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Fluid and osmolyte recovery in the common pond snail *Lymnaea stagnalis* following full-body withdrawal

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SUMMARY

The common pond snail *Lymnaea stagnalis* sacrifices 40–60% of its extracellular fluid (ECF) including solutes to the surrounding environment to fully retract into its shell. Our objectives were to determine recovery time following such ECF loss and characterize mechanisms involved in recovering Na^+, the primary cationic osmolyte in this snail. Pallial fluid was initially collected post-stimulation and again after a second stimulation on sub-groups of those snails 2 h to 10 weeks after initial sampling. Samples were analyzed for pH, osmotic pressure, and individual solute concentrations. Lost volume was recovered within 8 h with no significant changes in pH or total CO_2. Significant decreases in osmotic pressure, [Na^+] (43% loss), and [Cl^-] recovered in 48 h as a result of enhanced uptake from the water. Copper and total extracellular proteins took 5 weeks to recover. Measurements of Na^+ transport kinetics completed before and immediately after fluid loss revealed a near threefold increase in both affinity and capacity of the Na^+ uptake system. Sodium uptake was independent of ambient Cl^- and HCO_3^- in both control and fluid-depleted snails. Amiloride significantly reduced recovery-phase Na^+ uptake rates but did not influence baseline Na^+ flux. Recovery uptake was significantly reduced by amiloride, ethylisopropylamiloride, bafilomycin and ethoxzolamide indicating dependency upon Na^+/H^+ exchange, H^+ pump activity and H^+ from carbonic anhydrase-catalyzed CO_2 hydration. Thus enhanced uptake during recovery is likely *via* electrogenic Na^+/H^+ exchange and/or possibly a cation channel.

Key words: sodium uptake kinetics, pallial fluid chemistry, hemolymph, ion transport, active transport, osmoregulation.

INTRODUCTION

Predator avoidance in aquatic snails is facilitated by surfacing and emergence from the water in snails that have relatively fragile shells, and by hiding or possibly burial in the case of snails with shells of greater crush resistance (Alexander and Covich, 1991; McCarthy and Fisher, 2000; Rundle and Brönmark, 2001). The response is also highly dependent on the type of predator – i.e. shell-invading leeches and flatworms (Townsend and McCarthy, 1980; Brönmark and Malmqvist, 1986) *versus* shell-crushing sunfish or crayfish (Alexander and Covich, 1991; Brown, 1991). Additionally, and central to the present study, snails can perform graded withdrawal of soft tissue into their shells corresponding to the intensity of the predatory attack (Arshavsky et al., 1994). Pulmonate snails, such as the common pond snail *Lymnaea stagnalis* (L.), exhibit emergence behavior to escape predation but when the predatory threat is imminent, *L. stagnalis* can also complete full-body withdrawal by ejecting up to 60% of its total hemolymph volume (Schlichter, 1981), which averages 0.45 ml g^{-1} snail body mass (van Aardt, 1968). The extracellular fluid (ECF) enters the pulmonate cavity *via* the haemal pore and is then ejected into the environment through the pneumostome. Because this response can be achieved experimentally by stimulating the foot of the snail, and because the released fluids can be easily collected, the response provides a means by which internal conditions of the snail can be determined without sacrifice or puncture of the integument. Although the withdrawal response offers protection against predation, it is also potentially associated with reduced fitness. One possible cost of this escape response is that the snail becomes temporarily immobilized on the bottom during the initial recovery phase (personal observation) and is probably more vulnerable to benthic predators such as crayfish during that time. A, presumably, sub-lethal effect is the energetic expense associated with the needed recovery of fluids and osmolytes lost with the release of ECF.

Freshwater snails exhibiting the whole-body retraction and associated fluid loss have a physiological challenge associated with this escape response that may be of greater significance than for marine snails. Marine snails conform to ambient ion concentrations, thus the challenge of recovering ions lost due to retraction is minimal and mostly occurs in parallel with fluid recovery (Krogh, 1946). However, freshwater species are osmoregulators maintaining hyperosmotic conditions relative to the ambient waters. Thus, although both freshwater and seawater species lose fluids on complete full-body withdrawal, solute recovery of this magnitude (presumably as high as 60%), which requires active transport by freshwater species such as *L. stagnalis*, is probably of significant energetic cost for evading predation.

In an attempt to evaluate the severity of the consequence of ECF release, the primary goals of this investigation were to determine (1) the magnitude of electrolyte loss associated with the full-body withdrawal, (2) whether *L. stagnalis* recovers solutes to initial levels following ECF release and (3) the time required to such full recovery. These goals were pursued by completion of a time course study of hemolymph sampling (bleeding) to determine the time to recovery following the initial bleed in naïve, cultured snails.

Findings of relatively rapid recovery of ECF volume and osmotic pressure prompted investigations into the nature of this remarkable homeostatic response. Because Na^+ is the major cationic osmotic component of the ECF in these organisms (Schlichter, 1981), understanding the mechanism(s) of its recovery following ECF release is essential. It has long been recognized that *L. stagnalis*, like other freshwater organisms (Krogh, 1939; Krogh, 1946), obtains Na^+ from the surrounding freshwater by active carrier-mediated uptake (Greenaway, 1970), but the mechanism remains to be characterized. Consequently, our next objective was to (4) determine the Na^+ transport kinetics under basal (un-bled) and early recovery (bled) conditions to evaluate whether the accelerated uptake under fluid recovery conditions was due to altered transport kinetics of the Na^+ uptake pathways utilized to maintain homeostasis under basal conditions.

In all freshwater organisms examined to date, the process of Na^+ uptake is mediated at least in part by an electro-chemical gradient generated by the basolateral electrogenic Na^+,K^+-ATPase (Marshall and Grosell, 2006), which is responsible for low intracellular Na^+ concentrations and polarization of the cell membrane. Apical entry of Na^+ appears to be linked to H^+ extrusion either *via* direct Na^+/H^+ exchange as first proposed by Krogh (Krogh, 1939) or to H^+ extrusion *via* the vacuolar H^+-ATPase (Lin and Randall, 1991; Bury and Wood, 1999; Fenwick et al., 1999; Grosell and Wood, 2002). In the latter case, electrogenic H^+ extrusion further polarizes the apical membrane to facilitate Na^+ entry *via* a putative Na^+ channel, which so far has not been identified.

Our findings revealed greatly amplified Na^+ uptake in *L. stagnalis*, which can lose 30% or more of hemolymph Na^+ content in the response associated with whole-body withdrawal (Schlichter, 1981). One possible characteristic of pathways involved in Na^+ recovery could be dependency on availability of extracellular anions (de With, 1980; de With et al., 1987). Uptake of Na^+ in many freshwater organisms requires Cl^- (Kirschner, 2004) and the possibility of HCO_3^--dependent Na^+ uptake *via* a Na^+/HCO_3^--dependent carrier system exists (Perry et al., 2003). The observed stimulated Na^+ uptake associated with recovery from ECF release was attributed to both increased transport capacity and affinity. Because apical Na^+ channels or Na^+/H^+ exchange proteins likely would be the primary means by which Na^+ enters the cell, sensitivity of Na^+ uptake to amiloride was evaluated to provide insight into the mechanism(s) of enhanced Na^+ uptake following ECF loss. Furthermore, microtubule-dependent relocation of transport proteins from the cytoplasm to the epithelial membrane (Tresguerres et al., 2006) could also contribute to the rapid activation of the Na^+ uptake mechanism. Additionally, the enhanced transport may rely on availability of intracellular H^+ ions (Kirschner, 2004), which are produced by the hydration of CO_2, and would therefore be dependent upon carbonic anhydrase (CA). Therefore, our two final objectives were to (5) determine whether the amplified Na^+ uptake was Cl^-- or HCO_3^--dependent and (6) conduct a pharmacological assessment of transport mechanisms involved in Na^+ recovery.

