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Acute physiological responses of the freshwater snail *Elimia flava* (Mollusca: Pleuroceridae) to environmental pH and calcium

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ABSTRACT

The individual and interactive effects of environmental pH (7 [control], 6, 5, and 4) and calcium (0, 5, and 50 mg/L) were studied on hemolymph ions (pH, Ca^{2+}, total CO_2, Na^+, K^+) and osmolality in the freshwater snail, *Elimia flava*, over a 72-h exposure. All hemolymph factors strongly differed with environmental pH. Snails exposed to pH 4 were inactive and experienced more dramatic ionic disturbances than snails at pH 5, 6, and 7, including reduced hemolymph pH, depressed Na^+ concentrations, and increased Ca^{2+} and total CO_2 concentrations. There was an initial but transient increase in hemolymph K^+ over the 72 h exposure period. Environmental calcium ameliorated effects of acidification on hemolymph pH and Na^+, reducing the degree of depression in both variables irrespective of environmental pH or exposure time. In a separate experiment, effects of acidification on snail respiration were examined in which VO_2 was measured over 24 h in snails exposed to pH 7 and 4. Exposure to pH 4 caused a 64% reduction in oxygen uptake at 2 h and a maximum reduction (81%) at 11 h. Our results suggest that snails exposed to pH 4 cease interacting with the surrounding medium and use internal stores of $CaCO_3$ to buffer hemolymph acidification, whereas snails at pH 5 and higher appear to use environmental calcium as a buffer source. These results suggest an important role of environmental calcium in ameliorating the impacts of short-term, sublethal acidification.

1. Introduction

Natural and anthropogenic acidification of surface waters remains one of the most prominent ecological and environmental problems affecting the Northern Hemisphere during the 20th century (Galloway, 2001). Primary sources of surface water acidification include atmospheric deposition, acidic mine drainage (AMD), and the natural production of organic acids (Baker et al., 1991). Non-point source atmospheric deposition is caused by the industrial emissions of sulfur oxides and nitrogen oxides (Mason, 1989). In contrast, point-source AMD originates from the oxidation of sulfide minerals associated with coal mining activity (Herricks and Cairns, 1973). Exposure of aquatic organisms to low pH associated with AMD may be acute (e.g., occurring only during active mining or storm run-off), or chronic, with surface water chemistry often being altered long after mining has ceased. In the southeastern United States, low surface water pH (<3.0) can occur in streams that drain coal basins not actively mined for decades (Hyde, 1970; Dyer and Curtis, 1983; Black, 2005).

Within Alabama, acidification of surface waters by AMD has been problematic in streams within the Cahaba and Warrior Coal Fields (ADEM, 2008). Abandoned mine lands in this area account for ~211 ha and over 587 km of AMD-impacted streams, some with pH values as low as 3.7 (Black, 2005). Some stream remediation efforts have been successful, but much work is still needed to reclaim these acid-stressed environments.

Acidification of surface waters has profound consequences for freshwater communities (Weatherley and Ormerod, 1987, 1991; Guérold et al., 2000; Driscoll et al., 2003). Townsend et al. (1983) found a strong correlation between pH and species richness and densities of stream invertebrates and fishes. In the southeastern and northeastern United States, the diversity and abundance of benthic invertebrate species have been reduced by up to 50% in lake and stream habitats impacted by acid deposition (Townsend et al., 1983; Rosemond et al., 1992). Many invertebrate groups such as aquatic insects, crustaceans, and mollusks typically decrease or become extirpated in streams where mean pH decreases below 5.7 (Sutcliffe and Hildrew, 1989; Lewis et al., 2007).

Numerous studies have documented the physiological responses of fishes and crayfishes to acidic water, with the most commonly cited sources of mortality involving disruptions in blood–oxygen transport (Packer, 1979), blood acid–base status (McDonald et al., 1980; McMahon and Morgan, 1983), and ionic regulation (Vangenechten et al., 1989; Wood, 1989; Masson et al., 2002). The latter appears to be the most prevalent mechanism of toxicity. The crayfish *Procambarus clarkii* showed impaired hemolymph Na^+ and Cl^- regulation when

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exposed to acidic water of pH 5.2 (Zanotto and Wheatly, 1993). In addition, short-term exposure to naturally acidified water of pH 4.73 caused decreased survival and hemolymph Na^+ and Cl^- in *Gammarus fossarum* (Amphipoda), *Hydropsyche pellucidula* (Trichoptera), and *Dinocras cephalotes* (Plecoptera) (Felten and Guerold, 2006). In a related study, Havens (1992) reported significant decreases in body Na^+ content and survival of the cladoceran *Daphnia galeata mendotae* exposed to pH 4.5 for 24 h. Consequently, osmoregulatory capacity may be an effective way to monitor the health status of crustaceans (Lignot et al., 2000). Respiratory failure caused by acidic exposure is another source of physiological stress, observed in fishes (Laitinen and Valtonen, 1995), crayfishes (Patterson and DeFur, 1988) and amphipods (Felten and Guerold, 2001).

Comparatively few studies, however, have investigated physiological responses of aquatic molluscs exposed to acidic water. Mortality may be caused from electrolyte imbalance (Pynnonen, 1990) or shell dissolution (Mackie, 1987), the latter of which may increase vulnerability to pathogens (Kat, 1982) or predators (Vermeij and Covich, 1978; Stein et al., 1984). Longer-term exposure to acidic water can reduce snail populations because of decreased individual growth rates (Mackie, 1987), delayed development (Servos et al., 1985), or reproductive failure (Rooke and Mackie, 1984).

Molluscs survive acute acidification by mobilizing internal stores of $CaCO_3$ to buffer the increase in hemolymph H^+ ions caused by acid stress (Machado et al., 1988; Pynnonen, 1991). Freshwater bivalves exposed to acidic water for 24 h showed an initial decrease in hemolymph pH followed by an increase in hemolymph Ca^{2+} concentration, presumably originating from the large reserve of $CaCO_3$ in the shell and mantle (Pynnonen, 1991). $CaCO_3$ is mobilized from the shell to the hemolymph (Akberali et al., 1977, but see Machado et al., 1988), yielding increased hemolymph HCO_3^- and Ca^{2+} ions.

