shumway, S.E. 1977. Effect of salinity fluctuation on the osmotic pressure and Na^+, Ca^{2+} and Mg^{2+} ion concentrations in the hemolymph of bivalve molluscs. Mar. Biol. **41**(2): 153–177. doi:10.1007/BF00394023.

Siegel, S., and Castellan, J.N. 1988. Nonparametric statistics for the behavioral sciences. McGraw-Hill Book Company, New York.

Simmons, G.M., Jr., and Reed, J.R., Jr. 1973. Mussels as indicators of biological recovery zone. J. Water Pollut. Control Fed. **45**: 2480–2492. PMID:4769854.

Summerfelt, S.T., and Sharrer, M.J. 2004. Design implication of carbon dioxide production within biofilters contained in recirculating salmonid culture systems. Aquacult. Eng. **32**(1): 171–182. doi:10.1016/j.aquaeng.2004.03.006.

United States Army Corps of Engineers. 2014. The GLMRIS Report: Great Lakes and Mississippi River Interbasin Study. USACE, Washington, D.C.

Van Hassel, J.H., and Farris, J.L. 2006. A review of the use of unionid mussels as biological indicators of ecosystem health. *In* Freshwater bivalve ecotoxicology. CRC Press, Webster, N.Y. pp. 19–49.

Vanglandeghem, M.M., Wahl, D.H., and Suski, C.D. 2010. Physiological responses of largemouth bass to acute temperature and oxygen stressors. Fish. Manage. Ecol. **17**(5): 414–425. doi:10.1111/j.1365-2400.2010.00740.x.

Vaughn, C.C., and Hakenkamp, C.C. 2001. The functional role of burrowing bivalves in freshwater ecosystems. Freshw. Biol. **46**(11): 1431–1446. doi:10.1046/j.1365-2427.2001.00771.x.

Viarengo, A., and Canesi, L. 1991. Mussels as biological indicators of pollution. Aquaculture, **94**(2–3): 225–243. doi:10.1016/0044-8486(91)90120-V.

Wang, N., Ingersoll, C.G., Greer, I.E., Hardesty, D.K., Ivey, C.D., Kunz, J.L., Brumbaugh, W.G., Dwyer, F.J., Roberts, A.D., Augspurger, T., Kane, C.M., Neves, R.J., and Barnhart, M.C. 2007. Chronic toxicity of copper and ammonia

to juvenile freshwater mussels (Unionidae). Environ. Toxicol. Chem. **26**(10): 2048–2056. doi:10.1897/06-524R.1. PMID:17867874.

Wicks, L.C., and Roberts, M.J. 2012. Benthic invertebrates in a high-CO_2 world. *In* Oceanography and marine biology: an annual review. *Edited by* R.N. Gibson, R.J.A. Atkinson, J.D.M. Gordon, and R.N. Hughes. CRC Press, Boca Raton, Fla. pp. 127–188.

Widdows, J., and Johnson, D. 1988. Physiological energetics of *Mytilus edulis*: scope for growth. Mar. Ecol. Prog. Ser. **46**: 113–121. doi:10.3354/meps046113.

Widdows, J., Donkin, P., Staff, F.J., Matthiessen, P., Law, R.J., Allen, Y.T., Thain, J.E., Allchin, C.R., and Jones, B.R. 2002. Measurement of stress effects (scope for growth) and contaminant levels in mussels (*Mytilus edulis*) collected from the Irish Sea. Mar. Environ. Res. **53**(4): 327–356. doi:10.1016/S0141-1136(01)00120-9. PMID:11991207.

Williams, J.D., Warren, M.L., Jr., Cummings, K.S., Harris, J.L., and Neves, R.J. 1993. Conservation status of freshwater mussels of the United States and Canada. Fisheries, **18**: 6–22. doi:10.1577/1548-8446(1993)018<0006:CSOFMO>2.0.CO;2.