MATERIALS AND METHODS

Experimental snails

Common pond snails *Lymnaea stagnalis* were cultured from a stock originally procured from Dr Naweed Syed, University of Calgary, Canada. Cultures were reared in flow-through tanks supplied with dechlorinated, aerated Miami-Dade County, USA tapwater (Table 1) and were offered a continuous diet of Romaine lettuce, carrot and sweet potato. Snails reproduced readily under these conditions.

Table 1. Water chemistry for dechlorinated Miami–Dade county tapwater used for snail cultures

Characteristic	Value
$[Na^+]$	1.1
$[K^+]$	0.04
$[Ca^{2+}]$	0.31
$[Mg^{2+}]$	0.12
$[Cl^-]$	1.03
$[SO_4^{2-}]$	0.11
$[HCO_3^-]$	0.68
$[CO_3^-]$	0.01
[DOC]	199.1
[Total CO_2]	0.71
pH	7.69

All concentrations are in mmol l^{-1} except dissolved organic carbon (DOC), which is reported in μmol l^{-1} of carbon. Bicarbonate concentration and $[CO_3^-]$ were calculated from measured pH and the mean total $[CO_2]$. Values are taken from M. Grosell, R. Gerdes and K. V. Brix, manuscript in press.

Hemolymph sampling and time course for solute recovery

To determine the time necessary for the snails to recover solutes following loss of extracellular fluid (ECF), the foot of the snail was stimulated using a pipette tip to cause full retraction of the soft tissue into the shell and consequently ECF release. This procedure was performed with snails removed from the water to allow for ECF collection (which will subsequently be referred to as bleeding), after which the snails were immediately returned to the water. The released fluid obtained from the bleeding procedure was retained for later analysis. A total of 134 snails were weighed, bled and reweighed. A second stimulation was performed on subsets of the same 134 snails at 2 h (*N*=8), 4 h (*N*=8), 6 h (*N*=8), 8 h (*N*=8), 10 h (*N*=8), 12 h (*N*=8), 16 h (*N*=8), 18 h (*N*=8), 24 h (*N*=8), 48 h (*N*=8), 72 h (*N*=8), 1 week (168 h, *N*=10), 2 weeks (336 h, *N*=10), 5 weeks (*N*=10) and 10 weeks (*N*=9) following the initial bleeding.

Na^+ transport kinetics: radioisotope flux experiments

To determine whether rapid recovery of Na^+ post-bleeding was associated with stimulated Na^+ uptake capacity and/or enhanced affinity of the Na^+ transport system, Na^+ transport kinetics were determined pre- and post-bleeding in individual snails. Sodium-free artificial tapwater (ATW) with ion concentrations (except for Na^+ and Cl^-) similar to water from Miami-Dade county (Table 1) was used for these experiments and contained nominal concentrations of 100 μmol l^{-1} $MgSO_4$, 400 μmol l^{-1} $CaSO_4$ and 100 μmol l^{-1} $KHCO_3$. For uptake kinetic measurements, NaCl was added to achieve a range of nominal $[Na^+]$: 100, 250, 500, 1000 and 2000 μmol l^{-1}. Individual snails with wet masses between 0.5 and 1 g were randomly selected, weighed and placed in individual chambers containing 15 ml of the appropriate medium (*N*=10 snails each in individual chambers for each concentration). Equal amounts of radio-labeled ^{22}Na (2 μCi) were then added to each chamber and within 10 min the chamber medium was mixed using a micropipetter, and 5 ml of the water (initial) was removed for later analysis of ^{22}Na activity and $[Na^+]$. Snails were then allowed to flux for 1–3 h and all calculations were normalized for flux time. Each snail was then removed from its flux chamber, given a quick rinse in artificial ^{22}Na-free tapwater having the same Na^+

concentration as the flux medium, bled, rinsed again, and allowed to recover in individual chambers with dechlorinated tapwater (~1 mmol l^{-1} Na^+) for approximately 1 h. The flux process was then repeated with each snail being allowed to take up ^{22}Na from the same [Na^+] as was used in the initial flux in the un-bled condition but in fresh chambers and solutions including ^{22}Na isotope. For example a snail initially fluxed in 500 μmol l^{-1} Na^+ in the un-bled condition was subsequently allowed to flux under the bled condition with fresh medium containing 500 μmol l^{-1} Na^+.

Assessment of anion dependency in amplified Na^+ uptake post-bleeding

To determine whether Na^+ uptake under solute recovery conditions was dependent on anion availability in the ambient water, two flux experiments were completed to test Cl^- and HCO_3^- dependency. In the Cl^--dependency experiment, [Cl^-] was varied while maintaining nominal concentrations of Mg^{2+}, Ca^{2+}, K^+ and HCO_3^- as described above and maintaining 500 μmol l^{-1} [Na^+]. Control medium contained 500 μmol l^{-1} Na^+ as NaCl (500 μmol l^{-1} nominal [Cl^-], N=10) and two treatment media contained 500 μmol l^{-1} Na^+ as sodium gluconate (0 μmol l^{-1} nominal [Cl^-], N=10) or as Na_2SO_4 (0 μmol l^{-1} nominal [Cl^-], N=10). To test HCO_3^- dependency of enhanced uptake post-bleed, control medium contained 500 μmol l^{-1} Na^+ as sodium bicarbonate (500 μmol l^{-1} nominal [HCO_3^-], N=10) and three treatment media contained 500 μmol l^{-1} Na^+ as NaCl (100 μmol l^{-1} nominal [HCO_3^-], N=10), sodium gluconate (0 μmol l^{-1} nominal [HCO_3^-], N=10), and Na_2SO_4 (0 μmol l^{-1} nominal [HCO_3^-], N=10). Concentrations of ambient Mg^{2+}, Ca^{2+} and K^+ were maintained at nominal concentrations as listed in the initial kinetics experiment. The same flux procedures described above were completed on pre-weighed snails 1–1.5 g wet mass and each chamber contained 20 ml of medium for all controls and treatments. Snails were then bled, given a 1-h recovery in appropriate 500 μmol l^{-1} Na^+ medium, and were allowed to flux again for 2 h after ^{22}Na was added. Initial and final water samples for both sets of fluxes were analyzed for ^{22}Na activity and [Na^+] as described below. Additionally, 'Cl^--free' conditions were verified as described below.

Pharmacological assessment of mechanisms contributing to amplified Na^+ uptake post-bleeding

An affinity shift observed in bled relative to un-bled condition in Na^+ kinetics experiments indicated the possible use of two distinct transport pathways by which the snails take up Na^+, one under basal conditions and a second during recovery following fluid loss. We hypothesized that these transport pathways might display different pharmacological characteristics. Amiloride (*N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride: hydrate; Sigma-Aldrich), which inhibits Na^+ transport by blocking Na^+ channels at 0.34 μmol l^{-1} amiloride and other forms of Na^+ transport, including Na^+/H^+ exchangers, at 83.8 μmol l^{-1} amiloride (Kleyman and Cragoe, 1988; Masereel et al., 2003), was utilized in this experiment at concentrations that were sufficiently above the effective range of the drug for the Na^+ channel (used 10 μmol l^{-1} in our study) and the Na^+/H^+ exchanger (used 100 μmol l^{-1} in our study). For these experiments 30 un-bled and 30 bled snails with pre-bleed weights of 1–1.5 g were placed in individual chambers containing 20 ml of ATW (500 μmol l^{-1} nominal [Na^+] as NaCl) and either dimethyl sulfoxide [$(CH_3)_2SO$, DMSO, vehicle control, N=10], 10^{-5} mol l^{-1} amiloride (N=10), or 10^{-4} mol l^{-1} (N=10) amiloride dissolved in DMSO and allowed to recover for approximately 60 min prior to addition of ^{22}Na and commencement of 2-h flux as described above. In this experiment, separate snails were used for the bled and un-bled conditions. This allowed us to use amiloride under both basal and recovery conditions to evaluate whether different mechanisms were being utilized to maintain Na^+ homeostasis compared to recovery following fluid loss.

