J Comp Physiol B (2001) 171: 681–693
DOI 10.1007/s003600100219

ORIGINAL PAPER

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Ammonia uptake and its effects on ionoregulation in the freshwater crayfish *Pacifastacus leniusculus* (Dana)

Accepted: 30 June 2001 / Published online: 15 August 2001

Abstract Exposure of adult crayfish *Pacifastacus leniusculus* to Artificial Freshwater (AFW) media containing 1.5 m and 0.15 mmol.l^{-1} total ammonia [T_{amm}; 0.1×acute lethal concentration (24 h LC_{50}) and 0.01×24 h LC_{50}] and adjusted to pH 6.5, pH 8.2 and pH 10.5 resulted in significant increases in haemolymph ammonia over a 24-h period. Ammonia accumulated most rapidly at pH 10.5. These media were chosen to expose animals to a range of different un-ionised ammonia (UIA) [NH_3] and ionised ammonia [NH_4^+] concentrations. From comparisons of measured transepithelial potential differences (PD_{te}) with calculated Nernst potentials (PD_{NH4+}) for the known haemolymph-to-medium gradients of [NH_4^+], it was deduced that, in pH 8.2 and pH 6.5 AFW, NH_4^+ was not in thermodynamic equilibrium across the integument (presumably gill epithelium). In pH 10.5 AFW with 1.5 mmol.l^{-1} T_{amm} (predominantly NH_3), the accumulation of ammonia in the haemolymph was in the NH_4^+ form due to haemolymph pH regulation by the crayfish in this alkaline external medium. Measured net fluxes of ammonia (J_{amm}^{net}) were inwardly directed and maximal when [NH_3] was the main component externally, but were also significant at pH 8.2 with high [NH_4^+] ([NH_4^+]:[NH_3]≈20:1). Haemolymph Na^+ depletion was significant and, over the 24-h exposure period, most rapid in high [NH_3] medium but [Cl^-] was unaffected. However, paradoxically, sodium uptake (measured J_{Na}^{in} on immediate transfer to high T_{amm} medium) was not significantly inhibited when [NH_3] was the predominant ammonia species. In 1.5 mmol.l^{-1} T_{amm} (mainly [NH_4^+]), V_{Na}^{in} (the active component of J_{Na}^{in}) was significantly inhibited, particularly at low external [Na^+]. This inhibition could not be demonstrated as one of competition at an Na^+/NH_4^+ apical gill exchange site. The resultant net efflux of sodium from the animal showed that the ability of the animals to balance sodium losses at low external [Na^+] was severely affected. Longer exposure to pH 10.5 AFW with high [NH_3] (12 h) resulted in significantly increased J_{Na}^{out}, while not significantly affecting J_{Na}^{in}. Analysis of urinary Na^+ losses showed that, while urinary flow rate and water reabsorption was most likely unaffected by ammonia exposure, final urine [Na^+] was significantly elevated. The resulting urinary Na^+ loss accounted for 63% of the increased J_{Na}^{out} in high [NH_3] medium.

Keywords Ammonia net flux · Sodium uptake · Urinary losses · Freshwater crayfish · *Pacifastacus*

Abbreviations *AFW* Artificial Freshwater · ^{51}Cr-*EDTA* ethylenediaminetetra-acetic acid radioactively labelled with ^{51}Cr · J_{amm}^{net} net ammonia flux · J_{Na}^{in} sodium influx · J_{Na}^{out} sodium efflux · J_{Na}^{net} net sodium flux · *OC* osmotic concentration · pK'_{amm} ammonia dissociation constant · PD_{te} transepithelial potential difference · PD_{NH4+} calculated Nernst potential for NH_4^+ · P_{NH3} partial pressure of un-ionised ammonia · $\Delta[NH_3]/\Delta[NH_4^+]$ mean gradients for NH_3 and NH_4^+ T_{amm} · total ammonia · U/B urine-to-blood concentration ratio · *UIA*: un-ionised ammonia · V_{cl} clearance rate · V_{Na}^{in} apparent active uptake rate of sodium · V_u urine flow rate · *24 h* LC_{50} acute lethal concentration · *48 h* LC_{50} acute lethal concentration

Introduction

Ammonia is a common freshwater pollutant arising from various sources, notably with agricultural organic waste (slurry, silage liquors), in sewage and industrial

Communicated by G. Heldmaier

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effluents (Williams et al. 1986; National Rivers Authority 1992; Bloxham et al. 1999). It can also accumulate in aquaculture under intensive rearing conditions, particularly where there is re-cycling of water (Smart 1981). Both fish and crayfish aquaculture systems can suffer from elevated ammonia due to overcrowding or ammonia contamination of incoming water supply (Mead and Watts 1995). Williams et al. (1986) give mean values (1976 data) at 229 sites in the UK of 0.899 $mg.l^{-1}$ NH_3-N, with the ten most polluted sites in the range 13.1–42.8 $mg.l^{-1}$ NH_3-N (~0.8–2.5 $mmol.l^{-1}$). Far higher levels can occur locally at waste disposal sites and industrial effluent outfalls (up to 250 $g.l^{-1}$ NH_3-N (14.7 $mmol.l^{-1}$; Bloxham et al. 1999).

Ammonia occurs in natural waters in two forms; unionised ammonia (UIA; NH_3) and ionised ammonium (NH_4^+), the relative proportions of each depending on pH, temperature and ionic strength. The ammonia dissociation constant (pK^1_{amm}) and solubility values for ammonia of waters of different salinity and temperature have been published (Trussel 1972; Whitfield 1974; Emerson et al. 1975), as have constants for fish plasma (Cameron and Heisler 1983). Acute toxicity studies in freshwater have suggested that, in both fish and invertebrates such as crayfish, UIA concentrations are the determinants of mortality so that in alkaline conditions, mortality is greater for a given total ammonia concentration (T_{amm}) than in neutral pH waters (McCahon et al. 1991). Toxicity tests have tended to use relatively high concentrations of ammonium salts at natural water pH values to generate ranges of different NH_3 concentrations (Meade and Watts 1995). Field experiments studying the acute toxic effects of ammonia have shown that, if present in moderate concentrations but in alkaline waters, a rapid and high mortality of crayfish can occur (Foster and Turner 1993).

The high toxicity of UIA is attributed to its more rapid transfer across gill epithelia in fish and its accumulation within blood and tissues where it produces neurological effects and impairment of oxidative metabolism (Korsgaard et al. 1995 for review). However, less is known about its entry into crustaceans or its toxic effects in the group.

Some studies have concluded that high external NH_4^+ also has significant toxic effects. Thus, for crustaceans, Armstrong et al. (1978) proposed that it inhibited Na^+/NH_4^+ exchange processes at the gills, thus causing sodium depletion, while Lin et al. (1993) have shown that high [NH_4^+] causes osmoregulatory failure in early stages of *Penaeus japonicus*, mainly due to reductions in blood Na^+ levels. Other crustaceans show this reduction in haemolymph [Na^+] (Spaargaren 1990; Young-Lai et al. 1991). Early studies on crayfish by Shaw (1960) showed that external ammonia severely inhibited sodium influx This finding was important early evidence for the role of a Na^+/NH_4^+ apical exchange in the gills of aquatic animals. These actions may be important where the UIA component of T_{amm} is low and may involve uptake of ammonia in the NH_4^+ form.

There is an additional significance of ammonia as a toxicant due to the fact that it is the major end-product of nitrogen excretion in most aquatic species which are described as ammonotelic. In crustaceans 60–95% of total excreted nitrogen leaves the animal, mainly via the gills, as ammonia (Regnault 1987; Greenaway 1991 for reviews). Proposed mechanisms of ammonia excretion in crustaceans include diffusion of NH_3 down its partial pressure gradient between haemolymph and medium (ΔP_{NH3}), diffusion of NH_4^+ down an electrochemical gradient for ammonium (possibly paracellularly; Kormanik and Cameron 1981), and active excretion via a specific NH_4^+ channel or the Na^+/NH_4^+ exchanger (antiport) present in the apical membrane of gill epithelial cells. However, amiloride inhibition of ammonia net efflux is only partial or lacking in some species suggesting that diffusion of NH_3 is the major route here (Pressley et al. 1981). The use of reversed P_{NH3} gradients for investigating the mechanisms of ammonia excretion by gills of aquatic animals, mainly fish, (Cameron 1986; Wilson and Taylor 1992) elicited interpretations favouring an NH_4^+/H^+ exchange process in freshwater species. Recently, further support for ammonia excretion as NH_4^+ has come from ammonia flux measurements in isolated perfused gills of marine and freshwater crabs in which net effluxes of NH_4^+ against transepithelial concentration gradients of that ion of up to 300 $\mu mol.l^{-1}$ T_{amm} have been reported (Weihrauch et al. 1999). It is also suggested that, in habitats with high T_{amm}, the regulation of body ammonia is carried out by an active NH_4^+ extrusion mechanism. However, Wilkie (1997), in a well-argued and comprehensive review of ammonia excretion across fish gills, suggests that, in freshwater species, neither Na^+/NH_4^+ nor NH_4^+/H^+ exchanges are significant, and the primary route for ammonia excretion takes place via favourable P_{NH3} gradients. He also postulates that acidification of expired gill-water microlayer enhances these gradients.