External sources of calcium in freshwater systems also may be used to buffer the effects of acidic conditions. Snails appear tolerant of low pH so long as sufficient environmental calcium is available. In an extensive survey of 1000 Norwegian lakes, Økland (1983) found that total hardness (largely Ca^{2+}) and pH were the main factors determining snail distributions. Brown (1991) stated ~50% of all freshwater snail species require at least 25 mg/L environmental Ca^{2+}, and 95% require at least 3 mg/L. These data suggest that environmental Ca^{2+} may be a critical determinant of snail distributions, even if the exact mechanism explaining the interaction between Ca^{2+} and snail abundance is unknown (Lodge et al., 1987). Shells of freshwater snails typically comprise 95 to 99.9% $CaCO_3$ (White et al., 2007). A steady source of Ca^{2+} is needed not only for shell formation (Wilbur, 1972), but also for reproduction (Pynnonen, 1991), growth (Greenaway, 1971; Thomas et al., 1974), and acid–base regulation (de With et al., 1987). If environmental Ca^{2+} concentrations fall below minimum requirements, or if some factor suppresses Ca^{2+} uptake from the surrounding medium, some or all of the above processes could be inhibited.

Environmental Ca^{2+} supplements appear to reduce impacts of acidic water on both fish and crayfish. Hollett et al. (1986) reported significant losses of body Na^+ and increased mortality of juvenile crayfish, *Orconectes rusticus*, in treatments of low environmental pH and Ca^{2+}. Environmental Ca^{2+} supplements reduced toxicity of brown trout fingerlings exposed to low pH (Brown, 1981). Under decreased ambient Ca^{2+}, low pH caused increased fish mortality (McDonald et al., 1980; McDonald, 1983; McCormick and Jensen, 1992), decreased growth rates (Rogers, 1984), and disrupted plasma ion levels (McWilliams and Potts, 1978; McDonald et al., 1980; McDonald, 1983). In this context, Rogers (1984) hypothesized that, for fish, acid-induced interference with Ca^{2+} regulation was the most significant effect of environmental acidification; and, even if sufficient environmental Ca^{2+} is available, acidification can suppress Ca^{2+} uptake in crayfish (Malley, 1980), fish (Reader and Morris, 1988), and bivalves (Pynnonen, 1991). Glover and Wood (2005) suggested that, in fish, the environmental Ca^{2+} in sufficient concentrations in acid waters may protect against Na^+ depletion, a common source of mortality in acid-stressed freshwater organisms (Vangenechten et al., 1989; Wood, 1989; Masson et al., 2002; Felten and Guerold, 2006; Felton et al., 2008).

Freshwater molluscs appear particularly sensitive to environmental acidification (Raddum et al., 1988). However, few studies have examined physiological responses of molluscs exposed to acidic water, so the ultimate causes of mortality and the exact role of environmental Ca^{2+} in reducing acid toxicity are poorly known. In freshwater habitats, snails appear more tolerant of low pH so long as sufficient Ca^{2+} is available. For example, Økland (1983) reported that at a given pH high snail densities and species richness both were associated with high environmental Ca^{2+}. Such circumstantial evidence suggests that direct uptake and use of environmental Ca^{2+} is important in persistence of snails in acid-stressed habitats.

Our study reports on the results of experiments designed to determine the separate and interactive effects of acidic water and environmental Ca^{2+} on hemolymph ionic regulation and respiration of the freshwater snail, *Elimia flava*. Snails acclimated to control pH of 7.0 were exposed to 3 levels of acidification (pH 4, 5, and 6) and 3 levels of environmental Ca^{2+} (0, 5, and 50 mg/L Ca^{2+}) over a 72-h period. We sampled snail hemolymph over the study and measured changes in hemolymph pH, Ca^{2+}, total CO_2, Na^+, K^+ and osmolality. In a related experiment, we quantified snail respiration at pH 7 and 4 to assess if differences in snail respiratory/ventilatory activity (i.e., contact with environmental Ca^{2+}) accounted for differences in hemolymph physiology between acid-stressed vs. circumneutral pH environments.

2. Materials and methods

2.1. Experimental animal

Individuals of *E. flava* (Gastropoda: Pleuroceridae) were collected from Choctafaula and Chewacla creeks (Lee and Macon counties, respectively), southeast Alabama, from September 1993 to April 1994. Because of difficulty in extracting hemolymph from smaller individuals, only larger snails (~0.7 g wet mass) were used in experiments. Streamwater Ca^{2+} concentrations and pH levels in snail collection sites ranged from 16–20 mg/L (as $CaCO_3$) and 7–8, respectively.

Prior to experimentation, snails were held at least 24 h in 40-L aerated aquaria (~22 °C) under constant illumination from plant grow lamps. Snails were supplied with fresh periphyton (as biofilm on conditioned stream cobble rocks) 24 h prior to experimentation. It is important to use fed snails in experiments because starvation may affect rates of shell growth and calcium uptake (Greenaway, 1971).

2.2. Experimental conditions

Experiments were conducted in 20 L aerated plastic enclosures held under a natural light regime (16:8·h, L:D) at 22 ± 1 °C. Enclosures contained artificial pond water (APW) consisting of 0.5 mM NaCl, 0.2 mM $NaHCO_3$, and 0.05 mM KCl (described for freshwater bivalves by Dietz and Branton, 1975; Dietz and Byrne, 1990). Each snail (35–42/enclosure, depending on total wet weight) was provided ~0.5 L of APW, the minimum volume for optimal growth (see Thomas et al., 1974).

Experimental pH treatments were chosen based on the range of acid tolerance (i.e., minimum pH) for this species, determined empirically in the laboratory. Pilot 96-h LC_{50} toxicity bioassays indicated that *E. flava* was intolerant of pH<4.0 (unpublished data); hence, pH levels of 4.0, 5.0, 6.0, and 7.0 (control) were used in this study. These levels represent the range of ambient pH conditions found in most Alabama streams (O'Neil et al., 1987; Chandler, 1990; ADEM, 2008).

Snails were exposed to pH treatments for 72 h. Sulfuric acid, the primary acidic component of AMD, was used to acidify experimental water. Water pH was monitored 3–4 times daily during the exposure

period with a digital pH meter (Beckman Instruments model no. Φ-10, Fullerton, CA), and adjusted with 1% H_2SO_4 to maintain pH within ±0.20 U of the original treatment level. In addition, pH treatments were supplemented with $CaCO_3$ to obtain calcium treatment levels of 0 (i.e., no calcium supplement), 5, and 50 mg/L Ca^{2+} (equivalent to 12.5 and 125 mg/L $CaCO_3$, respectively). Calcium treatment levels were attained by titrating a dilute solution of H_2SO_4 into the treatment chamber until all $CaCO_3$ was dissolved. Calcium treatment levels used in this study are within the range of Ca^{2+} concentrations found in Alabama streams (O'Neil et al., 1987).