To provide further insight into the mechanism of Na^+ uptake, we used 100 μmol l^{-1} ethylisopropylamiloride ($C_{11}H_{18}ClN_7O$, EIPA; Sigma-Aldrich) dissolved in a final concentration of 1% DMSO to determine the possible role of a Na^+/H^+ exchanger in Na^+ uptake for un-bled (N=5) and bled (N=10) snails. EIPA is an NHE-specific amiloride analogue not believed to inhibit Na^+ channels (Kleyman and Cragoe, 1988). Additionally, we tested for effects of a proton pump inhibitor, bafilomycin ($C_{35}H_{58}O_9$; Sigma-Aldrich), on Na^+ uptake to evaluate potential H^+ pump involvement under both un-bled (N=7) and bled (N=10) conditions. Bafilomycin was dissolved in DMSO for a final concentration of 1 μmol l^{-1} bafilomycin and 0.1% DMSO.

To assess whether the rapid activation of Na^+ uptake was mediated by cellular trafficking or recruitment of transport proteins in vesicular compartments to the apical membrane, colchicine was employed. Colchicine is a microtubule disruptor that provides for a rudimentary assessment of possible transporter relocation from cytoplasmic compartments to the epithelial membrane. Un-bled snails were subjected to flux measurements employing the same procedure described above for 2 h in 20 ml of ATW containing ^{22}Na (2 μCi) and 500 μmol l^{-1} Na^+ as NaCl. Following the initial flux, snails were bled and allowed to recover for 1 h in ^{22}Na-free ATW with 500 μmol l^{-1} Na^+ as NaCl that was either drug free (control), had 1% DMSO (vehicle control), or that had 10^{-4} mol l^{-1} colchicine ($C_{22}H_{25}NO_6$, Sigma-Aldrich) dissolved in DMSO. Then a second ^{22}Na flux was performed in the same recovery chambers. Thus, fluxes were completed on the same group of snails (10 controls and 10 per treatment) first in the un-bled drug-free condition, then in the bled condition with colchicine exposure.

Regardless of the mechanism of Na^+ transport employed by freshwater organisms, cellular substrate in the form of H^+ appears to be important for Na^+ uptake. To test the possibility that an increase in cellular substrate availability through increased cellular hydration of CO_2 to form HCO_3^- and H^+ was involved in the rapidly activated Na^+ transport, a lipophilic carbonic anhydrase inhibitor, ethoxzolamide (6-ethoxy-2-benzothiazolesulfonamide, $C_9H_{10}N_2O_3S_2$, ETOX; Sigma-Aldrich), was employed. An experiment with control (ATW), 1% DMSO (vehicle control), and 10^{-4} mol l^{-1} ETOX dissolved in DMSO was performed on un-bled (N=10 for both controls and ETOX) and bled (N=8, 10 and 10, respectively) snails as described above for the amiloride experiment. Final DMSO concentration was maintained at 1% of total flux volume for the vehicle control and all pharmacological treatments with the exception of bafilomycin, for which DMSO concentration was 0.1% of total flux volume.

Analytical techniques and calculations for Na^+ kinetics experiments

Hemolymph samples were analyzed for pH, total CO_2, osmotic pressure, [Na^+], [Cl^-], [Ca^{2+}] and protein concentration. In addition, samples from selected time points were analyzed for [Cu], the central metal in gastropod respiratory proteins (Sminia, 1977). Within 1 h after collection, samples were analyzed for pH (Radiometer Analytical MeterLab PHM201, Cedex, France) and total CO_2 (Corning Carbon Dioxide Analyzer 965, Essex, UK),

which were used to determine partial pressure of CO_2 (P_{CO_2}) using the Henderson-Hasselbalch equation and pK_I and pK_{II} values of 6.135 and 9.61, respectively, as obtained from Truchot alignment nomograms (Truchot, 1976). Osmotic pressure was measured by vapor pressure osmometry (Wescor Vapro 5520, Logan, UT, USA), cation concentrations were determined by flame atomic absorption spectrophotometry (Varian SpectrAA220, Mulgrave, Victoria, Australia), and [Cl^-] was determined by a colorimetric reaction based on the method of Zall et al. (Zall et al., 1956). Total protein concentration in each hemolymph sample was determined by the Bradford protein assay using bovine serum albumin standards (Bradford, 1976). The [Cu] was determined using graphite furnace atomizer, atomic absorption spectrophotometry (Varian SpectrAA220 with a SpectrAA GTA110, Mulgrave, Victoria, Australia).

The initial and final water samples from flux experiments were analyzed for ^{22}Na activity (Packard Cobra II Auto-Gamma, Meriden, Connecticut, USA) with a window of 15–2000 keV and [Na^+] was measured as described above. Unidirectional Na^+ influx was calculated from the mean specific activity of the initial and final water samples, the snail mass and the total flux time as described previously (Grosell et al., 2000). Net flux values were obtained from the change in total [Na^+] during the flux period, snail mass and elapsed flux time, whereas efflux was determined as the difference between net flux and influx.

Samples of the stock test solutions (from anion-dependency experiments) of NaCl, sodium gluconate and Na_2SO_4 before use in flux experiments and initial and final flux waters for six randomly selected snails (three un-bled and three bled) from each treatment were analyzed for [Cl^-] and [SO_4^{2-}] using anion chromatography (DIONEX DX 120 PeakNet 6, Sunnyvale, CA, USA) to verify 'Cl^--free' conditions. Results for each analytical procedure are reported as mean ± s.e.m.

Statistical analyses

Time course data were analyzed using one-way repeated-measures ANOVA comparing initial hemolymph characteristics to values at later time points with statistically significant differences among means being evaluated by Student's *t*-test using multi-sample Bonferroni correction. Uptake kinetic constants were determined assuming Michaelis–Menten kinetics and using the non-linear regression function in SigmaPlot for Windows version 8.00:

$$J = (J_{max} \times [Na^+]) / (K_m + [Na^+]) .$$

Kinetic constants from snails in the bled and un-bled condition were subsequently compared using Student's *t*-test.

For the anion-dependency and pharmacological analyses, Student's *t*-tests were used to determine the significance of drug effects or ambient anion (Cl^- and/or HCO_3^-) availability on Na^+ uptake rates in bled and un-bled conditions. Tests for normality and equal variance were completed before selecting the statistical test. For pharmacological data sets that were normally distributed and/or exhibited homoscedasticity (i.e. influx data), we used a one-way ANOVA with Holm–Sidak test of multiple comparisons as a more powerful alternative for multiple comparison testing relative to a Tukey's test. To assess differences among non-parametric data sets (i.e. efflux, and some of the net flux sets), Dunn's test of multiple comparisons was employed following a Kruskal–Wallis Rank Sums test as the more appropriate test for unequal sample sizes. All analyses were completed using SigmaStat for Windows version 3.00 and comparisons were considered significantly different at $P<0.05$.

RESULTS

Solute recovery in extracellular fluid

All snails exhibited significant losses of extracellular fluid (ECF) volume, major ions and other solutes following the initial bleeding event. Of the 134 snails sampled at the initial time point, seven escaped or died over the course of the experiment. Data from the initial sampling of these snails were not included in the analyses.