Less is known about the effects of external ammonia on freshwater invertebrates and the mechanisms of its excretion. *Pacifastacus leniusculus*, the signal crayfish, originating from the northwestern USA has invaded many freshwater bodies in the UK. It is suggested to be relatively tolerant of many pollutants, including ammonia (Firkins 1993); more so than the native crayfish, *Austropotamobius pallipes*, with which Shaw (1960) worked. As a burrowing species it may be normally exposed to higher levels of ambient ammonia than other invertebrates and fish, an aspect of its biology which is being investigated. As a species used extensively in aquaculture, knowledge of its susceptibility to water ammonia levels will be of practical use. The physiology of the species has been studied extensively under many different environmental conditions (Wheatly and McMahon 1979, 1982; Wheatly 1989; Wheatly and Toop 1989; Harris and Coley 1991). Many comparisons can be made between normal and pollutant-exposed animals, making the species a useful model for the study of the impact of pollutants on freshwater invertebrates.

Materials and methods

Adult intermoult *P. leniusculus* (Dana) (mean mass 35.06 ± 2.06 g, $n = 95$) were obtained from crayfish farms or local streams and maintained in de-chlorinated, copper-free Leicester tapwater ($[Na^+] = 0.70$ mmol.l^{-1}, $[Cl^-] = 1.26$ mmol.l^{-1}, $[Ca^{2+}] = 2.50$ m equiv.l^{-1}) at 15 °C in batches of about 16 animals. Each batch was held in a 2-m long by 0.5-m wide and 0.25-m deep concrete tank under subdued lighting (natural day/night cycle). Animals were provided with short lengths of PVC drainpipe as shelters. They were fed weekly with parboiled potato and "Minced Morsals" dry dog food. Prior to the experiments the animals had been unfed for about 1 week.

Measurements of ammonia toxicity, ammonia net flux and sodium fluxes were carried out in an artificial freshwater medium (AFW; Harris and Coley 1991) to which different volumes of 100 mmol.l^{-1} NaCl stock solution were added to give the required Na^+ and Cl^- concentrations. The majority of the experiments were carried out in 1.0 mmol.l^{-1} $[Na^+]$. In studies with ammonia in the medium, volumes of 100 mmol.l^{-1} NH_4Cl stock solution (made up in AFW) were added to give the required concentrations. All media were pH-adjusted with KOH or H_2SO_4 to within ± 0.1 pH units of the required pH. This was monitored throughout using a Russell 1145 pH-combination glass microelectrode connected to an EIL 7050 pH meter.

Ammonia toxicity tests, and experiments to investigate the effects of ammonia on various haemolymph and urine constituents, were carried out on separate groups of animals held in individual, darkened chambers (800 ml), through which medium was pumped (~10 ml.min^{-1}) by means of peristaltic pumps (Minipump). These were supplied from 20-l polythene reservoirs containing the appropriate pH-adjusted AFW medium. Each chamber had a washed gravel substrate. Waste medium was allowed to overflow into the drainage system. The choice of test media and ammonia concentrations used was based on field observations of stream water chemistry made near a point source of chronic agricultural waste input (pig slurry) in Leicestershire, England. Relatively high T_{amm} values were recorded [3-year mean (1989–1992) = 0.71 ± mmol.l^{-1} (12.8 mg.l^{-1}), with peaks of up to 1.07 mmol.l^{-1} (19.3 mg. l^{-1})]. Water pH tended to alkalinity (average 8.2 ± 0.2). T_{amm} decreased in concentration with increasing distance from the source of pollution input (Coley 1992). The stream had previously held healthy populations of crayfish (in this case *Austropotamobius pallipes*) but recent surveys have failed to record any crayfish species.

Medium pH, T_{amm}, $[Na^+]$, $[Cl^-]$, and P_{O2} were monitored during all experiments (duration 4–24 h). Medium T_{amm} was determined (using 1-ml samples) by the salicylate-dichloroisocyanurate method of Reardon et al. (1966) This method was scaled down, and modified for use with both haemolymph and urine samples by the inclusion of a protein precipitation stage (Harris and Andrews 1985). Initially, this method was tested for accuracy and reliability by comparing measured values with those obtained using a GLDH enzymatic U/V method (Sigma). T_{amm} values of urine and blood samples measured using the two methods were in close agreement. Some medium T_{amm} measurements were made with an ammonia electrode (Orion 9512).

In all experiments haemolymph samples (0.1–0.2 ml) were exclusively pre-branchial and removed from the infra-branchial sinus by a 1-ml hypodermic syringe. Urine samples (~0.25 ml volume) were collected by gently tapping the opening of the nephropore and aspirating urine into a fine glass micropipette as it was released.

Medium, haemolymph and urine $[Na^+]$ were determined by flame photometry (Jenway PFP 7), after appropriate dilution. The $[Cl^-]$ of haemolymph (20-µl samples) was determined by Corning 925 titrator, and haemolymph osmolality by freezing-point depression (Roebling micro-osmometer).

Haemolymph and urine pH measurements were made immediately after collection using a Radiometer G299 A liquid junction capillary pH microelectrode connected to a Radiometer PHM73 blood gas analyser. Transepithelial potential differences (PD_{te}) were measured using calomel electrodes connected to 3 M KCl-filled glass microlectrodes via KCl/agar polythene bridges (10 cm in length) on an EIL Vibron 33B -S electrometer (input impedance ~10^{15} ohms) connected to a chart recorder. Animals were prepared for PD_{te} measurements as described previously (Harris and Coley 1991). Mean values of PD_{te} are given in millivolts, the sign being that of the haemolymph with respect to the external medium.

All fluxes are expressed per gram wet weight of animal. Ammonia net fluxes (J_{amm}^{net}) were determined on animals maintained in 200 ml AFW of appropriate composition in darkened Perspex flux chambers. Following transfer, 1-ml samples were removed at timed intervals (~hourly over 4–5 h) and stored at 5 °C for assay at the end of the experiment. J_{amm}^{net} (µmol.g^{-1}.h^{-1}) for each individual was calculated from the relationship:

$$J_{amm}^{net} = [T_{amm}]_i - [T_{amm}]_f.V/1000.t.W$$

where $[T_{amm}]_i$ is the initial, and $[T_{amm}]_f$ the final total ammonia (mmol.l^{-1}) in the water after time t (h). V is the volume of the medium (ml), and W is the wet mass of the animal (g). J_{amm}^{net} was found to be both positive i.e. net uptake of ammonia in high T_{amm} media, or negative where there was a net loss of ammonia from the animal in media with no added ammonia. T_{amm} includes both free, un-ionised ammonia $[NH_3]$ (UIA) and ionised ammonia $[NH_4^+]$. The concentrations of these forms in AFW media were calculated at different pH values using the pK′ for ammonia in freshwater at 15 °C of 9.490 (Cameron and Heisler 1983). Where animals were maintained in small volumes of medium, for example for measurement of J_{amm}^{net} and J_{Na}^{in} (see below), average $[NH_3]$ and $[NH_4^+]$ were calculated since medium pH changes occurred in the bath medium and these were different between individuals. Average pre-branchial haemolymph-to-water $[NH_3]$ and $[NH_4^+]$ gradients ($\Delta[NH_3]$ or $\Delta[NH_4^+]$; mmol.l^{-1}), were also calculated, using the pK' of a 200-mmol.l^{-1} NaCl solution (9.610), as an approximation of the ionic strength of *P. leniusculus* haemolymph, in the rearranged Henderson-Hasselbalch equation (Cameron and Heisler 1983; Wilson and Taylor 1992) and haemolymph pH and T_{amm} measured at the beginning and the end of the flux measurement periods.

Uni-directional sodium influxes (J_{Na}^{in}: µmol.g^{-1}.h^{-1}) were determined using ^{22}Na (NEN). Sodium-depleted animals (individuals placed in Na-free AFW for 24 h) were blotted dry, and their branchial chambers drained by shaking, before being placed in flux chambers containing 200 ml AFW with the required $[Na^+]$ and $[T_{amm}]$ (^{22}Na specific activity = 0.6–4 MBq.mmol^{-1} depending on medium $[Na^+]$). The medium was re-circulated past the animal (8–9 ml.min^{-1}) by peristaltic pump to ensure mixing. Samples of medium (1 ml) were removed for counting at 30-min intervals over a period of 4–5 h and replaced. ^{22}Na radioactivity was measured in a well-type NaI crystal scintillation detector connected to a Panax Reigate counter. Sodium influxes were calculated from the relationship:

$$M = A.[\ln(C_t/C_o)/t]$$

where C_o and C_t are the initial and final ^{22}Na counts (corrected for background), and A the total number of micromoles of Na^+ present in the external medium. M is the number of micromoles of Na^+ taken up by the animal in time t. Sodium influx, J_{Na}^{in}, was taken as: M/live mass of animal (g).

Medium specific activity greatly exceeded that of the animal during the experiment and A was not allowed to decrease by more than 10%.