2.3. Hemolymph measurements in acid-exposed snails

Snails were sacrificed by making a transverse incision with a razor blade across the side of the foot, just ventral to the operculum. Hemolymph was immediately collected with 100 µL microcapillary tubes at 0, 3, 6, 12, 24, 48, and 72 h of treatment exposure, and subsequently analyzed for pH, Ca^{2+}, HCO_3^-, Na^+, and K^+, and osmolality. The volume of hemolymph extracted varied with snail size (i.e., from 20–150 µL/snail) and a minimum volume of 250 µL was needed to complete all analyses, so hemolymph samples were pooled from 3 to 8 snails per treatment at each experimental time interval. Each treatment was replicated four times.

To minimize error from hemolymph-to-air exposure, hemolymph pH was determined immediately using 50 µL aliquots injected into a Radiometer G297-G2 capillary pH electrode and K497 reference electrode, thermostatted to the experimental temperature. Electrodes were connected to a Radiometer PHM-72 acid–base analyzer. Hemolymph total CO_2 (which at physiological pH is an approximate measure of HCO_3^-) also was immediately determined on 100 µL samples injected into a Corning 965 CO_2 analyzer calibrated to 15 mmol/L with Ciba-Corning Multi-cal standards. Remaining hemolymph was frozen for later analyses of Ca^{2+}, Na^+, K^+, and osmolality.

Calcium determinations were made on 40 µL hemolymph samples (diluted 1:10 with deionized water) using an Orion calcium-specific ion electrode connected to a Corning pH/ion meter 150. Hemolymph Na^+ (50 µL sample) and K^+ were determined simultaneously after dilution (1:200) using a Radiometer FLM3 flame photometer. Osmolality determinations were made on 10 µL samples using a Wescor Vapor Pressure Osmometer (Model 5100C) calibrated with Wescor 290 mmol/kg and 1000 mmol/kg standards.

A 2-factor repeated-measures ANOVA (Winer, 1971; SAS/TAT, 1989) was used to test for differences between pH and calcium treatments for each hemolymph factor. The Greenhouse–Geisser Epsilon statistic was used to quantify within-subject effects ($\alpha = 0.05$). If significant between-group differences were found, means for each hemolymph factor were further analyzed at 72 h with Tukey's multiple comparison test to determine where differences resided ($\alpha = 0.05$, SAS, 1989). In cases where calcium supplements had no significant effect on hemolymph factors, all calcium treatments at a given pH were combined prior to conducting multiple comparison tests, which increased treatment *N* from 4 to 12.

2.4. Oxygen uptake in acid-exposed snails

Snails exhibit an escape response from acid waters by withdrawing their foot into the shell and closing their operculum (M. Ewald, personal observations), ostensibly sealing them off from stressors in the ambient medium. Oxygen uptake (VO_2) was measured in snails and used as an indicator of ventilatory activity and, hence, the degree of contact with treatment water. Fresh snails, collected and handled similarly to those used in the main pH experiment, had their shells wiped with ethanol-soaked towels to remove biofilm and rinsed thoroughly in dechlorinated tap water immediately prior to measurements of VO_2. Snails were allowed to recover and acclimate to flow-through respirometry chambers until they re-emerged from their shells and were active. Chambers each consisted of a 25 mL Erlenmeyer flask fitted with a rubber stopper and an inlet and outlet port (22 gauge needles inserted to different depths in the chamber) for water circulation. Flask volume was reduced by the addition of glass beads, depending on the snail size. Water at pH 7 or 4 was pumped from an aerated 20-L reservoir using a Master Flex pump (Cole Parmer Instruments, Oak Park, IL, USA), and excurrent water was collected in a waste beaker. The pump was attached to a manifold, allowing 4 chambers to run simultaneously. Water pH was checked at 2- to 4-h intervals, and pH was adjusted as necessary.

Immediately prior to beginning the pH treatments, snails were maintained in circumneutral pH water and measured for oxygen uptake. Following this initial VO_2 measurement, the flow-through system for 2 of 4 chambers was changed to pH 4 water. VO_2 was measured for each snail ($N = 3$) at time intervals of 2, 10, 11, 12, 13, and 24 h to insure measurement of respiration during the 10- to 13-h time period in which acid-exposed snails had been observed to open and become exposed to effects of environmental pH. Following measurements of respiration, snail wet weights were measured to the nearest 0.01 g using an electronic analytical balance (Sartorius).

For measurement of VO_2, the flow-through circulation system was turned off and flasks were isolated and sealed. Water samples were taken using two 1-mL syringes. One sample was withdrawn from the chamber with one syringe, while air-saturated water was simultaneously injected with the other. Partial pressure of oxygen (PO_2) was measured by injecting 1 mL of chamber water into a blood gas analyzer (Radiometer PHM 73 and E5046/D616 electrode thermostatted to the experimental temperature). Respiration rates were measured as the decrease in dissolved oxygen over a 30-min period, after which flow-through conditions were resumed. PO_2 values were converted to molar concentrations using aqueous solubility coefficients (Dejours, 1975), and weight-specific VO_2 was calculated as µmol O_2/g/24 h using the equation in Jay (1985). Results were tested by repeated-measures ANOVA (SPSS, 2007) with the within-subjects

Table 1
Summary of two-factor (environmental pH and Ca) repeated-measures ANOVA for measured hemolymph ions of the snail *Elimia flava*.

Contrast	pH			Ca			HCO_3			Na			K			Osmolality		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Between-subject effects																		
pH	3	128.87	0.0001	3	13.61	0.0001	3	18.50	0.0001	3	49.89	0.0001	3	22.91	0.0001	3	4.51	0.0087
Ca	2	4.54	0.02	2	0.85	ns	2	2.71	ns	2	5.28	0.001	2	1.92	ns	2	0.55	ns
Interaction (pH × Ca)	6	3.04	0.02	6	0.83	ns	6	2.05	ns	6	1.27	ns	6	0.48	ns	6	0.51	ns
Within-subject effects																		
Time	6	7.32	0.0001	6	7.33	0.0001	6	13.97	0.0001	6	33.12	0.0001	6	4.68	0.001	6	2.28	ns
Time × pH	18	10.00	0.0001	18	6.78	0.0001	18	6.37	0.0001	18	20.33	0.0001	18	2.56	0.003	18	3.20	0.001
Time × Ca	12	1.19	ns	12	1.61	ns	12	2.56	0.01	12	2.51	0.01	12	0.93	ns	12	0.79	ns
Time × pH × Ca	36	1.35	ns	36	1.15	ns	36	1.53	ns	36	0.64	ns	36	0.62	ns	36	0.97	ns

ns = nonsignificant.

