Aquatic Toxicology 91 (2009) 302–311

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

High net calcium uptake explains the hypersensitivity of the freshwater pulmonate snail, *Lymnaea stagnalis*, to chronic lead exposure

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ARTICLE INFO

Article history:
Received 7 August 2008
Received in revised form 16 October 2008
Accepted 22 October 2008

Keywords:
Lead
Snails
Ionoregulation
Biotic ligand model

ABSTRACT

Previous studies have shown that freshwater pulmonate snails of the genus *Lymnaea* are exceedingly sensitive to chronic Pb exposure. An EC20 of <4 μg l^{-1} Pb for juvenile snail growth has recently been determined for *Lymnaea stagnalis*, which is at or below the current USEPA water quality criterion for Pb. We characterized ionoregulation and acid–base balance in Pb-exposed *L. stagnalis* (young adults ~1 g) to investigate the mechanisms underlying this hypersensitivity. After 21-day exposure to 18.9 μg l^{-1} Pb, Ca^{2+} influx was significantly inhibited (39%) and corresponding net Ca^{2+} flux was significantly reduced from 224 to −23 nmol g^{-1} h^{-1}. An 85% increase in Cl^- influx was also observed, while Na^+ ion transport appeared unaffected. Finally, a marked alkalosis of extracellular fluid was observed with pH increasing from 8.35 in the control to 8.65 in the 18.9 μg l^{-1} Pb-exposed group. Results based on direct measurement of Ca^{2+} influx in 1 g snails gave an influx nearly an order of magnitude higher (750 nmol g^{-1} h^{-1}) than in comparably sized fish in similar water chemistry. Under control conditions, specific growth rate in newly hatched snails was estimated at 16.7% per day over the first 38-day post-hatch and whole body Ca^{2+} concentrations were relatively constant at ~1100 nmol g^{-1} over this period. Based on these data, it is estimated that newly hatched snails have net Ca^{2+} uptake rates on the order of 7600 nmol g^{-1} h^{-1}. A model was developed integrating these data and measured inhibition of Ca^{2+} influx rates of 13.4% and 38.7% in snails exposed to 2.7 and 18.9 μg l^{-1} Pb, respectively. The model estimates 45% and 83% reductions in newly hatched snail growth after 30-day exposure in these two Pb-exposed groups. These results compare well with previous direct measurements of 47% and 90% reductions in growth at similar Pb concentrations, indicating the high net Ca^{2+} uptake is the controlling factor in observed Pb hypersensitivity.

1. Introduction

Historically, there has been a heavy emphasis on testing standard or model organisms in toxicity testing. Organisms such as the rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), and several daphnids (e.g., *Daphnia magna* and *Ceriodaphnia dubia*) are routinely used as models to the near exclusion of other families, classes, and even phyla that are commonly found in freshwater environments. As a result, a recent meta-analysis concluded that toxicity data sets used to derive water quality criteria for metals are so heavily biased towards model organisms that they are unrepresentative of real aquatic communities (Brix et al., 2005). This bias combines with a formidable challenge imposed upon environmental regulators by substantial interspecies metal sensitivity variation, which in many cases are several orders of magnitude (Bianchini et al., 2002; Grosell et al., 2002a,b, 2006a,b, 2007).

The problem of interspecies variation in sensitivity and intense testing of a few species has been further exacerbated by the development of biotic ligand models (BLMs) for a few standard test organisms to predict site-specific metal toxicity in aquatic environments with differing water chemistry (Di Toro et al., 2001; Paquin et al., 2002). These BLMs are now used in setting water quality criteria (USEPA, 2003) with an underlying assumption that the wide range of organisms in aquatic environments will respond similarly to changes in environmental conditions. It may be argued that teleost fishes and freshwater crustaceans will respond in a manner generally similar to the standard organisms (rainbow trout, daphnids) on which the BLMs are based. However, so far, there has been no effort made to validate the applicability of these models to more divergent phyla such as aquatic insects and mollusks, which may have substantially different physiological responses to metal exposure. A counter to this argument has been that at least for acute toxicity, these other phyla are generally insensitive and

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doi:10.1016/j.aquatox.2008.10.012

any differences in physiological responses will not impact the level of protection provided by setting water quality criteria at levels low enough to protect sensitive taxa. However, this assertion currently cannot be evaluated with respect to chronic metal toxicity in general or chronic Pb toxicity in particular due to the paucity of data available for a diverse range of organisms (Brix et al., 2005).

Borgmann et al. (1978) was the first to demonstrate the sensitivity of pulmonate snails to Pb. They estimated a no observable effect concentration (NOEC) of 12 $\mu g\,l^{-1}$ Pb for juvenile snail growth in *Lymnaea palustris*, which until recently was the most chronically sensitive species tested with Pb. However, we demonstrated that the congeneric species, *L. stagnalis*, is also unusually sensitive to chronic Pb exposure with an EC20 for juvenile snail growth of <4 $\mu g\,l^{-1}$ (Grosell et al., 2006a,b). This is of interest for several reasons. First, as mentioned above, freshwater gastropods have generally been considered insensitive to metals largely based on acute toxicity studies (Wurtz, 1962; Arthur and Leonard, 1970; Rehwoldt et al., 1973; Holcombe et al., 1984; Nebeker et al., 1986). Second, the ionoregulatory physiology of freshwater mollusks differs in a number of ways from the model organisms typically used to set water quality standards and derive BLMs. Finally, the observed effect level for Pb (<4 $\mu g\,l^{-1}$) is very close and potentially below the current water quality criteria for Pb (3.3 $\mu g\,l^{-1}$) normalized to the relevant test water hardness (USEPA, 1985).

The objective of the present study was to investigate the physiological mechanisms by which Pb affects juvenile snails. Lead has been demonstrated to impact both Ca^{2+}, Cl^- and Na^+ homeostasis in freshwater organisms, albeit at high concentrations (Rogers et al., 2003, 2005; Rogers and Wood, 2004). Large among-species variation in Ag and Cu toxicity in freshwater can be explained largely by different Na^+ turnover rates (Grosell et al., 2002a,b). Therefore, the possibility of Pb sensitive, high Na^+ uptake rates in snails was examined as an explanation for the observed hypersensitivity. Earlier studies revealed Na^+ uptake rates in *L. stagnalis* typical for similar sized freshwater organisms (Greenaway, 1970). To confirm these observations, Na^+ uptake rates and the sensitivity of Na^+ uptake to Pb exposure was also examined as part of the present study. Similarly, unidirectional Cl^- fluxes were measured in control and Pb-exposed snails. Furthermore, considering the likely influence of Pb on Ca^{2+} uptake and the extremely high Ca^{2+} requirements of these shell forming freshwater mollusks, Pb influence on Ca^{2+} homeostasis was characterized. Finally, since the exchange of Na^+ and Cl^- between freshwater organisms and their environment is intimately linked to the exchange of acid–base equivalents (Marshall and Grosell, 2006) and since shell formation requires CO_3^{2-} in addition to Ca^{2+}, the possible effect of Pb on acid–base balance was also examined.

