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Effects of sodium chloride exposure on ion regulation in larvae (glochidia) of the freshwater mussel *Lampsilis fasciola*

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ABSTRACT

The salinization of freshwater can have negative effects on ecosystem health, with heightened effects in salt-sensitive biota such as glochidia, the larvae of freshwater mussels. However, the toxicological mechanism underlying this sensitivity is unknown. Therefore, *Lampsilis fasciola* glochidia were exposed to NaCl (nominally 0.25 and 1.0 g/L) prepared in reconstituted moderately-hard water (control), as well as to a dilution of that water (1:4) with ultrapure reference water (diluted control). Unidirectional Na^+ influx (measured with ^{22}Na) was evaluated after 1, 3 and 48 h of exposure. In addition, unidirectional Cl^- influx (measured with ^{36}Cl), whole-body ion (Cl^- and Na^+) concentrations, and glochidia viability (measured as the ability to close valves) were assessed after 48 h of exposure. Significantly reduced glochidia viability (56%) was observed after exposure to 1.0 g/L NaCl. Na^+ influx was significantly higher in glochidia exposed to both 0.25 and 1.0 g/L NaCl for 1 h than in those kept under control conditions. After 3 and 48 h of exposure, differences in Na^+ influx rate between salt-exposed and control glochidia were generally reduced, indicating that larvae may be able to, at least temporarily, recover their ability to regulate Na^+ influx when exposed to elevated NaCl concentration. Compared to the moderately-hard water control, whole-body Na^+ and Cl^- concentrations were relatively unchanged in glochidia exposed to 0.25 g/L NaCl, but were significantly elevated in glochidia exposed to 1.0 g/L NaCl and the diluted control. While Na^+ influx rate had recovered to the control level after 48 h of exposure to 1.0 g/L NaCl, Cl^- influx rate remained elevated, being ~7-fold higher than the Na^+ influx rate. These findings suggest that the loss of viability observed when glochidia were exposed to a high NaCl concentration (1.0 g/L) could be caused by ionoregulatory disturbances mainly associated with an elevated Cl^- influx.

1. Introduction

Storage and use of salts for road de-icing in cold climates are significant sources of salt to freshwater environments. In fact, Canada alone uses more than 5 million tons of road salt per year (Environment Canada and Health Canada, 2001) and chloride levels as high as 4 g/L have been reported in water bodies in populated areas where road densities are high. In some regions of North America, the input of salts into freshwater from road runoff over the past 30 years has resulted in a steady increase in the chloride concentration of both surface water (Kaushal et al., 2005) and groundwater (Kelly et al., 2008; Kincaid and Findlay, 2009; Perera et al., 2009; Roy et al., 2015). Indeed, salinization of freshwater is believed to pose a risk to aquatic organisms (Environment Canada and Health Canada, 2001; Kaushal et al., 2005; Todd and Kaltenecker, 2012).

Freshwater mussels in their early life stages are particularly sensitive to salt (Bringolf et al., 2007; Gillis, 2011; Pandolfo et al., 2012; Valenti et al., 2006; Wang et al., 2007). In fact, chloride levels in some mussel habitats can reach levels which are acutely toxic to the larvae (glochidia) (Gillis, 2011). An analysis of long-term water quality data has revealed that even summertime chloride concentrations (not the elevated levels associated with influxes from snow-melt) in urban areas may pose a risk to endangered species of mussels (Todd and Kaltenecker, 2012). Glochidia exposed to high levels of waterborne contaminants, including sodium chloride (Beggel and Geist, 2015), lose the ability to attach to a host fish and thereby fail to complete their life cycle,

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which in turn can affect mussel recruitment (Cope et al., 2008). Despite the fact that acute toxicity studies have demonstrated the salt sensitivity of several species of freshwater mussels, the physiological mechanisms underlying this sensitivity in early life stages remains unknown.

Therefore, the goal of the present study was to evaluate the effects of NaCl exposure on glochidia and provide physiological information about salt sensitivity of the freshwater mussels. The impacts of sub-acute and acutely toxic levels of salt (NaCl) on the unidirectional influxes and whole-body concentrations of major ions (Na^+ and Cl^-) were examined in glochidia of the freshwater mussel, *Lampsilis fasciola*. This mussel is designated as a Species of Special Concern in Canada (COSEWIC, 2010) and was the most sensitive of the four glochidia species examined (EC50 range: 113–1430 mg Cl/L) in a previous study (Gillis, 2011).