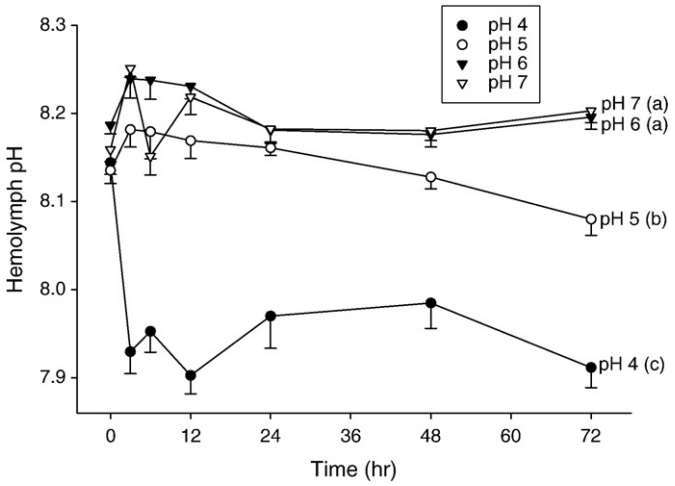

Fig. 1. Mean (±SE) hemolymph pH of the snail *Elimia flava* exposed to four environmental pH levels over 72 h ($n=12$). Lower case letters denote differences among means at 72 h of treatment exposure according to Tukey's multiple comparison test; means with the same letter are not significantly different ($\alpha=0.05$).

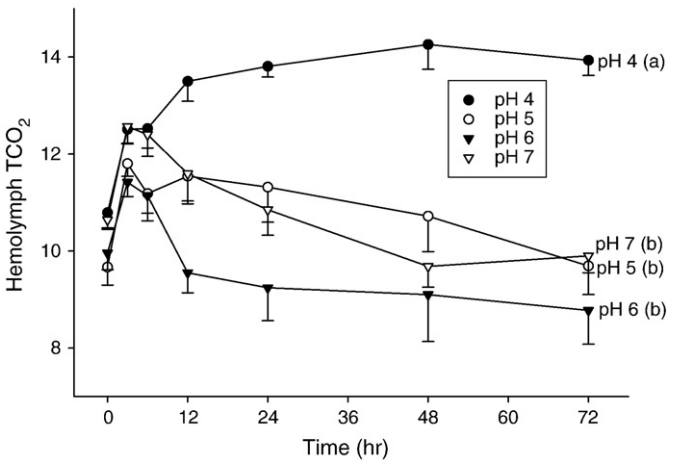

Fig. 3. Mean (±SE) hemolymph Total CO_2 (TCO_2) of the snail *Elimia flava* exposed to four environmental pH levels over 72 h ($n=12$). Lower case letters denote differences among means at 72 h of treatment exposure according to Tukey's multiple comparison test; means with the same letter are not significantly different ($\alpha=0.05$).

factor being time, the between-subjects factor being pH treatment, and the dependent variable being oxygen uptake rate.

3. Results

3.1. Effects of environmental acidification on snail hemolymph

Acid exposure, particularly pH 4, caused major disturbances in hemolymph ion concentrations. All hemolymph factors strongly differed with environmental pH ($p<0.0001$ for pH, Ca^{2+}, total CO_2, Na^+, K^+; $p<0.01$ for osmolality) and these differences increased over 72 h (i.e., significant pH × time interaction, $p<0.005$; Table 1).

Snails exposed to pH 4 showed rapid decreases in hemolymph pH, with the greatest rate of decrease occurring in the first 3 h of exposure (Fig. 1). Hemolymph pH for snails at pH 4 was significantly less ($p<0.05$) than values for other pH treatments at every interval over the 72-h experiment. Snails at pH 5 showed a more gradual decrease in hemolymph pH, which was significantly lower than pH 6 and 7 (control) at 72 h. Exposure to pH 6 caused no change in hemolymph pH over 72 h compared to controls (Fig. 1).

Snails at pH 4 showed dramatic increases in hemolymph Ca^{2+} and total CO_2 (TCO_2) over the study, reaching close to maximum concentrations after 12 h (Figs. 2 and 3, respectively). Ca^{+2} doubled within the first 12 h of acid exposure, and TCO_2 increased by approximately 40%. Both values remained elevated throughout the experiment and were significantly higher than controls at 72 h. In contrast, relative to controls, exposure to pH 5 and 6 had no effect on hemolymph Ca^{2+} or TCO_2 after 72 h (Figs. 2 and 3).

Hemolymph Na^+ concentrations were reduced by 50% in snails exposed to pH 4 by 72 h compared to control snails; the latter maintained constant hemolymph Na^+ throughout the experiment (Fig. 4). By 72 h, snails exposed to pH 5 and 6 also showed significant reductions (i.e., 11 and 9%, respectively) in hemolymph Na^+ concentrations, compared to controls (Fig. 4).

Hemolymph K^+ of snails exposed to pH 4 increased four-fold in 12 h relative to controls, and remained high through 48 h (Fig. 5). However, by 72 h, K^+ levels for snails at pH 4 decreased and were statistically indistinguishable from controls ($p>0.05$). Hemolymph K^+ of snails exposed to pH 5 and 6 never differed from controls over 72 h (Fig. 5).

Changes in hemolymph osmolality of snails at pH 4 appeared to reflect changes in individual constituent ions, decreasing rapidly by

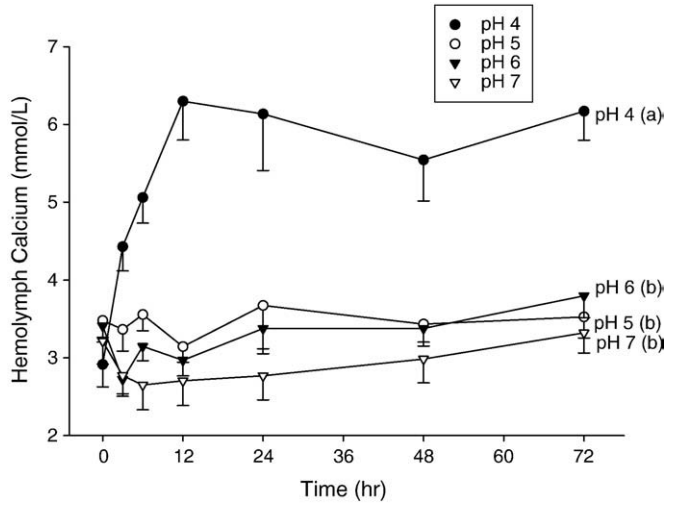

Fig. 2. Mean (±SE) hemolymph Ca^{2+} of the snail *Elimia flava* exposed to four environmental pH levels over 72 h ($n=12$). Lower case letters denote differences among means at 72 h of treatment exposure according to Tukey's multiple comparison test; means with the same letter are not significantly different ($\alpha=0.05$).

