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SALT AND WATER RELATIONS, AND NITROGEN EXCRETION, IN THE AMPHIBIOUS AFRICAN FRESHWATER CRAB *POTAMONAUTES WARRENI* IN WATER AND IN AIR

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Summary

Mechanisms of salt and water conservation, and nitrogen excretion, were investigated in the freshwater amphibious crab *Potamonautes warreni* from the High Veld of South Africa. Adaptations to fresh water were assessed as pre-adaptations to air-breathing, and nitrogen excretion was examined as a potential constraint to terrestrial excursions. *P. warreni* was able to regulate water and salt loss in water up to 40 % sea water, but not in 80 % sea water. The water permeability of *P. warreni* was low and, since 97 % of the haemolymph filtrate was reabsorbed in the antennal organ, urinary water loss was minimal (0.7 μl g^{-1} h^{-1}). The minimum equilibrium [Na] of *P. warreni* was low (0.116 mmol l^{-1}), as were the rates of both Na loss (0.22 μmol g^{-1} h^{-1}) and Ca loss (0.29 μmol g^{-1} h^{-1}). The low loss rates were due to urinary salt conservation of approximately 90 % or better and to low permeability (K'_{Na}=0.0025; K'_{Ca}=0.0521), and were compensated for by a high-affinity uptake mechanism (J_{max}=0.76 μmol g^{-1} h^{-1} and K_m=0.18 mmol l^{-1}). Acclimating *P. warreni* to low Na concentrations increased maximum net Na uptake rate to 1.77 μmol g^{-1} h^{-1}.

Nitrogen excretion in *P. warreni* was almost 100 % ammoniotelic, and there was no accumulation of haemolymph or urinary ammonia or urea when in air. *P. warreni* was unable to excrete ammonia to air, but in water the rate of excretion was nearly 70 μmol kg^{-1} h^{-1}. Crabs in amphibious conditions showed pulses of elevated NH_3 excretion (350 μmol kg^{-1} h^{-1}) when subsequently submerged, while for crabs breathing air for 3 days this pulse reached 4.9 mmol kg^{-1} h^{-1}. Air-breathing *P. warreni* with artificially irrigated branchial chambers excreted double the amount of NH_3 *via* the gills compared with crabs from amphibious conditions.

Water and salt conservation form useful pre-adaptations to terrestrial forays. While the relatively low water loss extends the duration of emersion, *P. warreni* is required to return briefly to water to excrete stored nitrogenous waste. The nature of the store remains to be determined.

Key words: *Potamonautes warreni*, crab, nitrogen excretion, salt, water, air-breathing.

Introduction

Freshwater crabs of the superfamily Potamoidea are widely distributed throughout tropical and subtropical Australasia (Parathelphusoidea), South America (Psuedothelphusoidea), Europe and Africa (Potamonidea). Early studies of salt and water balance in *Potamon* sp. examined the marine ancestry (e.g. Duval, 1925; Drilhorn-Courtois, 1934) and while some survive seawater immersion, e.g. *P. potamios* (Warburg and Goldenberg, 1984), most are obligate freshwater crabs (Harris and Micallef, 1971; Shaw, 1959*b*).

Physiological adaptations to fresh water, primarily low urinary water loss and salt reabsorption mechanisms, appear to pre-adapt freshwater crabs to life on land (Wolcott, 1992) and may permit extended terrestrial forays by potamid crabs. Conversely, since aquatic crabs excrete their nitrogenous waste as ammonia (NH_3/NH_4^+) across the gill epithelia into the water (for a review, see Greenaway, 1991), their movement onto land may thereby be limited. Thus, the duration of terrestrial excursions may not ultimately be limited by respiratory or water balance issues but rather by impediments to the elimination of nitrogen in air. Therefore, nitrogen excretion, together with water and ion balance in the Potamonidea, warrants further consideration with respect to the transition from water- to air-breathing.

Many of the Potamoidea exploit the terrestrial environment to some extent (MacMillen and Greenaway, 1978; Gherardi *et al*. 1988, 1989; Gherardi and Micheli, 1989; Warburg and Goldenberg, 1984; van Aardt, 1990, 1993). These freshwater crabs maintain a relatively high haemolymph osmotic pressure (e.g. Shaw, 1959*b*) and, since the urine is iso-osmotic with the haemolymph, it represents a potential route of salt loss. When

immersed, the crabs replace salts by high-affinity uptake mechanisms (Greenaway, 1981), but the fact that salt losses are minimised by significant reductions in urine flow is also important (e.g. Shaw, 1959*b*; Harris, 1975; Greenaway, 1980). While reduced urine flow might be an adaptive mechanism for water conservation during air-breathing, when the crabs are submerged they suffer an influx of water that exceeds urine flow and requires extra-renal water excretion (Shaw, 1959*b*; Thompson, 1970; Harris, 1975; Greenaway, 1980). However, the more terrestrial species can withstand substantial desiccation (Dandy and Ewer, 1961; Greenaway and MacMillen, 1978; Warburg and Goldenberg, 1984; Greenaway, 1988) and, although evaporative losses over the respiratory surfaces and integument can be significant, these freshwater crabs often exhibit relatively reduced water permeability in air, e.g. *Holthuisana transversa* (Greenaway and MacMillen, 1978; Greenaway, 1980). The urinary flow is reduced approximately 50 % in *P. edulis*, and considerably more in *Holthuisana transversa* (Greenaway, 1981), apparently resulting from water resorption in the antennal gland (Harris, 1975). If nitrogen excretion is dependent on urinary flow, it will thus be compromised during air-breathing.