Net sodium flux rates (J_{Na}^{net} ; µmol.g^{-1}.h^{-1}) was determined from the change in medium $[Na^+]$ (2-ml samples of original and final medium from each animal chamber) and calculated using the same relationship as for J_{amm}^{net} above. Sodium efflux (J_{Na}^{out}) was obtained by rearranging the formula: $J_{Na}^{net} = J_{Na}^{in} - J_{Na}^{out}$ (Kirschner 1982).

The apparent active uptake of sodium V_{iNa}^{in} was calculated as $J_{Na}^{in} - J_{Na}^{in(p)}$, where $J_{Na}^{in(p)} = J_{Na}^{out}.\exp(-FE/RT).[Na_{out}]/[Na_{in}]$.

Here, $[Na_{out}]$ = medium $[Na^+]$, $[Na_{in}]$ = haemolymph $[Na^+]$ (mmol.l^{-1}), F = 96500 °C.mol^{-1}, T = 288 K, R = 8.314 J.mol^{-1}, E = PD_{te} (after Taylor and Harris 1986).

Urine flow rates were determined by measuring the clearance rate of ethylenediaminetetra-acetic acid radioactively labelled with ^{51}Cr (^{51}Cr-EDTA). Concentrated ^{51}Cr-EDTA solution (Amersham; 50µl, 0.74 MBq) was injected at the base of the fifth pereiopod and allowed to distribute in the haemocoel for 1.5 h. An initial haemolymph sample was taken from the limb on the opposite side of the body, diluted with 1 ml distilled water and counted for ^{51}Cr radioactivity. Samples were taken at 12-h intervals for 96 h. Urine samples for ^{51}Cr-EDTA counting were taken at similar intervals. Haemolymph ^{51}Cr-EDTA activity was plotted semi-logarithmically against time and, from the slope of the resulting linear plots, the rate constant of clearance (k; h^{-1}), determined. ^{51}Cr-EDTA clearance (V_{cl}; ml.kg^{-1}.h^{-1}) was calculated as described in Harris and Andrews (1982). Urine flow rates(V_u; ml.kg^{-1}.h^{-1}) were obtained from the relation: $V_{cl}/U/B_{Cr\text{-}EDTA}$, the divisor being the ratio of urine:blood ^{51}Cr-EDTA radioactivity at the termination of the clearance measurement period.

All data are expressed as means ± 1 SEM. The number of experimental animals is given in parenthesis. Differences between means were tested for significance by one-way analysis of variance and multiple range tests (Student-Newman-Keuls procedure), or paired sample Student's t-tests (two-tailed) where appropriate.

Results

Ammonia toxicity

Exposure of adult *P. leniusculus* to AFW medium containing ammonia and adjusted to pH 8.2 (to simulate field conditions; see above), gave 24 h and 48 h LC_{50} values of, respectively, 15.0 ± 2.6 mmol.l^{-1} and 4.9 ± 1.1 mmol.l^{-1} T_{amm} (n = 24). From the nomogram given in Cameron and Heisler (1983), the UIA (NH_3) concentrations of these levels of T_{amm} were calculated as 0.91 mmol.l^{-1} and 0.30 mmol.l^{-1}, respectively, at pH 8.2 and 15 °C. Animals exposed to ammonia became rapidly disorientated and sluggish, often remaining inverted for the duration of the exposure. This state persisted even following removal from the ammonia-containing medium and transfer to flowing AFW with no ammonia added. Severely affected animals, with no eye withdrawal reflex but some slow scaphognathite activity, often did not recover from ammonia exposure. On the basis of these data, T_{amm} levels in AFW of 0.1×24 h LC_{50} and 0.01×24 h LC_{50} (1.5 mmol.l^{-1} and 0.15 mmol.l^{-1} T_{amm}) were used in experiments to investigate the uptake and sub-lethal effects of ammonia.

Haemolymph ammonia changes following exposure to ammonia

The mean T_{amm} of pre-branchial haemolymph of *P. leniusculus*, maintained in AFW with no added ammonia (< 0.005 mmol.l^{-1} T_{amm}), was 0.30 ± 0.02 (24) mmol.l^{-1}. Some differences were seen in haemolymph levels between batches of animals but generally T_{amm} values were in the range 0.25–0.35 mmol.l^{-1}. These levels were maintained over the 24-h experimental period in AFW media adjusted to pH 10.5, 8.2 and 6.5. Following transfer to media with 1.5 mmol.l^{-1} T_{amm} added (0.1×24 h LC_{50}), haemolymph T_{amm} increased. Over 24 h, the increase was most rapid, and to a significantly higher concentration (0.93 ± 0.20 mmol.l^{-1}) in pH 10.5 AFW (containing $[NH_4^+]$ = 0.13 mmol.l^{-1} and $[NH_3]$ = 1.37 mmol.l^{-1}; i.e. a ~0.1:1.0 concentration ratio; P < 0.001; Fig. 1) Smaller, but significant, increases in haemolymph T_{amm} were seen in pH 8.2 and pH 6.5 media (containing $[NH_4^+]$ = 1.43 mmol.l^{-1} and 1.499 mmol.l^{-1}; $[NH_3]$ = 0.07 mmol.l^{-1} and 0.001 mmol.l^{-1}, respectively; P < 0.05). Significant increases in haemolymph T_{amm} were seen also in pH 8.2 and pH 10.5 media with 0.15 mmol.l^{-1} T_{amm} added (0.01×24 h LC_{50}). The latter data are not shown for reasons of space.

In these experiments, different haemolymph-to-medium gradients of $[NH_3]$ and $[NH_4^+]$ were created by changing the medium pH while maintaining T_{amm} constant. In order to calculate these gradients, haemolymph pH was also measured during exposure to each medium (Table 1). The mean haemolymph pH of animals maintained in AFW with no added ammonia was 7.505 ± 0.022 (18). Following 24 h exposure to AFW of pH 6.5, 8.2 and 10.5, no significant change in haemolymph pH was seen. However, exposure to media containing 1.5 mmol.l^{-1} T_{amm} resulted in a significant change only at pH 8.2 where an acidosis was seen (P < 0.01; Table 1).

P. leniusculus appears to take up ammonia particularly rapidly when NH_3 predominates (alkaline condi-

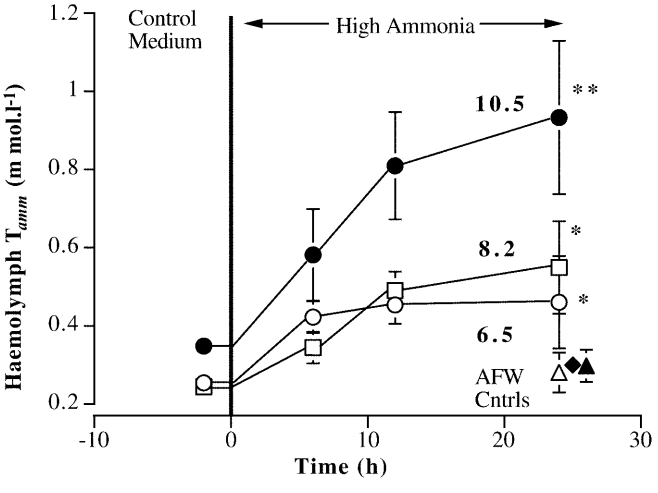

Fig. 1 Changes in haemolymph total ammonia (T_{amm}) in *Pacifastacus leniusculus* exposed to flowing artificial freshwater (AFW) medium containing 1.5 mmol.l^{-1} T_{amm} and pH-adjusted to produce different concentrations of NH_3 (un-ionised ammonia, UIA) and NH_4^+. Thus, the $[NH_3]$:$[NH_4^+]$ ratio is 0.001:1.499 mmol.l^{-1} in pH 6.5 (*open circles*), 0.07:1.43 mmol.l^{-1} in pH 8.2 (*open squares*), and 1.37:0.13 mmol.l^{-1} in pH 10.5 (*filled circles*). Control animals (AFW Controls) measured at 24 h are shown in same order of pH, *open triangle*, *filled diamond* and *filled triangle*. *Significant increases compared to mean in control medium of same pH; **significantly higher T_{amm} compared to all other groups at 24 h. Means ± 1 SEM (n = 6–12)

Table 1 Haemolymph pH in *Pacifastacus leniusculus* before and after 24 h exposure to different pH-adjusted artificial freshwater (AFW) media without (control) and with 1.5 mmol.l^{-1} T_{amm} (+amm). Means ± 1 SEM; $n = 6$)

Time (h)	pH 6.5		pH 8.2		pH 10.5	
	Control	+amm	Control	+amm	Control	+amm
0	7.399 ± 0.026	7.403 ± 0.016	7.566 ± 0.023	7.522 ± 0.021*	7.531 ± 0.023	7.686 ± 0.094
24	7.383 ± 0.070	7.375 ± 0.031	7.530 ± 0.030**	7.230 ± 0.065*, **	7.515 ± 0.048	7.615 ± 0.033

*Significant difference between 0 and 24 h values
**Significantly different from controls after 24 h exposure to ammonia

tions). However, some uptake also occurs in media where NH_4^+ is the major component but, in both situations, haemolymph T_{amm} remained below that of the medium.