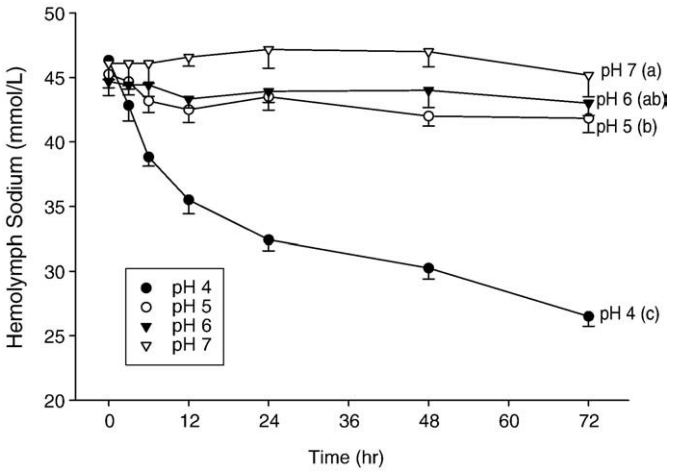

Fig. 4. Mean (±SE) hemolymph Na^+ of the snail *Elimia flava* exposed to four environmental pH levels over 72 h ($n=12$). Lower case letters denote differences among means at 72 h of treatment exposure according to Tukey's multiple comparison test; means with the same letter are not significantly different ($\alpha=0.05$).

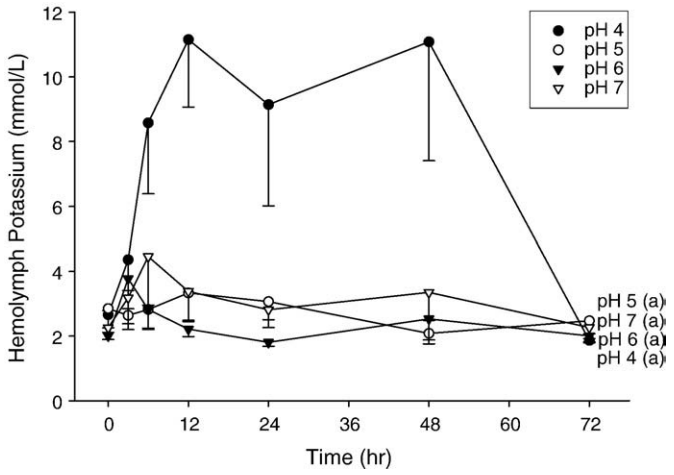

Fig. 5. Mean (±SE) hemolymph K^+ of the snail *Elimia flava* exposed to four environmental pH levels over 72 h ($n=12$). Lower case letters denote differences among means at 72 h of treatment exposure according to Tukey's multiple comparison test; means with the same letter are not significantly different ($\alpha=0.05$).

48 h and becoming significantly lower than controls. Osmolality of snails at pH 5 also was significantly lower than controls after 72 h, whereas osmolality for snails at pH 6 did not differ from controls (Fig. 6). Combined, the osmotic and ionic data show that at low pH, especially pH 4, solutes were being leached from hemolymph.

3.2. Effects of environmental Ca^{2+} on snail hemolymph

Calcium supplements significantly affected snail hemolymph pH and Na^+ concentrations ($p<0.05$; Table 1). Snails in treatments lacking calcium supplements (i.e., 0 mg/L Ca^{2+}) had lower hemolymph pH and Na^+ than snails at either 5 or 50 mg/L Ca^{2+}, irrespective of environmental pH or exposure time (Fig. 7A, B). Of these two hemolymph factors, pH appeared sensitive only to presence or absence of environmental Ca^{2+}, rather than to differences in Ca^{2+} concentration, as hemolymph pH of snails at 5 mg/L Ca^{2+} was similar to that of snails at 50 mg/L Ca^{2+} (Fig. 7A). In contrast, although somewhat variable, hemolymph Na^+ generally showed a gradient of sensitivity to Ca^{2+}, and increased with increasing environmental Ca^{2+} (Fig. 7B).

Environmental Ca^{2+} significantly influenced responses of hemolymph pH to environmental pH (i.e., significant pH×Ca interaction; Table 1). Snails at pH 4 and 5 with zero environmental Ca^{2+} had lower

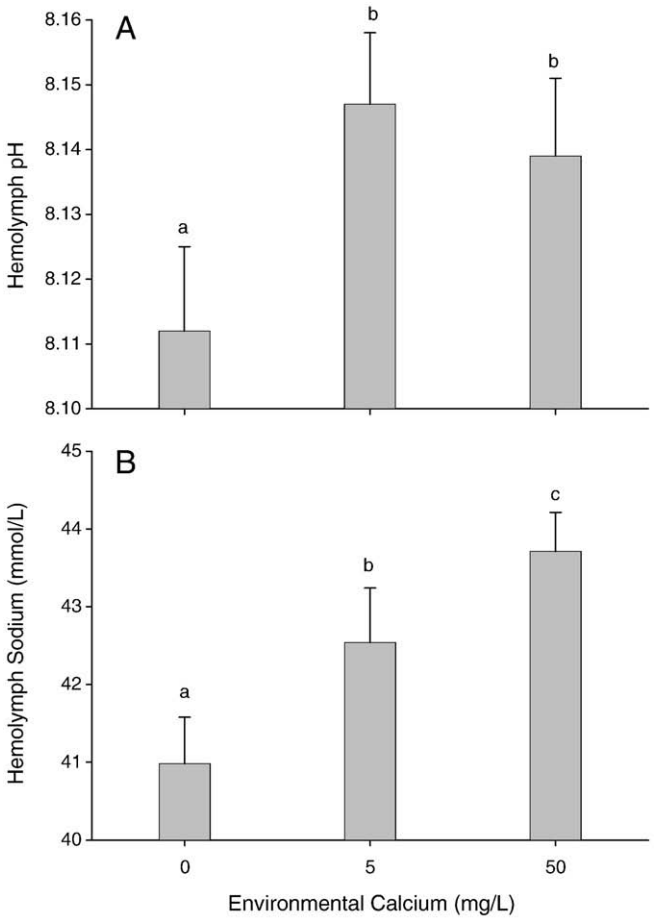

Fig. 7. Mean (±SE) hemolymph (A) pH, and (B) Na^+ of the snail *Elimia flava* exposed to three environmental calcium concentrations ($n=112$). pH treatments and time intervals were combined for each Ca treatment to illustrate the strong effects on environmental Ca across pH and time. Lower case letters denote differences among means according to Tukey's multiple comparison test (means with the same letter are not significantly different at $\alpha=0.05$).

hemolymph pH than treatments with 5 or 50 mg/L Ca^{2+}. Across all pH levels, hemolymph pH depended on the presence or absence of environmental Ca^{2+} rather than its concentration. Relative to controls (pH 7), environmental Ca^{2+} at pH 6 had no effect on hemolymph pH.