2. Materials and methods

2.1. Experimental animals

Adult snails were obtained from an in-house culture maintained in flow through dechlorinated City of Miami tap water ($[Na^+]$ ~1.1, $[Ca^{2+}]$ ~0.31, $[Mg^{2+}]$ ~, $[Cl^-]$ ~1.03, $[HCO_3^-]$ ~0.68 mmol l^{-1}, [DOC] ~200 $\mu mol\,l^{-1}$, pH ~7.7) at room temperature. The culture was fed a mix of lettuce, carrots and sweet potatoes and egg masses were transferred from the main culture tanks to static-renewal nursery tanks for hatching and juvenile growth phase before they were reintroduced to the main culture.

2.2. General experimental procedures

Young adult snails (~1 g) were used for ionoregulation and acid–base balance studies and were not fed for 48 h prior to experimentation. For juvenile growth rate experiments, newly hatched snails were used and allowed unlimited access to food. Lead exposures were performed in dechlorinated City of Miami tap water under flow-through conditions as described previously (Grosell et al., 2006a,b). At the end of experiments snails were blotted dry on paper towels after which total body mass was determined to the nearest 0.1 μg on an analytical balance (Mettler, Toledo).

2.3. Na^+ uptake kinetics in Lymnaea

To first examine the possibility of *Lymnaea* having high Na^+ turnover rates, perhaps explaining hypersensitivity to Pb, Na^+ uptake kinetics for unexposed snails were determined. For these experiments, an artificial freshwater was used (Ebanks and Grosell, 2008) and Na^+ concentrations were varied by addition of NaCl to yield a total of 6 test concentrations (59.4 ± 1.7, 88.3 ± 5.5, 139.7 ± 5.9, 146.6 ± 6.1 and 490.7 ± 7.6 μM Na^+). For each of these Na^+ concentrations 10 snails (mass, 1.57 ± 0.08 g) were rinsed in the appropriate test solution and placed in individual plastic beakers containing 20 ml of test solution. Snails were allowed to recover from handling for 10 min before ^{22}Na was added to the individual flux chambers (0.02 μCi of ^{22}Na, as NaCl; Amersham Biosciences, specific activity >100 mCi/mg Na^+) and an initial water sample of 1 ml was obtained from each flux chamber 5 min after isotope addition. After 1–2 h (exact time recorded) of ^{22}Na incubation a final water sample was obtained and snails were removed from flux chambers for wet weight determination.

2.4. Prolonged exposure of snails to Pb

To evaluate the influence of lead exposure on unidirectional ion fluxes, two experimental series were performed in which three replicates per treatment contained 10 individual snails in 1 l tripour beakers receiving a continuous flow (6–8 ml min^{-1}) of the appropriate test solution. Details of the test system have been described elsewhere (Grosell et al., 2006a,b). In the first experimental series, snails were exposed to <0.5 (control), 2.7 and 18.9 $\mu g\,l^{-1}$ Pb for 21 days, followed by a series of unidirectional flux experiments. In the second experimental series, snails were exposed to <0.5 (control), 1.3 and 7.5 $\mu g\,l^{-1}$ Pb for 14 days, followed by measurements of acid–base balance and extracellular fluid ion homeostasis. Differences in Pb concentrations between the two experiments were unintentional and the result of well known difficulties in maintaining Pb in solution due to sorption to the sides of exposure chambers.

2.5. Unidirectional ion flux measurements

Snails used for ion flux experiments weighed 1.07 ± 0.08 g and were fluxed in medium obtained from the flow through exposure mixing chamber containing the appropriate Pb concentrations (<0.5, 2.7 and 18.9 $\mu g\,l^{-1}$). To determine Cl^- uptake and loss rates, 10 snails from each treatment were placed in individual beakers each containing 20 ml of dechlorinated tap water and were allowed to recover from handling for 10 min prior to the initiation of flux measurement. Each beaker was covered with Parafilm® and gently aerated through polyethylene tubing. At the onset of Cl^- flux measurements a total of 0.02 μCi ^{36}Cl (as NaCl; Amersham Biosciences, specific activity >3 mCi/g) was added to each beaker and a 5 min equilibration period was allowed before water samples were obtained for ^{36}Cl radioactivity and total Cl^- concentrations measurements. This 5 min equilibration period allows for any adsorption of Cl^- (radioactive or not) to occur such that differences between this initial water sample and the final water sample can be ascribed to uptake (or excretion) by the organism. After a total flux time of 2–3 h (exact time for individual snails recorded) a

second water sample was obtained from each beaker after which the snails were removed from the flux beaker, rinsed, blotted dry and weighed.

Unidirectional transport rates of Na^+ and Ca^{2+} were determined simultaneously in 10 individual snails per treatment as outlined for the Cl^- transport studies. For these measurements, 0.02 μCi of ^{22}Na (as NaCl; Amersham Biosciences, specific activity >100 mCi/mg Na^+) and 0.02 μCi of ^{45}Ca (as $CaCl_2$; Amersham Biosciences, specific activity 5–50 mCi/mg Ca^{2+}) were added to each individual beaker at the onset of flux measurements and a water sample was obtained after 5 min of equilibration and immediately before termination of the experiments. At the end of these flux measurements snails were weighed as described above.

For Cl^-, Ca^{2+} and Na^+ flux measurements, including the Na^+ uptake kinetic experiment, the specific activity of the relevant isotope in the water samples at the beginning and end of the flux period was determined from measured radioactivity and corresponding total ion concentrations. Unidirectional ion uptake rates (nmol g^{-1} h^{-1}) were determined from the depletion of radioactivity from the water for the ion in question and the average specific activity of this ion over the flux period, the individual snail mass and the time elapsed during the flux period as described previously (Grosell et al., 2000). Net flux rates were determined in a similar manner but simply from the change in total concentration of the relevant ion from the beginning to the end of the flux period. Unidirectional efflux rates were determined as the difference between influx and net flux (Grosell et al., 2002a,b).

2.6. Hemolymph and tissue ion concentrations

Acid–base balance and ion concentrations in extracellular fluids and tissue ion concentrations were determined in snails (0.52 ± 0.02 g) after 14-day exposure to <0.5, 1.3 and 7.5 μg l^{-1} Pb. Snails were maintained in individual beakers as for the flux experiments above prior to sampling. A predatory avoidance reflex in *L. stagnalis* which expels substantial volumes of extracellular fluid in response to stimulation of the foot was utilized to obtain extracellular fluid samples as described in Ebanks and Grosell (2008). In brief, individual snails were gently removed from their exposure medium, carefully blotted dry and held upside down while stimulated to retract into their shell and thereby release extracellular fluid. This procedure results in accumulation of expelled extracellular fluid in the vacated outer shell cavity, which can easily be obtained by a pipette. Collected extracellular fluid was transferred to micro-centrifuge tubes and a subsample was transferred to a separate 0.5 ml tube for immediate pH measurements. Following pH measurements, the subsamples were used for measurements of total CO_2 concentrations. The remaining extracellular fluid samples were frozen at −20 °C and stored for later analysis of ionic composition.

Following sampling of extracellular fluids, snails were euthanized by freezing at −20 °C and stored for later analysis of tissue ion concentrations and shell mass. Upon thawing, soft tissue was separated from the shell by dissection, weighed and digested in 1N H_2SO_4 at 70 °C overnight prior to analysis of tissue Cl^-, Na^+ and Ca^{2+}. The mass of the remaining shell was also determined.