Ammonia fluxes

In AFW media (no added ammonia), a net efflux of ammonia was observed. Manipulation of medium pH resulted in differences in these rates of net efflux (J_{amm}^{net}). Rates were lowest in pH 10.5 medium (–0.011 ± 0.003 μmol.g^{-1}.h^{-1}; $n = 6$, the minus sign indicating a net efflux from the animal), and highest in pH 6.5 (–0.067 ± 0.005 μmol.g^{-1}.h^{-1}; Fig. 2). Transfer into media containing 1.5 mmol.l^{-1} T_{amm} resulted in large net influxes of ammonia, particularly in pH 10.5 AFW. Measured over 5 h, J_{amm}^{net} was significantly higher in this medium compared to pH 8.2 containing ammonia ($P < 0.001$). In contrast, in pH 6.5 AFW, J_{amm}^{net} was outwardly directed and very much reduced (–0.002 ± 0.028; $n = 6$) compared to control animal rates in pH 6.5. Initially, at least, all ammonia fluxes in media containing 1.5 mmol.l^{-1} T_{amm} were probably the resultants of large influxes of ammonia from the high external T_{amm} and outwardly directed effluxes of metabolically derived ammonia. For example, in pH 6.5 AFW with ammonia, some influx may have occurred, as suggested by the increase in haemolymph T_{amm} (Fig. 1). However, this may be due to an accumulation of metabolic ammonia. The highest rates of ammonia net influx occurred in media containing the highest [NH_3]. Calculated mean gradients of UIA and ammonium ion between pre-branchial haemolymph and external medium ($\Delta[NH_3]$ and $\Delta[NH_4^+]$) are also given in Fig. 2. Changes in pH occurred in the small volumes of medium used for flux measurements, probably caused by release of acidic equivalents from the animals. Therefore it was necessary to calculate for each individual an average $\Delta[NH_3]$ and $\Delta[NH_4^+]$ for the experiments, using the initial and final medium and haemolymph pH values.

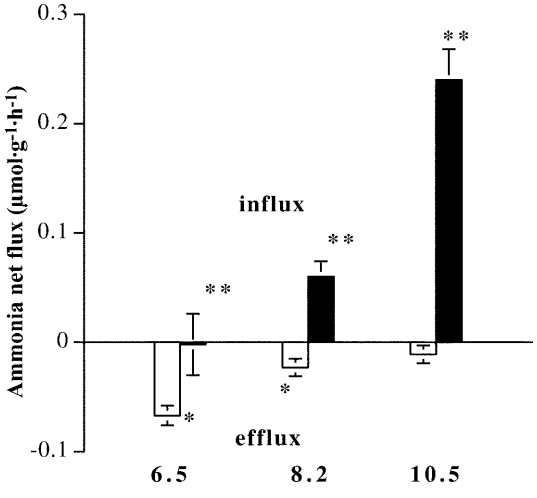

Fig. 2 Ammonia net fluxes in *P. leniusculus* in different pH-adjusted AFW media, without T_{amm} (*open squares*) and with (*filled squares*) 1.5 mmol.l^{-1} T_{amm}. In pH 6.5 with added ammonia, the calculated mean haemolymph-to-medium gradients $\Delta[NH_4^+] = -1.141$ mmol.l^{-1}, $\Delta[NH_3] = 0.003$ mmol.l^{-1}; in pH 8.2, $\Delta[NH_4^+] = -1.095$ mmol.l^{-1} and $\Delta[NH_3] = -0.010$ mmol.l^{-1}; in pH 10.5 $\Delta[NH_4^+] = -0.818$ mmol.l^{-1} and $\Delta[NH_3] = -0.235$ mmol.l^{-1}. The negative sign refers to a higher concentration of that ammonia component in the medium compared to the haemolymph. *Control means significantly different from each other; ** ammonia-exposed means significantly different from each other and from control net flux rates. Means ± 1 SEM, $n = 6$ for each treatment

Sub-lethal effects of ammonia

Haemolymph electrolyte and osmotic concentrations

The haemolymph [Na^+] of *P. leniusculus* in pH 8.3 and pH 10.5-adjusted AFW (no added ammonia) was relatively constant over the 24-h exposure period {mean of measurements at all sampling intervals = 205.7 ± 4.5 mmol.l^{-1} [Na^+] (24)}. However, in pH 6.5 AFW there was a steady and significant decline in haemolymph [Na^+] ($P < 0.01$; Fig. 3a).

Following exposure to media containing 1.5 mmol.l^{-1} T_{amm}, large reductions in haemolymph [Na^+] were observed, most notably in the pH 10.5 AFW medium. Animals showing a high degree of sodium depletion generally showed poor survival after removal from high-ammonia medium. Furthermore, survivors required a long period in low ammonia media before haemolymph [Na^+] was restored. The decreases in haemolymph [Na^+] seen in pH 8.2 and pH 6.5 media containing ammonia were smaller but significant ($P < 0.05$; Fig. 3b). In addition, animals maintained for 24 h in pH 10.5 AFW containing 0.15 mmol.l^{-1} T_{amm}, (0.01×24 h LC_{50}) showed a significant decrease in haemolymph [Na^+] (to 190.6 ± 6.7 mmol.l^{-1} [Na^+]; data not shown).

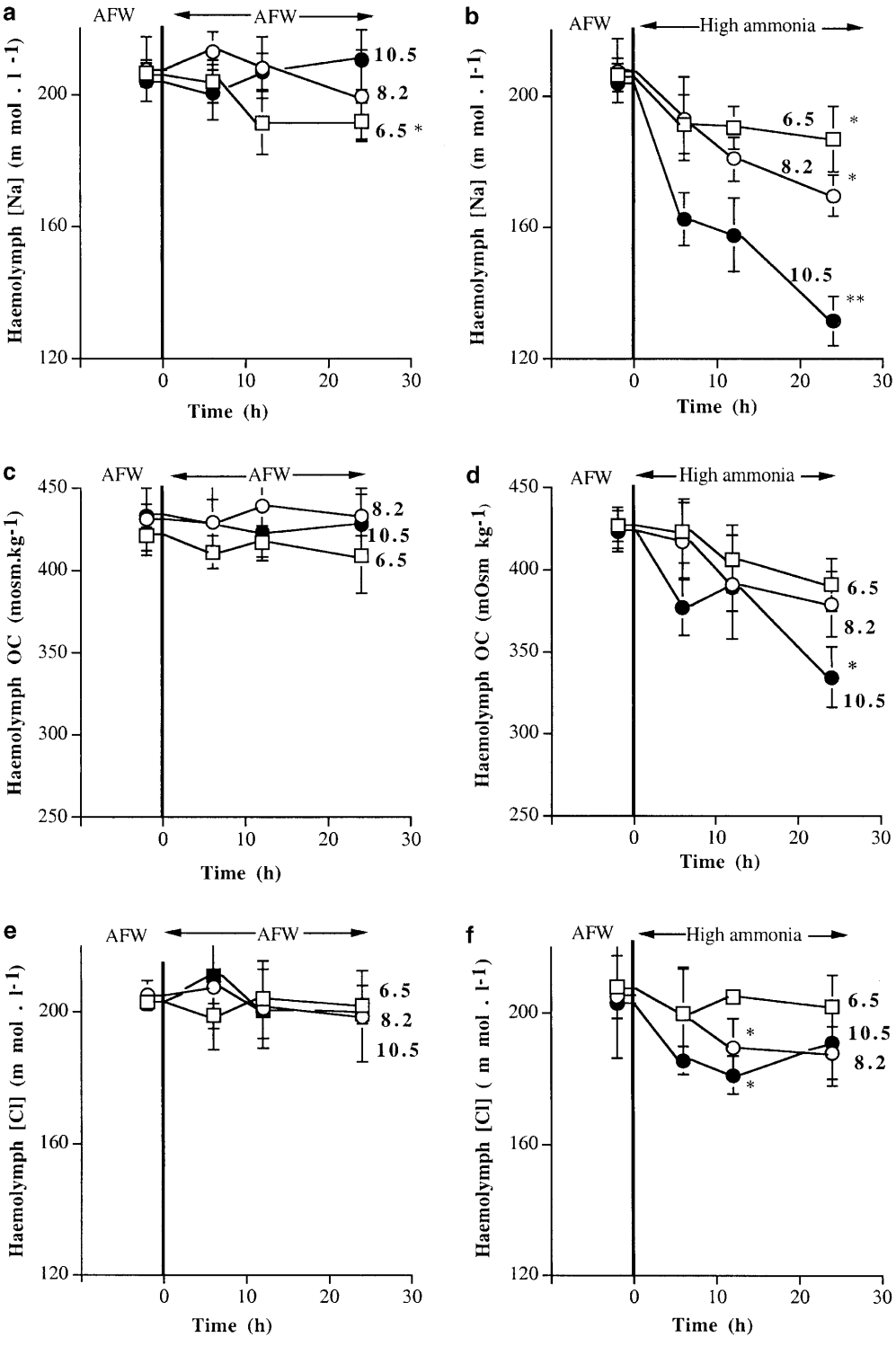

Fig. 3a–f Changes in haemolymph [Na^+], [Cl^-] and osmotic concentrations in *P. lenuisculus* during exposure to flowing AFW medium containing 1.5 mmol.l^{-1} T_{amm} and pH-adjusted to produce different concentrations of NH_3 (UIA) and NH_4^+. *Symbols* as for Fig. 1. Means ± 1 SEM, $n = 12$

Haemolymph osmotic concentration (OC) remained relatively constant in pH-adjusted AFW (Fig. 3c) but following 24 h exposure to 1.5 mmol.l^{-1} T_{amm} showed a significant decrease in pH 10.5 AFW ($P < 0.01$) (Fig. 3d).