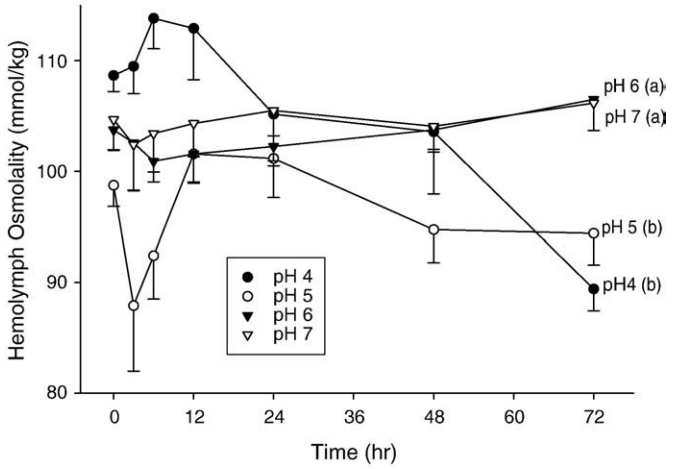

Fig. 6. Mean (±SE) hemolymph osmolality of the snail *Elimia flava* exposed to four environmental pH levels over 72 h ($n=12$). Lower case letters denote differences among means at 72 h of treatment exposure according to Tukey's multiple comparison test; means with the same letter are not significantly different ($\alpha=0.05$).

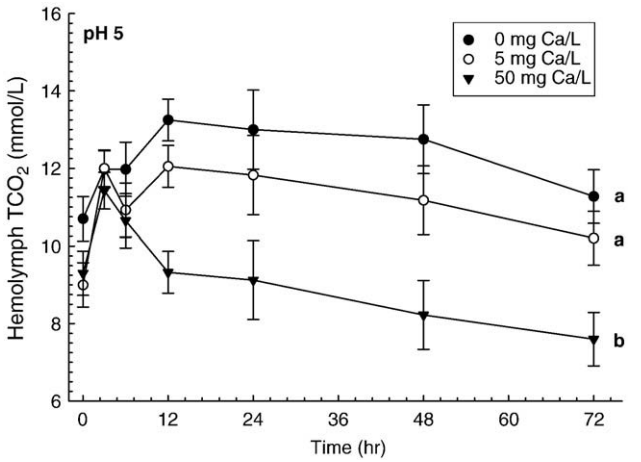

Fig. 8. Mean (±SE) hemolymph total CO_2 (TCO_2) of the snail *Elimia flava* exposed to pH 5 and three calcium levels over 72 h ($n=4$). Lower case letters denote differences among 72 h means according to Tukey's multiple comparison test; means with the same letter are not significantly different at $\alpha=0.05$.

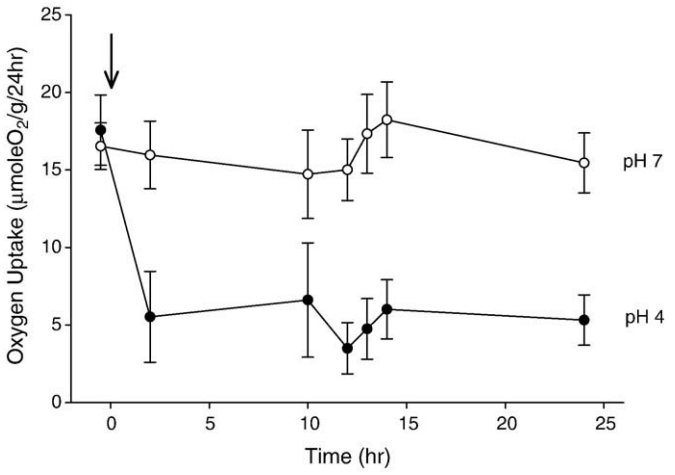

Fig. 9. Mean (±SE) oxygen uptake of the snail *Elimia flava* exposed to two environmental pH levels over 24 h ($N=3$). Arrow indicates start point of pH exposure.

Hemolymph TCO_2 and Na^+ were the only factors that showed variable responses to environmental Ca^{2+} levels over the 72-h study (i.e., significant $Ca^{2+} \times$ time interaction; $p<0.05$).

Snails at pH 4 showed a gradual increase in TCO_2 over time compared to controls, irrespective of environmental Ca^{2+}. In contrast, TCO_2 levels for snails at pH 5 strongly depended on environmental Ca^{2+} (Fig. 8). Snails at 0 or 5 mg/L Ca^{2+} showed significantly higher hemolymph TCO_2 than snails at 50 mg/L Ca^{2+}; this pattern occurred at 12 h and persisted through 72 h. Compared to controls, exposure to pH 6 had no effect on hemolymph TCO_2 at any level of environmental Ca^{2+}.

Snails exposed to pH 4 showed a rapid decrease in Na^+ compared to controls (pH 7), irrespective of environmental Ca^{2+}, whereas snails at pH 5 and 6 maintained Na^+ levels similar to controls. Within each pH level, snails in treatments without Ca^{2+} supplements showed consistently lower Na^+ over the 72-h experiment compared to snails with high Ca^{2+} supplements (i.e., 50 mg/L Ca^{2+}).

3.3. Effects of acid exposure on oxygen uptake

Oxygen uptake rates of pre-exposed snails (i.e., at pH 7) were approximately 17 μmol O_2/g/24 h (Fig. 9). No differences in VO_2 occurred for snails at pH 7 over the 24-h experiment ($p=1.00$). After transfer, rates of VO_2 were significantly lower for snails at pH 4 than at pH 7 ($F=13.48$, $p=0.004$, Table 2, Fig. 9). Exposure to pH 4 resulted in a 64% reduction in VO_2 after 2 h ($p=0.004$) and a maximum reduction of 81% at 11 h ($p<0.0001$). After 24 h, oxygen uptake of snails at pH 4 remained at minimal levels, and was significantly lower than control snails ($p=0.004$). At no time, however, did VO_2 cease entirely for snails at pH 4.

