



Investigations into the mechanism of lead toxicity to the freshwater pulmonate snail, *Lymnaea stagnalis*

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ABSTRACT

The freshwater pulmonate snail, *Lymnaea stagnalis*, is the most sensitive aquatic organism tested to date for Pb with an estimated EC20 for juvenile snail growth of $3 \mu\text{g l}^{-1}$. A previous study supported the hypothesis that this hypersensitivity to Pb was due to an extremely high Ca^{2+} uptake rate needed to support shell formation. The current study sought to build upon this working hypothesis and develop a mechanistic predictive model for inhibition of snail growth as a function of Pb exposure. Initial experiments confirmed previous predictions that juvenile snails have net Ca^{2+} uptake rates of $7000\text{--}8000 \text{ nmol g}^{-1} \text{ h}^{-1}$, approximately 100-fold higher than observed in a typical freshwater fish. However, an initial time course study revealed that the onset of growth inhibition occurs at least 4 d prior to inhibition of net Ca^{2+} flux in Pb-exposed snails indicating the latter is not the primary mechanism of action. Qualitative observations during this experiment indicated snail feeding was inhibited in a dose-dependent manner. A subsequent experiment demonstrated that when food is withheld from snails for even 24 h, net Ca^{2+} uptake is significantly (~50%) reduced. A second time course study demonstrated quantitatively that snail feeding is inhibited by Pb exposure by up to 98% at relatively high Pb concentrations ($57 \mu\text{g l}^{-1}$) but no inhibition was observed at $\leq 10 \mu\text{g l}^{-1}$ Pb indicating feeding inhibition is not causing observed growth effects at concentrations approximating the EC20 of $3 \mu\text{g l}^{-1}$ Pb. A final experiment testing whether Pb-induced growth effects are related to inhibition of carbonic anhydrase activity in the snail mantle also failed to demonstrate an effect. We conclude that while both feeding and net Ca^{2+} uptake in snails are affected by Pb exposure, they appear to be secondary effects. The primary mechanism of action explaining *L. stagnalis* hypersensitivity to Pb remains to be identified.

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1. Introduction

Previous studies have demonstrated that members of the freshwater pulmonate snail genus *Lymnaea* are unusually sensitive to chronic waterborne lead (Pb) exposure (Borgmann et al., 1978; Grosell et al., 2006), with *Lymnaea stagnalis* being the most sensitive freshwater species tested to date with an estimated EC20 for growth of $<4 \mu\text{g l}^{-1}$, the lowest concentration tested (Grosell et al., 2006). Recent studies have also demonstrated that *L. stagnalis* is either the most sensitive or second most sensitive freshwater species tested in chronic exposures to Co, Cu and Ni (De Schampelaere et al., 2008; Schlekot et al., 2010; Brix et al., 2011). Given their high sensitivity to these metals in chronic exposures, it is important to explicitly consider freshwater gastropods in

revisions to water quality criteria (WQC) for metals including WQC based on the biotic ligand model (BLM).

Important in the development of any BLM is an understanding of the mechanisms underlying observed toxicity to a given organism. The biology of pulmonate snails such as *L. stagnalis* is different from most aquatic organisms used in BLM development in several important respects. First, as a pulmonate, *L. stagnalis* lacks gills and utilizes a combination of aerial and cutaneous respiration, with aerial respiration being the dominant mode (Jones, 1961). Lacking gills, pulmonate snails transport ions across the skin predominantly around the foot (Schlichter, 1982). The snail mantle is also a potentially important tissue as this is where Ca^{2+} and HCO_3^- are secreted for shell formation (Boer and Witteveen, 1980).

Lead is a Ca^{2+} antagonist in acute exposures to fish (Richards and Playle, 1998; Rogers et al., 2003) with inhibition of Ca^{2+} uptake and less pronounced inhibition of Na^+ and Cl^- uptake (Rogers et al., 2005) as the primary mechanism of action at high ($100\text{--}2000 \mu\text{g l}^{-1}$) Pb exposures. In contrast, the chronic toxicity of Pb to fish appears largely unrelated to disruption of Ca^{2+} homeostasis with developmental abnormalities (Davies et al., 1976;

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Holcombe et al., 1976) and neurotoxicological effects (Rademacher et al., 2005; Mager et al., 2008, 2010) being the primary mechanisms of action.

The mechanisms underlying acute and chronic Pb toxicity to invertebrates have not been extensively studied. Specific to chronic Pb exposures and *L. stagnalis*, we previously proposed that the hypersensitivity of *L. stagnalis* is driven by their high Ca²⁺ demand to support shell formation, particularly during early life stages when specific growth rates (SGR) are on the order of 15–20% d⁻¹ (Grosell and Brix, 2009). Exposure of adult *L. stagnalis* to 19 µg l⁻¹ Pb for 21 d resulted in a 39% inhibition of Ca²⁺ uptake, no effect on Ca²⁺ efflux, and no net Ca²⁺ uptake after accounting for diffusive loss. Significant declines in both hemolymph and soft tissue Ca²⁺ were also observed as well as a physiological cascade of ionoregulatory and acid–base balance disturbances. Notably, no inhibition of Na⁺ influx was observed in snails exposed to 19 µg l⁻¹ Pb (~5× the growth EC20) unlike in previous studies on fish that have shown effects on Na⁺ homeostasis, albeit at higher Pb concentrations (Rogers et al., 2005). This suggests that under these conditions the inhibition of Ca²⁺ uptake leads to reduced shell formation and, consequently, reduced growth. Based on these data, a simple model was developed to predict SGR in Pb-exposed snails:

$$\text{SGR}_{\text{Pb}} = \text{SGR}_{\text{con}} \times (1 - \% \text{ inhibition Ca}_{\text{net}}^{2+}) \quad (1)$$

where SGR_{Pb} is the predicted specific growth rate in Pb-exposed snails, SGR_{con} is the measured specific growth rate (% d⁻¹) in control animals and % inhibition Ca²⁺_{net} is fractional inhibition of net Ca²⁺ flux measured in Pb-exposed animals relative to the control. Data on inhibition of net Ca²⁺ flux available for adult snails exposed to 3 and 19 µg l⁻¹ Pb was highly predictive of 30-d growth in juvenile snails exposed to the same concentrations in a separate study (Grosell et al., 2006; Grosell and Brix, 2009).

Recently, Esbaugh et al. (2011, in press) characterized the acute and chronic toxicity of Pb in a series of natural waters collected across North America in order to provide data for parameterizing BLMs for several model test species including *L. stagnalis*. Concurrent with Esbaugh et al. (in press), the initial objective of the study described in this paper was to evaluate whether the mechanistic model described in Grosell and Brix (2009) could be used to predict chronic Pb toxicity to *L. stagnalis* in the natural waters being tested by Esbaugh et al. (in press). Specifically, we hypothesized that short-term (e.g., 24 h) exposure to Pb followed by measurements of net Ca²⁺ flux (where net Ca²⁺ flux equals the sum of Ca²⁺ influx (positive value) and Ca²⁺ efflux (negative value)) could be used to predict long-term (e.g., 30 d) impacts on snail growth.

The model developed in Grosell and Brix (2009) has several important assumptions that needed to be tested. First, it was assumed that Pb-induced inhibition of net Ca²⁺ uptake measured in adult snails also occurs to the same degree in juvenile (1–30 d old) snails used in standard chronic toxicity tests with this species. Second, the high net Ca²⁺ uptake for juvenile snails (7600 nmol g⁻¹ h⁻¹) estimated in Grosell and Brix (2009) was based on SGR and whole body Ca²⁺ concentrations rather than direct measurements. Finally, the model assumes that inhibition of net Ca²⁺ uptake by Pb is rapidly (<24 h) induced, most likely by blocking Ca²⁺ channels in the apical membrane of epithelial cells responsible for Ca²⁺ uptake from water (Rogers et al., 2003; Rogers and Wood, 2004).