The significant loss of nearly 30% of ECF, evident from fluid volumes obtained at the second bleeding at 2 h, was regained in 8 h as indicated by the increase in body mass (Fig. 1A), but fluid composition did not completely return to initial conditions during the first 2 weeks of the time course. Despite substantial fluid loss, osmotic pressure was generally 9–10% lower for the first 18–24 h, but rarely significantly so (Fig. 1B). Na^+ and Cl^- were the major ionic constituents of the initial osmotic pressure value of 109.8 mOsmol kg^{-1}. Na^+ took 24–48 h to recover to initial values from a 29% loss (Fig. 1C); and Cl^- also recovered from a significant loss of 39% in the same timeframe, though with greater variability (Fig. 1D). Ca^{2+} contributed much less to osmotic pressure and showed no significant change in concentration over the experimental period (Fig. 1E). Though total CO_2 concentration (mainly HCO_3^-) was much greater than that of Ca^{2+}, it also did not vary significantly during the time examined (Fig. 1F). Additionally, snail ECF had brief periods of significant alkalosis and increases in P_{CO_2} during earlier time points (first 24–72 h) and though both returned to initial levels concurrently or before the other parameters, including [Cu] and total protein concentration, there was significant acidosis and significantly elevated P_{CO_2} levels at 10 weeks (Table 2). The significant loss of Cu (Fig. 2A) and total proteins (Fig. 2B) did not recover over the 2 week sampling period and subsequent experiments revealed recovery by 5 weeks after the initial fluid loss. Surprisingly, plasma protein concentration exceeded initial values at 10 weeks.

Na^+ transport kinetics

Sodium uptake rates increased with increasing ambient [Na^+] in a hyperbolic pattern characteristic of Michaelis–Menten kinetics in

Table 2. Mean pH and P_{CO_2} for extracellular fluid samples taken from snails at the initial time point and sub-sets of the initial group re-sampled at time intervals listed

Time point (h) (*N*)	pH	P_{CO_2} (Pa)
0 (118)	8.34±0.01	385.3±13.33
2 (3)	8.37±0.09	269.3±85.33
4 (3)	8.32±0.05	380.0±8.00
6 (8)	8.45±0.05*	328.0±48.00
8 (8)	8.33±0.04	341.3±46.66
10 (8)	8.43±0.04*	364.0±25.33
12 (8)	8.35±0.02	414.6±32.00
16 (8)	8.40±0.05	413.3±85.33
18 (7)	8.27±0.03	490.6±32.00*
24 (8)	8.31±0.03	400.0±36.00
48 (8)	8.34±0.02	380.00±22.66
72 (7)	8.43±0.09*	292.0±44.00
168 (10)	8.24±0.02	413.3±28.00
336 (10)	8.23±0.04	388.00±37.33
5 weeks (10)	8.21±0.04	361.3±30.66
10 weeks (8)	8.02±0.06*	596.00±100.00*

Asterisks indicate time points for which data were significantly different from time 0 mean. Smaller sample sizes at 2 h and 4 h were due to insufficient fluid volumes to complete all analyses at these time points. Values are means ± s.e.m.

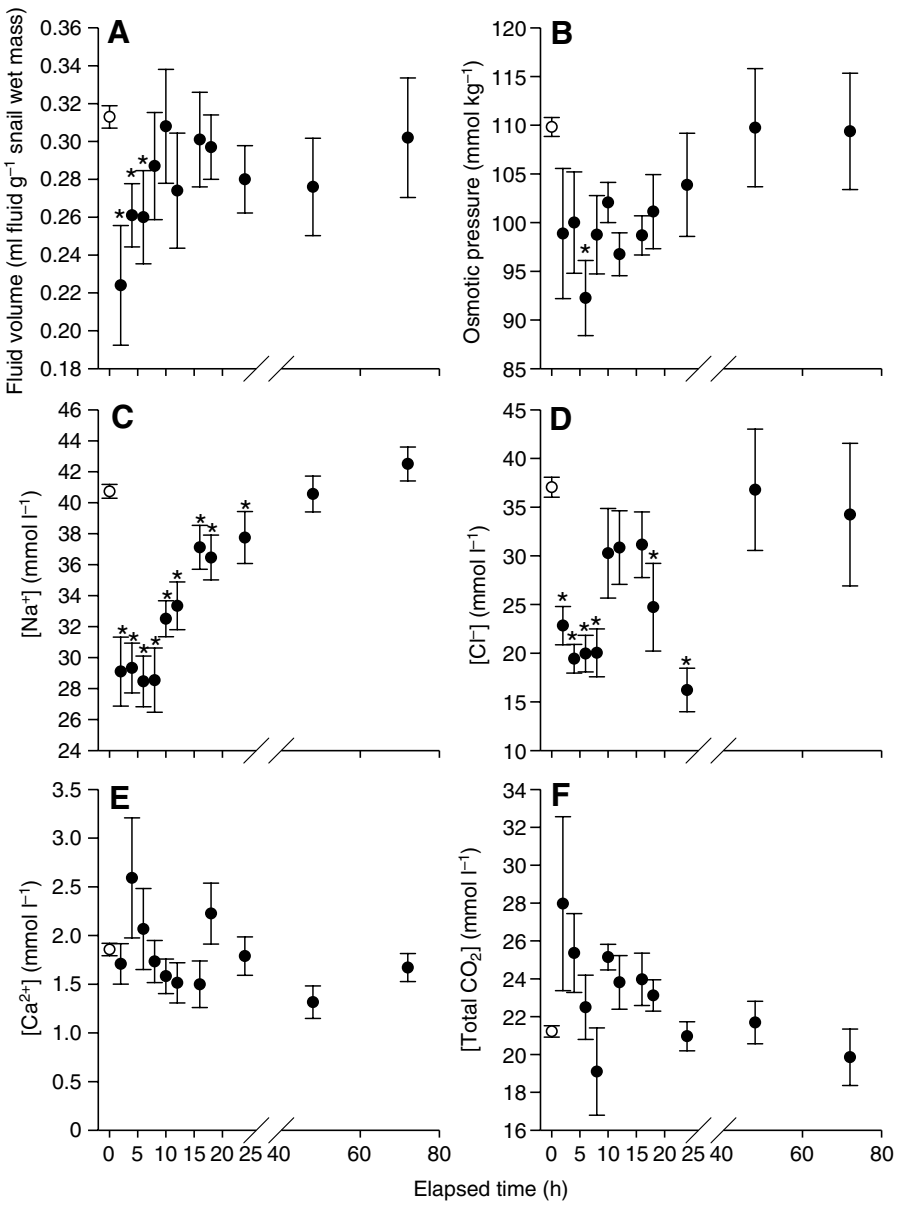

Fig. 1. Fluid volume released during bleeding (A), osmotic pressure (B), and the concentrations of the main electrolytes (C) Na^+, (D) Cl^-, (E) Ca^{2+} and (F) total CO_2; in hemolymph from *L. stagnalis* taken from all individuals at the initial time point (open circles) and again from subsets of the same initial snails in groups of eight individuals at any one time point between 2 and 72 h (closed circles) following initial sampling. Asterisks indicate values significantly different from initial time point. Values are means ± s.e.m. (N=114 at t_0 and N=8 at later time points).

snails under both bled and un-bled conditions (Fig. 3); however, snails in the bled condition had significantly greater capacity (3.1-fold) and affinity (threefold) for Na^+ relative to the un-bled condition. In both cases, the affinity value (apparent K_m) was well below the [Na^+] in which the snails were reared (~1 mmol l^{-1}; Fig. 3). Flux values recorded at 2 mmol l^{-1} Na^+ did not conform to the saturation kinetics observed at flux concentrations below the rearing concentration of 1 mmol l^{-1}. Therefore, values recorded for snails in the un-bled and bled condition that were fluxed at nominal [Na^+] of 2 mmol l^{-1} were excluded from the regression analysis but are included in Fig. 3.