2. Material and methods

2.1. Mussels

Gravid wavyrayed lampmussels *L. fasciola* (Rafinesque 1820) were collected between July and September (2010) from the Grand River (Ontario, Canada), under a Canadian Species at Risk permit (SECT 73 SARA C&A 10-014). Mussels were transferred to the laboratory in Burlington (Ontario, Canada) and held at 10 °C (± 2 °C) under a 16 h:8 h light:dark cycle in reconstituted moderately-hard water (ASTM, 2006). The relatively cold temperature was required to prevent release of glochidia. Water was renewed weekly and mussels were fed (1.2×10^{10} algal cells per mussel per day) with a commercial shellfish diet (Instant Algae Shellfish Diet 1800®, Richmond Hill, ON, Canada). For experiments, glochidia were collected by gently flushing the gills of gravid females with a water-filled syringe (ASTM, 2006).

2.2. Salt exposure

Salt exposures were based on the ASTM (2006) method for conducting toxicity tests with early life stages of freshwater mussels. Four exposure treatments were tested as follows: (i) diluted control, which is reconstituted moderately-hard water diluted 1:4 with ultrapure reference water; (ii) control (reconstituted moderately-hard water); (iii) nominally 0.25 g/L NaCl; and (iv) nominally 1.0 g/L NaCl. Exposure solutions of NaCl were prepared by adding certified ACS grade (Fisher Scientific, Downsview, ON, Canada) sodium chloride (NaCl) to the control solution. The nominal concentrations of Na^+ and Cl^- in these solutions were 0.013, 0.052, 0.150 and 0.445 g/L, and 0.0009, 0.003, 0.155 and 0.611 g/L, respectively. In molar units, they corresponded to 0.57, 2.28, 6.54 and 19.37 mmol/L for Na^+ and 0.028, 0.11, 4.37 and 17.21 mmol/L for Cl^-, respectively. Solutions were prepared 24 h before being used in exposures, and were stored in the dark at 4 °C.

Glochidia viability was determined at the beginning ($t=0$) and after 48 h of exposure. Viability refers to the ability of larvae to close their valves after addition of a concentrated salt solution (240 g/L NaCl). Glochidia viability was calculated according to ASTM (2006).

Glochidia were pooled from five gravid females, each one with glochidia viability > 90%, and assigned to the various treatments. Exposures were conducted in 250-mL glass beakers ($n=3$ per treatment) at 21 °C under a 16 h:8 h light:dark cycle. After 48 h of exposure, glochidia viability was assessed in each replicate beaker by determining the mean viability of three sub-samples (~100 glochidia in each sub-sample).

2.3. Na^+ and Cl^- influx rates

Two independent time series experiments were conducted to determine the time course of unidirectional chloride (^{36}Cl; Amersham, Little Chalfont, UK) and sodium (^{22}Na; NEN-Dupont, Boston, MA, USA.) influx in glochidia under control conditions (moderately-hard water). ^{36}Cl and ^{22}Na experiments were performed separately but followed the same procedures. Glochidia were pooled from five gravid females and subdivided into samples to assess Na^+ (130 larvae per sample) and Cl^- (400 larvae per sample) influx. The time series experiment for Na^+ was created by adding 0.125 μCi ^{22}Na to each larvae sample. Radioactivity of the samples was measured 7, 15, 30, 60, 120, 180 and 240 min (8 samples per treatment) after the addition of ^{22}Na. For the Cl^- time series experiment, the same procedures were adopted, however with 0.125 μCi ^{36}Cl added to each glochidia sample. In view of the high cost of ^{36}Cl relative to ^{22}Na, the Cl^- influx was measured at only three time points (15, 60 and 180 min; 8 samples per treatment). At the end of each experimental time, samples were vacuum filtered (~10 s) using polycarbonate membrane filters (GE Polycarbonate, 8.0 μm, GE Water and Process Technologies, Trevose, PA, USA) and then rinsed with 5 ml of a cold NaCl solution containing a ten-fold higher NaCl concentration than the corresponding exposure solution in order to remove any radioisotope loosely bound to the external surface of the glochidia. The polycarbonate membrane was then removed from the filter and placed in a glass scintillation vial for subsequent measurement. In order to adjust for any isotope that remained bound to the filter paper after rinsing, a correction factor was determined for each exposure concentration using blank samples (experimental media with no glochidia containing 0.125 μCi of ^{22}Na or ^{36}Cl). These samples were filtered and rinsed as described above. For each exposure solution, the radioactivity measured in the filter paper without glochidia was subtracted from that measured in the filter paper with glochidia samples. This correction typically amounted to less than 5% of the radioactivity in a sample. ^{22}Na and ^{36}Cl uptake tests were also run with dead larvae; no uptake above the background blank could be detected.