Only one species, *Birgus latro*, has adopted uricotelism so that nitrogen excretion is independent of urine flow (Greenaway and Morris, 1989). Most land crabs have remained ammoniotelic, but may either add NH_4^+ to the primary urine (e.g. DeVries and Wolcott, 1993; DeVries *et al.* 1994) or to urine within the branchial chamber (Wolcott, 1991; Greenaway and Nakamura, 1991; Varley and Greenaway, 1994). Excretion of nitrogen as urea is of little importance (e.g. Wolcott, 1991). It appears that nitrogen excretion is dependent on the rate of urine production and flow to the branchial chamber and it seems probable, therefore, that freshwater land crabs must curtail nitrogen excretion while in air, as intimated for *Holthuisana transversa* (Linton and Greenaway, 1995). A similar situation is found in the freshwater *Cardisoma hirtipes*, which apparently stores waste nitrogen until it can be cleared by an extra- renal process to water (Dela-Cruz and Morris, 1998).

Potamonautes warreni (Calman) is abundant in tributaries of the Vaal River in the High Veld of South Africa and was selected for its clear predilection to breathe air (Dandy and Ewer, 1961; van Aardt, 1990, 1993; Adamczewska *et al.* 1997). Much of the freshwater habitat of *P. warreni* drains karst rocks and provides water with a relatively high mineral content. In addition, the low barometric pressure (approximately 85 kPa) and sub-zero temperatures during winter provide an atypical environment for land crabs, and terrestrial excursions are likely to be challenging for *P. warreni*. The ability of *P. warreni* to breathe and exercise in air is reassessed elsewhere (Adamczewska *et al.* 1997), but there are relatively few data concerning the salt, water and nitrogen regulation of these potamonautid crabs. The current study examines the ion regulatory abilities of *P. warreni* in naturally hard water and determines the effects of exposure to soft water on ion loss and uptake. The ion and water balance mechanisms are assessed both as pre-adaptations to life on land and as limitations of nitrogen excretion that potentially constrain the duration of terrestrial excursions by *P. warreni*.

Materials and methods

Potamonautes warreni were collected from the Mooi River in Potchefstroom, South Afrca. The crabs were captured during May and June using beef liver baits suspended outside the burrow openings. The crabs (mass 37–104 g) were housed in the laboratory within individual containers at 25 °C with a 12 h:12 h light:dark regime. The containers held filtered Mooi River water (MRW) at a depth of approximately 2 cm and a plastic pipe which allowed the animals to choose between air- and water-breathing. The crabs were fed pelleted pet food and the water was replaced once each week for a period of at least 3 weeks before experiments. All determinations were made at 25 °C in artificial Mooi River Water (AMRW) unless otherwise stated. The AMRW was based on measured water values (given below) and contained (in mmol l^{-1}): KCl, 0.04; NaCl, 1.02; $MgCO_3$, 1.98; $CaCO_3$, 1.77 and $CaCl_2$, 0.12.

Osmotic and ionic regulation by P. warreni

The basic regulatory abilities of *P. warreni* were assessed using groups of six crabs submerged in water of different salinities (1, 5, 10, 20, 40 and 80 % sea water) with AMRW as a control. The crabs were transferred from AMRW to saline water, and a 200 µl haemolymph sample was immediately taken from the infra-branchial sinus. This process of sampling was repeated after 1, 2 and 3 weeks, and then all crabs were returned alive to the Mooi River. The osmotic pressure of the haemolymph was determined immediately (using a Knauer freezing point depression osmometer). The remaining sample was denatured, by mixing 1:1 with 0.1 mmol l^{-1} HNO_3, and was transported to Sydney for further analysis. The Cl concentration was determined using a chloride titrator (CMT10, Radiometer) and Mg, Ca, K and Na were measured by atomic absorption spectrophotometry (AAS; GBC 906, GBC Melbourne). To suppress interference, samples for measurement of Na and K were diluted with 5.9 mmol l^{-1} $CsCl_2$, while Mg and Ca samples were diluted with 7.2 mmol l^{-1} $LaCl_3$. Samples of Mooi River water were similarly analysed and, in addition, Ca concentrations were determined after treatment with NHO_3 to mobilise any Ca present as $CaCO_3$.