2.7. Growth rates in juvenile Lymnaea and Ca^{2+} requirements

To assess growth rates in newly hatched *L. stagnalis*, 1 l dechlorinated tap water was placed in a glass container and a few small pieces of sweet potato was introduced to precondition the container with respect to surface associated microbial communities which we suspect is the primary food source for newly hatched snails. After a 5-day preconditioning period, egg masses in advanced state of development (snail embryos clearly visible) were introduced. Juvenile snails were collected and their individual masses determined 48 h post-hatch and every 2–4 days thereafter for a total of 38 days. Specific growth rates were calculated for each sampling interval from the change in mass between two sampling points.

2.8. Analytical techniques

Water samples for determination of Pb exposure concentrations were collected three times per week, passed through a 0.45 μm cellulose nitrate syringe filter (Acro-disc, Pall Life Sciences, MI, USA) and acidified by addition of HNO_3 (Fisher Scientific, Trace metal grade) to a final concentration of 1%. Lead concentrations were analyzed by graphite furnace atomic absorption (Varian 220Z, Varian, Walnut Creek, CA, USA).

Concentrations of Cl^- in extracellular fluid, tissue digests and water samples were determined using anion chromatography (DIONEX DX 120, Sunnyvale, California, USA) while Na^+ and Ca^{2+} concentrations were analyzed by atomic absorption spectrophotometry (VarianAA 220FS, Mulgrave, Victory, Australia). Extracellular fluid pH was determined in 20–50 μl subsamples using an Accumet micro pH electrode coupled to a Radiometer 220 pH meter (Radiometer, Copenhagen, Denmark). Concentrations of total CO_2 in hemolymph samples were analyzed using a Corning Carbon Dioxide Analyzer 965 (Essex, England). Gamma radioactivity from ^{22}Na was analyzed using an automated gamma counter (Packard Cobra II Auto-Gamma, Meriden, Connecticut, USA). Beta radioactivity from ^{36}Cl was determined using an automated scintillation counter (TmAnalytical Beta Tract 6895) as was the combined activity from ^{22}Na and ^{45}Ca. Cross calibration of the gamma counter and beta counter using a set of samples containing only ^{22}Na allowed for subtraction of the ^{22}Na radioactivity from the combined activity determined by the scintillation counting for determination of ^{45}Ca radioactivity alone for samples containing both ^{45}Ca and ^{22}Na (Grosell and Jensen, 1999).

2.9. Data presentation and statistical evaluation

Data are presented as mean ± S.E.M. and $n = 10$ in all cases. Statistical evaluation consisted of Student's two-tailed t-tests and results were considered statistically different at $p < 0.05$. All analyses were performed using a commercial software package (SPSS, 2002).

3. Results

No Pb-induced mortality was observed during the 14–21 days of Pb exposure or during the flux experiments.

3.1. Na^+ uptake kinetics and unidirectional flux results for Na^+, Ca^{2+}, and Cl^-

Uptake of Na^+ from the water adheres to saturation kinetics in *L. stagnalis* with a maximal uptake rate (V_{max}) of 512 ± 59 nmol g^{-1} h^{-1} and an affinity constant (K_m) of 97 ± 32 μM (Fig. 1). Unidirectional fluxes and net Na^+ flux was unaffected by Pb exposure at least and the end of the exposure period (21 days), when the Na^+ flux was performed (Fig. 2).

Unidirectional Ca^{2+} uptake rates were suppressed by Pb exposure in a dose-dependent manner with a statistically significant reduction (39%) in Ca^{2+} uptake in snails exposed to 18.9 μg l^{-1} Pb for 21 days (Fig. 3). In the absence of a significant effect on unidirectional Ca^{2+} efflux, the reduced Ca^{2+} influx translates to a significantly reduced net Ca^{2+} uptake from 224 nmol g^{-1} h^{-1} in the control to a net flux not significantly different from 0

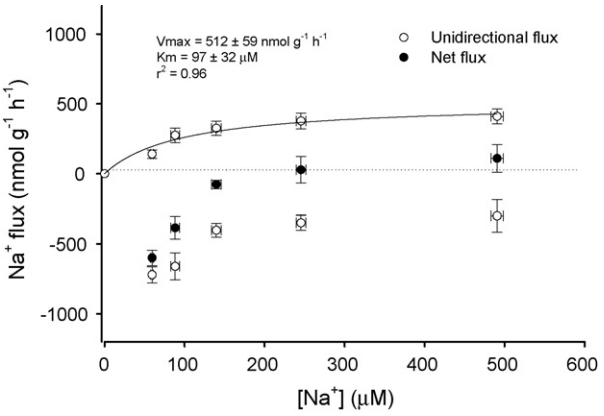


Fig. 1. Uptake kinetics in young adult *Lymnaea stagnalis*, mean ± S.E.M., $n = 10$ in all cases. Open symbols, positive values represent unidirectional Na^+ uptake, while open symbols, negative values represent unidirectional efflux and closed symbols represent the resulting net flux.

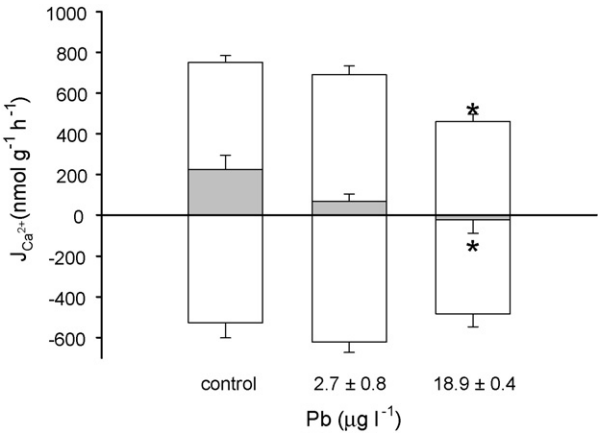


Fig. 3. Ca^{2+} influx, efflux and net flux in *L. stagnalis* exposed to <0.5 (control), 2.7 or 18.9 µg l^{-1} Pb for 21 days. Positive open bars represent influx, negative open bars represent efflux, and shaded bars represent net flux in nmol g^{-1} h^{-1}. Data presented as mean ± S.E.M. ($n = 10$). (*) Statistically significant difference from the control ($\alpha < 0.05$).

(−23 nmol g^{-1} h^{-1}) in animals exposed to the highest Pb concentration (Fig. 3). In contrast, unidirectional Cl^- uptake was significantly elevated (86%) relative to the control in snails exposed to 18.9 µg l^{-1} Pb for 21 days. This resulted in an increased net Cl^- uptake to approximately 150 nmol g^{-1} h^{-1} in snails exposed to the highest Pb concentration (Fig. 4) compared to near 0 in control snails.

3.2. Hemolymph and tissue ion concentration results

The clear effects of Pb on unidirectional Ca^{2+} uptake and net Ca^{2+} transport is reflected in the soft tissue Ca^{2+} concentrations which show dose-dependent reductions at 1.3 and 7.5 µg l^{-1} Pb (Fig. 5A). Interestingly, it appears that extracellular fluid Ca^{2+} concentrations are protected and remain unaltered by Pb exposure (Fig. 5B) despite the substantial changes in soft tissue Ca^{2+} concentrations and unidirectional Ca^{2+} uptake. A third significant Ca^{2+} pool is the shell of *L. stagnalis*, which was also apparently unaffected by Pb exposure. In control snails the shell accounted for 25.0 ± 0.9% of the total snail mass compared to 24.1 ± 0.7% and 24.1 ± 0.9% in snails exposed to 1.3 and 7.5 µg l^{-1} Pb, respectively (data not shown).