For ^{22}Na and ^{36}Cl, radioactivity measurements were performed using a Gamma counter (1480 3 in. crystal Auto Gamma Counter, Perkin Elmer Wizard, Fremont, CA, USA) and a scintillation counter (Tri-carb liquid scintillation analyzer, Perkin Elmer, Illinois, USA), respectively. Prior to determining Cl^- radioactivity, 4 ml of scintillation fluid (UltimaGold AB, Packard Bioscience, Groningen, The Netherlands) was added to each sample, and the samples were held in the dark for 2 h prior to counting. Quench correction was performed through internal standardization. Data were expressed as mmol Na^+ or Cl^-/glochidia/min following the equation:

$$J_{in} = \text{cpm x}(1/\text{SA})\text{x}(1/\text{number of glochidia})$$

where, cpm is counts per minute, and SA is the specific activity of the experimental medium. The specific activity was calculated using the following equation:

SA=radioactivity concentration (cpm/L)/ion concentration in the sample (mmol/L). Based on the results of the time series experiment, an exposure (or flux period) of 60 min was chosen for subsequent measurements of unidirectional influx rates.

Unidirectional Na^+ and Cl^- influx rates were determined for glochidia exposed to the experimental NaCl concentrations described above (see Section 2.2). The Na^+ influx rate was measured in glochidia exposed to the NaCl concentrations for 1, 3 and 48 h by adding 0.125 μCi ^{22}Na to the samples (8 samples for each treatment). The same procedure was adopted to assess Cl^- influx rate, but again due to the high cost of ^{36}Cl, it was measured only after 48 h of exposure. Unidirectional Cl^- influx rate in the diluted control treatment was excluded because of the very low Cl^-

concentration (0.0009 g/L). Sample filtration, washing and radioactivity measurement procedures were performed as described above.

2.4. Whole-body ion concentrations

Following NaCl exposure, glochidia viability was determined on a subsample of exposed glochidia as described above. The remaining NaCl-exposed glochidia were filtered and then rinsed with 5 ml nanopure water. The wash procedure did not exceed 5 s, and was confirmed not to affect viability. Sodium was analyzed by atomic absorption spectrophotometry (SpectrAA 220FS Atomic Absorption Spectrometer, Varian, Toronto ON, Canada), while chloride was determined using the colorimetric method described by Zall et al. (1956). Certified NaCl standards (Radiometer-Copenhagen, Denmark) were employed for QA/QC. Sodium and chloride accumulation tests were also conducted with dead larvae; no accumulation of Na^+ or Cl^- could be detected. Data were expressed as μmol Na^+ or Cl^-/mg dry weight. Dry weight was determined after the samples were dried (50 °C) for 24 h. Results were corrected for measured glochidia viability in the corresponding treatment in order to consider only living glochidia.

2.5. Data presentation and statistical analysis

Data were expressed as means ± standard error ($N=8$, where N represents the number of glochidia samples (of ~100 glochidia each) for each treatment). Na^+ and Cl^- influxes in the time series experiments were analyzed by non-linear regression analyses (exponential rise to maximum), yielding the constants of maximum influx and the time to half maximum ($t_{0.5}$) (SigmaPlot 11, Systat Software, USA). Cl^- influx data after NaCl exposure were evaluated by linear regression analysis. Comparisons of influx rates were made following NaCl exposure and between NaCl concentrations using one-way analysis of variance (ANOVA) followed by the Tukey's test. Data were log-transformed when necessary to meet ANOVA assumptions (data normality and homogeneity of variances). The significance level adopted was 95% ($\alpha=0.05$). Linear regression analyses, one-way ANOVA and Tukey's test were performed using the software Statistica 7.0 (Stat Soft, USA).