Haemolymph and urine salt composition

Haemolymph and urine samples were taken from animals that had access to either air or water, simulating the field condition, and also from crabs that had been either submerged in MRW or emersed for 4 days. Crabs in the emersed condition were held with 2 mm of MRW in the base of their containers. Urine samples were obtained by gently deflecting the nephropore flap and drawing released urine into a fire-drawn pipette. The samples were analysed in Potchefstroom as above (Varian AAS).

Water turnover

P. warreni previously held in MRW with access to air were drilled using a fine dental drill through the carapace immediately dorsal to the pericardium, but not through the underlying hypodermis, and allowed at least 24 h recovery. Crabs were injected, *via* the prepared opening, with tritiated water (185 kBq μl^{-1}) at a dosage of 2.33 MBq 100 g^{-1}. After a period of 1 h, an initial haemolymph sample was taken to establish the water content of the crabs by dilution of the tritium label. Two groups of crabs (N=6 in each case) were prepared: the first was held for 4 days in buckets on mesh platforms above 2 l of filtered MRW; the second was held in similar buckets but immersed in river water for 2 days. Haemolymph (10 μl) was taken from air-breathing crabs daily and from water-breathing crabs at intervals of between 6 and 12 h. This provided water exchange rates for air- or water-breathing *P. warreni.* In submerged crabs, isotopic exchange between internal tritiated water and external water adds an exchange component that is independent of the water requirements. The radioactivity of the samples was determined using a liquid scintillation counter (Beckman, USA). The rate exchange fraction (K) and the biological half-life of the isotope ($t_{1/2}$) were derived as described previously for crustaceans (Greenaway, 1980):

$$K = \ln(H_0/H_1)/t \quad (1)$$

$$t_{1/2} = \ln 2/K, \quad (2)$$

where t is elapsed time in hours and H is the specific activity at time t. Extrapolation of this plot to the y intercept was used to derive the dilution of label in body water at time zero:

$$E_T = \frac{200(W_2 - W_1)\ln(H_1W_1/H_2W_2)}{(M_1 + M_2)\ln(W_2/W_1)/t} \quad (3)$$

(Nagy and Costa, 1980), where E_T is tritium efflux (ml 100 g^{-1} day^{-1}), M is body mass (g), W is the total body water (ml) and subscripts 1 and 2 refer to initial and subsequent values (see Greenaway, 1994).

Urine production and clearance

Rates of ^{51}Cr-labelled EDTA clearance, urine production and fluid resorption within the urinary system were determined in *P. warreni* that had free access to either air or water (amphibious). Crabs prepared similarly to those for injection with tritiated water were injected with ^{51}Cr-labelled EDTA (nominal activity 3.7 kBq μl^{-1}) at 740 kBq 100 g^{-1}. The crabs were returned to amphibious conditions and haemolymph was sampled (25 μl) at 12 h intervals for 3 days. The radioactivity of the sample was determined as for tritium. Clearance (ml h^{-1}) was calculated as K×EDTA space, determined from dilution of the injected ^{51}Cr-labelled EDTA (Greenaway *et al.* 1991). The ratio of specific activity between haemolymph and urine (U:H) allowed calculation of fluid resorption. Reference to haemolymph and urine ion concentrations allowed the clearance and resorption of specific ions to be determined.

Na balance

The minimum Na equilibrium concentration of *P. warreni* was determined according to Greenaway (1989) by placing eight crabs into plastic containers containing 200 ml of Na-free AMRW. The containers held plastic mesh inserts to ensure that the crabs remained submerged. A small net diffusive loss occurred until the [Na] of the water reached that with which the crab could maintain equilibrium. During the following 6 days, the water was replaced daily, and 2 ml samples were taken at 8 h intervals to determine the minimum Na concentration with which *P. warreni* could maintain Na balance. This treatment was continued without water sampling for a further week to produce Na-depleted crabs.

Unidirectional Na uptake from AMRW was determined in *P. warreni* previously held in amphibious conditions. Crabs (N=6) were placed in the same containers as used previously for minimum equilibrium experiments containing 200 ml of AMRW labelled with sufficient ^{22}Na as to provide 12 000 cts min^{-1} for a 200 μl sample measured using the Beckman scintillation counter. After transferring the towel-dried crabs into the experimental chambers, the initial sample was taken at 2 min and further samples were taken thereafter at 10, 20, 30 and 40 min. This procedure was carried out with Na concentrations of 0, 0.025, 0.1, 0.5, 0.6, 1 and 2 mmol l^{-1} in AMRW. Since the crabs invariably either added small amounts of Na to the water or caused a slight dilution when transferred, the exact start and end values of [Na] were determined. This facilitated the calculation of unidirectional Na influx J (μmol g^{-1} h^{-1}) using the special solution equation of Shaw (1963):

$$y = y_0 \exp\left(-\frac{J}{A}t\right), \quad (4)$$

where y is the concentration of ^{22}Na in the water at time t, y_0 is the concentration of ^{22}Na at time zero and A is the amount of Na in the water. The resulting uptake rate *versus* water [Na] data were analysed by Lineweaver–Burk and by direct linear plot, which gave a similar maximal uptake rate (J_{max}) and affinity (K_m) for each of the crabs. Mean uptake rates and affinities were calculated and used to derive a general rate curve for Na uptake dependent on [Na].