The current study tested these assumptions and, based on observed results, undertook a series of additional experiments to explore the underlying mechanisms of chronic Pb toxicity to *L. stagnalis*.

2. Methods and materials

2.1. Experimental animals

Adult snails were obtained from an in-house culture maintained in flow-through dechlorinated City of Miami tap water ([Na⁺]=1.14, [Ca²⁺]=0.51, [Mg²⁺]=0.09, [Cl⁻]=1.03, [HCO₃⁻]=0.68 mmol l⁻¹, [DOC]=200 µmol l⁻¹, pH=7.8) at 23–25 °C. 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 before they were used in toxicity studies.

2.2. Characterization of age-dependent specific growth rates and net Ca²⁺ flux

This experiment was initiated with newly hatched (<24 h old; 0.35 mg mean wet weight) snails. Ten snails were initially held individually under static renewal conditions (100% water change daily) in 30 ml polypropylene cups with 20 ml of dechlorinated City of Miami tap water. At Day 20, snails were transferred to 250 ml containers with 200 ml of dechlorinated tap water, again under static renewal conditions. On Day 35, snails were transferred to 1 l containers under continuous flow-through conditions for the remainder of the experiment. Through Day 14, snails were fed a mix of sweet potato and lettuce and then a diet of only lettuce. Fresh food was provided daily and feeding rates were adjusted to ensure that snails always had access to food.

Net Ca²⁺ flux rates in individual snails were determined on Days 1, 2, 3, 5, 11, 15, 20, 28, 35, 42, 49, 56, and 70. Snails were placed in individual containers that ranged from 1.5 ml centrifuge tubes to 1 l beakers depending on the size of the snail. Snails were allowed to recover from handling for 10 min prior to flux initiation. Each container was gently aerated through polyethylene tubing and covered as appropriate for the container. At the onset of flux measurements, a water sample (0.2–2 ml depending on flux volume) was collected for measurement of Ca²⁺ concentration. After a total flux time of 2–8 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 to the nearest 0.01 mg on an analytical balance (Mettler, Toledo). The flux volume and duration for different sized snails were based on preliminary studies with the intent of ensuring that waterborne Ca²⁺ concentrations differed by at least ~50 µM Ca²⁺ over the flux period but did not drop below 200 µM (from an initial Ca²⁺ concentration of ~510 µM). A methods control experiment (without animals) demonstrated no loss of Ca²⁺ to test container walls or Ca–Pb interactions during an 8 h flux period.

Net Ca²⁺ flux rates were determined as the change in total Ca²⁺ concentration from the beginning to the end of the flux period, accounting for the duration of the flux period and the mass of the snail. Specific growth rates were determined using snail wet weights of whole snails (including shell) and calculated as previously described (Grosell and Brix, 2009).

2.3. Initial time course characterizing Pb inhibition of net Ca²⁺ flux

This experiment was initiated with juvenile snails (7–10 d old, 10.7 mg mean wet weight). Snails were exposed to nominal concentrations of 0, 6, 12.5, 25, 50 and 100 µg l⁻¹ Pb. Eight snails were exposed for each treatment individually in 250 ml polypropylene cups with 200 ml of water. Daily 100% water changes were performed with fresh test solutions. Test solutions were prepared 24 h prior to use to allow for equilibration. Net Ca²⁺ fluxes (in the presence of Pb) were performed on snails as described above

after 8 h, and 1, 2, 4 and 7 d of exposure. Water samples were collected at test initiation and on Days 1–4, and 7 for measurement of dissolved Pb (passed through 0.45 μM filter and acidified with trace metal grade HNO_3).

A second experiment was performed in which snails were exposed to higher Pb concentrations (0, 50, 100, 200, 400, 600 $\mu\text{g l}^{-1}$ Pb nominal) for 24 h after which net Ca^{2+} flux was determined. Water samples for measurement of dissolved Pb were collected at the beginning and end of this experiment.

2.4. Effects of starvation on net Ca^{2+} flux and low waterborne Ca^{2+} on snail feeding

Based on qualitative observations of reduced feeding by snails in the initial Pb time course experiment, a set of experiments were performed to evaluate the effect of starvation on net Ca^{2+} flux and the effects of low waterborne Ca^{2+} on snail feeding rates. In the starvation experiment, eight juvenile snails (~ 1 month old, 51 mg mean wet weight) were held individually in 250 ml containers with 200 ml of water under static renewal conditions. An initial measurement of net Ca^{2+} flux was performed (6 h duration) and then food was withheld for the remainder of the experiment (5 d) with daily measurements of net Ca^{2+} as previously described.

In a second experiment, juvenile snails (~ 6 weeks old, 169 mg mean wet weight) were exposed to either control conditions or an artificial water with the same ionic composition as dechlorinated tap water with Mg^{2+} (as MgSO_4) replacing Ca^{2+} ($[\text{Na}^+] = 1.12$, $[\text{Ca}^{2+}] = 0.002$, $[\text{Mg}^{2+}] = 0.62$, $[\text{K}^+] = 0.08$, $[\text{Cl}^-] = 0.63$, $[\text{HCO}_3^-] = 0.64$ mM, $[\text{DOC}] = 110 \mu\text{M}$, $\text{pH} = 7.8$). Eight snails were exposed to control conditions, or “ Ca^{2+} -free” water, for 5 d. Daily water changes were performed and snails were fed daily as previously described. Water samples were collected daily for measurement of Ca^{2+} in both the control and “ Ca^{2+} -free” water.

At the end of the exposure period, lettuce ingestion rates were measured in each snail. Preliminary experiments were performed to evaluate how to best characterize ingestion rates. Simple wet weight measurements of lettuce before and after a feeding period were found inaccurate due to variable water absorption by lettuce in test containers (data not shown). Instead, the relationship between lettuce wet weight (prior to being placed in test containers) and area was determined. Area of lettuce was determined by photographing the lettuce and digitally analyzing the image using the free area analysis software Image J (available at <http://rsb.info.nih.gov/ij>). For the feeding rate experiment, individual pieces of lettuce were photographed and then placed with snails. Snails were allowed to feed on the lettuce for 24 h, after which the lettuce was removed and re-imaged. Differences in area before and after were then used to estimate the mass of lettuce consumed (see Supplemental file for further details and example results).

2.5. Definitive time course experiment of Pb inhibition of net Ca^{2+} flux and feeding rates

Based on results from experiments described above, a second definitive time course experiment was performed to characterize Pb inhibition of net Ca^{2+} flux rates along with concurrent measurements of snail feeding rates over the course of a 16-d exposure. Total ammonia (T_{amm}) and urea flux rates were also measured at the end of the exposure as a potential indicator of snail metabolic rates. This experiment was initiated with juvenile snails (10–14 d old, 22.6 mg mean wet weight). Snails were exposed to nominal concentrations of 0, 6, 12.5, 25, 50 and 100 $\mu\text{g l}^{-1}$ Pb. Eight snails were exposed for each treatment individually in 250 ml polypropylene cups with 200 ml of water. Daily 100% water changes were performed with

fresh test solutions. Test solutions were prepared 24 h prior to use to allow for equilibration.

Net Ca^{2+} fluxes (in the presence of Pb) were measured on snails as described above after 4, 8, 12 and 16 d of exposure using the methods previously described. Snail feeding rates were characterized on Days 8 and 16 using the same methods described earlier. On Day 16, initial and final water samples were collected for analysis of T_{amm} and urea at the same time as those collected for the characterization of net Ca^{2+} flux. Water samples were also collected approximately every other day to measure dissolved Pb in each treatment ($n = 11$ per treatment).