3. Results

3.1. Toxicity test

Glochidia viability prior to exposure ($t=0$) was 93 ± 4%. After 48 h of exposure, viability was similar in glochidia kept under the control condition or exposed to either the diluted control or 0.25 g/L NaCl (84 ± 8%, 84 ± 4% and 74 ± 3%, respectively) (Fig. 1). However, viability of glochidia exposed to 1.0 g/L NaCl for 48 h was significantly lower (56 ± 2%) than of those subjected to the other treatments.

3.2. Time course kinetics of Na^+ and Cl^- influxes

The time courses of Na^+ and Cl^- influxes from ^{22}Na and ^{36}Cl incubations fitted to an "exponential rise to maximum" curves (Fig. 2). The maximum uptake for Na^+ was estimated as 2.0×10^{-5} mmol Na^+/glochidia and the time to half maximum influx ($t_{0.5}$) was 19 min. The maximum Cl^- uptake was estimated as 6.0×10^{-6} mmol Cl^-/glochidia (~30% of that found for Na^+) and the $t_{0.5}$ was estimated as 8 min (~60% lower than that found for Na^+). Based on these results, a 1-h incubation time was adopted for the radioisotope influx experiments, chosen as a compromise, representing a period over which uptake was reasonably linear with time and yielded sufficient radioactivity (cpm) in the glochidia for good resolution.

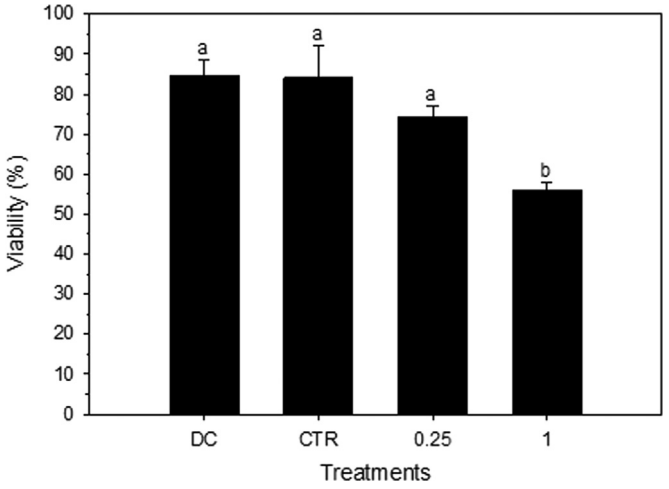

Fig. 1. Viability (%) of *Lampsilis fasciola* glochidia after 48-h exposure to different treatments. DC=diluted control solution; CTR=control solution; 0.25=0.25 g/L NaCl solution; and 1.0=1.0 g/L NaCl solution. Data are expressed as mean ± standard error ($n=8$). Mean values sharing the same letter are not significantly different ($p<0.05$).

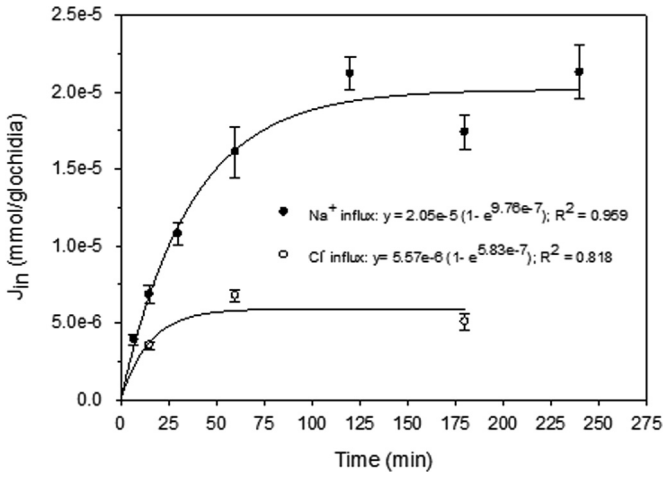

Fig. 2. Unidirectional Na^+ and Cl^- influxes in *Lampsilis fasciola* glochidia incubated in ^{22}Na and ^{36}Cl, respectively. ^{22}Na-exposed glochidia were sampled after 7, 15, 30, 60, 120, 180 and 240 min of exposure. ^{36}Cl-exposed glochidia were sampled after 15, 60 and 180 min of exposure. Data are expressed as mean ± standard error ($n=8$). The maximum influx and time to half maximum influx ($t_{0.5}$) were significantly lower for Cl^- influx than for Na influx ($p<0.05$).