Tissue Cl^- concentration was significantly reduced after exposure to 1.3 µg l^{-1} Pb, but apparently normal after exposure to 7.5 µg l^{-1} Pb (Fig. 6A). A similar pattern of effects at the low but not the high Pb concentration was observed for extracellular Cl^- concentrations but in contrast to the tissue Cl^- concentrations, extracellular Cl^- was elevated compared to controls after 14 days of exposure to 1.3 µg l^{-1} (Fig. 6B). Exposure for 14 days to both Pb concentrations resulted in a substantial reduction (30–40%) in soft tissue Na^+ concentrations and a slight (5%), yet significant, reduction in extracellular fluid Na^+ concentrations after exposure to 7.5 µg l^{-1} Pb (Fig. 7).

The extracellular fluid of *L. stagnalis* contains high concentrations of total CO_2 (>20 mM) and is highly alkaline compared to most other freshwater aquatic organisms (pH > 8.3) (Fig. 8). Exposure to Pb had a dose-dependent effect on hemolymph pH resulting in an increase of nearly 0.3 pH units in snails exposed to 7.5 µg l^{-1} Pb for 14 days (Fig. 8A). In agreement with this observation is slightly elevated (though not statistically significant at 7.5 µg l^{-1} Pb) hemolymph total CO_2 following the 14 days of Pb exposure (Fig. 8B).

3.3. Growth rate in juvenile L. stagnalis

Individual mean mass of *L. stagnalis* reached nearly 60 mg at 38-day post-hatch with a biphasic pattern of relative specific growth rate being very high immediately post-hatch (28% per day),

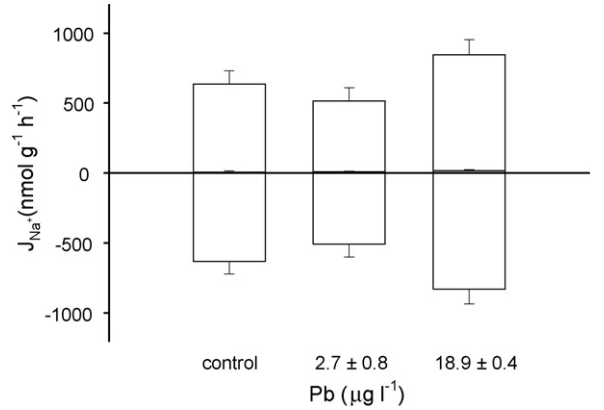


Fig. 2. Na^+ influx, efflux and net flux in *L. stagnalis* exposed to <0.5 (control), 2.7 or 18.9 µg l^{-1} Pb for 21 days. Positive open bars represent influx, negative open bars represent efflux, and shaded bars represent net flux in nmol g^{-1} h^{-1}. Data presented as mean ± S.E.M. ($n = 10$).

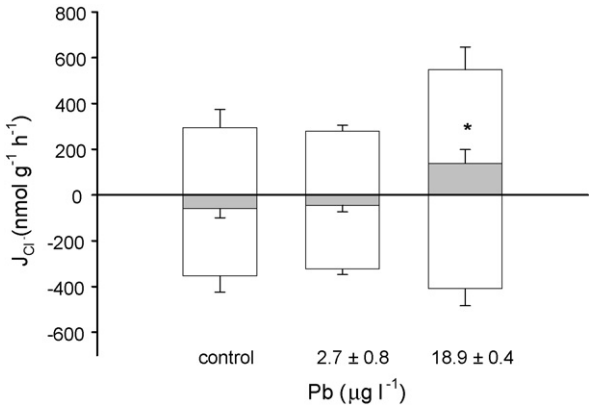


Fig. 4. Cl^- influx, efflux and net flux in *L. stagnalis* exposed to <0.5 (control), 2.7 or 18.9 µg l^{-1} Pb for 21 days. Positive open bars represent influx, negative open bars represent efflux, and shaded bars represent net flux in nmol g^{-1} h^{-1}. Data presented as mean ± S.E.M. ($n = 10$). (*) Statistically significant difference from the control ($\alpha < 0.05$).

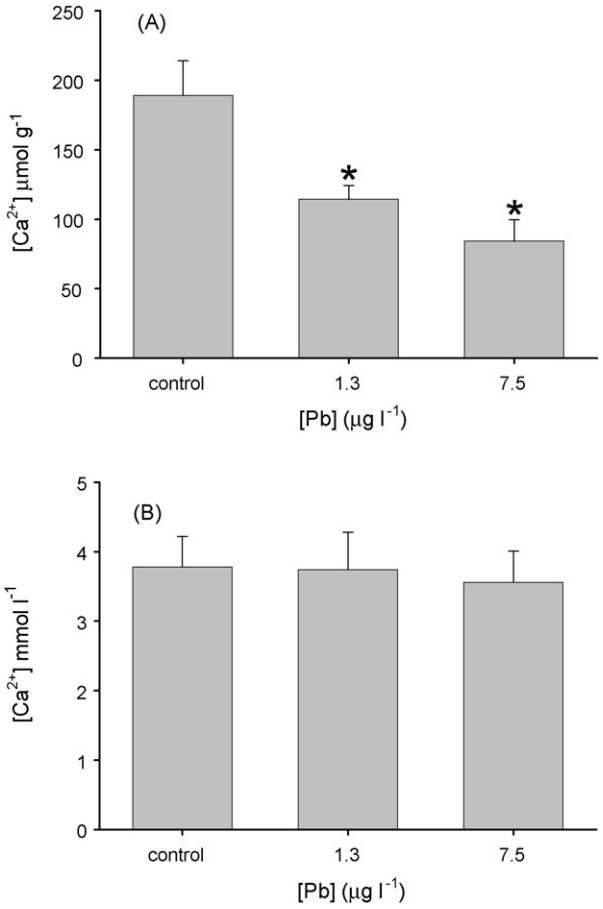


Fig. 5. (A) Ca^{2+} concentrations ($\mu mol\ g^{-1}$) in soft tissue of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\ l^{-1}$ Pb for 14 days. (B) Ca^{2+} concentrations ($mmol\ l^{-1}$) in hemolymph of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\ l^{-1}$ Pb for 14 days. All data presented as mean ± S.E.M. ($n = 10$). (*) Statistically significant difference from the control ($\alpha < 0.05$).