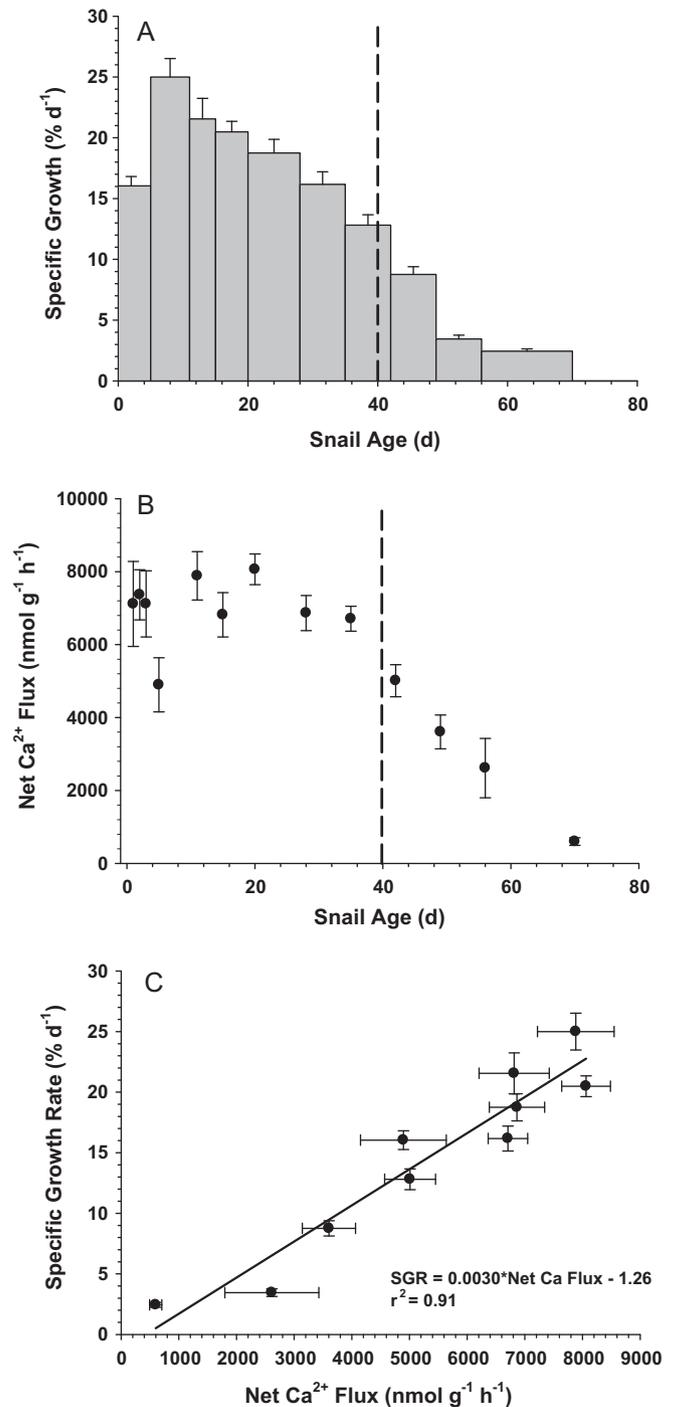


Fig. 1. Age-dependent *L. stagnalis*: (A) growth, (B) net Ca^{2+} flux, and (C) the correlation between growth and net Ca^{2+} flux. The vertical dashed line in A and B indicates the onset of reproduction. Data presented as mean \pm SEM ($n = 8$).

2.6. Effect of Pb on carbonic anhydrase activity in snail mantle

A final hypothesis we tested was that Pb either directly or indirectly inhibited carbonic anhydrase (CA) activity in the snail mantle (the primary tissue responsible for shell formation), which would in turn affect shell formation, growth and the animals' demand for Ca^{2+} (Ebanks et al., 2010a; Brix et al., 2011). In this experiment, juvenile snails (200–400 mg wet weight) were exposed to either 0 or $10 \mu\text{g l}^{-1}$ Pb (nominal) for 7 d ($n=12$ per treatment). Water was changed and snails were fed daily as previously described. At the end of the exposure period, the snail mantle was excised and flash frozen in liquid N_2 for subsequent analysis of CA activity. Two snail mantles were pooled for each assay replicate ($n=6$) in order to obtain sufficient biomass for the analysis.

Carbonic anhydrase activity was measured using the electrometric delta pH method (Henry, 1991). The reaction medium consisted of 2.5 ml of buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris base; Sigma, MO, USA) kept at 4°C . The reaction was started by adding $100 \mu\text{l}$ of CO_2 saturated Milli-Q water using a gas tight Hamilton syringe. The reaction rate was measured over a pH change of 0.15 units (+10 mV). To calculate the true catalyzed reaction rate, the uncatalyzed reaction rate was subtracted, and the buffer capacity of the reaction medium was used to convert the rate from mV into mol H^+ per unit time. The pH was measured using a PHC4000 combined pH electrode (Radiometer Analytical,

Lyon, France) attached to a PHM220 lab pH meter (Radiometer Analytical, Lyon, France). Mantle tissue (14–36 mg) was homogenized (4 ml reaction buffer for 1 g tissue) on ice using a motor driven homogenizer, and briefly centrifuged (1 min \times 10,000 rpm) to pellet cellular debris. In all cases, duplicate assays were performed with $30 \mu\text{l}$ of tissue homogenate used per assay. All results were normalized to total protein levels as detected using the Bradford assay (Sigma) with bovine serum albumin standards and measured using a plate spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.7. Analytical chemistry

Water samples for determination of Pb exposure concentrations were passed through a $0.45 \mu\text{m}$ 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) with a practical quantitation limit of $0.25 \mu\text{g l}^{-1}$ Pb.

Concentrations of Ca^{2+} in water samples were determined by atomic absorption spectrophotometry (VarianAA 220FS, Mulgrave, Victoria, Australia) with a practical quantitation limit of $2 \mu\text{M Ca}^{2+}$. Total ammonia in water was measured by a micro-modified colorimetric method (Verdouw et al., 1978). Urea in water was measured using the method of Rhamatullah and Boyde (1980).