3.3. Effects of NaCl exposure on unidirectional Na^+ and Cl^- influx rates

For all treatments, unidirectional Na^+ influx rate was significantly higher after 1 h than after either 3 or 48 h of exposure to NaCl (Fig. 3). Although the Na^+ influx rates in the diluted control and control solutions were similar, they were both significantly lower than those observed in glochidia exposed to 0.25 and 1.0 g/L NaCl for 1 h or glochidia exposed to 1.0 g/L NaCl for 3 h or glochidia exposed to 0.25 g/L NaCl for 48 h (Fig. 3).

Unidirectional Cl^- influx rate in glochidia exposed to 1.0 g/L NaCl for 48 h was significantly higher than that observed in those maintained in the control solution or exposed to 0.25 g/L NaCl. Cl^- influx rates were comparable to Na^+ influx rates in glochidia kept in the control solution and in those exposed to 0.25 g/L NaCl. However, Cl^- influx rate was ~7-fold higher than the Na^+ influx

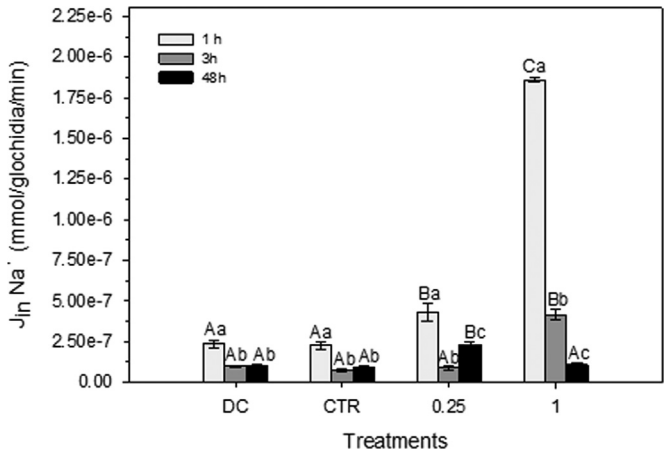

Fig. 3. Unidirectional Na^+ influx rate in *Lampsilis fasciola* glochidia exposed to different treatments for 1 h (light gray bars), 3 h (dark gray bars) and 48 h (black bars). DC=diluted control solution; CTR=control solution, 0.25=0.25 g/L NaCl solution; 1.0=1.0 g/L NaCl solution. Data are expressed as mean ± standard error (n=8). Different lower case letters indicate significantly different mean values among times of exposure for the same treatment. Different upper case letters indicates significantly different mean values among treatments for the same exposure time ($p < 0.05$).

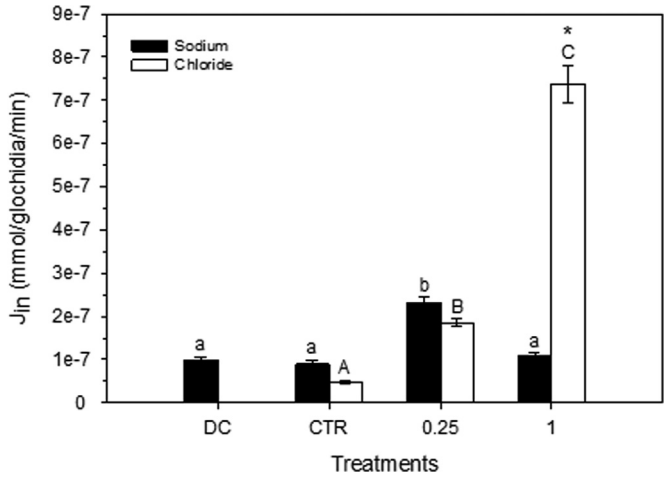

Fig. 4. Unidirectional Na^+ (black bars) and Cl^- (gray bars) influx rates in *Lampsilis fasciola* glochidia after 48 h of exposure to different treatments. DC=diluted control solution; CTR=control solution; 0.25=0.25 g/L NaCl solution; and 1.0=1.0 g/L NaCl solution. Data are expressed as mean ± standard error (n=8). Different lower and upper case letters indicate significantly different mean values among treatments for Na^+ and Cl^-, respectively ($p < 0.05$). An asterisk indicates a significant difference between Na^+ and Cl^- influx rates for the same treatment ($p < 0.05$).

rate in glochidia exposed to 1 g/L NaCl (Fig. 4).