The maximum net Na uptake was determined in the crabs depleted of Na (above) by transferring them to distilled water for 1 min, towelling them dry and placing them in AMRW containing 0.5 mmol l^{-1} Na, from which the uptake of Na was monitored. Water samples were taken at 1 min and thereafter at 15 min intervals for 90 min, and the decrease in water [Na] was determined. A linear plot was always obtained during the first 1 h.

Maximum rates of Na and Ca loss were determined in crabs transferred from containers with MRW to either Na- or Ca-free AMRW. The maximum Na loss rate was determined in Na-depleted *P. warreni* to assess whether acclimation to low-Na water induced a reduction in Na permeability. The amount of Na appearing in the water was monitored and measured as for

net Na uptake (above). Haemolymph samples were taken from these crabs so that the permeability constant K' (Shaw, 1961; Sutcliffe, 1975; Greenaway, 1981) could be derived for Na and Ca.

Nitrogen excretion

Haemolymph and urine concentrations of ammonia and urea were determined (Boehringer test kit 942546) in *P. warreni* that had either been allowed free access to air and water or had been held in air unable to submerse for 4 days. Excretion to air by the gaseous volatilisation of NH_3 was determined in crabs previously held in amphibious conditions, using a smaller version of chambers described by Greenaway and Morris (1989). The evolved NH_3 was trapped in 0.1 mol l^{-1} HCl as NH_4^+, and the concentration was determined using an Orion ammonia electrode and 920A meter. The basal rate of ammonia (defined as NH_3/NH_4^+) excretion to water by crabs held in amphibious conditions was determined by completely submerging them in 200 ml of water for 1 h and determining the increase in ammonia during (a) the initial 30 min and (b) the subsequent 30 min (Boehringer test kit). The excretion rate was similarly determined for *P. warreni* that had been held completely submerged for 24 h or alternatively had been held in air and unable to submerge for 96 h.

Branchial ammonia excretion was believed to occur in *P. warreni*, and specific experiments were designed to assess ammonia excretion by this route. The branchial chambers of 12 crabs were catheterised by inserting small sections of PE tubing (inside diameter 0.5 mm) through drilled and cauterised holes in the branchiostegite on each side. After 48 h of recovery, these tubes were connected to a peristaltic pump (Ismatec VP, Switzerland) which perfused the branchial chambers with artificial urine at 1.09 ml min^{-1}. The crabs were held in small buckets with the bases replaced by stainless-steel mesh, any fluid overflowing from the branchial chambers passed through the mesh into a funnel from which 1 ml fractions were collected at 1, 2, 5 and 60 min. Two groups of crabs were treated in this way; the first had been maintained in amphibious conditions prior to placement in the perfusion apparatus, the second was held in air for 24 h prior to starting perfusion.

Data analysis

All data are presented as means ± S.E.M. unless otherwise stated. Statistical analysis was by one- or two-factor analysis of variance (ANOVA), except for the initial experiments where crabs were exposed to sea water of different strengths for 3 weeks, in which case a repeated one-way measures analysis was used. Homogeneity of variances was confirmed by Bartletts χ^2-test.

Results

Osmo- and ionoregulation

In general, the osmotic pressure in the haemolymph of *P. warreni* was regulated by crabs kept in lower-salinity water (Fig. 1A), but increased after 1 week of exposure to 80 % sea water (SW). *P. warreni* maintained haemolymph [Na] when in water concentrations up to and including 40 % SW, but was unable to prevent increases after 2 of weeks of exposure to 80 % SW (Fig. 1B). The regulation of haemolymph [Cl] failed after only 1 week in 80 % SW (Fig. 1C). Haemolymph [Mg] also increased after only 1 week in 80 % SW, while crabs held in 40 % SW did not show an increase until the third week of exposure (Fig. 1D). Haemolymph [K] increased after 2 weeks in the group exposed to 80 % SW only (Fig. 1E), while there was no statistical change in haemolymph [Ca] in any treatment even after 3 weeks (Fig. 1F).

Water content and exchange by tritiated water

The relative body water content of *P. warreni* with access to water, determined by tritiated water dilution, was 74.3±2.3 % compared with 74.7±3.1 % in crabs breathing air. The clearance of tritiated water by *P. warreni* occurred with a half-life of $t_{1/2}$=104.1±12.4 h in air-breathing crabs and $t_{1/2}$=3.43±0.36 h in water-breathing crabs. This corresponded to efflux rates of 5.09±0.78 μl g^{-1} h^{-1} in air and 156.1±12.3 μl g^{-1} h^{-1} in water, and apparent relative permeability constants (K') of −0.019±0.005 during air-breathing and −0.211±0.020 during water-breathing.