4. Discussion

4.1. Effects of acidification

4.1.1. Strongly acidic conditions (pH 4)

Results of our study demonstrate that the ability of *E. flava* to regulate its short-term hemolymph osmotic and ionic concentrations is significantly disrupted by a strongly acidic environment. Significant hemolymph acidosis occurred in snails exposed to pH 4. The increase in H^+ ions may have been produced internally, from an accumulation of acidic anaerobic end products, or externally, from H^+ influx from the environment, or a combination of these sources. Increased influx of H^+ across thin epithelial surfaces (e.g., gills) during acid exposure has been reported for freshwater fish and crayfish. In addition, changes in hemolymph pH were accompanied by changes in hemolymph ion concentrations (e.g., loss of Na^+), so it is possible that changes in the strong ion difference could have contributed to acidosis (Stewart, 1978); however, because we did not quantify all major strong ions we could not test this hypothesis. For trout (*Salmo trutta*), gill membrane permeability to H^+ ions is strongly dependent on external pH (McWilliams and Potts, 1978). Acidosis in the crayfish *Orconectes propinquus* is attributable to massive influx of acidic equivalents (H^+, NH_4^+), rather than from internal sources (Wood and Rogano, 1986); in the latter study, there was no evidence of lactic acid production. However, molluscs, unlike fishes or crayfishes, can behaviorally avoid acidic environments by closing their opercula (snails), or valves (bivalves) to the surrounding medium. As a result, internal changes in pH caused by environmental H^+ influx may be exacerbated by production of acidic end products of anaerobic metabolism during the time snails engage in an escape response. This situation may be particularly true for snails during the initial hours of acid exposure. Bivalves in harsh environments (e.g., exposure to air or low pH water) can maintain valve closure for extended periods (hours to days) to avoid contact with the medium (Machado et al., 1988; Pynnonen, 1991; Byrne and McMahon, 1994). Complete valve closure induces anaerobic respiration, which causes accumulation of acidic anaerobic end products (Byrne and McMahon, 1994). In our study, virtually all snails exposed to pH 4 were inactive (i.e., with tightly closed opercula) and immobile for the entire 72 h. Oxygen uptake rates over the first 24 h for snails at pH 4 were significantly reduced to a level indicating a corresponding reduction in exchange with the surrounding medium, with the highest rate of reduction occurring within 2 h of exposure. Snails had reduced contact with the medium during this inactive period, so it is possible that the initial acidosis resulted at least in part from internal sources (e.g., anaerobic metabolism; Packer, 1979). Given that ventilation never entirely ceased and that the mineral acid load at pH 4.0 was much higher than what could have been produced by snail metabolism, it is likely that hemolymph acidosis was caused primarily by the environmental proton load.

Snails at pH 4 experienced rapid and dramatic increases in hemolymph Ca^{2+} and TCO_2, which persisted throughout the experiment. Previous studies on a wide range of freshwater and terrestrial invertebrate species suggest that increased influx of Ca^{2+} from the environment is an unlikely source of Ca^{2+} increase. For example, the freshwater unionid bivalves *Anodonta anatina* and *Unio tumidus* both showed decreased uptake of environmental ^{45}Ca from low pH water (pH 4–4.5; Pynnonen, 1991). At pH 5.2, the crayfish *P. clarkii* also showed decreased net uptake of Ca^{2+} and basic equivalents (OH^-, HCO_3^-) (Zanotto and Wheatly, 1993). Last, in a related study on postmolt crayfish (*Orconectes virilis*), Malley (1980) reported Ca^{2+} uptake was inhibited in moderately acid water (pH<5.7), which ceased entirely at pH 4.0. In the terrestrial crab, *Gecarcinus lateralis*, which has no source of external calcium, hemolymph Ca^{2+} levels rose 7 mM in response to a hypercapnic acidosis (Henry et al., 1981).

Elevated hemolymph Ca^{2+} following acid exposure may be explained by dissolution of internal calcium sources. For freshwater and terrestrial crustaceans, this source would be from the calcareous carapace (Henry et al., 1981; Morgan and McMahon, 1982; Wood and Rogano, 1986). In this case, increased proton concentrations solubilized $CaCO_3$, yielding Ca^{2+} and HCO_3^-, the latter of which could be used

Table 2
Summary of repeated-measures ANOVA for oxygen uptake by the snail *Elimia flava* exposed to two environmental pH levels (pH 4 vs. 7) over 24 h.

	df	*F*	*p*
Between-subject effects			
pH	1	13.48	0.004
Within-subject effects			
Time	6	4.87	<0.0001
Time × pH	6	4.21	0.001

to buffer the hemolymph acidosis. The freshwater bivalve, *Anodonta cygnea*, exposed to acidic water showed increased hemolymph Ca^{2+} that apparently originated from shell–mantle $CaCO_3$, as environmental calcium was eliminated as a possible Ca^{2+} source (Machado et al., 1988). In that study, exposure to acidification (pH 3) for 12 days resulted in a significant hemolymph acidosis and a doubling of hemolymph Ca^{2+}, which was attributable to solubilization of calcareous microspherules in the mantle. In our study, internal decalcification from shell $CaCO_3$ stores also was the most likely mechanism accounting for increased hemolymph Ca^{2+} in *E. flava*: at pH 4 snail hemolymph Ca^{2+} doubled (from ~3 to 6 mmol/L after 72 h), across all Ca^{2+} treatments, even for those with zero environmental calcium.

Exposure to pH 4 for 72 h caused a severe decrease (~50%) in hemolymph Na^+, which may, in part, explain the 90% mortality rate that occurs between 72 and 96 h exposure (unpublished data). Freshwater fish exposed to acidic conditions showed similar physiological responses in which passive Na^+ efflux rapidly increased while active influx decreased, leading to net Na^+ loss in the blood and reduction of body Na^+ (Packer and Dunson, 1970, 1972; McWilliams and Potts, 1978; McDonald et al., 1980). If plasma Na^+ or Cl^- concentrations are depressed 30% below normal, death in trout will occur within hours (Wood, 1989). McDonald (1983) reported similar findings for rainbow trout, but emphasized that it was the rate, rather than the amount of Na^+ efflux, that determined lethality. Similar responses have been observed for ranid tadpoles exposed to low pH (2.5–3.5), in which animals died when Na^+ levels fell below 50% of initial body Na^+ levels (Freda and Dunson, 1984).

Exposure to pH 4 caused a four-fold increase in snail hemolymph K^+ by 12 h; however, K^+ returned to levels similar to controls by 72 h. Rapid initial increases in extracellular hemolymph K^+ may occur because of intracellular K^+ leakage into the hemolymph, and the subsequent decreases observed at 72 h may be attributable to K^+ leakage from the hemolymph to the surrounding medium. Diffusional efflux of K^+ may result from electrochemical imbalances created at the cellular membrane when extracellular Na^+ is lost to the environment. Na^+ and K^+ loss might also be a result of the breakdown of structural and/or functional integrity of both epithelial and endothelial membranes as a result of exposure to high proton concentrations. This pattern would result in the loss of function of excitable tissue (nerve, muscle, and heart) and may be the ultimate source of mortality for acid-stressed snails.