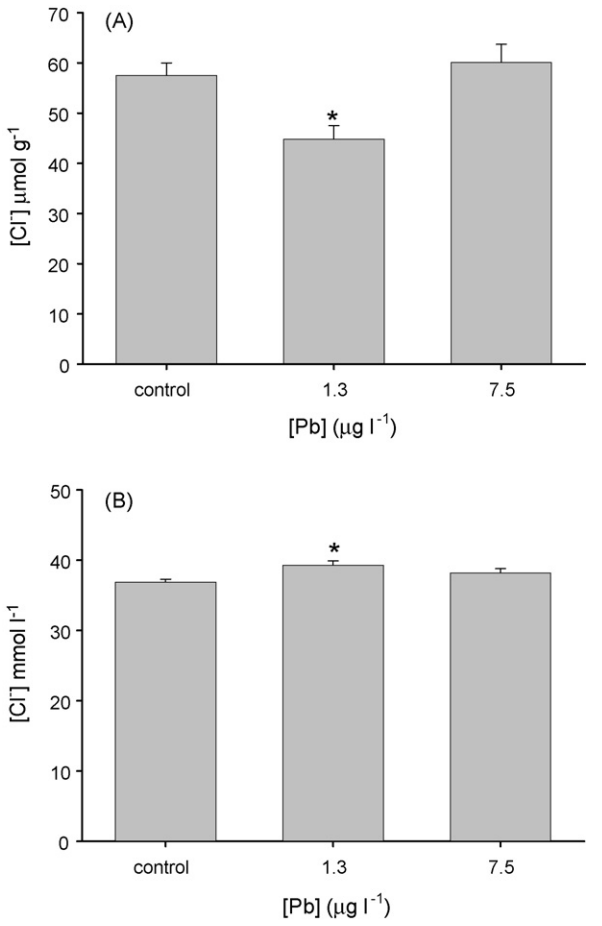


Fig. 6. (A) Cl^- concentrations ($\mu mol\ g^{-1}$) in soft tissue of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\ l^{-1}$ Pb for 14 days. (B) Cl^- concentrations ($mmol\ l^{-1}$) in hemolymph of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\ l^{-1}$ Pb for 14 days. All data presented as mean ± S.E.M. ($n = 10$). (*) Statistically significant difference from the control ($\alpha < 0.05$).

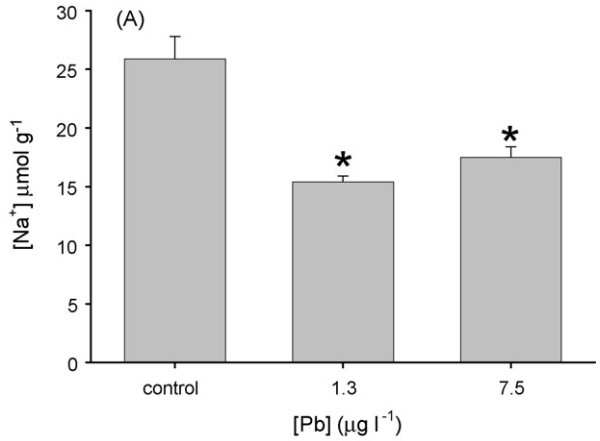
declining to approximately day 18 post-hatch to increase again through the rest of the observation period for an overall mean of 16.7% per day for the entire period. The reason for the apparent biphasic pattern of specific growth rates is unknown but may reflect a switch from a microbial to a herbivorous diet during early juvenile development.

4. Discussion

4.1. Comparison to other studies

The only other extensive characterization of Pb effects on ionoregulation in aquatic organisms was performed by Rogers and colleagues using the rainbow trout, *Oncorhynchus mykiss*. Their experiments were conducted on juvenile trout (1–3 g), but unlike our studies, were focused on acute toxicity using relatively high aqueous Pb concentrations ($1\ mg\ l^{-1}$). Similar to our results, Rogers et al. (2003) demonstrated that acute Pb exposure ($1\ mg\ l^{-1}$) reduced Ca^{2+} influx by ~65%. In a second study, Rogers and Wood (2004) demonstrated this inhibition of Ca^{2+} uptake was a result of both competition at apical voltage-independent Ca^{2+} channels and inhibition of a basolateral Ca^{2+}-ATPase. Rogers et al. (2003) also demonstrated an approximately 50% reduction in Na^+ uptake and corresponding 40% reduction in Na^+/K^+-ATPase activity. This result is dissimilar to what we observed in snails, although we did observe a 30–40% reduction in Na^+ concentration in the soft tissue and a slight (5%) reduction in the hemolymph (Fig. 7). Finally, Rogers et al. (2003) observed a 50% reduction in Cl^- influx and Rogers et al. (2005) showed that this was a result of inhibition of carbonic anhydrase, which facilitates the hydration of metabolic CO_2 to HCO_3^-, providing substrate for apical Cl^-/HCO_3^- exchange. This result is effectively the opposite of what we observed in snails, where Cl^- uptake was stimulated by ~85% relative to the control (Fig. 4).

To understand the apparent similarities and differences in physiological responses to Pb exposure between snails and rainbow trout, we developed a physiological model of snail ionoregulatory processes (Fig. 9). This model was developed based on a number of previous studies that investigated either single or multiple ion transport processes in *L. stagnalis* (Van der Borght and Van Puymbroeck, 1966; Zylstra et al., 1978; De With et al., 1980, 1987; Schlichter, 1981; De With and Van der Schors, 1982; Ebanks and Grosell, 2008). In this model, major cations (Na^+, Ca^{2+}) and anions (Cl^-) are taken up at the integument via transport systems identical or similar to those of fish. Namely, Ca^{2+} uptake is driven by a basolateral Ca^{2+}-ATPase and an apical Ca^{2+} channel (Flik and Verboost, 1993). Sodium uptake is driven by a basolateral Na^+/K^+-ATPase and an apical Na^+/H^+ exchanger which appears to be electrogenic and rely in part on a V-type H^+ ATPase (Ebanks and Grosell, 2008). Chloride uptake in *L. stagnalis* is similar to most fish, occurring via Cl^-/HCO_3^- exchange De With et al. (1980).



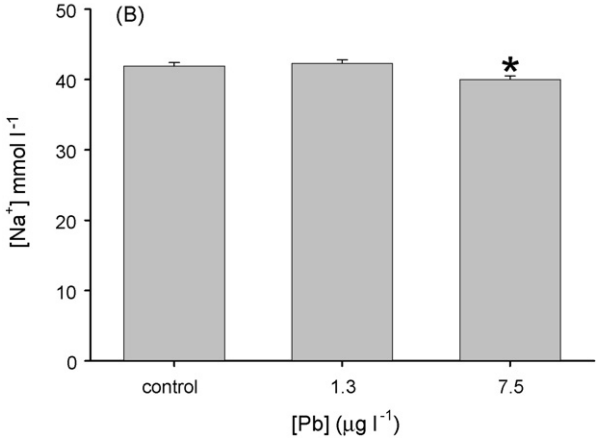
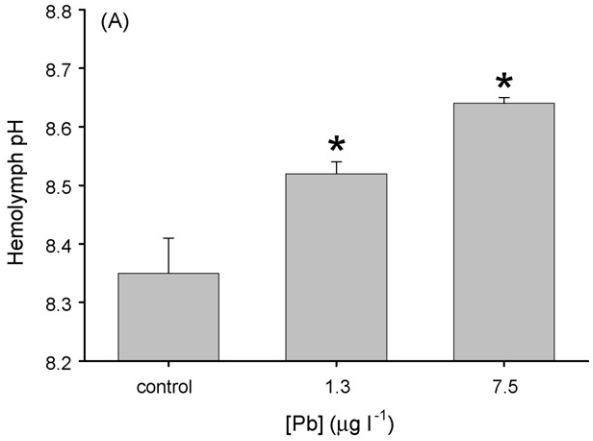


Fig. 7. (A) Na^+ concentrations ($\mu mol\, g^{-1}$) in soft tissue of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\, l^{-1}$ Pb for 14 days. (B) Na^+ concentrations ($mmol\, l^{-1}$) in hemolymph of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\, l^{-1}$ Pb for 14 days. All data presented as mean ± S.E.M. ($n = 10$). (*) Statistically significant difference from the control ($\alpha < 0.05$).