Contents and clearance rates of haemolymph and urine ions

Mooi River water sampled in June 1995 contained (in mmol l^{-1}): K, 0.044±0.002; Na, 1.02±0.01; Ca, 1.88±0.05; Mg, 1.98±0.10 and Cl, 1.30±0.12. When the water was acidified to mobilise $CaCO_3$, [Ca] increased to 1.98±0.04 mmol l^{-1}.

The [Cl] and [Na] of the urine of *P. warreni* held in amphibious conditions were similar to the concentration in the haemolymph (Table 1), while [Ca], [Mg] and [K] were all significantly elevated in the urine compared with the haemolymph (Table 1). The extracellular space (haemolymph) determined by ^{51}Cr-EDTA dilution was 37.9 % body mass. The ^{51}Cr-EDTA clearance (primary urine production) was 620 μl h^{-1}, but urine flow was only 39.5 μl h^{-1} because of a U:H ratio in ^{51}Cr-EDTA of 32.9 (Table 2). Analysis of haemolymph and urine from *P. warreni* before and after 4 days of breathing air showed that air-breathing induced no change in the osmolality of either haemolymph or urine (Table 1). However, the osmotic pressure of the urine was significantly lower than that of the haemolymph in water-breathing crabs. The elevated levels of Mg, Ca and K in the urine compared with the haemolymph persisted, although the osmotic pressure in air-breathing animals tended to be greater in the haemolymph than in the urine (Table 1). Using the values for urine production (^{51}Cr-EDTA) and final urinary flow, together with haemolymph and urine salt concentrations, the clearance and excretion of Na, Cl, Ca, Mg and K could be calculated (Table 3). NaCl was more than 90 % reabsorbed in the antennal gland, while more than 80 % of K, Mg and Ca were reabsorbed. Whether the crabs were breathing air or water made no difference to the extent of reabsorption.