4.1.2. Moderate and weakly acidic conditions (pH 5 and 6)

Compared to pH 4, exposure to pH 5 and 6 caused proportionally less severe acid–base and ionic imbalances in *E. flava* hemolymph. Snails at pH 5 showed an 8% loss of Na^+ after 72 h and moderate, albeit significant, decreases in hemolymph pH and osmolality. However, hemolymph K^+, TCO_2, and Ca^{2+} were not different from controls (pH 7) over the 72-h experiment, so these factors appeared unaffected by more moderately depressed environmental pH. Our results revealed that pH 6 had no physiological effects on the hemolymph factors we measured, but others have suggested that water at or below this pH could have important long-term ecological consequences for snails. In a survey of 1000 Norwegian lakes, Økland (1983) found a decline in the number of snail species in lakes below pH 6, and suggested that this value may set the lower limit of physiological tolerance for snails exposed to chronic acidification.

Our study was designed only to examine physiological effects of acute exposure to acidification; thus, it is possible that chronic (i.e., weeks/months) exposure to pH at or below 6 could produce more significant disruptions in physiological processes than we observed. Hemolymph imbalances observed at pH 5 were so gradual over 72 h that *E. flava* may acclimate to prolonged exposure to less acidic environments and never experience the drastic hemolymph disturbances observed for snails at pH 4. Acute exposure to pH 6 had no effect on *E. flava*, but chronic exposure might have produced more significant stress. It is also possible, however, that species such as *E. flava* are more tolerant of weakly acidic conditions and show no physiological effects from pH 6, irrespective of exposure duration. For example, in acid-stressed macroinvertebrate communities in the Rybinsk Reservoir in Russia, several mollusks could tolerate water from pH 5.02 to 5.95, and one species, *Lymnaea ovata*, occurred at pH <5 (Berezina, 2001). It is also possible that mortality resulting from chronic exposure to pH 5 occurs during a more sensitive stage in the life cycle (e.g., larval or juvenile).

4.2. Effects of environmental Ca2+

4.2.1. Effects on Na+

At pH 4, environmental Ca^{2+} ostensibly played no role in hemolymph Na^+ regulation, as Na^+ decreased irrespective of environmental Ca^{2+} concentration. High levels of environmental H^+ also could have interfered with any possible ameliorating effects of environmental Ca^{2+}. Moreover, at 72 h, hemolymph Na^+ was not different among environmental Ca^{2+} treatments at any environmental pH level. However, there was a trend for snails in treatments lacking environmental Ca^{2+} to show lower hemolymph Na^+ than those with supplemental Ca^{2+}; this result held for almost every pH–time combination, including controls (pH 7). In addition, irrespective of pH treatment and time of exposure, hemolymph Na^+ tended to increase with increasing environmental Ca^{2+} (Fig. 7). Research on juvenile *O. rusticus* crayfish revealed significant losses of Na^+ and increased mortality in low ambient Ca^{2+} treatments, irrespective of pH level (Hollett et al., 1986). For fish, reductions in environmental Ca^{2+} increased passive efflux of Na^+ (Potts and Flemming, 1971; Eddy, 1975), whereas increases in Ca^{2+}, in contrast, reduced efflux rates of Na^+ (Eddy, 1975). Supplemental Ca^{2+} can benefit Na^+ regulation in acidic environments. For trout, environmental Ca^{2+} minimized the effect of low pH by decreasing the rate of passive Na^+ efflux at the gill (McWilliams and Potts, 1978; McWilliams, 1982). For tadpoles, increased Ca^{2+} in treatment water lowered sensitivity to low pH by reducing Na^+ efflux (Freda and Dunson, 1985). *Daphnia magna* exposed to low pH and high environmental Ca^{2+} exhibited severely inhibited Na^+ influx, explained by competitive interactions at a $2Na^+/H^+$ exchanger (Glover and Wood, 2005). Taken together, results of these and our studies provide compelling evidence that, at sublethal acidification, environmental Ca^{2+} plays a key role in Na^+ regulation for a wide array of freshwater animals.

Most of the above studies identified Ca^{2+} as instrumental in hemolymph ion balance, but only one study (McDonald et al., 1980, for rainbow trout) specifically linked low Ca^{2+}, rather than other ions, with ionoregulatory failure and mortality at low pH. At pH 4.3, trout in acidified hard water (i.e., high Ca^{2+}) showed major blood acidosis but only minor depressions in plasma ion levels. In contrast, acidified soft water (i.e., low Ca^{2+}) caused only minor blood acidosis but major plasma ion losses (primarily Na^+ and Cl^-). In addition, acidified soft water treatments caused higher mortality than hard water treatments (50 vs 11%, respectively, over 6 days); this evidence suggests that in low Ca^{2+} water ionoregulatory failure, as a result of the acid–base disturbance, is the primary toxic mechanism of low pH for fish (McDonald et al., 1980). In our study, snails at pH 4 experienced both ionoregulatory failure and severe acidosis, irrespective of environmental Ca^{2+}. The rate of increase in hemolymph H^+ was most severe in the initial 3 h of exposure to pH 4, after which hemolymph acidosis remained consistently depressed. Significant mortality occurred only after 72 h (M. Ewald, unpublished data), so death was most likely attributable to concurrent decreases in extracellular Na^+ and intracellular K^+, rather than hemolymph acidosis.

4.2.2. Effects on total CO_2

Similar to the case for hemolymph Na^+, environmental Ca^{2+} had little influence on hemolymph TCO_2 at pH 4, as the latter increased irrespective of environmental Ca^{2+} levels. As stated previously, snails at pH 4 were completely inactive with closed opercula and minimal contact with the external medium, as indicated by the rapid reduction

in VO_2 after exposure, through 72 h. As a result, the absorption of H^+ from the ambient water into the hemolymph may have been restricted and the initial acidosis could have resulted from a combination of protons originating from both external and internal sources. Observed increases in hemolymph TCO_2 were produced by internal sources, most likely decalcification of the calcareous shell by the reaction: $CaCO_3 + H^+ \rightarrow HC\bar{O}_3 + Ca^{2+}$. The doubling of hemolymph Ca^{2+} during the first 12 h of exposure to pH 4 suggests both Ca^{2+} and HCO_3^- originate from the shell.

4.3. Conclusion

Many studies have emphasized the detrimental effects of acidification on a variety of freshwater organisms; however, surprisingly few have investigated the role of environmental calcium in ameliorating physiological stress of acidic water. In our study, environmental Ca^{2+} appeared to provide no benefit to *E. flava* at pH 4, as snails experienced severe acidosis and lost hemolymph Na^+ and intracellular K^+, irrespective of environmental Ca^{2+}. However, for snails at pH 5, ionic and acid/base disturbances decreased with increasing environmental Ca^{2+}, indicating the beneficial effects of calcium at less toxic pH levels.

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