In addition to increased uptake rates with respect to ambient [Na^+] and whether snails were fluid-depleted, bled snails also exhibited a general increase in unidirectional Na^+ efflux relative to when they were under un-bled conditions. Nevertheless, there was consistently less net Na^+ loss and generally a net gain of Na^+ under recovery conditions (Table 3) with few exceptions. Although snails fluxed at 100 μmol l^{-1} Na^+ conformed to the aforementioned general trend of increased influx, they still experienced a net loss of Na^+ under bled conditions, albeit less loss than under pre-bleed conditions.

Assessment of anion dependency in Na^+ transport

The changes in transport kinetics equated to a general four- to fivefold increase in Na^+ uptake rate for bled snails in 500 μmol l^{-1} Na^+. This increase was neither Cl^- (Fig. 4) nor HCO_3^- dependent (Fig. 5). When 500 μmol l^{-1} Na^+ was made available to the snails in Cl^--free medium (sodium gluconate and sodium sulfate) *versus* the NaCl control, there was no significant difference in Na^+ uptake rates for either the bled or un-bled condition. However, there was significantly greater unidirectional efflux of Na^+ in un-bled snails in 0 μmol l^{-1} nominal [Cl^-] medium relative to those in 500 μmol l^{-1} Cl^-, which resulted in significant net Na^+ loss relative to NaCl control (Table 4). For snails under recovery conditions, there was no apparent correlation between Cl^- availability and unidirectional Na^+ efflux or net flux. The Cl^- concentration in the NaCl stock solution was 490.3 μmol l^{-1}, in the sodium gluconate stock was 10.1 μmol l^{-1} Cl^-, and in the sodium sulfate stock was

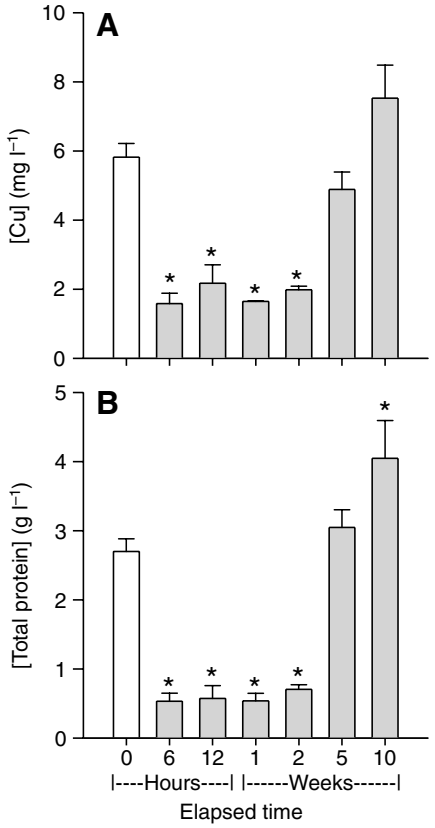

Fig. 2. (A) Copper and (B) total protein concentrations in hemolymph of *L. stagnalis* as a function of time. Individuals were bled at time 0 (open bars; *N*=54 for Cu, *N*=53 for proteins) and sub-groups (closed bars) were bled again at 6 h (*N*=8 for Cu and *N*=6 for proteins), 12 h (*N*=7 for Cu and *N*=8 for proteins), 1 week (*N*=10 for Cu and total proteins), 2 weeks (*N*=10 for Cu and total proteins), 5 weeks (*N*=10 for Cu and total proteins) and 10 weeks (*N*=9 for Cu and total proteins). Asterisks indicate values significantly different from initial time point values. Values are means ± s.e.m.

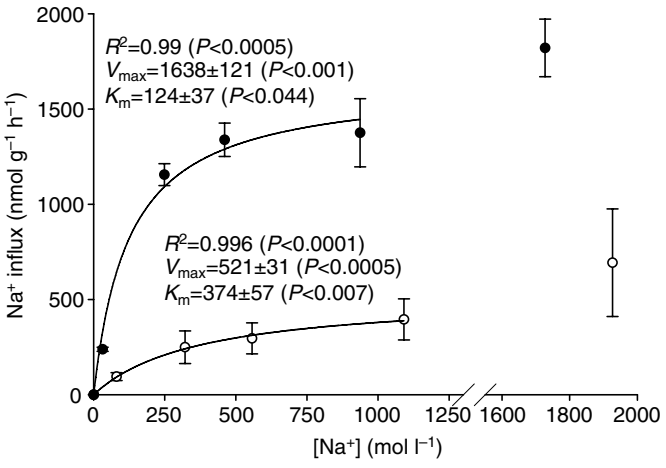

Fig. 3. Sodium uptake rates (nmol g^{-1} h^{-1}) as a function of ambient Na^+ ion concentration, by the pond snail *L. stagnalis.* Values are for bled (closed circles) and un-bled (open circles) snails. Regression lines are Michaelis–Menten curves (SigmaPlot 8.0 for Windows). Uptake values at 2 mmol l^{-1} [Na^+] were excluded from the regression analysis because they did not conform to the saturation kinetics observed at and below the rearing concentration of 1 mmol l^{-1}. *N*=10 for each data point. Values are means ± s.e.m.

Table 3. Sodium efflux and net flux in pond snail *Lymnaea stagnalis* under un-bled and bled conditions with varying ambient [Na^+]

Nominal [Na^+] (μmol l^{-1})	Condition (*N*)	Efflux (nmol g^{-1} h^{-1})	Net flux (nmol g^{-1} h^{-1})
100	Un-bled	–226±14†	–131±19
	Bled (10)	–277±9†	–39±9‡
250	Un-bled (9)	–361±57	–111±33
	Bled (10)	–932±57	223±34
500	Un-bled (10)	–446±57	–150±35
	Bled (10)	–1045±91	293±38
1000*	Un-bled (9)	–456±99	–61±23
	Bled (9)	–1163±151	212±71
2000	Un-bled (8)	–735±256	–41±38
	Bled (10)	–1518±107	303±53

*Concentration at which snails were reared and to which all statistical comparisons of Na^+ effects were made (1000 μmol l^{-1}). Values (means ± s.e.m.) for un-bled compared to bled condition within treatment concentration were significantly different except 100 μmol l^{-1} Na^+ concentration.
†Significantly lower efflux rate compared with respective condition in 1000 μmol l^{-1} treatment.
‡Significantly lower net flux rate than bled snails in 1000 μmol l^{-1} treatment.

below detection limit (~2 μmol l^{-1}). The mean [Cl^-] in snail flux chambers with ^{22}Na isotope were 534.1±27.88 μmol l^{-1} (maximum 698.3 μmol l^{-1}), 41.1±5.01 μmol l^{-1} (maximum 72.9 μmol l^{-1}) and 60.4±16.96 μmol l^{-1} (maximum 177.1 μmol l^{-1}) for NaCl, sodium gluconate and sodium sulfate, respectively. Bicarbonate availability did not affect Na^+ uptake in snails in the bled or un-bled condition provided with 500 μmol l^{-1} Na^+ and 0, 100 or 500 μmol l^{-1} HCO_3^- (Fig. 5). With the exception of the NaCl (100 μmol l^{-1} HCO_3^-), un-bled snails had significantly greater unidirectional efflux rates than when they were in the bled condition (Table 5). There was an unexpected higher basal Na^+ uptake rate for the bicarbonate dependency experiment with no significant differences in uptake rates from the different sources of Na^+ for un-bled snails (Fig. 5, Table 5). However, there was still

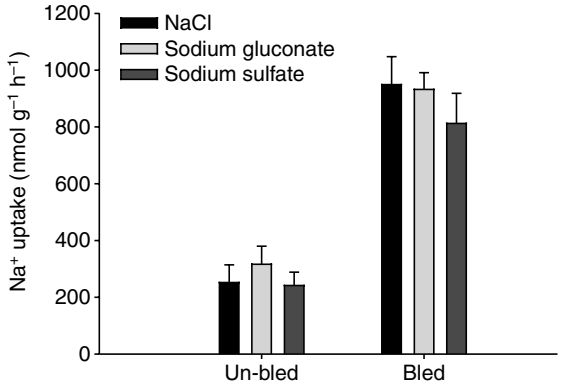

Fig. 4. Assessment of Cl^- dependency for Na^+ uptake by un-bled and bled *L. stagnalis* as tested under control (with NaCl) and treatment (with sodium gluconate or sodium sulfate) conditions. Each treatment medium contained 500 μmol l^{-1} of Na^+. Differences between un-bled and bled conditions within treatments were significantly different. There were no significant differences among Na^+-treatment groups in un-bled or bled conditions. *N*=10 snails in all cases. Values are means ± s.e.m.