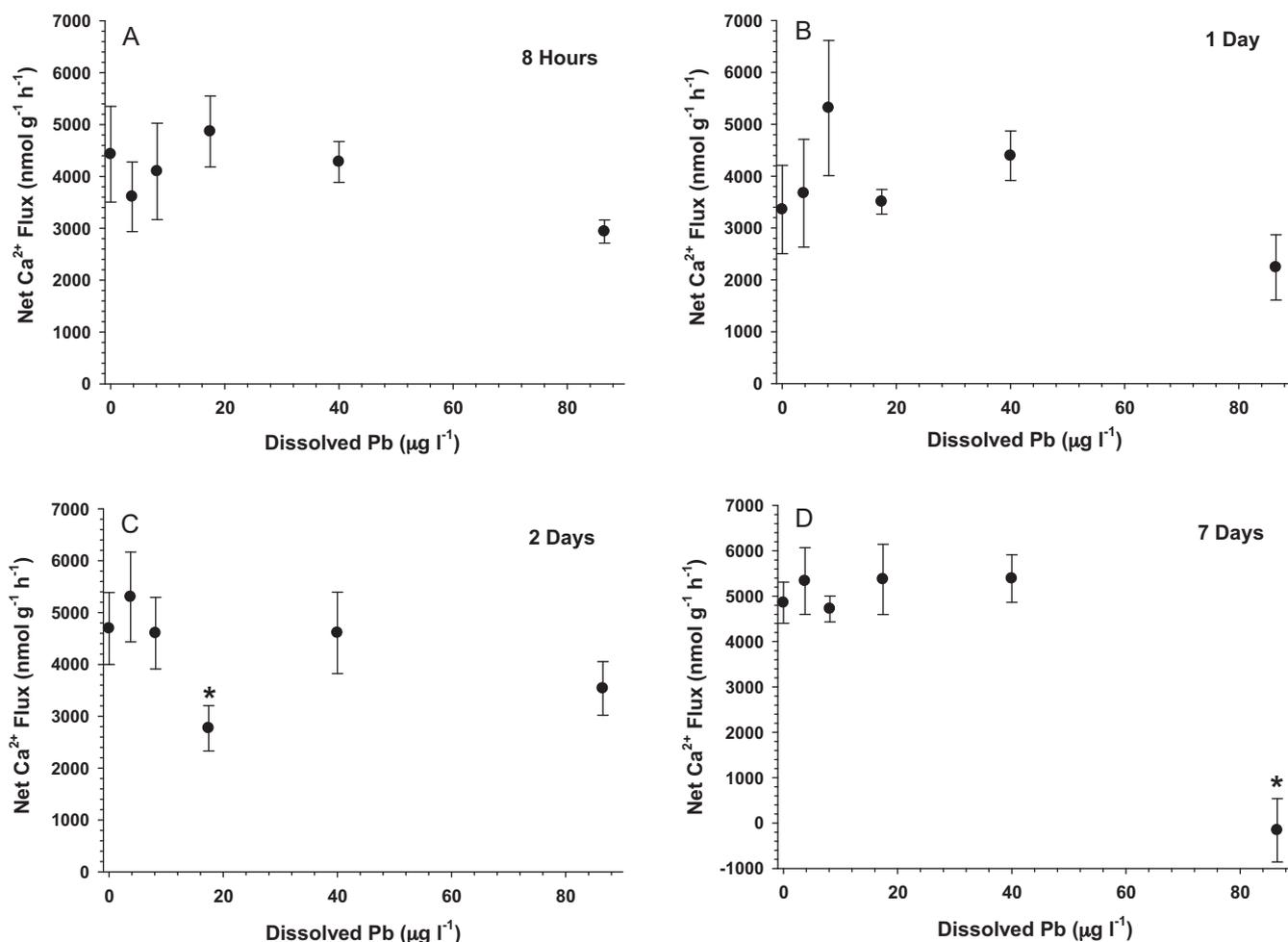


Fig. 2. Effect of Pb on net Ca^{2+} flux in juvenile *L. stagnalis* after: (A) 8 h, (B) 1 d, (C) 2 d, and (D) 7 d of exposure. Data presented as mean \pm SEM ($n=8$). *Statistically significant difference from the control ($p \leq 0.05$).

2.8. Data analysis

All analyses were performed on measured test concentrations. Data are presented as mean \pm SEM and $n=8$ in all cases except for the CA assay, where $n=6$. The effect of Pb on snail growth ($EC_{20} \pm 95\%$ CI) was estimated using the linear interpolation method (USEPA, 2002). Statistical evaluation of data from physiological studies consisted of Student's two-tailed t -tests and results were considered statistically different at $p \leq 0.05$. Chronic toxicity data were analyzed using ToxCalc (ToxCalc, 1996). All other analyses were performed using SigmaStat (SPSS, 2006).

3. Results

3.1. Age-dependent specific growth rates and net Ca^{2+} flux

As has been previously characterized, juvenile snail SGRs were relatively high on the order of 16 – 25% d^{-1} . Initial SGRs for the first 5 d post-hatch were $16 \pm 0.8\%$ d^{-1} , increased to $25 \pm 1.5\%$ d^{-1} over the next 5 d, and gradually declined until the end of the study (Fig. 1a). The initial increase in SGR corresponded with snails switching feeding substrates from primarily sweet potato to a primarily lettuce diet (both were provided to snails). Observations suggest snails feed on bacterial growth on the sweet potato surface during early development, as snail radulae are not sufficiently large to masticate lettuce leaf until snails are ~ 6 d old. Snails began laying egg masses at 40 d and SGR rapidly declined to $\sim 3\%$ d^{-1} after that point. Corresponding net Ca^{2+} fluxes were ~ 7000 – 8000 $nmol\ g^{-1}\ h^{-1}$ through the first 20 d of snail development, confirming our earlier hypothesis regarding net Ca^{2+} flux rates in juvenile snails (Grosell and Brix, 2009). Similar to SGR, net Ca^{2+} flux rates gradually declined as the snails aged, dropping to 599 $nmol\ g^{-1}\ h^{-1}$ in 70-d old snails (Fig. 1b). A strong correlation ($r^2=0.91$) between SGR and net Ca^{2+} flux rates was observed over the course of the experiment (Fig. 1c).

3.2. Initial time course characterizing Pb inhibition of net Ca^{2+} flux

The initial time course study revealed no significant effect of dissolved Pb on net Ca^{2+} uptake in juvenile snails after 1 d of exposure (Fig. 2). A statistically significant ($p < 0.05$) effect was observed after 2 d exposure, but this was a relatively modest effect and occurred only in an intermediate Pb treatment ($17\ \mu g\ l^{-1}$ Pb) and not at higher Pb concentrations (Fig. 2c). A strong concentration–response relationship was not observed until 7 d of exposure when net Ca^{2+} flux was -160 $nmol\ g^{-1}\ h^{-1}$ (i.e., net Ca^{2+} loss) in the highest Pb treatment ($87\ \mu g\ l^{-1}$ Pb), but no significant effects were observed in any of the lower Pb treatments (Fig. 2d).

A second experiment exposing juvenile snails to higher Pb concentrations for 24 h led to results relatively consistent with the previous experiment. After 24 h exposure, net Ca^{2+} uptake was moderately (37%) but significantly inhibited at $69\ \mu g\ l^{-1}$ Pb. In contrast, after 24 h in the first experiment, net Ca^{2+} uptake was inhibited by 33% at $87\ \mu g\ l^{-1}$ Pb, but the inhibition was not statistically significant. Higher concentrations of dissolved Pb (173 and $259\ \mu g\ l^{-1}$) led to increasing net Ca^{2+} uptake inhibition (61% and 85%) after 24 h exposure in the second experiment (Fig. 3).

3.3. Effects of starvation on net Ca^{2+} flux and low waterborne Ca^{2+} on snail feeding

Starvation was shown to have a rapid and significant effect on net Ca^{2+} uptake in snails (Fig. 4a). After only 1 d without food, snail net Ca^{2+} uptake rates were reduced by 40% ($p < 0.05$). A similar reduction occurred at 2 d, and the rates continued to decline up

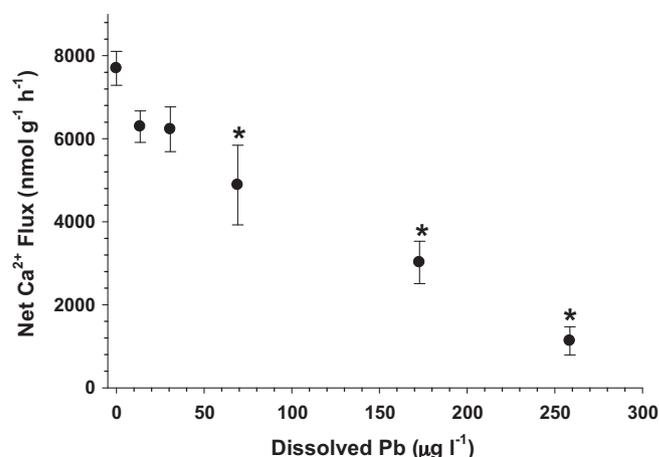


Fig. 3. Effect of high Pb concentrations on net Ca^{2+} flux in juvenile *L. stagnalis* after 24 h of exposure. Data presented as mean \pm SEM ($n=8$). *Statistically significant difference from the control ($p \leq 0.05$).