3.4. Whole-body Na^+ and Cl^- concentration

The whole-body Na^+ concentration of glochidia held in the control solution was 0.15 ± 0.02 μmol Na^+/mg dry weight, which was significantly lower than that observed in all other treatments, including the diluted control solution (Fig. 5). Na^+ concentration of glochidia from the diluted control solution and the 0.25 g/L NaCl treatment were similar (0.25 ± 0.04 and 0.23 ± 0.01 μmol Na^+/mg dry weight, respectively), but significantly different than that observed in glochidia exposed to the other treatments. Glochidia from the 1.0 g/L NaCl treatment had a whole-body Na^+ concentration of 0.35 ± 0.01 μmol Na^+/mg dry weight. Whole-body Cl^- concentrations followed the same pattern as whole-body Na^+ concentration. Glochidia maintained in the control solution exhibited significantly lower Cl^- concentration (0.29 ± 0.02 μmol

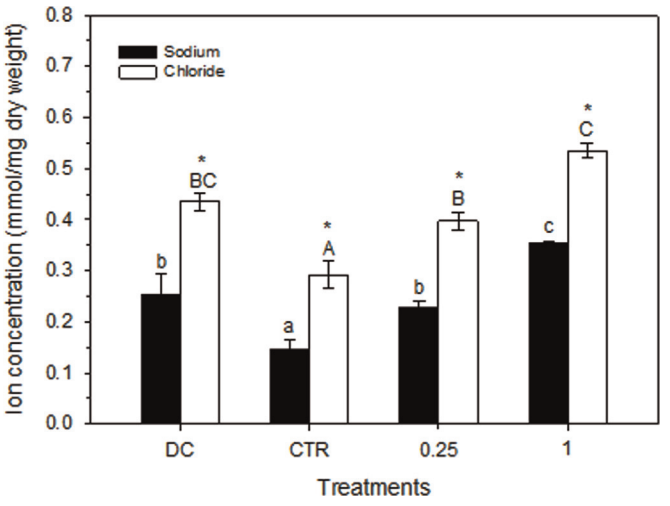

Fig. 5. Whole-body Na^+ (black bars) and Cl^- (gray bars) concentrations in *Lampsilis fasciola* glochidia after 48 h of exposure to different treatments. DC=diluted control solution; CTR=control solution; 0.25=0.25 g/L NaCl solution; 1.0=1.0 g/L NaCl solution. Data are expressed as mean ± standard error (n=3). Different lower and upper case letters indicate significantly different mean values among treatments for Na^+ and Cl^-, respectively ($p < 0.05$). An asterisk indicates a significant difference between Na^+ and Cl^- influx rates for the same treatment ($p < 0.05$).

Cl^-/mg dry weight) than those from the other treatments. Whole-body Cl^- concentration was similar in glochidia subjected to the diluted control solution and the 0.25 g/L NaCl treatment (0.43 ± 0.01 and 0.40 ± 0.01 μmol Cl^-/mg dry weight, respectively). The highest Cl^- concentration was observed in larvae exposed to 1.0 g/L NaCl (0.53 ± 0.01 μmol Cl^-/mg dry weight).

4. Discussion

In the present study, we used radiolabelling techniques to assess time-dependent kinetics of unidirectional Na^+ and Cl^- uptake in addition to determining their whole-body concentrations in freshwater mussel larvae. Our analysis was limited to whole-body measurements due to the small size (approx. 0.5 mm diameter) of glochidia. While information on major ion composition of glochidia is scarce, Na^+ and Cl^- are the major ions of the hemolymph of adult Unionid mussels (Zheng and Dietz, 1998).