Table 4. Assessment of Cl^- dependency of Na^+ efflux and net flux

Na compound (nominal [Cl^-]; μmol l^{-1})	Condition (*N*)	Efflux (nmol g^{-1} h^{-1})	Net flux (nmol g^{-1} h^{-1})
NaCl (500)	Un-bled (9)	–232±28	19±54
	Bled (10)	–362±38	587±124
Sodium gluconate (0)	Un-bled (10)	–457±32*	–141±59†
	Bled (10)	–267±17	665±55
Sodium sulfate (0)	Un-bled (10)	–595±35*	–354±64†
	Bled (10)	–326±29	487±89

Values (means ± s.e.m.) for un-bled compared with bled condition within treatment concentration were significantly different.
*Significantly greater unidirectional efflux than un-bled snails in 500 μmol l^{-1} Cl^-.
†Significantly greater net Na^+ loss relative to un-bled snails in 500 μmol l^{-1} Cl^-.

Table 5. Assessment of HCO_3^- dependency of Na^+ efflux and net flux

Na compound (nominal [HCO_3^-]; μmol l^{-1})	Condition (*N*)	Efflux (nmol g^{-1} h^{-1})	Net flux (nmol g^{-1} h^{-1})
$NaHCO_3^-$ (500)	Un-bled (9)	–426±60	69±83
	Bled (9)	–200±46	574±80
NaCl (100)	Un-bled (7)	–240±17	263±58
	Bled (9)	–223±43	571±62
Sodium gluconate (0)	Un-bled (10)	–468±31	137±48
	Bled (9)	–168±21	545±30
Sodium sulfate (0)	Un-bled (9)	–443±63	57±76
	Bled (10)	–194±62	551±108

Values (means ± s.e.m.) for un-bled compared to bled condition within treatment concentration were significantly different except unidirectional efflux within the 100 μmol l^{-1} [HCO_3^-].

a net gain of Na^+ across all HCO_3^- concentrations and ECF-level conditions.

Pharmacological assessment of Na^+ uptake

Of the five drugs tested, all except colchicine elicited significant reductions of post-bleed Na^+ uptake. In the amiloride experiment, bled snails treated with amiloride exhibited a concentration-dependent suppression of the amplified Na^+ uptake, which was significant at the 100 μmol l^{-1} amiloride concentration, compared to DMSO-control snails (Fig. 6). Sodium uptake in un-bled control snails was not affected by amiloride and there was no significant effect of DMSO on Na^+ uptake in un-bled or bled snails (Fig. 6). DMSO control, bled snails had significantly less unidirectional Na^+ efflux and significantly greater net Na^+ uptake than bled control snails (Table 6).

For the experiments to test efficiency of EIPA, bafilomycin and ETOX, bled snails in DMSO vehicle control exhibited significantly greater unidirectional Na^+ efflux than non-DMSO bled controls (Table 7). However, there was no significant effect of DMSO on net Na^+ flux. EIPA-treated, bled snails had

Table 6. Effect of a Na^+ transport inhibitor amiloride on Na^+ efflux and net flux

Drug	Condition (*N*)	Efflux (nmol g^{-1} h^{-1})	Net flux (nmol g^{-1} h^{-1})
Control	Un-bled (9)	–617±54	–389±49
	Bled (8)	–587±214	685±86
1% DMSO control	Un-bled (10)	–559±65	–269±56
	Bled (10)	–154±26*	1162±68†
10 μmol l^{-1} amiloride	Un-bled (10)	–648±69	–358±64
	Bled (10)	–408±281	976±126
100 μmol l^{-1} amiloride	Un-bled (9)	–421±47	–157±65
	Bled (9)	–64±127	988±104†

*Significantly less unidirectional efflux relative to un-bled snails in DMSO. For all other within treatment efflux comparisons, un-bled and bled condition values were not significantly different.
†Significantly greater net uptake relative to bled control. Net flux values for un-bled compared to bled condition within treatments were significantly different.
Values are means ± s.e.m.

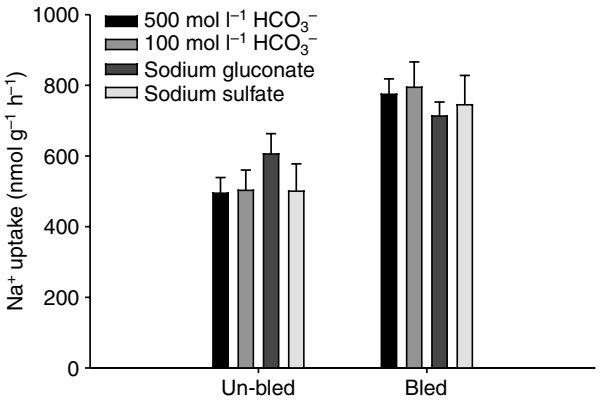

Fig. 5. Assessment of HCO_3^- dependency for Na^+ uptake by bled and un-bled *L. stagnalis* as tested under control (sodium bicarbonate) and treatment (NaCl, sodium gluconate or sodium sulfate) conditions. Each treatment medium contained 500 μmol l^{-1} of Na^+. Differences between un-bled and bled conditions within treatments were significantly different. There were no significant differences among sodium treatment groups in un-bled or bled conditions. *N*=10 in all cases except control (NaCl, *N*=8) and sodium gluconate treatment (*N*=9). Values are means ± s.e.m.

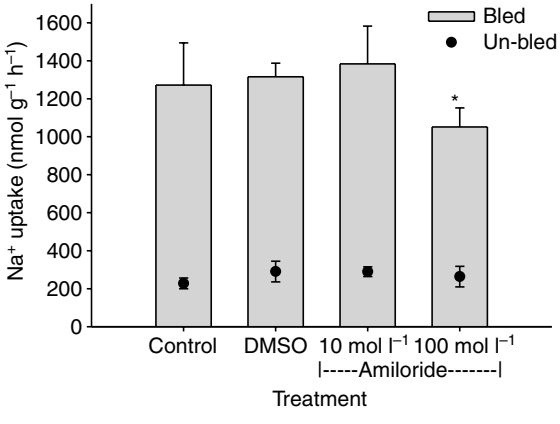

Fig. 6. Effect of 1% dimethyl sulfoxide (DMSO) and two amiloride concentrations on un-bled (closed circles) and bled (gray bars) snails. *N*=10 for each concentration/treatment. Both un-bled and bled snails were exposed to DMSO or amiloride. Asterisk indicates value significantly less than DMSO control value. Differences between un-bled and bled conditions within treatments were significantly different. There was no significant difference between uptake rates in regular control relative to rates in the DMSO carrier control medium. Values are means ± s.e.m.