Following ammonia exposure, haemolymph [Cl^-] showed a different pattern of changes compared to both [Na^+] and OC. After 12 h exposure [Cl^-] was significantly lower than initial values in pH 10.5 AFW, with a smaller reduction seen in pH 8.2 ($P < 0.05$). However, at 24 h, haemolymph [Cl^-] appeared to have stabilised at a higher level, but was not fully restored to pre-exposure levels. No significant changes in [Cl^-] were seen in the haemolymph of animals maintained in AFW adjusted to the corresponding pHs but with no added ammonia (Figure 3e, f).

Sodium fluxes

Na^+-depleted *P. leniusculus* transferred to pH 8.2 AFW containing 1.0 mmol.l^{-1} [Na^+] showed a mean net influx of sodium (J_{Na}^{net}) (Table 2). When challenged by 1.5 mmol.l^{-1} T_{amm} in pH 8.2 AFW (initial [NH_4^+] = 1.43; average $\Delta[NH_4^+] = -1.095$ mmol.l^{-1}; minus sign indicating lower haemolymph concentrations), there was significant reduction in influx (J_{Na}^{in}; $P < 0.001$). There was also a 46% increase in J_{Na}^{out} ($P < 0.05$) which, with the reduction in J_{Na}^{in}, produced a net efflux of sodium.

A similar pattern of response was seen in pH 6.5 medium with ammonia (initial [NH_4^+] = 1.499; average $\Delta[NH_4^+] = -1.141$ mmol.l^{-1}). In pH 6.5 with no added ammonia reduced J_{Na}^{in} and an elevated J_{Na}^{out} were seen, in comparison to fluxes in pH 8.2, resulting in a small net efflux. The addition of ammonia resulted in a significantly lower mean J_{Na}^{in} ($P < 0.05$) and a higher rate of efflux, giving a high J_{Na}^{net} (-0.144 ± 0.067 μmol.g^{-1}.h^{-1}).

Thus acidic conditions alone appeared to depress influx. The addition of ammonia (as NH_4^+) appeared to reduce influx further and, at the same time, to increase efflux above that seen with elevated [H^+] alone.

Transfer to pH 10.5 AFW with added ammonia (predominantly un-ionised; initially [NH_3] = 1.37; average $\Delta[NH_3]$ and $\Delta[NH_4^+] = -0.235$ mmol.l^{-1} and -0.819 mmol.l^{-1}, respectively), had no significant effect on either J_{Na}^{in} or J_{Na}^{out}, with only a slightly reduced net influx (Table 2).

PD_{te} measured between haemolymph and medium in pH-adjusted AFW, with and without 1.5 mmol.l^{-1} T_{amm}, are also shown in Table 2. The addition of ammonia to pH 6.5 and pH 8.2 AFW media had no significant effect on either the sign (always haemolymph negative) or amplitude of PD_{te}. However, there was a significant increase in haemolymph negativity in pH 10.5 medium ($P < 0.01$).

The effects of ammonia on sodium uptake following a longer period of ammonia loading was investigated by measuring sodium fluxes in animals that had been exposed for 12 h to pH 10.5 AFW containing 1.5 mmol.l^{-1} T_{amm}. These animals had accumulated about 0.6 mmol.l^{-1} T_{amm} in their haemolymph. Sodium fluxes were then measured over a 4-h period in the same medium and are shown in Fig. 4. J_{Na}^{in} showed no significant differences between short-term and 12 h exposed animals but J_{Na}^{out} showed a significant increase ($P < 0.05$) and J_{Na}^{net} was significantly increased and was a net efflux ($P < 0.001$). Thus longer exposure to ammonia, and therefore a higher internal T_{amm}, appeared to be correlated with an elevated efflux of sodium and, therefore, greater sodium depletion.

The effect of ammonia on sodium uptake

The acute effect of external ammonia on sodium uptake kinetics was investigated by measuring the J_{Na}^{in} of groups of Na^+-depleted *P. leniusculus* in pH 8.2 AFW in the presence and absence of 1.5 mmol.l^{-1} T_{amm} over a

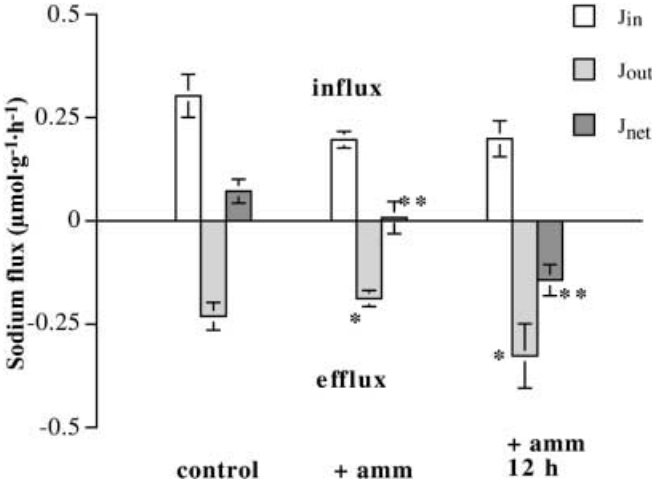

Fig. 4 Rates of sodium flux [J_{Na}^{in} + value, J_{Na}^{out} – value and J_{Na}^{net} (*filled squares*); all in μmol.g^{-1}.h^{-1}] in *P. leniusculus* following 12 h exposure to pH 10.5 AFW medium containing 1.5 mmol.l^{-1} T_{amm}. (+amm 12 h). Fluxes measured in pH 10.5 medium with no added ammonia (control) and immediately following transfer to AFW containing ammonia (+amm) are also shown. Means ± 1 SEM, $n = 6$

Table 2 Sodium fluxes in *P. leniusculus* in AFW with no added ammonia (Control) and ACW containing 1.5 mmol.l^{-1} T_{amm} (+amm) in media of different initial pH. A *negative sign* before a flux value indicates net efflux from the animal. Means ± 1 SEM (n = number of individuals measured; $n = 6$ for all flux measurements). Measured transepithelial potentials (PD_{te}; mV, sign = haemolymph) are also shown

	Medium					
	pH 6.5		pH 8.2		pH 10.5	
	Control	+amm	Control	+amm	Control	+amm
Flux μmoles g^{-1}.h^{-1}						
J_{Na}^{in}	0.234 ± 0.070*	0.150 ± 0.053*	0.316 ± 0.044**	0.079 ± 0.026**	0.285 ± 0.092	0.197 ± 0.058
J_{Na}^{out}	0.243 ± 0.049	0.295 ± 0.081	0.138 ± 0.024[a]	0.201 ± 0.012[a]	0.244 ± 0.058	0.188 ± 0.019
J_{Na}^{net}	–0.010 ± 0.056	–0.144 ± 0.0.067	0.178 ± 0.024[b]	–0.122 ± 0.018[b]	0.041 ± 0.040	0.008 ± 0.039
PD_{te} (mV)	–3.85 ± 1.89(4)	–5.15 ± 1.52(4)	–5.43 ± 0.35(7)	–6.14 ± 0.54(5)	–5.05 ± 3.36(6)	–10.65 ± 1.34(6)[c]

*, **, [a–b]Significantly different means
[c]A PD_{te} significantly different from other ammonia-treated groups

range of medium [Na^+] from 0.04 mmol.l^{-1} to 2.21 mmol.l^{-1} (Fig.5). At this pH the initial [NH_4^+] was, therefore, 1.43 mmol.l^{-1} and the average $\Delta[NH_4^+] = -1.095$ mmol.l^{-1}. From the calculated J_{Na}^{in} at each medium [Na^+] and measured PD_{te} values, the passive component of J_{Na}^{in}, $J_{Na}^{in(p)}$ was obtained and subtracted from J_{Na}^{in} to obtain V_{Na}^{in}, the rate of apparent active uptake of sodium (Harris and Coley 1991). $J_{Na}^{in(p)}$ was relatively small in these low [Na^+] media, averaging only 0.42% and 0.78% of total J_{Na}^{in}, in medium with and without ammonia, respectively. The relationship between V_{Na}^{in} and external [Na^+] in this range was typically non-linear (Fig.5a). We assumed that the pooled data showed a hyperbolic relationship typical of both sodium and chloride active uptake in all aquatic crustaceans studied. Thus, using the Lineweaver-Burke graphical method (Michal 1974; graphs not shown), we obtained the uptake constants V_{Na}^{max} (=0.629 μmol.g^{-1}.h^{-1}) and K_m (=0.733 mmol.l^{-1} [Na^+]). Here K_m is the [Na^+] at which $V_{Na}^{in} = V_{Na}^{max}/2$ (the linear regression was $y = 1.165x + 1.588$; $r = 0.994$). For ammonia-exposed animals, $V_{Na}^{max} = 0.224$ and $K_m = 0.224$ ($y = 2.066x + 4.468$; $r = 0.928$). In media with low [Na^+], there were significant reductions in V_{Na}^{in} in the presence of [NH_4^+] (by 67–75% in the range 0.30–0.70 mmol.l^{-1}; $P < 0.001$). With increased [Na^+] (>1.5 mmol.l^{-1}), V_{Na}^{in} showed some increases, but remained significantly lower when ammonia was present in media of up to 2.20 mmol l^{-1} [Na^+]. At this [Na^+], V_{Na}^{in} values with and without ammonia were not significantly different.