until 5 d, when the experiment was terminated. After 5 d without food, net Ca^{2+} uptake rates were reduced by 98% in starved snails. In the experiment exposing snails to “ Ca^{2+} -free” water, measured Ca^{2+} concentrations averaged $15\ \mu M$ over the course of the experiment due to net diffusive loss of Ca^{2+} into the water by the snails. Snail feeding rates were 380 ± 48 and 368 ± 54 $mg\ g^{-1}\ d^{-1}$ in the

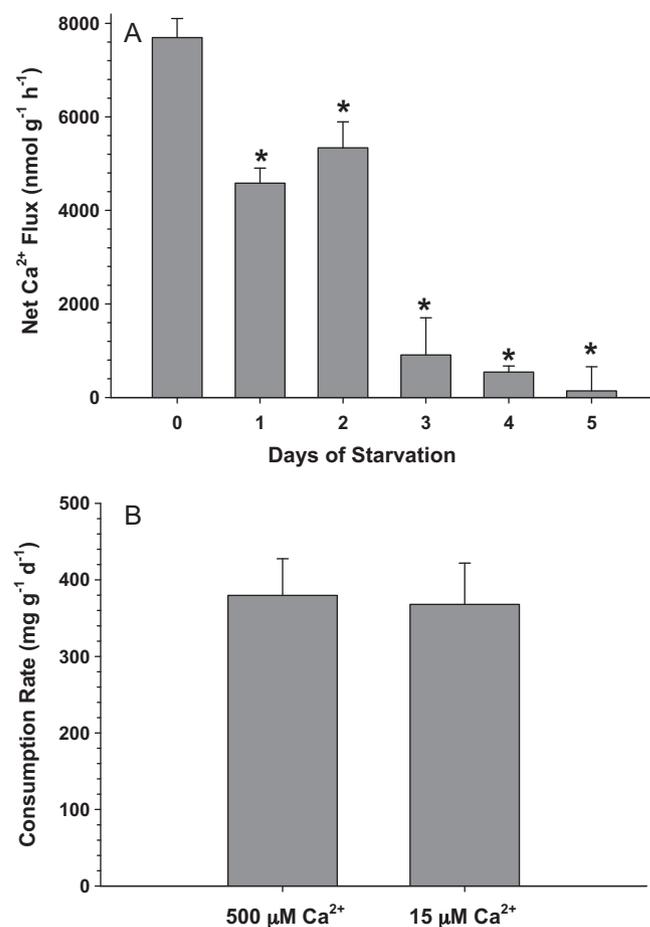


Fig. 4. (A) Net Ca^{2+} flux in juvenile *L. stagnalis* as a function of days of starvation and (B) Effect of 96 h exposure to low waterborne Ca^{2+} on food consumption rate. Data presented as mean \pm SEM ($n=8$). *Statistically significant difference from the control ($p \leq 0.05$).

control and “Ca²⁺-free” water, indicating that the inability to take up Ca²⁺ from water does not affect snail feeding rates (Fig. 4b).

3.4. Definitive time course experiment of Pb inhibition of net Ca²⁺ flux, feeding rates, and ammonia excretion

The definitive time course studied revealed a moderate effect of Pb on juvenile snail growth after 4 d of exposure with the severity of growth inhibition increasing after 8 d, where it reached an asymptote through the end of the exposure at 16 d (Fig. 5). The estimated EC₂₀ at 8 d of exposure was 3.2 μg l⁻¹ Pb and 3.5 μg l⁻¹ Pb after 16 d

of exposure (Fig. 5e), similar to those previously estimated in 14-d and 30-d exposures (Grosell et al., 2006; Esbaugh et al., in press).

Consistent with the initial time course experiment, no significant effects on net Ca²⁺ uptake were observed in snails after 4 d of exposure, although there was a statistically insignificant 50% reduction in net Ca²⁺ uptake in the highest treatment (Fig. 6a). After 8 d of exposure, a relatively strong dose–response relationship was observed for net Ca²⁺ flux, with significant effects ($p < 0.05$) at 24 and 57 μg l⁻¹ Pb (Fig. 6b). The concentration–response relationship was less pronounced after 12 d of exposure (Fig. 6c), and by 16 d of exposure net Ca²⁺ uptake was comparable to the control