Na^+ and Cl^- influxes in *L. fasciola* glochidia in reconstituted moderately-hard water followed saturation-type kinetics over time. Na^+ influx reached a maximum value between 75 and 100 min while Cl^- influx achieved a maximum value 50 min after addition of the radioisotope. The same pattern of response was observed in isolated gill tissue of the freshwater mussel, *Ligumia substrata* (Dietz and Graves, 1981) where the plateau for Na^+ influx was observed after 60 min. Although a direct comparison cannot be made, the lowest difference between Na^+ and Cl^- influx (~4-fold) observed in this study in *L. fasciola* glochidia supports what was previously described for isolated gill tissue (3-fold) of *L. substrata* (Dietz and Graves, 1981).

After the first hour of exposure, glochidia showed a higher Na^+ influx rate than that measured at 3 and 48 h. These elevated values may be a result of the possible physiological stress caused by transferring the larvae from the marsupial environment in the maternal gill to the experimental solutions. In fact, there is evidence that adult freshwater bivalves do not strongly regulate the osmotic and ionic concentrations of their extracellular compartment (hemolymph). The adults hyper-regulate ion concentrations in their hemolymph when in media with osmolalities below 100 mOsmol/kg H_2O, but they are ionoconformers when

acclimated to osmolalities above 100 mOsmol/kg H_2O (Jordan and Deaton, 1999). In the present study, glochidia were transferred from the marsupia to moderately-hard water. Assuming that the internal ion concentrations of glochidia are similar to those reported for adult mussel hemolymph (Dietz, 1979), then this transfer to a dilute environment would likely trigger an adjustment of their internal ion concentrations. Physiological stress in mussels can stimulate the release of endogenous serotonin, which is directly associated with an increase in Na^+ uptake (Dietz et al., 1982). Indeed, elevated Na^+ influx has been associated with an increase in serotonin in the gills of both marine (*Mytilus edulis*) and freshwater mussels (*L. substrata*) (Dietz and Graves, 1981; Catapane et al., 1979).

Recovery of Na^+ influx to background levels in glochidia appears to occur 3 h after salt exposure. It is known that freshwater mussels can take hours or days to regulate Na^+ flux rates after hypo- or hyperosmotic shock (Dietz and Branton, 1975, 1979). In the present study, no significant difference in Na^+ influx was observed between 3 and 48 h of exposure in glochidia kept under control condition, thus indicating that 3 h is enough time for glochidia to recover their ionic steady-state following a change in external Na^+ and Cl^- concentrations. This may be important in allowing animals to recover from sudden salt pulses associated with precipitation events. Therefore, glochidia of *L. fasciola* appear to be able to achieve an ionic balance faster than adults of other bivalve species, such as *Dreissena polymorpha* (48 h) and *Corbicula fluminea* (12 h) (Gainey, 1978; Horohov et al., 1992). This is likely due to the high uptake rates observed in the early life stage of *L. fasciola*. For example, Na^+ uptake rate was shown to be higher in neonates than in adult daphnids (Bianchini and Wood, 2008). A lower affinity for Na^+ combined with a higher maximum capacity of Na^+ uptake was observed in neonate daphnids, which is likely associated with ontogenetic changes, anatomical and/or structural modifications in the ion-transporting cells and organs (Bianchini and Wood, 2008).

At all exposure times, no significant difference in Na^+ influx rate was observed between glochidia exposed to the diluted control condition and those kept under control condition. Similar Na^+ influxes were also observed in gills of pondwater-exposed and salt-depleted mussels (*L. substrata*) (Dietz and Graves, 1981). Under hypo-osmotic conditions, reduced unidirectional efflux of ions combined with an increased active uptake of ions through the Cl^-/HCO_3^- and Na^+/H^+ transporters have been observed in adult freshwater mussels (Dietz and Branton, 1979).