Net sodium fluxes, J_{Na}^{net}, over this [Na^+] range are shown in Fig.5b. Sodium-depleted animals in AFW with no added ammonia showed a net influx of Na^+ in media concentrations above ~0.30 mmol.l^{-1} [Na^+]. However, in pH 8.2 AFW containing 1.5 mmol^{-1} T_{amm}, crayfish showed a net efflux of Na^+ in all media with <1.5 mmol^{-1} [Na^+]. Sodium loss rates (J_{Na}^{out}) in animals in pH 8.2 AFW with added ammonia were not significantly different from those of crayfish in AFW medium alone (J_{Na}^{out} in AFW over medium [Na^+] range investigated = 0.226 ± 0.030 μmol.g^{-1}.h^{-1}; with ammonia = 0.196 ± 0.019 μmol.g^{-1}.h^{-1}).

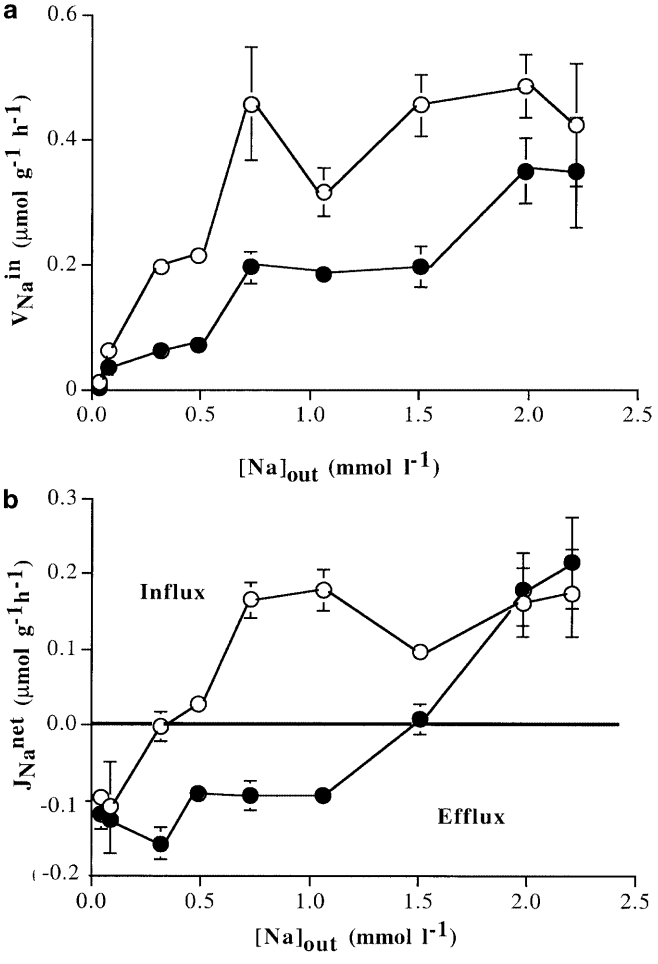

Fig. 5 Rates of apparent active uptake of Na^+ (V_{Na}^{in}; **a**) and net efflux (J_{Na}^{net}; **b**) by sodium-depleted *P. leniusculus* in a range of medium [Na^+], with and without added ammonia. *Open circles* control animals in AFW, *filled circles* animals in 1.5 mmol.l^{-1} T_{amm} AFW, both at pH 8.2. Mean ± 1 SEM (n = 6–12)

The effect of external ammonia on urine composition and production rate

The mean T_{amm} of urine collected from the nephropore of *P. leniusculus* maintained in Leicester tapwater was 3.26 ± 0.29 mmol.l^{-1} (24). Following 24 h exposure to AFW containing 1.5 m mol^{-1} T_{amm}, urine ammonia was found to increase significantly ($P < 0.001$). This occurred in all AFW media containing ammonia but the highest urine T_{amm} was seen in pH 10.5 medium (Table 3). Urine T_{amm} remained at a constant level in all AFW media which had no added ammonia. U/B ratios for ammonia increased from 6.63 ± 1.06 (6) in control animals in pH 10.5 AFW to 12.17 ± 0.94 (12) after ammonia exposure. Increases in urine T_{amm} appeared to follow those of the haemolymph, with the highest levels found in animals exposed to pH 10.5 AFW with ammonia. Lesser increases were seen in pH 6.5 and pH 8.2 media. In all cases urine T_{amm} exceeded that of the haemolymph over the 24-h sampling period.

The mean [Na^+] of urine collected from crayfish maintained in pH 8.2 AFW was 6.35 ± 2.01 mmol.l^{-1} with a [Na^+] U/B ratio of 0.031 ± 0.005 (18). Significant increases in urine [Na^+] occurred following transfer to ammonia-containing media. Thus urine sampled after 24 h in pH 10.5 and pH 8.2 AFW with 1.5 mmol.l^{-1} T_{amm} had, respectively, ~two-fold and six-fold higher [Na^+] (Table 3). These increases, in combination with concurrent decreases in haemolymph [Na^+] (Fig. 4a), resulted in much higher [Na^+] U/B ratios. At 24 h in pH 10.5 AFW medium these had risen to a mean of 0.277 ± 0.091 (6); an order of magnitude higher than normal.

Urine pH values, measured initially and 24 h after transfer to ammonia-containing medium, are also shown

Table 3 Urine parameters in *P. leniusculus* before and after 24-h exposure to different pH-adjusted AFW media containing 1.5 mmol.l^{-1} T_{amm}. Means ± 1 SEM ($n = 6$ except where indicated). ^{51}CrEDTA measured in pH 8.2 and pH 10.5 media only. *U/B* is the urine-to-blood concentration ratio for a given parameter (T_{amm}, Na^+ or CrEDTA). All other terms and units are as indicated

Treatment	pH 6.5		pH 8.2		pH 10.5	
Time (h)	0	24	0	24	0	24
Urine T_{amm} (mmol.l^{-1})	2.89 ± 0.53	4.09 ± 0.70**	3.92 ± 0.41	4.45 ± 0.41**	2.31 ± 0.37*	11.57 ± 0.89*,**
U/B $_{Tamm}$	11.58 ± 2.12	8.83 ± 1.51	13.29 ± 1.38	8.11 ± 0.75	6.63 ± 12.17	12.17 ± 0.94
Urine [Na^+] (mmol.l^{-1})	4.50 ± 1.50	2.64 ± 0.62	5.45 ± 0.52	11.15 ± 0.99**	6.35 ± 1.02*	36.40 ± 11.90* **
U/B $_{Na}$	0.023 ± 0.007	0.014 ± 0.003	0.056 ± 0.005	0.031 ± 0.006	0.017 ± 0.003	0.277 ± 0.091**
pH	7.344 ± 0.076	7.699 ± 0.080*	7.522 ± 0.021	7.244 ± 0.066**	7.508 ± 0.060	7.548 ± 0.096
^{51}CrEDTA clearance (V_{cl}) (ml. kg^{-1}. h^{-1})	–	–	3.93 ± 1.01	2.00 ± 0.27	4.89 ± 1.24 (5)	5.08 ± 1.96(5)
U/B $_{CrEDTA}$	–	–	3.07 ± 0.34	3.55 ± 0.48	3.43 ± 1.20	3.17 ± 0.95
Urine flow rate (V_u) (ml. kg^{-1}. h^{-1})	–	–	1.29 ± 0.17	0.64 ± 0.06	1.37 ± 0.37(5)	1.62 ± 0.78(5)

*Significantly different from controls
**Significantly different from other ammonia-exposed groups

in Table 3. As can be seen, exposure to pH 6.5 AFW resulted in little change in haemolymph pH but ammonia exposure was accompanied by significant alkalisation of the urine. In contrast, in pH 8.2 AFW containing ammonia, a significant reduction in urine pH occurred ($P < 0.01$). No significant changes in urine pH were observed in pH 10.5 AFW with ammonia.

Urine flow rates were measured in *P. leniusculus*. acclimated to pH 8.2 and 10.5 AFW media and, in the same individuals, following 24 h exposure to these media containing 1.5 mmol.l^{-1} T_{amm}. Table 3 shows ^{51}CrEDTA clearance rates (V_{cl}), U/B ratios, and urine flow rates (V_u) calculated from these data. There were no significant changes in either Vcl or Vu following exposure to ammonia over this period, nor did ^{51}CrEDTA U/B ratios alter, suggesting that water reabsorption in the antennal gland was unaffected by this treatment.