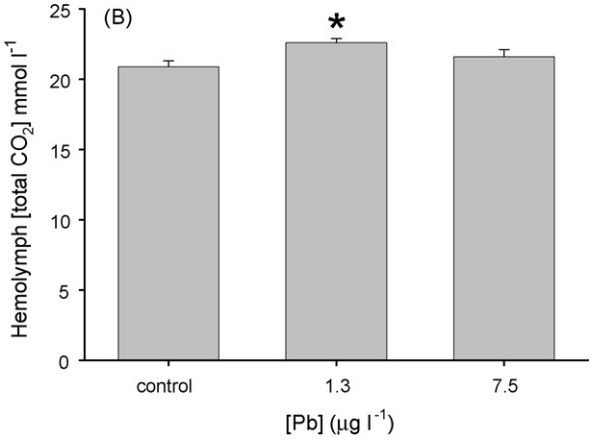


Fig. 8. (A) Hemolymph pH of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\, l^{-1}$ Pb for 14 days. (B) Hemolymph total CO_2 ($mmol\, l^{-1}$) of *L. stagnalis* exposed to <0.5 (control), 1.3 or 7.5 $\mu g\, l^{-1}$ Pb for 14 days. All data presented as mean ± S.E.M. ($n = 10$). (*) Statistically significant difference from the control ($\alpha < 0.05$).

Unlike fish, snails possess an excreting epithelium, the mantle, which is responsible for shell formation. At the mantle, Ca^{2+} is excreted along with HCO_3^- resulting in the formation of $CaCO_3$ for the shell with a resulting H^+ as a byproduct. Bicarbonate needed for this process is likely provided at least in part by carbonic anhydrase (CA) facilitated hydration of metabolic CO_2. The H^+ produced in $CaCO_3$ formation is then transported back into the snail to maintain an alkaline pH for shell formation (Fig. 9). As discussed later the exact fate of the H^+ produced by this process is uncertain.

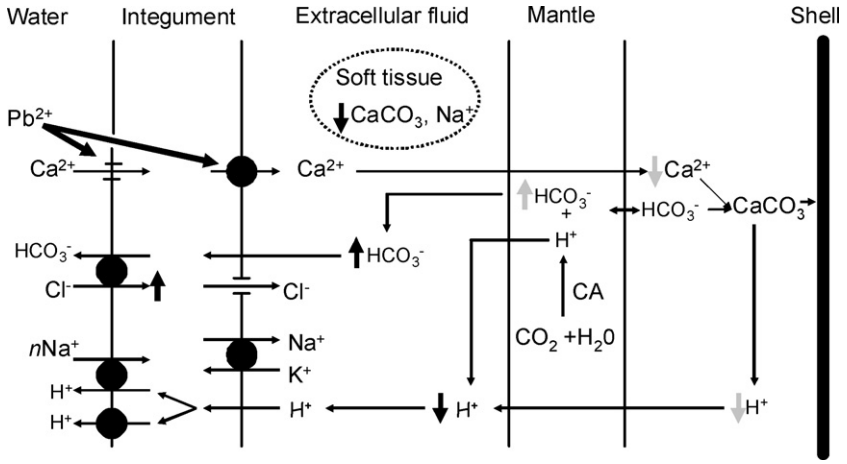


Fig. 9. Conceptual model of ion transport processes in *L. stagnalis* and the observed and hypothesized effect of Pb on these processes. Thin black arrows are ion transport process under baseline conditions. Thick black arrows indicate observed up- or down-regulation of specific ions or transport processes in response to Pb exposure. Thick gray arrows indicate hypothesized up- or down-regulation of specific ions or transport processes in response to Pb exposure.

4.2. Effects of Pb on Na^+ homeostasis

Our hypothesis of Pb-sensitive, high rate Na^+ uptake as a possible explanation for hypersensitivity in *L. stagnalis* was proven false by the present study. A review summarizing >70 studies on Na^+ uptake has shown that Na^+ uptake rates scale with body mass (Grosell et al., 2002a,b). According to the relationship between Na^+ uptake and body mass from this review a 1.5 g freshwater organism (approximate mass of snails in this experiment) is expected to have a Na^+ uptake rate of ~900 nmol g^{-1} h^{-1}. Even the maximal Na^+ uptake rate observed in *L. stagnalis* does not exceed this value and high Na^+ turnover rates therefore cannot account for Pb hypersensitivity or for hypersensitivity to other metals in this organism. Furthermore, the lack of inhibition of Na^+ influx and unchanged Na^+ efflux following Pb exposure does not support interactions with Na^+ transport pathways as the explanation for Pb hypersensitivity. The possibility that Na^+ influx was inhibited early during Pb exposure via reduced Na^+/H^+ exchange as a compensatory response to systemic alkalosis but subsequently recovered might explain the statistically significant (30–40%) reduction in soft tissue Na^+ concentrations and slight (5%) reduction in the hemolymph. We cannot exclude that the timing of our sampling might have been such that recovery of soft tissue Na^+ concentrations had yet to occur, or was in progress while extracellular Na^+ was returned to near control levels after 2–3 weeks of exposure.

A perhaps more likely explanation for the depleted intracellular Na^+ concentrations (soft tissue Na^+) is compensatory intracellular pH (pH_i) regulation in response to the systemic alkalosis induced by Pb exposure (see below). Maintenance of pH_i in the face of extracellular alkalosis would be served by cellular retention of H^+ and thus reduced cellular Na^+ entry via Na^+/H^+ exchange. In this context it should be noted that the intracellular Na^+ pool is small compared to the extracellular pool and that effects of small changes in intracellular Na^+ may therefore not influence extracellular Na^+ sufficiently to yield statistical significance. In any case, it seems that Na^+ homeostasis is affected to some extent by Pb exposure but that this effect is secondary and compensatory to the alkalosis induced by Pb exposure.

4.3. Effects of Pb on Ca^{2+} homeostasis

The observed Ca^{2+} uptake inhibition in *L. stagnalis* was similar to that observed in rainbow trout, albeit at a significantly lower (50-fold for comparable level of inhibition) Pb concentration. This likely means that a component of the snail Ca^{2+} uptake system (apical channel and/or basolateral Ca^{2+}-ATPase) is more sensitive to Pb than the corresponding system in trout. In addition to the higher sensitivity of the Ca^{2+} uptake pathways in snails, it is important to keep in mind that the overall Ca^{2+} uptake rate in snails is substantially higher than in trout. For example, Rogers and Wood (2004) measured Ca^{2+} influx on the order of 100 nmol g^{-1} h^{-1} for 1–3 g rainbow trout. In comparison, 1 g snails in water with a similar Ca^{2+} concentration, had a Ca^{2+} influx of approximately 750 nmol g^{-1} h^{-1}.