Table 7. Effect of ethylisopropylamiloride (EIPA), ethoxzolamide (ETOX) and bafilomycin on unidirectional Na^+ efflux and net flux in snails under un-bled and bled conditions

Drug	Condition (*N*)	Efflux (nmol g^{-1} h^{-1})	Net flux (nmol g^{-1} h^{-1})
Control	Un-bled (10)	–559±30	–257±49
	Bled (8)	–136±23‡	505±58
1% DMSO control	Un-bled (10)	–440±27	–249±35
	Bled (10)	–384±38	411±97
EIPA	Un-bled (5)	–552±68	–424±62
	Bled (10)	–266±64	230±29§
ETOX	Un-bled (10)	–2534±195*	–2057±209†
	Bled (10)	–208±36	360±44
Bafilomycin	Un-bled (7)	–890±147*	–767±164
	Bled (10)	–125±57‡	256±40

*Significantly greater unidirectional Na^+ efflux compared with un-bled DMSO vehicle control.
†Significantly greater net Na^+ loss compared with un-bled DMSO vehicle control.
‡Significantly less unidirectional Na^+ efflux compared with bled DMSO vehicle control.
§Significantly less net Na^+ uptake compared with bled DMSO vehicle control.
Values are presented as mean ± s.e.m.

significantly reduced net (Fig. 7) and net (Table 7) Na^+ uptake rates relative to DMSO controls. Bled snails treated with bafilomycin had significantly lower unidirectional Na^+ influx (Fig. 7) rates compared with the DMSO controls. Bled snails treated with ETOX had significantly reduced Na^+ uptake rates (Fig. 7); however there was no significant effect of ETOX on unidirectional efflux or net flux in bled snails. The system responsible for the amplified Na^+ uptake observed in bled snails displayed no apparent sensitivity to colchicine for unidirectional influx, efflux or net flux (Table 8).

In un-bled snails, there was a significant increase in unidirectional Na^+ influx in DMSO carrier-control snails but the increase was not great enough to cause a significant effect in Net Na^+ flux (Fig. 7). There was no significant effect of EIPA on un-bled snails (Fig. 7, Table 7). Un-bled snails treated with the proton pump inhibitor, bafilomycin had significantly greater unidirectional Na^+ efflux rates relative to DMSO carrier controls; however the effect on net Na^+ flux was not statistically significant (Table 7). Also, ETOX-treated, un-bled snails had significantly greater unidirectional influx (Fig. 7) and efflux, with a resulting net Na^+ loss compared to DMSO control snails (Table 7). Un-bled snails were not treated with colchicine as we were interested in transporters that were possibly mobilized to facilitate the observed amplified uptake in bled snails.

Table 8. Effect of colchicine on unidirectional Na^+ influx, efflux and net flux in snails under bled condition

Drug	Condition (*N*)	Influx (nmol g^{-1} h^{-1})	Efflux (nmol g^{-1} h^{-1})	Net flux (nmol g^{-1} h^{-1})
Control	Bled (10)	870±122	–241±40	628±120
1% DMSO control	Bled (10)	813±81	–254±55	559±74
Colchicine	Bled (9)	900±87	–280±64	620±120

There were no significant differences among controls and colchicine treatment. Values are means ± s.e.m.

DISCUSSION

The naïve *L. stagnalis* utilized in the time course experiment exhibited a remarkable ability to recover significant losses of extracellular fluid (ECF) volume, major and some minor ions in a relatively short period of time despite its hypo-osmotic environment. Our non-lethal sampling technique required that the snails be air-exposed to allow collection of the ECF; however, these snails do exhibit the same full-body retraction while submerged (personal observation). The major osmolytes recovered from a 30% loss in 1–2 days. Initial concentrations of the major ions, Na^+ and Cl^-, in pallial fluid of *L. stagnalis* were 40.73±0.445 and 37.04±1.027 mmol l^{-1}, respectively and were slightly lower than those previously reported in hemolymph, which were approximately 49 mmol l^{-1} Na^+ (Burton, 1968) and approximately 44 mmol l^{-1} Cl^- (de With and van der Schors, 1982) from the same species. Based on Schlichter (Schlichter, 1981), we expected higher ion concentrations in pallial fluid relative to hemolymph values reported in the literature.

With respect to other ions measured, there were no significant changes in $[Ca^{2+}]$ or total CO_2 (mostly HCO_3^-) though pH and P_{CO_2} data indicated possible respiratory acidosis and partial compensation. Additionally, significant losses of protein and Cu, a component of the respiratory pigment hemocyanin, did not recover until 5 weeks after the initial fluid loss. Therefore, it was clear that the major osmotic constituents in these snails are recovered quickly; but the delayed recovery of some potentially important components such as Cu and plasma proteins indicates that repeat sampling may influence hemolymph chemistry significantly.

L. stagnalis exhibited saturation kinetics of Na^+ uptake in relation to ambient sodium concentrations, and displayed amplified Na^+ uptake in the bled condition similar to previously reported uptake rates of 132 nmol g^{-1} h^{-1} for un-bled snails acclimated to artificial tapwater containing 350 μmol l^{-1} Na^+ (Greenaway, 1970). Additionally, Na^+ uptake was 363±0.27 nmol g^{-1} h^{-1} for the Greenaway study in which snails were bled in a similar manner to that used in our study but acclimated to 350 μmol l^{-1} $[Na^+]$ and maintained in a 280 μmol l^{-1} Na^+ solution for a 1 h ^{22}Na flux. We measured unidirectional influx rates of 249±86 nmol g^{-1} h^{-1} and 1155±57 nmol g^{-1} h^{-1} for ambient $[Na^+]$ of 321 μmol l^{-1} $[Na^+]$ and 248 μmol l^{-1} $[Na^+]$ for snails under un-bled and bled conditions, respectively. Though the absolute flux values are different between the present and the earlier studies, the approximately threefold increase in the Greenaway (Greenaway, 1970) study was similar to the fourfold increase in our study. One point for consideration is that in the Greenaway study, different snails were utilized for the un-bled and bled snail experiments but flux values presented in the present study are for the same snails in the un-bled and bled conditions. Net flux rates observed in snails fluxed in 100 μmol l^{-1} nominal $[Na^+]$ indicated continued net loss of sodium even under post-bled conditions, which suggested a possible lower threshold of 100–250 μmol l^{-1} $[Na^+]$ below which the snails may be unable to recover from a bleeding event.

Four- to fivefold increases in sodium uptake rates of post-bleed snails relative to basal conditions provide some insight into the mode of relatively fast recovery observed in the time course. The total amount of extracellular Na^+ in naïve snails estimated from the measured hemolymph $[Na^+]$ and the reported volume of 0.45 ml hemolymph g^{-1} (van Aardt, 1968) is 18.3 μmol g^{-1}. This is comparable to 10.5 μmol g^{-1}

snail 2 h post-bleeding estimated from the $[Na^+]_{ECF}$ at that time and a reduction in hemolymph volume of 0.089 ml g^{-1} (the difference between volume released by naïve snails and snails re-bled 2 h after first bleed). Comparing these numbers reveals a remarkable 43% extracellular Na^+ loss and illustrates the need for Na^+ recovery from the environment. As such, the elevated net Na^+ uptake of 212 nmol Na^+ g^{-1} h^{-1} observed post-bleeding in snails allowed to flux in 1000 μmol l^{-1} Na^+, which is similar to the acclimation concentration (Table 3), if constant over the 48 h required for recovery, amounts to 10.2 μmol g^{-1} and thus accounts almost perfectly for the observed return to normal hemolymph $[Na^+]$ 48 h post-bleeding.