Discussion

Previous ammonia toxicity tests on crayfish have exposed animals to media of constant pH containing relatively high T_{amm} (50–400 mg.l^{-1} as NH_4Cl or NH_4SO_4) to produce the required concentrations of UIA [NH_3]. Generally UIA is regarded as the more toxic ammonia species in aquatic organisms. However, as can be seen from this study, sub-lethal physiological effects are produced at concentrations of one-tenth or lower than the T_{amm} used in such acute toxicity tests. Our 24 h LC_{50} and 48 h LC_{50} values for [NH_3] of 0.91 mmol.l^{-1} and 0.30 mmol.l^{-1} (12.76 mg.l^{-1} and 4.1 mg.l^{-1} NH_3-N, respectively) are broadly within the range reported for juvenile and adult crayfish of various species (~0.4–0.6 mmol.l^{-1}; Firkins 1993; Liu et al. 1995; Lourey and Mitchell 1995; Rouse et al. 1995).

Our experiments maintained a constant T_{amm}, but, by using different medium pHs, altered the ratios of ionised ammonia to UIA concentrations ([NH_4^+]/[NH_3]) significantly. They showed that significant changes in haemolymph composition (particularly Na^+) occurred under these conditions. Thus, even when elevated T_{amm} occurs in near to neutral pH conditions, such as sewage discharge or landfill leachate (Bloxham et al. 1999), sublethal effects may be occurring in aquatic crustaceans such as crayfish. These effects may affect fitness and long-term survival. Using a moderate T_{amm}, (1.5 mmol.l^{-1} ≡ 27.0 mg.l^{-1}), relative to previous toxicity studies on crayfish, to simulate the T_{amm} and pH changes seen in farm waste pollution episodes, we observed significant physiological effects at this and, sometimes, at lower concentrations. Interestingly, field experiments simulating farm waste pollution incidents, in which the Atlantic crayfish *A. pallipes* was exposed to a combination of high pH (9.0) and quite modest T_{amm} (calculated as 31.3 mg.l^{-1}), showed a high mortality. Under these conditions a high [NH_3] pulse would be generated (0.41 mmol.l^{-1}; ≡ 7.0 mg.l^{-1} [NH_3]; Foster and Turner 1993). The toxic effects of ammonia in *P. leniusculus* are characteristic of those seen in many aquatic animals including convulsions, lack of coordination and loss of equilibrium. Among other effects, excess tissue ammonia is known to cause glutaminase inhibition leading to reductions in concentrations of key neurotransmitters (Korsgaard et al. 1995). Also, elevated plasma ammonia has been found to be associated with reduced swimming performance in trout by lowering anaerobic capacity (Beaumont et al. 1995).

J_{amm}^{net} in media containing no added ammonia were always net effluxes, and were similar in magnitude to those reported by Wheatly (1989) for this species. These efflux rates appeared to be reduced in highly alkaline conditions (pH 10.5), compared to those in pH 8.2 and pH 6.5 AFW. In pH 6.5, J_{amm}^{net} was significantly higher than in pH 8.2. A similar inhibition of ammonia net efflux in alkaline conditions has been reported in fish

(Wilkie and Wood 1991). In *P. leniusculus* this would tend to exacerbate the haemolymph accumulation of ammonia when transferred to alkaline medium containing high ammonia levels. However, the increase in J_{amm}^{net} in pH 6.5 AFW medium in *P. leniusculus* is in contrast to that seen in previous studies on the effect of acid waters on crayfish (Wood and Rogano 1986; Mauro and Moore 1987). Where such increases occur these are explained as arising due to enhanced blood-to-water [NH_3] gradients (ΔNH_3) in acid conditions. However, the ΔNH_3 calculated here showed only minor differences (respectively, 0.0018 mmol.l^{-1} and 0.00192 mmol.l^{-1}, favouring net ammonia efflux) and do not explain the two-fold increase in J_{amm}^{net} observed when moving from pH 8.2 to pH 6.5. Further work is necessary to elucidate the pathway of ammonia excretion in *P. leniusculus*.

In external media containing 1.5 mmol.l^{-1} T_{amm}, the normal Δ[NH_3] between medium and haemolymph was reversed and was large (e.g. pH 10.5 AFW). Under these conditions *P. leniusculus* showed the highest rate of ammonia net influx. It would appear that, as in fish, the gills of crayfish are more permeable to NH_3 than to NH_4^+ and that rapid diffusive entry of NH_3 takes place. However, net influx of ammonia also occurs in conditions in which ionised ammonia largely predominates but at a slower rate. It is known from studies in fish that ammonia in NH_3 form is highly lipid-soluble and diffuses through gill membranes rapidly (Heisler 1990; Wilkie 1997). A fuller analysis would be required to ascertain if a linear relationship exists between the [NH_3] gradient (strictly P_{NH3}) and ammonia influx (Heisler 1990). Presumably NH_3 is protonated to NH_4^+ following entry, either intracellularly in the gill epithelium, or within the haemolymph of *P. leniusculus*, although no significant haemolymph alkalosis was observed after 24 h. In the haemolymph, T_{amm} accumulated most rapidly, and to the highest concentrations, under conditions of high medium [NH_3] but no steady state level appeared to have been reached after 24 h. Significant accumulation of ammonia also occurred in pH 6.5 and pH 8.2 AFW in which NH_4^+ predominates, but in pH 6.5 there appears to be a levelling off of T_{amm} at a [NH_4^+] of about 45% of external T_{amm}. The electrochemical gradients of NH_4^+ were calculated using the 24-h values of haemolymph T_{amm}, haemolymph pH and measured transepithelial potential differences (PD_{te}). These gave Nernst potential differences (PD_{NH4+}), required to maintain haemolymph-to-water [NH_4^+] gradients at thermodynamic equilibrium, of +23.4 mV and +28.9 mV in pH 6.5 and pH 8.2 AFW with ammonia, respectively. Measured PD_{te} gave values of –5.15 mV and –6.14 mV. It would appear that in neither medium was NH_4^+ in thermodynamic equilibrium across the integument after 24 h. However, further studies would be necessary to demonstrate an NH_4^+ extrusion mechanism, possibly by Na^+/NH_4^+ exchange across the gills (Weihrauch et al. 1999). In pH 10.5 AFW with ammonia, the apparent accumulation of NH_4^+ in the haemolymph was against an electrochemical gradient (PD_{NH4+} = –47.7 mV compared to PD_{te} of –10.6 mV). This is due to "ammonia trapping". The form of ammonia diffusing inward is uncharged NH_3 which is protonated internally to NH_4^+ (Wilson and Taylor 1992). The driving force for NH_4^+ accumulation is the relatively large pH difference between medium and pH maintained during the experiment. Crayfish appear to be relatively tolerant to short-term exposure to alkaline pH media (Jay and Holdich 1977), and able to maintain acid-base status in alkaline conditions by rapid respiratory and metabolic adjustments (Ellis and Morris 1995).

The link between ammonia excretion and sodium ion uptake, in which an Na^+/NH_4^+ coupled exchange occurred across gill epithelia, was demonstrated originally by Krogh (1939) and subsequently confirmed in many species (Shaw 1960; Maetz and Garcia-Romeu 1964). Elevated external ammonia was found to reduce J_{Na}^{in} markedly in crayfish (Shaw 1960), although the effect of this inhibition on haemolymph [Na^+] was not shown. Studies on marine crustaceans have shown significant blood Na^+ depletion in the presence of external ammonia, suggesting either an inhibition of Na^+ uptake or increased Na^+ permeability (Spaargaren 1990; Young-Lai et al. 1991; Lin et al. 1993). Since Na^+ is a major osmotic effector in the haemolymph of crustaceans, osmoregulatory failure has been proposed as a major toxic effect of high external ammonia (Armstrong et al. 1978). Exposure of *P. leniusculus* to external media in which either NH_4^+ or NH_3 predominated was correlated with a depletion of haemolymph Na^+ and the effect, over 24 h at least, appears to be greatest in the presence of un-ionised ammonia (NH_3). As in *A. pallipes* (Shaw 1960), chloride ion regulation in *P. leniusculus* seems to be unaffected by high external ammonia. This reduction in haemolymph [Na^+] can account for a large part of the osmotic concentration decrease. In pH 10.5 medium, a reduction of 72 mmol. l^{-1} [Na^+] is accompanied by a fall of 88 mosmol.kg^{-1} and in pH 8.2 medium, the values are, respectively, 38 mosmol.kg^{-1} and 47 mosmol.kg^{-1} after 24 h exposure. Other components are reduced but these have yet to be identified.