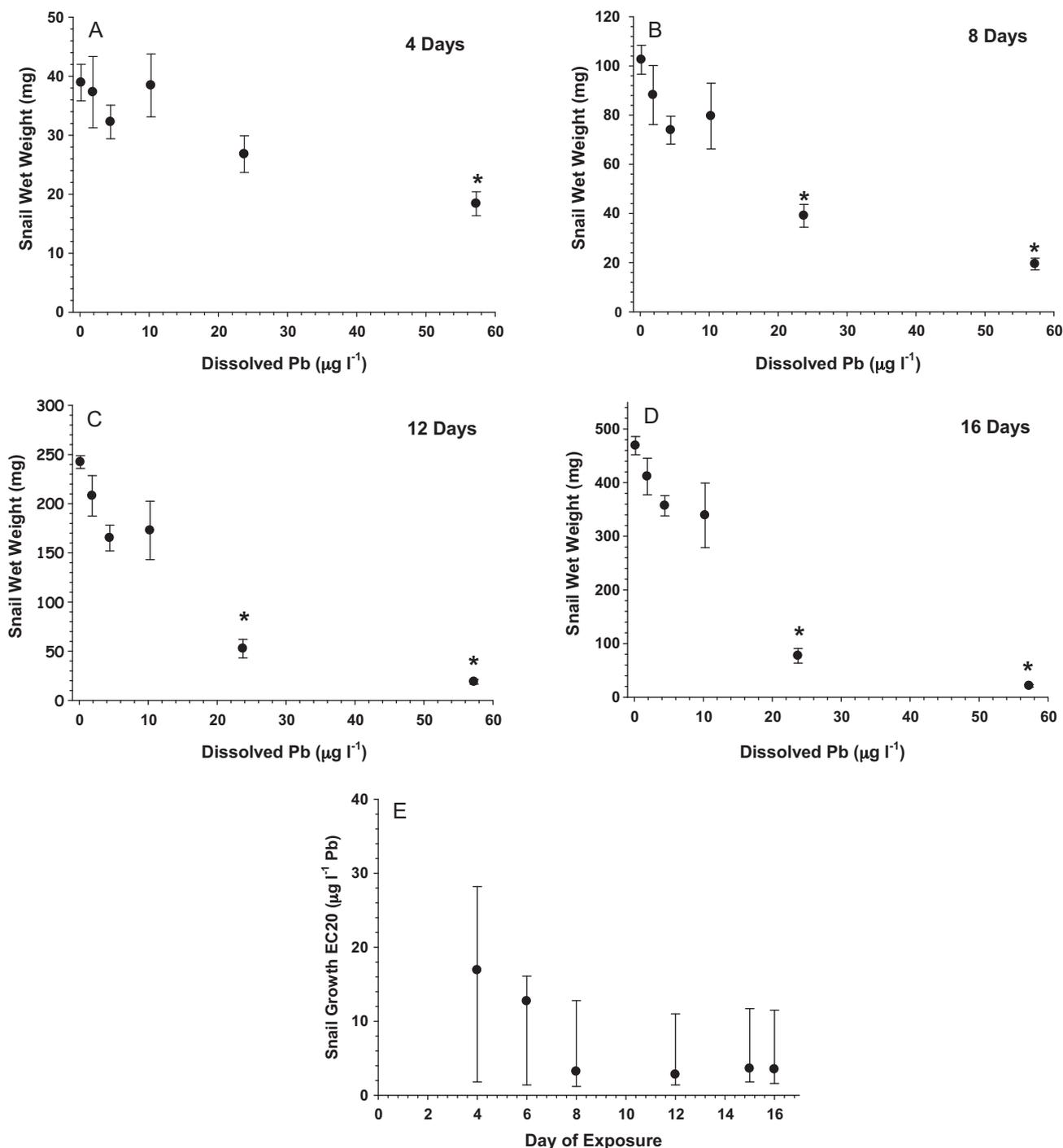


Fig. 5. Effect of Pb exposure on juvenile *L. stagnalis* growth after: (A) 4 d, (B) 8 d, (C) 12 d and (D) 16 d of exposure. (E) Estimated EC₂₀s for snail growth as a function of exposure to Pb (error bars = 95% CI). Data presented as mean ± SEM ($n = 8$). *Statistically significant difference from the control ($p < 0.05$).

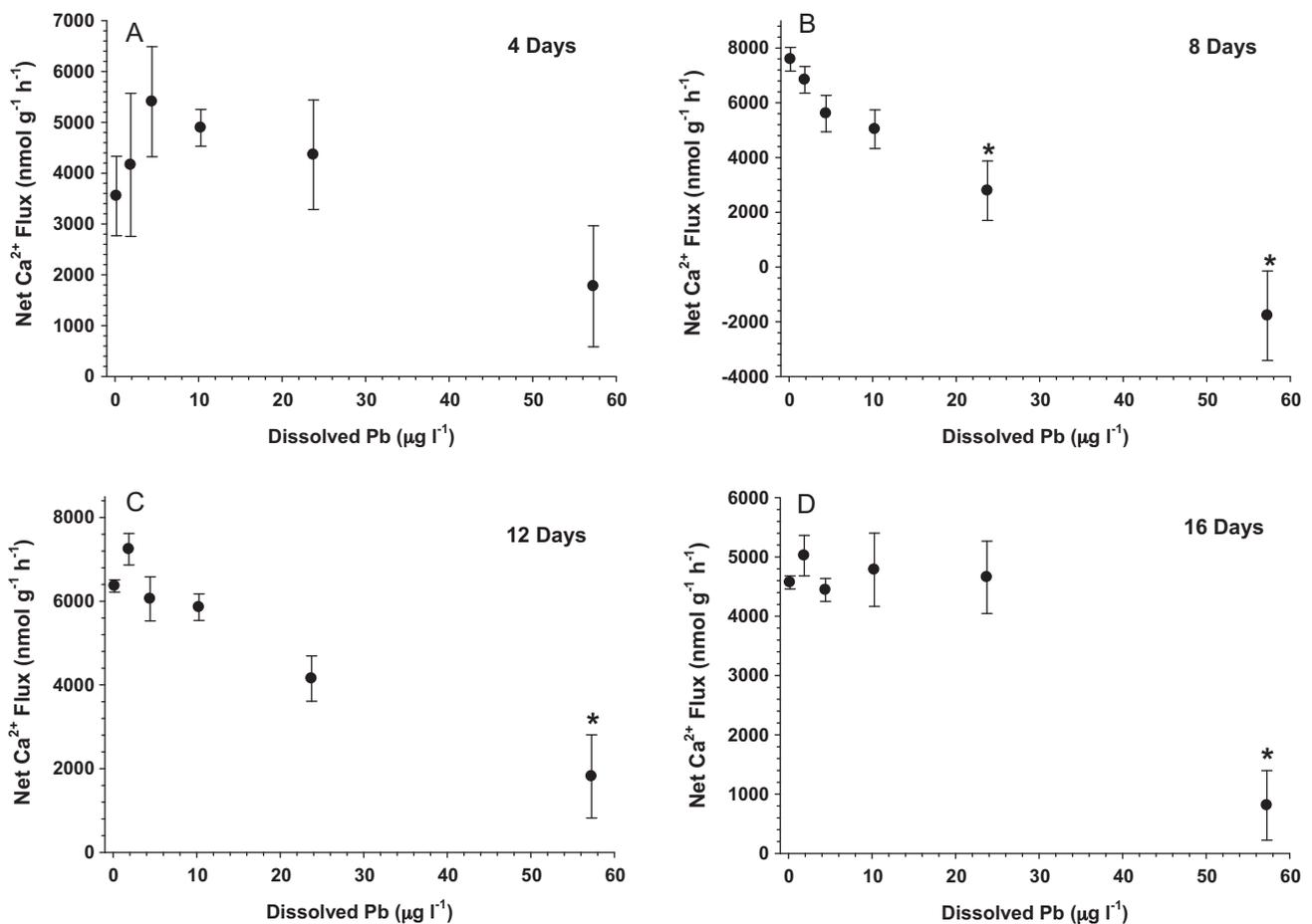


Fig. 6. Effect of Pb exposure on juvenile *L. stagnalis* net Ca²⁺ flux after: (A) 4 d, (B) 8 d, (C) 12 d and (D) 16 d of exposure. Data presented as mean \pm SEM ($n = 8$). *Statistically significant difference from the control ($p \leq 0.05$).

in all treatments except the highest ($57 \mu\text{g l}^{-1}$) Pb, where an 82% reduction ($p < 0.05$) was observed (Fig. 6d).

Food consumption rates during this experiment were monitored at 8 and 16 d of exposure. After 8 d, food consumption rates exhibited a concentration–response relationship mirroring that of the corresponding dose response for net Ca²⁺ flux, with significant feeding inhibition (47% and 94% reduction relative to control) at 24 and $57 \mu\text{g l}^{-1}$ Pb (Fig. 7a). Unlike net Ca²⁺ flux, feeding rates decreased slightly further after 16 d in the two highest treatments (Fig. 7b).

Ammonia excretion rates after 16 d showed a biphasic pattern, with excretion increasing in a concentration-dependent manner up to $24 \mu\text{g l}^{-1}$ Pb and then decreasing at $57 \mu\text{g l}^{-1}$ Pb, although no treatment was statistically different from the control (Fig. 8). There was no detectable urea excretion in any of the treatments (data not shown).

3.5. Effect of Pb on carbonic anhydrase activity in snail mantle

Exposure of young adult snails to $9 \mu\text{g l}^{-1}$ Pb (approximately the EC₅₀ for growth) for 7 d had no significant effect on carbonic anhydrase activity in the snail mantle (Fig. 9).

4. Discussion

The objective of the current study was to test several key assumptions of the toxicity model we previously developed to predict chronic Pb toxicity to *L. stagnalis* (Grosell and Brix, 2009). This included assumptions regarding net Ca²⁺ uptake rates in juvenile

snails and the time course of Pb inhibition of net Ca²⁺ flux. Consistent with our model assumptions, we were able to demonstrate that juvenile *L. stagnalis* do have extraordinarily high rates of net Ca²⁺ uptake, on the order of $7000\text{--}8000 \text{ nmol g}^{-1} \text{ h}^{-1}$. This contrasts to a Ca²⁺ influx rate of $60\text{--}100 \text{ nmol g}^{-1} \text{ h}^{-1}$ in a typical fish such as *Oncorhynchus mykiss* (Rogers et al., 2003). Specific growth rates for juvenile snails reached a maximum of $25\% \text{ d}^{-1}$ and then steadily declined over time. This pattern in SGR differs from our previous observations, in which SGR was initially high ($28\% \text{ d}^{-1}$), declined until snails were ~ 20 d old and then increased again (Grosell and Brix, 2009). The high SGRs observed in this and our previous study contrast with that of other researchers. For example, De Schampelaere et al., 2008 observed a maximum SGR of $9\% \text{ d}^{-1}$ in a chronic toxicity study on Co. There are several methodological differences (e.g., frequency of water changes, feeding rates) that likely explain observed differences in SGR.