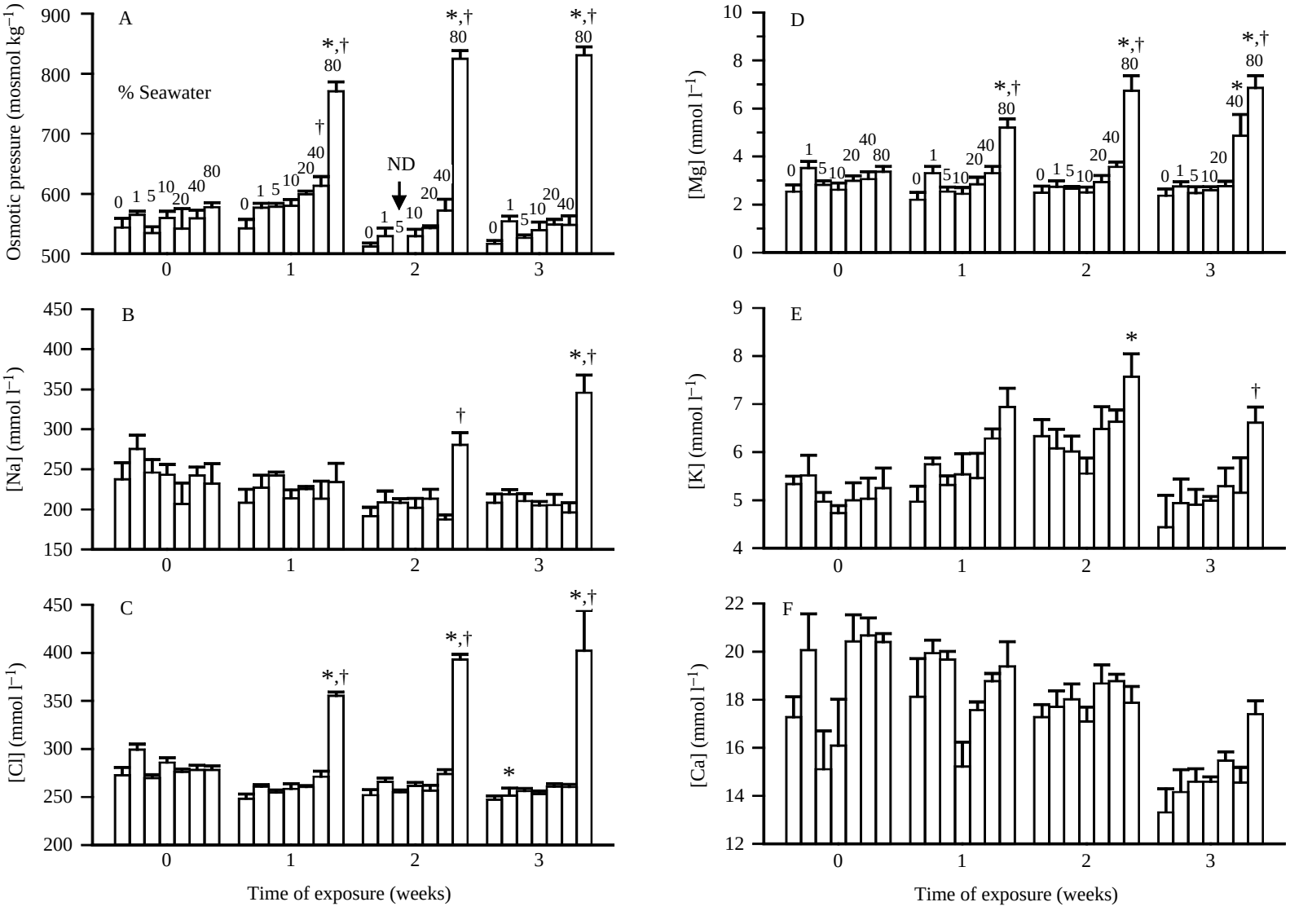

Fig. 1. The haemolymph osmotic and salt status of *Potamonautes warreni* subjected to 3 weeks of exposure to salinities between 0 and 80 % sea water (SW) (see text for exact composition). (A) The osmolality, (B) [Na], (C) [Cl], (D) [Mg], (E) [K] and (F) [Ca]. Six crabs were subjected to each salinity and the results were analysed by repeated-measures methods at each salinity after 0, 1, 2 and 3 weeks of exposure. Bars represent means from six animals and the error bars represent S.E.M. (total N=42). †Significantly different from 0 % SW value during the same week; *significantly different from the value for the same %SW in week 0.

Table 1. *Summary of ion concentrations and osmolality in the haemolymph and urine of* Potamonautes warreni *before and after 4 days of breathing air*

	Haemolymph			Urine		
	Water	Air	Amphibious	Water	Air	Amphibious
Osmolality (mosmol kg^{-1})	635.8±32.9[1]	557.7±19.7	ND	487.7±61.5[1]	528.5±17.7	ND
Cl	271.5±7.3	260.8±6.1	269.5±7.7	289.7±13.4	258.7±11.9	291.0±14.3
Na	238.0±12.3	217.4±5.7	244.5±18.7	240.5±13.0	238.1±22.1	257.9±14.3
Ca	13.01±0.97[2]	13.35±0.41[3]	16.12±1.49[10]	21.10±2.57[2]	25.14±2.55[3]	22.19±1.83[10]
Mg	4.69±0.28[4]	4.84±0.55[5]	3.23±0.77[9]	11.08±2.09[4]	12.37±1.29[5]	9.50±2.20[9]
K	4.55±0.16[6]	5.02±0.12[7]	6.05±0.40[8]	12.93±1.28[6]	14.05±1.28[7]	10.5±1.74[8]
U–H ion gap	43.6	46.9	48.4			

Values are means ± S.E.M. and N=6 for each value.
The statistical analysis revealed no difference between treatments.
Superscripted numbers indicate significant differences between corresponding haemolymph and urine values. ND, not determined.
U–H ion gap is the difference in the total measured ion concentration (mmol l^{-1}) of the haemolymph subtracted from that of the urine.
Ion concentrations are given in mmol l^{-1}.

Table 2. *Relative haemolymph volume in* Potamonautes warreni *by* ^{51}*Cr-EDTA dilution*

Haemolymph (% mass)	Urine production (µl h^{-1})	Filtration (% body mass day^{-1})	H (cts min^{-1})	U (cts min^{-1})	U:H	Urine flow (µl h^{-1})
37.88±2.42	620±120	25.12±4.29	1666±495	32324±6363	32.9±7.7	39.5±13.5

Values are means ± S.E.M. (N=6).
H, haemolymph; U, urine.
Urine production, haemolymph filtration, urine:haemolymph ratio of label and final urine flow determined from EDTA clearance and from urinary and blood concentrations.

Table 3. *Clearance of primary haemolymph ions by filtration at the antennal gland, excretion at the nephropore and the percentage reabsorption in the gland*

	Clearance		Excretion		% Reabsorbed	
	Water	Air	Water	Air	Water	Air
Cl	1.72±0.22	1.81±0.24	0.118±0.016	0.115±0.017	93.2±0.20	93.7±0.21
Na	1.53±0.25	1.51±0.47	0.098±0.016	0.103±0.015	93.5±0.4	93.0±0.6
Ca	0.081±0.009	0.093±0.013	0.009±0.001	0.011±0.002	89.3±1.7	88.0±1.2
Mg	0.030±0.004	0.034±0.007	0.004±0.001	0.005±0.001	85.0±2.4	83.1±2.0
K	0.029±0.004	0.035±0.005	0.005±0.001	0.006±0.001	81.8±1.9	82.1±1.8

Values are means ± S.E.M. (N=6).
Rates are given as µmol g^{-1} h^{-1}.