Our observations suggest that Pb-exposed adult snails mobilized Ca^{2+} stores in their soft tissue in order to maintain extracellular fluid Ca^{2+} and provide Ca^{2+} for shell formation and/or maintenance, resulting in the decrease in soft tissue Ca concentration in Pb-exposed snails (Figs. 5 and 9). Several studies have characterized Ca^{2+} deposits located throughout the soft tissues, particularly along the alimentary canal, in the digestive gland and in the mantle (Carriker and Bilstad, 1946; Greenaway, 1971a). Greenaway et al. (Greenaway, 1971b) also showed that when *L. stagnalis* is held in Ca-free water for up to 5 weeks, snails will mobilize shell Ca^{2+} in order to maintain hemolymph Ca^{2+} concentrations and that the rate of mobilization is comparable to the Ca^{2+} efflux rate. However, based on the measured net loss rate in snails exposed to the high Pb treatment (23 nmol g^{-1} h^{-1}), it is estimated that snails would only lose ~8 μmol g^{-1} of Ca^{2+} over the course of a 2-week exposure. Comparison with the measured 105 μmol g^{-1} Ca^{2+} lost from the soft tissue suggest that this Ca^{2+} pool could easily compensate for inhibited Ca^{2+} uptake and some of this pool may have also been used to sustain shell maintenance and growth. This conclusion is further supported by our data which show relative shell mass was not influenced by Pb exposure (Section 3.2 and Fig. 5B). Mobilization of Ca^{2+} from the soft tissues would likely have acid–base balance implications, as discussed below.

We hypothesize that the inhibited Ca^{2+} uptake ultimately resulted in a decrease in Ca^{2+} available, despite mobilization of internal Ca^{2+} stores, for shell formation at the mantle, and that this explains the effects on juvenile snail growth observed at very low Pb concentrations (Grosell et al., 2006a,b). The noted combination of Pb sensitive Ca^{2+} uptake pathway(s) and the extremely high Ca^{2+} requirements renders juvenile *L. stagnalis* (and perhaps many other freshwater gastropods) highly sensitive compared to fish.

4.4. Effects of Pb on acid–base balance and Cl^- transport

There were two direct indicators of an acid–base balance disturbance when *L. stagnalis* was exposed to Pb; a marked alkalosis with hemolymph pH increasing from 8.3 in control snails to 8.6 in the high Pb treatment and a slight but statistically significant increase in hemolymph total CO_2. The net result of these two effects is an increase in hemolymph HCO_3^- and we hypothesize this lead to a compensatory response in which the snails increased Cl^-/HCO_3^- exchange in an attempt to reduce excess HCO_3^- (Fig. 9).

There are two possible mechanisms to elicit these responses. In one scenario, the lack of Ca^{2+} at the mantle decreased or eliminated (depending on the extent of inhibition) $CaCO_3$ formation. This caused a decrease in elimination of H^+ liberated during $CaCO_3$ formation and possibly an excess of HCO_3^- still being produced by the CA-facilitated hydration of CO_2 (Fig. 9). Rogers et al. (2005) measured a significant inhibition of CA by Pb in rainbow trout, which if it occurred in snails would reduce or eliminate formation of excess HCO_3^- but we propose that this likely did not occur in snail mantle epithelia for two reasons. First, Rogers et al. demonstrated CA inhibition at Pb concentrations an order of magnitude higher than those used in the snail experiments. Second, Pb is believed to be entering the snail at the integumental ion exchange epithelia near the base of the snail foot and is unlikely to travel to the mantle epithelia, to target CA at the site of $CaCO_3$ formation, in significant concentrations without being sequestered.

The elevated accumulation of HCO_3^- formed by CA hydration but not consumed in $CaCO_3$ formation and the reduction in H^+ liberation from reduced $CaCO_3$ precipitation combines to produce the observed metabolic alkalosis (Fig. 8A) and compensatory increase in Cl^-/HCO_3^- exchange. Note that this compensation is incomplete since Pb-exposed snails still exhibit a significant alkalosis.

An additional process which may also explain the observed alkalosis involves the snails mobilizing Ca^{2+} from their soft tissue stores in response to inhibition of Ca^{2+} uptake by Pb. Mobilization of Ca^{2+} from the soft tissue results in mobilization of Ca^{2+} and consumption of H^+ for $CaCO_3$ titration or mobilization of both Ca^{2+} and CO_3^{2-} or HCO_3^- if $CaCO_3$ is simply dissolving as a function altered Ca^{2+} gradients. In the latter case both Ca^{2+} and CO_3^{2-}/HCO_3^- would be transported into the snail hemolymph. Regardless of how Ca^{2+} is mobilized, it would result in the observed maintenance of hemolymph Ca^{2+} concentrations, along with an increase in hemolymph pH and total CO_2 (primarily HCO_3^-) either from H^+ consumption in the $CaCO_3$ storage compartments or direct elimination of CO_3^{2-} from these storage compartments. With the

observations presented in the present study we are unable to determine which of these two processes are occurring but both may account for the observed alkalosis during Pb exposure. Similar to the reduced $CaCO_3$ precipitation for shell formation discussed above, increased Cl^-/HCO_3^- exchange would be a compensatory response to the alkalosis arising from $CaCO_3$ mobilization.

4.5. Explanation for sensitivity of snails to Pb

The primary objective of this experiment was to explore possible physiological reasons for the high sensitivity of juvenile snails to Pb. We have shown that Pb exposure inhibits Ca^{2+} influx and net flux, which leads to a cascade of secondary ionoregulatory and acid–base balance disturbances in the snails. While this cascade of effects is of great interest and provides important insights on the overall ionoregulation and acid–base balance of the snail, the ultimate cause of their high sensitivity to Pb appears to be the extraordinarily high Ca^{2+} demand of the animals combined with high Pb sensitivity of their Ca^{2+} uptake pathway.

As discussed, in 1 g snails, the Ca^{2+} influx rate is 750 nmol g^{-1} h^{-1} compared with a similar sized rainbow trout where Ca^{2+} influx is 80–100 nmol g^{-1} h^{-1}. While this is certainly a significant difference, it grossly underestimates the Ca^{2+} demand of the newly hatched snails (0.2 mg) used in the chronic toxicity test which demonstrated their hypersensitivity (Grosell et al., 2006a,b). Flux measurements are difficult to perform on such small animals. However, we characterized the specific growth rate (SGR) of newly hatched snails and measured levels as high as 28% per day in newly hatched snails with a mean SGR of 16.7% per day (Fig. 10). We have previously determined whole body Ca^{2+} in juveniles snails to be ~1100 μmol g^{-1} and relatively constant in snails ranging from 0.2 to >200 mg in size (Grosell et al., 2006a,b). From these data, the net Ca^{2+} demand of newly hatched snails can be estimated as

$$\frac{\mathrm{SGR} \times \mathrm{WB}[Ca^{2+}]}{24\,\mathrm{h\,day^{-1}}} \quad (1)$$

where SGR is the specific growth rate (% per day) and WB[Ca^{2+}] is the whole body Ca^{2+} concentration (nmol g^{-1}). Using the mean SGR of 16.7% per day, the estimated net Ca^{2+} flux for these snails would be 7674 nmol g^{-1} h^{-1}, which would necessitate an even higher Ca^{2+} influx than observed in larger snails. For comparison, the net Ca^{2+} flux in 1–3 g rainbow trout is 70 nmol g^{-1} h^{-1} (Rogers and Wood, 2004), two orders of magnitude lower.