Because the post-bled uptake rate increase was attributed to significant increases of both capacity and affinity of the system, it was necessary to consider increased activity of transporters already active under basal conditions and the likely activation (translation, trafficking or recruitment) of dormant and perhaps distinct transporters. However, the first step was to consider whether the system was dependent on availability of anions in ambient media. Environmental Cl^- may facilitate Na^+ uptake through exchange of excess cytoplasmic HCO_3^- to maintain favorable conditions for continued H^+ production *via* CA-mediated CO_2 hydrolysis to be used in three known pathways for Na^+ uptake in freshwater invertebrates. This could be *via* electroneutral apical Na^+/H^+ exchange, electrogenic Na^+/H^+ exchange (Ahearn et al., 1994), and/or an epithelial Na^+ channel, which functions in conjunction with an apical H^+ pump. To determine whether any of these systems was utilized to maintain Na^+ homeostasis or for recovery, we assessed the possible use of these pathways under basal and recovery conditions. Previous studies have determined that Na^+ uptake can occur in the absence of ambient Cl^- availability (Krogh, 1938; Krogh, 1939; Kirschner, 2004), although the rate may be reduced relative to conditions that provide Cl^- (de With et al., 1987). Considering this evidence that Na^+ uptake can occur in Cl^--free conditions, we were not surprised to find a lack of dependency on Cl^-. We also evaluated whether this system was dependent on environmental availability of HCO_3^- to allow uptake *via* Na^+/HCO_3^- co-transport. The higher Na^+ uptake that was observed in un-bled snails in this experiment relative to other un-bled snails used in preliminary studies may have been due to any number of factors including, but not limited to, possible seasonal fluctuations in baseline Na^+ flux rates or changes in feeding patterns. After finding no significant dependency of the system on ambient anion availability, we attempted to characterize possible transporters involved in the Na^+ uptake under recovery and control conditions.

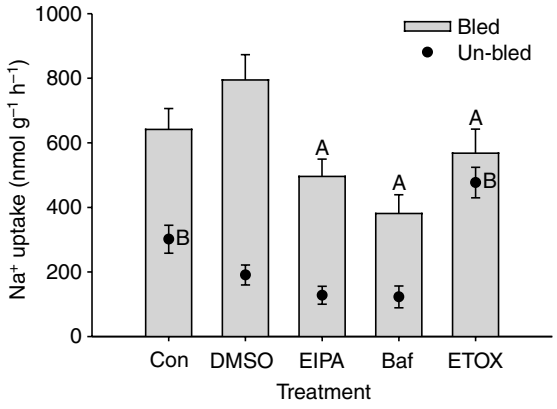

Fig. 7. Effect of 1% dimethyl sulfoxide (DMSO), 100 μmol l^{-1} ethylisopropylamiloride (EIPA), 100 μmol l^{-1} ethoxzolamide (ETOX) and 1 μmol l^{-1} bafilomycin (Baf) on un-bled (closed circles) and bled (gray bars). *N*=10 snails for each except un-bled EIPA (*N*=5), un-bled bafilomycin (*N*=7), and bled non-DMSO control (*N*=8). Both un-bled and bled snails were treated as indicated. [A]Bled snails in drug treatments had significantly lower Na^+ uptake rates than those in DMSO carrier control. [B]Un-bled snails in experimental control and those treated with ETOX had significantly greater unidirectional influx rates relative to those in the DMSO carrier control medium. Values are means ± s.e.m.

Pharmacological assessment of possible Na^+ transport proteins facilitating the increased Na^+ uptake rates in bled *L. stagnalis* revealed that the amplified uptake observed during the recovery phase is at least partially attributed to an amiloride-sensitive pathway (i.e. Na^+ channels and/or Na^+/H^+ exchange) and it appears that an electrogenic Na^+/H^+ exchanger may be the greater contributor to the enhanced Na^+ uptake associated with full-body withdrawal. Evidence for the involvement of an NHE transporter include no significant reduction in Na^+ recovery uptake rates, at the low amiloride concentration (10 μmol l^{-1}), which is assumed sufficient to inhibit the Na^+ channels (Kleyman and Cragoe, 1988), but a reduction with the higher concentration (100 μmol l^{-1}), which targeted Na^+/H^+ exchange (Kleyman and Cragoe, 1988). Also there was a significant reduction in Na^+ uptake in bled snails treated with EIPA, a more specific NHE inhibitor (Masereel et al., 2003). Furthermore, in bled snails the proton pump inhibitor, bafilomycin, caused the greatest reduction of any of the drugs used, which indicates the importance of the electrochemical gradient established by H^+ extrusion *via* a H^+ pump in the recovery phase system. The latter observation suggests that an electrogenic NHE-like protein may facilitate the amplified Na^+ uptake. Electrogenic NHE in gill, renal and gastrointestinal tissues of freshwater and marine species from both superphyla of invertebrates, Protostomia and Deuterostomia, has been reported to exchange $2Na^+$ for $1H^+$ (Ahearn et al., 1994; Ahearn et al., 2001). Thus the potential for an electrogenic NHE-type transport exists. However, the potential involvement of a cation channel in the enhanced Na^+ influx observed during the recovery phase cannot be excluded. Regardless of the mode of Na^+ entry, this mechanism appears to be limited by availability of cellular substrate in the form of excess H^+ as indicated by the reduced Na^+ uptake by bled snails that were treated with a CA inhibitor, ethoxzolamide. The lack of an effect of colchicine on Na^+ uptake following bleeding indicated that the amplified uptake observed in bled animals was not likely due to relocation of cytoplasmic transport proteins to the apical membrane.

The Na^+ channel and Na^+/H^+ inhibitor (amiloride), more specific Na^+ channel blocker (EIPA), proton pump inhibitor (bafilomycin) and CA inhibitor (ETOX) were all tested on un-bled snails, but only ETOX had a significant effect on net Na^+ transport. Interestingly, the significant effect of the ETOX treatment on all transport parameters measured in un-bled snails indicates a link between intracellular $[H^+]$ and Na^+ flux. Inhibition of CA results in elevated molecular CO_2 in the integument epithelial cells which presumably will tend to acidify these cells. Enhanced compensatory H^+ extrusion by NHE exchange is suggested by the significantly elevated Na^+ influx (Fig. 7). The resulting Na^+ gain appears to be more than fully compensated by elevated unidirectional Na^+ efflux. It appears that maintaining acid–base balance under conditions of CA inhibition occurs at the cost of significant Na^+ loss.

We conclude that though ECF chemistry is not completely restored to naïve, pre-bleed conditions, the noteworthy ability of this species to mobilize compensatory mechanisms on time scales of less than 3 h after significant fluid loss, is worthy of further study. Additionally, *L. stagnalis* recovers significant losses in volume first; then, and possibly more importantly, recovers major solutes from hypo-osmotic media in a matter of hours following fluid recovery. Finally, the ethoxzolamide sensitivity and the modest amiloride sensitivity of the system responsible for the Na^+ recovery indicates that the recovery process is at least partially dependent upon cellular substrate in the form of protons and likely occurs *via* an electrogenic Na^+/H^+ anti-porter.

Implications of findings

The consequences of the prolonged periods of reduced protein and Cu concentrations in the ECF are unknown, but if we assume the hemocyanin (and with that Cu) is one of the proteins lost with the ECF, impaired O_2 uptake and delivery may be one consequence. Such respiratory impairment might pose a need for more frequent surfacing for these facultative air-breathing snails following ECF loss, which could render them more susceptible to predation.

Furthermore, the energetic cost of the greatly increased Na^+ (and Cl^-) uptake following a substantial loss of ECF might be significant to these snails and is clearly worthy of investigation.

LIST OF ABBREVIATIONS

ATW	artificial tapwater
Baf	bafilomycin
CA	carbonic anhydrase
DMSO	dimethyl sulfoxide
ECF	extracellular fluid
EIPA	ethylisopropylamiloride
ETOX	ethoxzolamide
P_{CO_2}	partial pressure of carbon dioxide

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