Sodium balance

The minimum equilibrium [Na] (lowest sustainable [Na] in the water) of *P. warreni* submerged in AMRW was determined as 0.116±0.023 mmol l^{-1}. Analysis of unidirectional Na uptake from ^{22}Na labelling experiments under equilibrium conditions gave J_{max}=0.76±0.14 µmol g^{-1} h^{-1} and K_m=0.18±0.05 mmol l^{-1} (see Fig. 2 for the Michaelis–Menton plot). Maximum net Na uptake in Na-depleted crabs was higher at 1.77±0.25 µmol g^{-1} h^{-1}. Maximum Na loss from *P. warreni* maintained with access to AMRW was 0.22±0.04 µmol g^{-1} h^{-1}, while K' was 0.0025±0.0004. Depleting crabs of Na did not reduce Na permeability or Na loss, which was 0.28±0.06 µmol g^{-1} h^{-1}. The maximum rate of Ca loss was similar at 0.29±0.06 µmol g^{-1} h^{-1}, but with a greater permeability since K'=0.0521±0.0052.

Nitrogen excretion

P. warreni were unable to excrete ammonia to air as the rate of NH_3/NH_4^+ evolution was less than 1 µmol kg^{-1} h^{-1} (Table 4). Holding *P. warreni* in humid air did not change the haemolymph or the urine concentration of either ammonia or urea, which were all similar (Table 5). However, crabs held submerged for the previous day excreted NH_3 to the water at nearly 70 µmol kg^{-1} h^{-1} (Table 4). Interestingly, crabs allowed

Table 4. *The ammonia excretion rate of* Potamonautes warreni *previously held in either amphibious conditions (access to air and water), submerged for 24 h or emersed in humid containers for 24 or 96 h*

History and conditions	Rate of NH_3/NH_4^+ excretion ($\mu mol\ kg^{-1}\ h^{-1}$)
Excretion to air (24 h)	
Previous access to air and water (*N*=6)	0.95±0.26
Excretion to water	
Submerged for previous 24 h (*N*=8)	69.9±14.6
Previous access to air and water (*N*=8)	
0–30 min immersion	348.9±141.0
30–60 min immersion	65.7±20.0
Emersed 96 h (*N*=8)	
0-30 min immersion	4895.0±860.2
30–60 min immersion	74.7±56.0

Values are means ± S.E.M.

Excretion rates to air were measured for animals from amphibious conditions. All other crabs were placed in AMRW and the rate of NH_3/NH_4^+ excretion to water was determined over the times shown.

All groups showed different rates except those submerged for 30 min or longer.

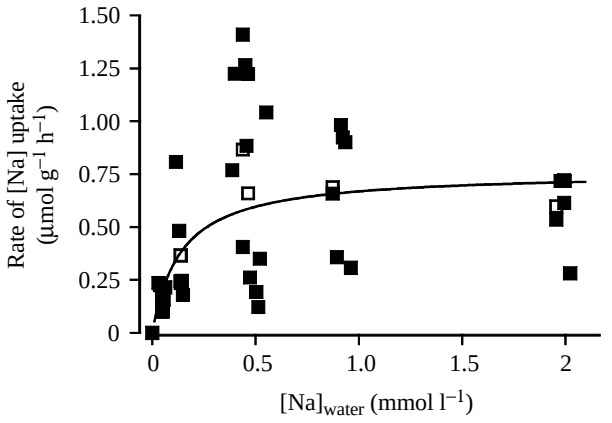

Fig. 2. Unidirectional Na uptake in *Potamonautes warreni* acclimated to artificial Mooi River Water, determined by ^{22}Na uptake at seven water Na concentrations. Filled squares show individual data points, open squares show mean values for each water [Na]. These data for six individuals were analysed by Lineweaver–Burk and direct linear plot to provide mean J_{max} and K_m values for Na uptake. The fitted Michaelis–Menton curve was derived from the mean of these values. Note that the symbols for the two lowest concentrations are closely adjacent.

free access to air or water prior to the experiment showed a significantly higher rate, approaching 350 $\mu mol\ kg^{-1}\ h^{-1}$ within the first 30 min of submergence. Crabs held in humid air showed a similar but much greater burst of elevated NH_3 excretion during the initial 30 min when given access to water (4.9 $mmol\ kg^{-1}\ h^{-1}$). In both of these groups of crabs, the rates