Whole-body Na^+ and Cl^- concentrations were significantly higher in glochidia subjected to the diluted control and those exposed to elevated NaCl concentrations (0.25 and 1.0 g/L) than in those kept under control condition (moderately-hard water) for 48 h. As previously mentioned, under hypo-osmotic conditions a reduced unidirectional efflux of ions and increased active uptake of ions occurs. These findings are in agreement with the fact that freshwater bivalves are able to retain/or maintain Na^+ and Cl^- and survive even in extremely ion-poor conditions (Dietz and Branton, 1975; Dietz, 1978). However, hyper-concentrations of NaCl in exposure solutions can stimulate the uptake of both Na^+ and Cl^-, thus resulting in increased concentrations of these ions in the hemolymph of the oyster *Crassostrea brasiliana* (Machado et al., 2002). Furthermore, the maintenance of higher Na^+ and Cl^- whole-body concentrations in salt-exposed glochidia could be a result of a lower ion efflux due to the similarity between the concentration of ions present in the (glochidia's) hemolymph and the external medium (Zheng and Dietz, 1998). In the present study, the concentrations of Na^+ and Cl^- in 1.0 g/L NaCl exposure solution were both ~17 mmol/L, which are close to those observed in the hemolymph of adult Unionid mussels (~20 mmol/L Na^+ and ~12 mmol/L Cl^-; Dietz, 1979).

Increased whole-body Na^+ and Cl^- concentrations could interfere with the functioning of excitable tissues (*e.g.* nervous tissues), becoming a limiting factor for animal survival (Horohov et al., 1992). However, viability was not significantly affected in glochidia exposed to the diluted control (84%) and 0.25 g/L NaCl (74%). Viability was only significantly reduced in glochidia exposed to 1.0 g/L NaCl (56%). This effect could be associated with the significant increase in Cl^- influx observed in glochidia exposed to this level of salt exposure. Cl^- influx rate increased linearly ($R^2=0.99$) with increasing Cl^- concentration in the exposure medium. Although the Cl^- transport system in mussels is saturable (Dietz and Branton, 1979; Zheng and Dietz, 1998), the linear increase in the Cl^- influx observed in the present study indicates passive diffusion, which does not appear to be occurring for Na^+ influx (Wilcox and Dietz, 1995). Furthermore, it has been demonstrated that Na^+ and Cl^- uptake in adult freshwater mussels are independent (Dietz, 1978; Dietz and Branton, 1979; Dietz and Hagar, 1990), which may explain the different unidirectional Na^+ and Cl^- influx rates observed in the time kinetic experiments of the present study, as well as in the glochidia exposed to 1.0 g/L NaCl.

A previous study demonstrated that adult freshwater mussels exposed to Cl^- concentrations higher than 20 mM do not open their valves (Zheng and Dietz, 1998). Similar valve closing behavior has been reported in freshwater mussels and other bivalves exposed to different waterborne contaminants (Cope et al., 2008; Curtis et al., 2000; Gilroy et al., 2014; Salánki and Balogh, 1989), and is thought to be an avoidance mechanism for limiting exposure to poor water quality (Hellou, 2011). No studies in the literature report a similar mechanism in freshwater mussel larvae. Furthermore, glochidia valves have pores, which may interfere with the efficiency of controlling ion balance (Schwartz and Dimock, 2001). In general, in freshwater animals an increased sensitivity to Cl^- is associated with the higher energy expenditure required to maintain the ionic gradient between the internal and external media (Morgan and Iwama, 1998; Venkatachari and Vasantha, 1979; Zheng and Dietz, 1998), once homeostatic mechanisms are overwhelmed. In fact, in fish exposed to high NaCl solutions, toxicity is a consequence of osmoregulatory failure, a syndrome in which dysfunction of Cl^- regulation plays a significant role (De Boeck et al., 2000).

Recent studies have reported that early life stage freshwater mussels have a heightened sensitivity to salt exposure, though the physiological mechanism underlying this sensitivity was unknown. The current study has demonstrated that glochidia exposed to elevated Cl^- experience a significant increase in Cl^- influx at concentrations where viability is reduced, indicating that the mode of action of chloride toxicity in glochidia is ionoregulatory disturbance. Reduced glochidia survival could negatively impact freshwater mussel recruitment and ultimately mussel populations. In fact, there is a growing body of evidence that early life stage freshwater mussels may be at risk from chloride exposure in their natural habitats (Blaskeslee et al., 2013; Gillis, 2011; Todd and Kaltenecker, 2012). The recent Canadian Water Quality Guideline for protection of aquatic life for chloride (CCME, 2011) along with adaptation of best practices for road salt use and storage (Environment Canada, 2004) should lead to a reduction in surface water chloride levels which will help protect these imperiled organisms.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.09.003:

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