The critical parameter, at least with respect to chronic growth in snails, is the net flux of Ca^{2+} which provides the substrate for shell formation. It is possible to develop a simple model describing the potential effect of Ca^{2+} inhibition by Pb on newly hatched snails if we accept a few assumptions. The key to this model is data showing that whole body Ca^{2+} concentrations do not vary with snail size over the range of 0.2–200 mg. As a result, the Ca^{2+} net flux needed for shell formation is directly proportional to growth of the snail. Viewed another way, the inhibition of snail growth will be directly proportional to the inhibition of Ca^{2+} net flux. To estimate net Ca^{2+} influx in the newly hatched snails, an assumption is made that Ca^{2+} efflux is comparable to that measured in adult snails (500 nmol g^{-1} h^{-1}). Under this assumption, the influx rate in newly hatched snails would need to be 8174 nmol g^{-1} h^{-1} to achieve a net flux rate of 7674 nmol g^{-1} h^{-1}, the rate needed to sustain the 16.7% per day growth rate observed under control conditions. Note that this assumption is conservative and the effects described below would be even larger if Ca^{2+} efflux is higher in newly hatched snails compared to larger snails which would be expected from a simple surface area to volume ratio.

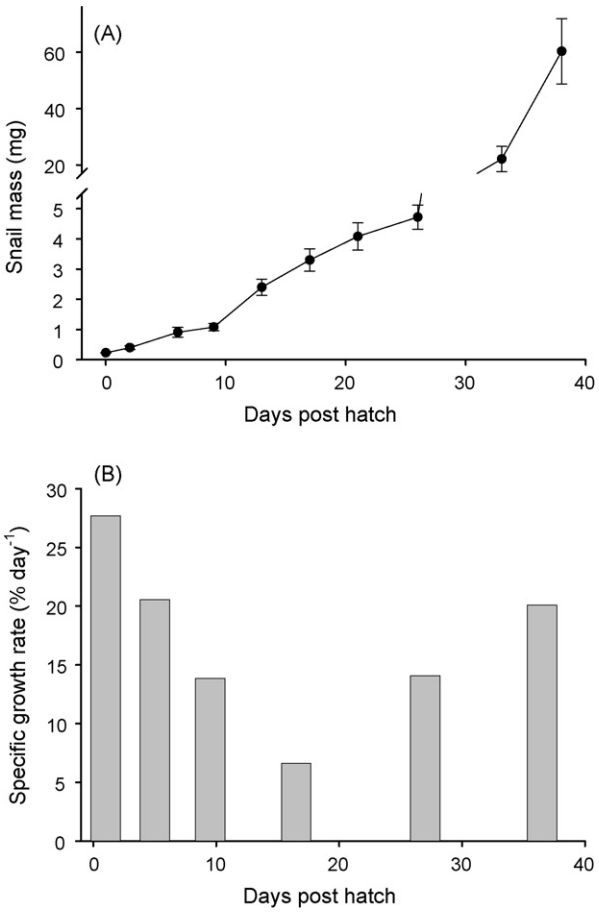


Fig. 10. (A) Change in mean weight (mg wet weight) of newly hatched *L. stagnalis* over 38 days. (B) Estimated specific growth rate (% per day) derived from data in A.

From our previous work (Grosell et al., 2006a,b), two important data points are available for comparison to the current study. At the end of the 30-day exposure, we previously observed a 47% reduction in growth in snails exposed to 4.3 μg l^{-1} Pb and a 90% growth reduction in snails exposed to 16.2 μg l^{-1} Pb. These exposure concentrations approximate the 2.9 and 18.9 μg l^{-1} Pb treatments in the current study. To evaluate the potential role of inhibited Ca^{2+} uptake on this growth effect we first consider that in the current study Ca^{2+} influx was reduced by 13.4% and 38.7% at the two Pb exposures, respectively. Assuming that inhibition of Ca^{2+} influx occurred within a few hours (Rogers and Wood, 2004), and that this inhibition was throughout the 30-day exposure period, the %inhibition of Ca^{2+} net flux in newly hatched snails can be calculated as

$$\frac{(Ca^{2+}_{in}\,I) - Ca^{2+}_{eff}}{Ca^{2+}_{net}} \quad (2)$$

where Ca^{2+}_{in} is the estimated Ca^{2+} influx rate of 8176 nmol g^{-1} h^{-1}, I is the fractional inhibition of Ca^{2+} influx, Ca^{2+}_{eff} is the efflux rate (assumed to be constant at 500 nmol g^{-1} h^{-1}) and Ca^{2+}_{net} is the net Ca^{2+} flux under control conditions. Using this formula, the reductions in net Ca^{2+} transport in the two treatments are 14.2% and 41.2%, respectively. Because a reduction in Ca^{2+} net transport is directly proportional to a reduction in the SGR, the inhibited SGR can be estimated as

$$\mathrm{SGR_{con}}(1 - \%\mathrm{inhibition}\ Ca^{2+}_{net}) \quad (3)$$

where SGR_{con} is the control SGR. In this case, the resulting inhibited SGRs are 14.3 and 9.8% per day for the 2.7 and 18.9 $\mu g\,l^{-1}$ Pb treatments, respectively. Starting with newly hatched 0.2 mg snails, the %reduction in snail mass relative to the control in these two treatments after a 30-day exposure is estimated to be 45% and 83%, respectively. This is in close agreement with measured growth inhibitions of 47% and 90% in comparable Pb concentrations, and we believe provides strong evidence for inhibition of Ca^{2+} influx being the driving factor for reduced growth in Pb-exposed snails.

5. Conclusions

Overall, the current study provides a mechanistic basis for the previously observed high sensitivity of freshwater pulmonate snails to Pb exposure. The central mechanism for this sensitivity is the extraordinarily high sensitivity of Ca^{2+} uptake pathways combined with very high Ca^{2+} demand of the snails which when inhibited by Pb, triggers a complex cascade of secondary ionoregulatory and acid–base disturbance. More importantly, inhibition of the net flux of Ca^{2+} appears to be directly proportional to the specific growth rate in newly hatched snails allowing for reasonable estimates of growth inhibition if effects on Ca^{2+} net flux are known. Further data development characterizing the relationship between Pb inhibition of Ca^{2+} net flux and water chemistry would provide an interesting opportunity to develop a mechanistic, physiologically based BLM for chronic Pb toxicity to the most sensitive organism tested to date.

Finally, as initially discussed, a major concern with current development of BLM-based water quality criteria is that they may not adequately characterize the responses of whole phyla that have not been considered in the development of these models. The results of this study suggest these concerns may be warranted. The inhibition of Ca^{2+} by Pb effectively controls chronic toxicity in *L. stagnalis*, likely other freshwater gastropods, and even perhaps mollusks in general. Therefore, from a BLM perspective, Ca^{2+} concentrations in the aquatic environment will be a major determining factor in the chronic toxicity of Pb to snails. This is a result of not only inhibition of Ca^{2+} uptake by Pb, but equally important, the effect of Ca^{2+} concentrations in the environment on Ca^{2+} net uptake and SGR in snails, independent of Pb (Mackie and Flippance, 1983; Madsen, 1987). In contrast, while ambient Ca^{2+} concentrations have a strong effect on acute Pb toxicity to fish and crustaceans, there is limited or no effect of ambient Ca^{2+} on chronic Pb toxicity to fish (Davies et al., 1976; Grosell et al., 2006a,b; Mager et al., 2008) or the daphnid *Ceriodaphnia dubia* (E. M. Mager, K. V. Brix, R. Gerdes and M. Grosell, unpublished data). Given this, application of a chronic Pb BLM based on these taxa is unlikely to be predictive or potentially protective of toxicity to freshwater snails which appear to be the most sensitive taxa in aquatic communities.

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