Despite the strong correlation observed between net Ca²⁺ uptake rates and SGR (Fig. 1c), results from the initial time course study suggested that Pb inhibition of Ca²⁺ uptake may not be the primary mechanism of action for observed reductions in snail growth. Inhibition of net Ca²⁺ flux was only observed at 7 d exposure to $87 \mu\text{g l}^{-1}$ Pb, a concentration far higher than the EC₂₀ for growth. The second experiment with higher Pb concentrations showed inhibition of Ca²⁺ net uptake within 24 h at concentrations ranging between 70 and $259 \mu\text{g l}^{-1}$ Pb. Effects on net Ca²⁺ uptake at higher Pb concentrations are more consistent with observations on *O. mykiss*, where Pb inhibited Ca²⁺ uptake within 12 h when exposed to $290 \mu\text{g l}^{-1}$ Pb (Rogers and Wood, 2004), but suggests *L. stagnalis* is somewhat more sensitive than *O. mykiss* in

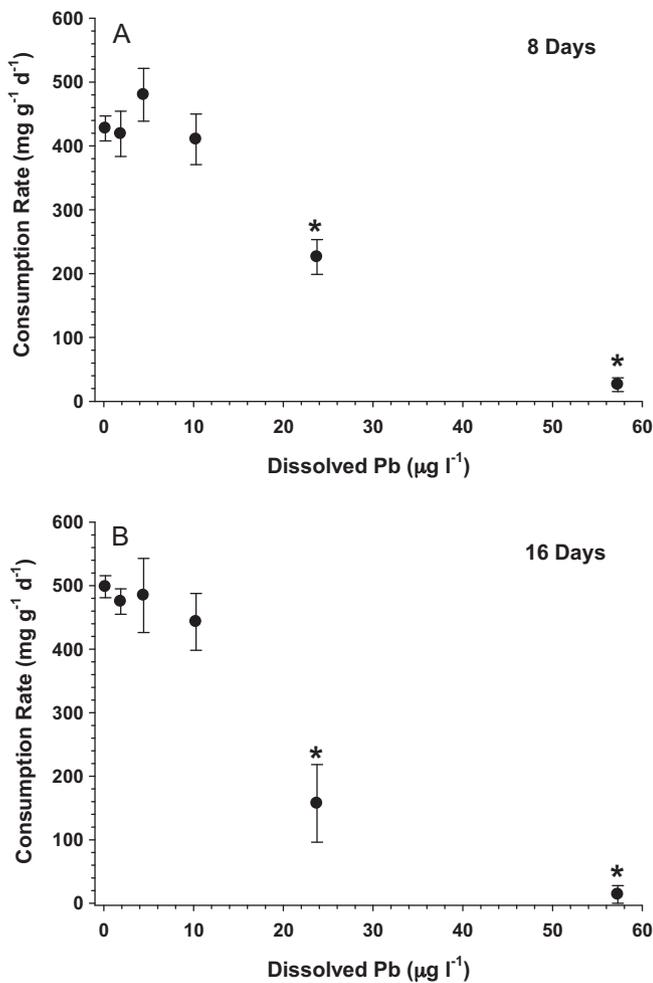


Fig. 7. Effect of Pb exposure on juvenile *L. stagnalis* food consumption rate after: (A) 8 d and (B) 16 d of exposure. Data presented as mean \pm SEM ($n=8$). *Statistically significant difference from the control ($p \leq 0.05$).

this regard. Importantly, the current data invalidate our previous model assumption that Ca^{2+} net uptake inhibition occurs relatively rapidly (within 24 h) at Pb concentrations approximating the EC20 ($3 \mu\text{g l}^{-1}$) as might be expected if Pb were blocking Ca^{2+} uptake at an apical Ca^{2+} channel or $\text{Ca}^{2+}:\text{H}^{+}$ exchanger (Ebanks et al., 2010a).

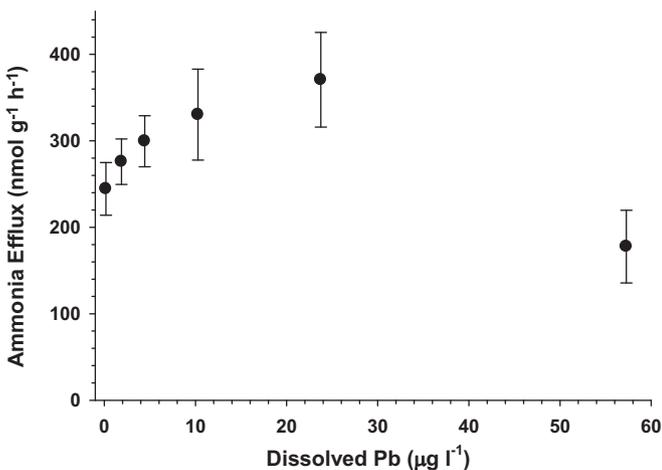


Fig. 8. Effect of Pb exposure on juvenile *L. stagnalis* total ammonia efflux after 16 d of exposure. Data presented as mean \pm SEM ($n=8$). *Statistically significant difference from the control ($p \leq 0.05$).

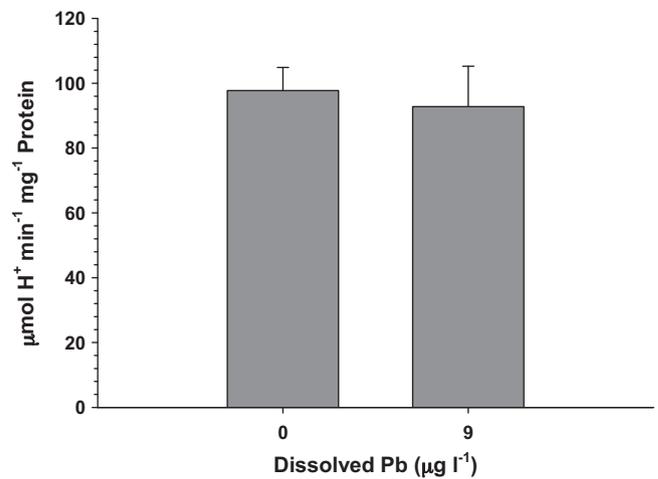


Fig. 9. Carbonic anhydrase activity in the mantles of *L. stagnalis* after exposure to $9 \mu\text{g l}^{-1}$ Pb for 7 d. Data presented as mean \pm SEM ($n=6$). *Statistically significant difference from the control ($p \leq 0.05$).

An alternative explanation for the mechanism of action at low Pb concentrations is that Pb is inhibiting a basolateral Ca^{2+} -ATPase. This might explain the delay in inhibition observed in the initial time course study. Rogers and Wood (2004) were able to demonstrate a significant inhibition in Ca^{2+} -ATPase activity in *O. mykiss* exposed to $1140 \mu\text{g l}^{-1}$ Pb after 96 h exposure. While species-specific differences in sensitivity are possible, given the Ca^{2+} -ATPase in *O. mykiss* is less sensitive to Pb than the apical Ca^{2+} channel, we considered this an unlikely possibility. Rather, qualitative observations during the initial time course study suggested juvenile snails were consuming less lettuce in a concentration-dependent manner. Given this observation and the obvious link between feeding and growth, we pursued the hypothesis that Pb was inhibiting the feeding response in snails leading to growth reduction and that observed inhibitions of net Ca^{2+} uptake were a secondary effect. This hypothesis was supported by a previous experiment by De With (1980) who observed that starved snails had reduced Ca^{2+} concentrations in the hemolymph and soft tissues, and increased hemolymph HCO_3^{-} , Cl^{-} and pH. All of these observations are consistent with those in Pb-exposed snails and attributed to a physiological cascade prompted by inhibition of Ca^{2+} uptake (Grosell and Brix, 2009).