If Na^+/NH_4^+ counter ion exchange across the gill epithelium is inhibited by elevated external ammonia in *P. leniusculus*, as predicted by previous studies, the greatest reduction in J_{Na}^{in} would be expected in media containing ionised ammonia. Our experiments show that the form NH_4^+ does have the greatest effect on J_{Na}^{in} and that when [NH_3] is high (but at constant T_{amm}) J_{Na}^{in} is less inhibited. There appears to be a reduction in J_{Na}^{in} in the presence of low pH, confirming many previous studies on the effects of increased water acidity on crayfish (Wood and Rogano 1986; McMahon and Stuart 1989). However the reduction in J_{Na}^{in} seen in pH 6.5 AFW with added ammonia is greater than that seen in pH 6.5 medium alone suggesting a specific NH_4^+ impairment of influx. No major change in PD_{te} was seen, suggesting that influx changes may be due to a slowing of the Na^+/NH_4^+ apical exchange which is thought to be driven by

the $[NH_4^+]$ gradient normally existing between gill cell cytoplasm and external water (Kirschner 1991). An alternative explanation is that ammonia acts to inhibit J_{Na}^{in} by reducing cellular $[H^+]$ and, consequently, reducing the rate of outwards electrogenic proton pumping. The resulting reduced depolarisation of the apical cell membrane (inside negative) would reduce the rate of Na^+ entry (Avella and Bornancin 1989). However, in *P. leniusculus* J_{Na}^{in} is least affected when NH_3, which would bind intracellular H^+ and reduce proton pump activity, is diffusing most rapidly inwards, i.e. pH 10.5 AFW. In this species inhibition of J_{Na}^{in} is greatest in high $[NH_4^+]$ medium. Furthermore, allowing animals to accumulate ammonia for 12 h, which might decrease intracellular $[H^+]$ further, did not reduce J_{Na}^{in}.

Inhibition of V_{Na}^{in} (the presumed active component of influx, J_{Na}^{in}) seen here bears a marked similarity to that reported in *A. pallipes* in 1.0 mmol.l^{-1} T_{amm} in a medium in which presumably NH_4^+ would predominate (pH not indicated, but presumably around 7.5; Shaw 1960). This inhibition was greatest in low $[Na^+]$ media. Normally sodium-depleted *P. leniusculus* can balance diffusive losses of Na^+ in ~0.30 mmol.l^{-1} $[Na^+]$ and has a K_m for sodium uptake of 0.73 mmol.l^{-1}. This indicates an uptake system with a lower affinity for Na^+ than *A. pallipes* ($K_m = 0.15$ mmol.l^{-1}; Shaw 1959). Exposure to 1.5 mmol.l^{-1} T_{amm} reduces the ability of *P. leniusculus* to regulate its haemolymph sodium in low $[Na^+]$ media. Balance is only achieved in a five-fold higher medium $[Na^+]$. With increased medium $[Na^+]$, V_{Na}^{in} of ammonia-exposed animals tends towards V_{Na}^{max} in control animal (no added ammonia). Shaw (1960) showed that an ammonia/external sodium ratio of 10:1 would produce an inhibition of J_{Na}^{in} of about 80%. In *P. leniusculus* the effect is less, but at a lower ammonia:sodium ratio (3:1). Elevated $[NH_4^+]$ did not appear to affect sodium efflux rates on immediate exposure, confirming the findings of Shaw (1960) in *A. pallipes*, but longer-term exposure to ammonia did result in increased J_{Na}^{out}. This will be discussed further below.

In comparison with the inhibition of chloride uptake by external nitrite in crayfish (Jensen 1990; Harris and Coley 1991), the interaction of ammonia with Na^+ uptake in *P. leniusculus* appears not to be one of simple competitive inhibition and a fuller investigation is required to determine its nature. In the presence of ammonia, the relationship between $1/V_{Na}^{in}$ and $1/[Na^+]$ is not linear in the lower range of medium $[Na^+]$. V_{Na}^{in} values are depressed below expected levels, suggesting that there is a changing interaction between NH_4^+ and Na^+ at the presumed single-substrate apical Na^+/NH_4^+ exchange sites as external $[Na^+]$ increases.

If exposed to high $[NH_4^+]$ in media with relatively low $[Na^+]$ the effect of ammonia on sodium balance will lead to significant sodium depletion. The consequences of this could be: (a) stimulation of maximal Na^+ uptake rate by activating full sodium pump capacity to overcome the impairment caused by ammonia, or (b) lowering of haemolymph Na^+ which could change transmembrane ionic gradients and, possibly functioning, of excitable cells. Ammonia accumulation affects neuromuscular systems directly by inhibiting glutaminase thus decreasing key neurotransmitter levels (Beaumont et al. 1995; Korsgaard et al. 1995). The former (a) would have an energy cost; while (b) may reduce fitness by affecting behaviour and movement. *P. leniusculus* exhibits burrowing behaviour in the Midlands area of the UK, constructing deep burrows in clay river banks. The possibility that the T_{amm} of burrow water may be elevated in comparison to open water is being investigated since this may result in sub-lethal toxic effects if burrow irrigation currents were weak.

The inhibition of J_{Na}^{in} was found to be maximal where NH_4^+ is the predominant ammonia species present externally. However, the extent of haemolymph depletion over 24 h was greatest in alkaline conditions where NH_3 predominated (and where ammonia influx rates were highest and J_{Na}^{in} least inhibited). This apparent contradiction may be explained by examining the different period of exposure used in the two experiments. J_{Na}^{in} was measured over a period of 4–5 h, whereas significant haemolymph Na^+ depletion is evident only after 12 h. We examined the possibility that longer-term ammonia exposure (mainly as UIA) affected Na^+ losses in the urine in addition to the diffusive losses at the gills.

Following longer-term exposure, ammonia accumulates in the haemolymph to high levels (~0.90 mmol.l^{-1}) and probably to even higher concentrations in the antennal gland lumen. Haemolymph buffering ensures that this ammonia will mainly be in the form of NH_4^+. Its accumulation to these levels could inhibit Na^+/NH_4^+ exchange at internal uptake sites, particularly in the antennal gland tubule where T_{amm} exceeds that of the haemolymph by a significant factor. Bladder urine T_{amm} U/B ratios obtained in our control animals exceeded those previously reported (3.09 ± 0.6; Wheatly and Toop 1989). With increasing haemolymph T_{amm}, urine ammonia levels also increased so that U/B ratios remained relatively high. U/B_{Tamm} exceeds U/B_{CrEDTA} confirming the findings of Wheatly and Toop (1989), and supporting the suggestion that the antennal gland secretes ammonia into the tubular urine during processing.

Classically crayfish produce a dilute urine by withdrawal and reabsorption of cations and anions (Riegel 1972). External ammonia does not appear to increase either the urine filtration rate or the final urine flow rate in *P. leniusculus* but significantly elevated $[Na^+]$ was found in the final urine. In fish increases in urine flow rate during ammonia exposure have been reported (Lloyd and Orr 1969). From urinary flow rates and urine $[Na^+]$ values, the urinary Na^+ loss in animals exposed to pH 10.5 AFW without added ammonia can be calculated as 0.008 μmol.g^{-1}.h^{-1}. This is 3.6% of the total efflux, J_{out} ($= 0.244$ μ.mol.g^{-1}.h^{-1}). Following 24 h exposure to a medium containing 1.5 mmol.l^{-1} T_{amm} , this increased over seven-fold to 0.060 μmol.g^{-1}.h^{-1}. This value is closer to rates of urinary Na^+ efflux reported in

normal animals by Wheatly and Toop (1989). In the present study, 63% of the increase in J_{out} measured in ammonia-containing medium can be accounted for as being due to this greater Na^+ loss in the urine.

In conclusion, elevated water [ammonia] has multiple sub-lethal effects on crayfish in addition to direct toxic action. It penetrates the integument (most probably the gill epithelium) most rapidly as un-ionised ammonia (NH_3) so that, if accompanied by alkaline conditions, even a moderate T_{amm} can result in an ammonia build-up in the haemolymph due to favourable P_{NH3} gradients maintained by the animals' acid-base homeostasis. Even if present mainly in ionised form (NH_4^+), sub-lethal effects will be seen, notably an inhibition of Na^+ uptake. Reduced sodium influx, particularly at low external [Na^+] will cause a net efflux of Na^+ and therefore depletion of the haemolymph. Crayfish living in low ion "soft" waters may be particularly susceptible to elevated ammonia in this respect. Pulses of elevated T_{amm} ($\sim$0.25 mmol.l^{-1}) in similar water conditions have been reported as a diurnal occurrence downstream from upland farms arising from dairy washings (Schofield et al. 1990). Even higher field levels have been reported in leachates of landfill sites (6–15 mmol.l^{-1}; Bloxham et al. 1999). In the latter study, the heart rate of *P. leniusculus* exposed to 1 mmol.l^{-1} T_{amm} at pH 10.5 showed a significant increase, even over a relatively short (4 h) exposure period. It is unlikely that the degree of perturbation of haemolymph ionic composition within that time would cause changes in excitable cell functioning, either in cardiac cells or cardiac neural networks, but such possibilities should be investigated.

Accumulation of ammonia in the haemolymph over the longer-term (12–24 h) is accompanied by an increase its filtration and (probably) secretion into the antennal gland lumen. The resulting higher urinary T_{amm} is accompanied by elevated urinary [Na^+] thus raising Na^+ U/B ratios and increasing renal sodium loss rates, This accounts for a significant part of the increased $J_{Na}{}^{out}$ seen following ammonia exposure, and the depletion of haemolymph [Na^+]. Thus both short-term episodes and longer-term exposure to relatively low water T_{amm} can have severe sub-lethal and toxic effects in *P. leniusculus* which is regarded as a physiologically robust freshwater crayfish species (Holdich et al. 1995). The implications of these findings to the effects of ammonia on more sensitive crayfish species, and other freshwater macroinvertebrates, should be investigated.

Acknowledgements All experiments performed in this study comply with UK regulations and do not fall within the Animals (Scientific Procedures) Act 1986.

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