Initial experiments supported this hypothesis. A simple experiment in which snails were starved revealed a 40% reduction in net Ca^{2+} flux within 24 h and 98% reduction after 5 d without food (Fig. 4a). We also demonstrated that an inability to take up Ca^{2+} from the water due to low environmental Ca^{2+} did not reduce snail feeding rates (Fig. 4b). This suggested that any direct inhibition of Ca^{2+} uptake by Pb, either apically or basolaterally, would not result in reduced feeding rate because of insufficient Ca^{2+} to accommodate shell growth. The significant reduction in net Ca^{2+} flux after 24 h without food is also worth noting from a methodological perspective. It is often standard practice in various physiological and toxicological studies not to feed animals for 24–48 h prior to experimentation. It should be noted that in the case of *L. stagnalis* and likely other herbivorous gastropods, this will result in significant changes in both ionoregulatory and acid–base status which would probably not be considered the “typical” physiological state of the animal.

Results from the detailed time course experiment revealed a relatively complex pattern of effects. The lack of any inhibition of net Ca^{2+} uptake after 4 d exposure to $\leq 24 \mu\text{g l}^{-1}$ Pb, despite an EC20 for growth of $18 \mu\text{g l}^{-1}$ at this time point, provides further evidence that inhibition of Ca^{2+} uptake is not the primary mechanism of

toxicity. Similarly, while inhibition of snail feeding showed a strong concentration–response relationship after 8 and 16 d of exposure, there was no indication that snails were feeding at reduced rates in Pb treatments approximating the EC20 for growth. Given that net Ca^{2+} flux rates at 16 d were comparable to the control in the $24 \mu\text{g l}^{-1}$ Pb treatment, despite a 47% reduction in feeding rate, suggests that this level of feeding inhibition does not affect net Ca^{2+} flux and that reduced feeding likely only contributed to reduced net Ca^{2+} flux in the highest Pb treatment ($57 \mu\text{g l}^{-1}$ Pb).

We also measured ammonia and urea excretion rates after 16 d exposure to Pb as a possible indicator of protein metabolism, which would presumably be linked to any observed inhibition of feeding. The biphasic pattern observed in ammonia excretion as a function of Pb exposure (Fig. 8) does not provide any clear evidence that protein catabolism is significantly reduced even when snail feeding is inhibited by 98%. It is also not readily apparent why ammonia excretion rates tend to increase relative to the control except in the highest treatment.

Reduction in ammonia excretion in the highest Pb treatment may be the result of the near complete inhibition of feeding in this treatment. Additionally, in *L. stagnalis*, Ca^{2+} uptake at the apical membrane is via a voltage dependent calcium channel and possibly via an electrogenic $\text{Ca}^{2+}/2\text{H}^{+}$ exchanger with an apical H^{+} -ATPase providing the electrogenic motive force (Ebanks et al., 2010a). Excretion of these protons may facilitate ammonia excretion through proton trapping (Wilkie, 1997). Hence, the elimination of net Ca^{2+} uptake in the highest Pb concentration and consequent reduction in acid excretion may also contribute to the drop in ammonia excretion in the high Pb treatment.

Measurements in the current experiment did not detect any urea excretion. This differs from previous studies which indicate ~30% of N excretion in *L. stagnalis* is as urea (Friedl and Bayne, 1966; Bayne and Friedl, 1968). Previous measurements in our lab (unpublished data) on adult snails are consistent with these studies and suggest there may be a developmental shift in modes of N excretion as snails age.

The inhibition of Ca^{2+} uptake may also be a secondary effect in response to the direct effect of Pb on some other protein or system in the snail. We considered this possibility and hypothesized that inhibition of Ca^{2+} uptake may have been the result of a direct inhibition of CA in the snail mantle (Rogers et al., 2005). Carbonic anhydrase in the snail mantle hydrates CO_2 to form HCO_3^- and H^+ (Freeman, 1960). The HCO_3^- is presumably utilized for shell formation (CaCO_3) while the proton may be exchanged apically for calcium (Ebanks et al., 2010b). Hence, inhibition of CA would not only eliminate the need for Ca^{2+} for shell growth, but would also significantly reduce the availability of H^+ to drive Ca^{2+} uptake across the apical membrane. While this hypothesis seemed plausible, we were unable to detect any inhibition of CA in the snail mantle of Pb-exposed animals (Fig. 9). This suggests inhibition of CA is not the primary mechanism of action of Pb toxicity, although issues with dilution of tissue homogenates in performing this assay have been previously noted (Brix et al., 2011).

5. Conclusions

The current study indicates that our previous hypothesis that *L. stagnalis* is hypersensitive to Pb because of its extremely high Ca^{2+} demand is most likely incorrect. Clearly, net Ca^{2+} flux, snail feeding rates, and snail growth are all interlinked. Inhibition of net Ca^{2+} uptake is observed at concentrations ~8-fold higher than the EC20 for growth. At Pb concentrations ~20-fold higher than the EC20 for snail growth, Pb is inhibiting snail feeding, which leads to reduced net Ca^{2+} uptake. At slightly higher concentrations, there appears to be rapid (within 24 h) direct inhibition of Ca^{2+} uptake.

However, the current study suggests that at concentrations approximating the EC20 for snail growth, neither of these processes is the primary mechanism of action. This conclusion is consistent with the recent study by Esbaugh et al. (in press), which demonstrated varying waterborne Ca^{2+} concentrations had no effect on chronic Pb toxicity to *L. stagnalis* which might be expected if inhibition of Ca^{2+} uptake was the primary mechanism of action.

Additional hypotheses regarding the primary mechanism(s) of action are entirely speculative at this point. However, we can make the following observations. First, juvenile growth in *L. stagnalis* is sensitive to several different metals (Co, Cu, Pb, Ni) three of which (Co, Cu, Pb) are known to disrupt Ca^{2+} metabolism (Ni has not been studied in this respect) (De Schampelaere et al., 2008; Grosell and Brix, 2009; Brix et al., 2011). It is worth noting that Cu is generally not considered a Ca^{2+} antagonist (Wood, 2001). Surprisingly, *L. stagnalis* is relatively insensitive in chronic Zn exposures (De Schampelaere and Janssen, 2010), despite Zn being a well known Ca^{2+} antagonist (Wood, 2001). Second, all four metals to which *L. stagnalis* is sensitive (Co, Cu, Pb, and Ni) are not regulated (i.e., accumulate significantly) in *Lymnaea* (Amiard and Amiard-Triquet, 1979; Pyatt et al., 1997, 2003; Croteau and Luoma, 2009), while the considerably less toxic Zn is regulated (Desouky et al., 2003). Finally, in addition to the current study for Pb, Co and Cu have also been shown to inhibit feeding in *L. stagnalis* (De Schampelaere et al., 2008; Das and Khangarot, 2011), but has not been evaluated for Ni or Zn. Together, these observations suggest the potential for a common mechanism of action for the metals to which *L. stagnalis* is sensitive. One possible mechanism of action consistent with these attributes is neuroendocrine disruption (Geraerts, 1992; Li and Geraerts, 1992; Lagadic et al., 2007). Although speculative, we suggest this may be a mechanism worth investigation in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.11.007.

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