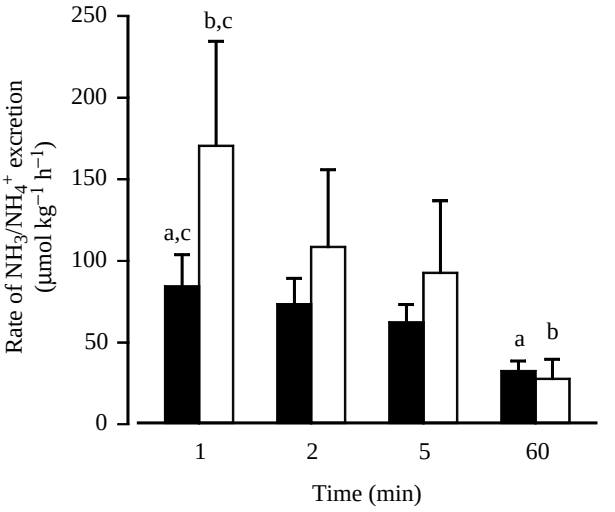

Fig. 3. The ammonia (NH_3/NH_4^+) excretion rate ($\mu mol\ kg^{-1}\ h^{-1}$) of *Potamonautes warreni* with artificially perfused branchial chambers. Rates of excretion to branchial perfusate were determined for 1 min intervals, terminating at 1, 2, 5 and 60 min for each of six crabs in each of the two treatments. In treatment 1 (filled columns), the crabs were allowed free access to air or water, as they chose. In treatment 2 (open columns), the crabs were held in humid air for 24 h prior to the perfusion. Bars represent mean values. Error bars represent S.E.M. Values sharing a common letter are significantly different from one another.

quickly decreased to those of previously submerged crabs (Table 4).

Given the evidence for an extra-renal site for NH_3 excretion, the branchial chambers of crabs were perfused to determine the rates of NH_3 loss, presumably *via* the gills. *P. warreni* previously held in humid air for 24 h excreted double the amount of NH_3 through the gills during the first minute of perfusate flow compared with crabs held in amphibious conditions (Fig. 3). Both groups of crabs showed a progressive decline in NH_3 excretion over the 1 h of perfusion to 32.5±6.3 $\mu mol\ kg^{-1}\ h^{-1}$ for amphibious and 27.7±12.1 $\mu mol\ kg^{-1}\ h^{-1}$ for 24 h air-breathing crabs (Fig. 3).

Discussion

Mechanisms of ion and water regulation by *P. warreni* were generally typical of the potamid crabs (Table 6). Reabsorption of urinary water and thereby ions allowed high-affinity, trans-integumental ion pumps to compensate for diffusive losses. These conservation mechanisms pre-adapt *P. warreni* to air-breathing, but deprive the crab of a fluid vehicle for nitrogen excretion. *P. warreni* is ammoniotelic, but suspends nitrogen excretion while in air and requires periodic access to water for branchial ammonia excretion.

Adaptation to fresh water and response to hypersaline water

P. warreni appears to have relatively better ion regulation in hypersaline water than related species. For example, the increase in haemolymph ion levels and osmolality of *P.*

Table 5. *Summary of NH_3/NH_4^+ and urea concentrations in the urine and haemolymph before and after 4 days in air*

	[NH_3/NH_4^+]		[Urea]	
	Haemolymph	Urine	Haemolymph	Urine
Water (pre-emersion)	0.203±0.079	0.139±0.077	0.247±0.219	0.380±0.224
4 days in air	0.291±0.280	0.093±0.037	0.162±0.168	0.179±0.146

Values are means ± S.E.M. (*N*=6).
Concentrations are given as mmol l^{-1}.

Table 6. *Sodium balance indices for* Potamonautes warreni *compared with other selected freshwater decapod crustaceans*

Species	Urine flow rate (% body mass day^{-1})	Minimum equilibrium concentration (mmol l^{-1})	K_m (mmol l^{-1})	K'_{Na}	K'_{Ca}	Total loss (mmol l^{-1} haemolymph h^{-1}) Na	Ca	J_{max} (μmol g^{-1} h^{-1})	Source
Potamonautes warreni (Na-depleted)	1.60	0.116	0.18	0.003	0.052 0.73	0.59	0.75 ≈2.0	0.76	This study
Potamon niloticus	–	0.5	0.05	0.011	–	2.82	–	1.8*	Shaw (1959*b*)
Potamon edulis	0.58	–	–	0.008	–	2.1	–	–	Harris (1975)
Holthuisana transversa	0.47	0.001	0.18	0.002	–	0.50	–	1.2*	Greenaway (1981)
Pseudothelphusa jouyi	0.36	0.13	0.10	0.028	–	6.7	–	3.0*	Thompson (1970)
Metapaulias depressus	4.39	0.17	0.20	0.062	–	17.2	–	9.9*	Thompson (1970)
Cardisoma hirtipes	8.59	0.117	0.26	0.008	0.096	11.5	1.26	1.67	Greenaway (1989)
Eriocheir sinensis	18.7	–	≈1	0.018	–	5.5	–	1.8*	Shaw (1961)
Austropotamobius pallipes	4.99	0.04	0.25	0.003	0.015	0.51	0.18	0.78*	Bryan (1960); Shaw (1959*a*)

K′ is the relatively permeability constant (ion loss rate/haemolymph concentration).
Some values are from Table 3 in Greenaway (1989).
*Values for converted from mmol l^{-1} blood h^{-1} as provided in Greenaway (1981).

warreni in 80 % sea water was continued after 3 weeks, whereas in *H. transversa* (Greenaway, 1981) this was largely completed after 2 days and in *P. edulis* (Shaw, 1961; Harris and Micallef, 1971) the increase continued for 12 days. In particular, *P. warreni* exhibited relatively smaller increases in [Ca] and [Mg] than those that occurred in *H. transversa* (Greenaway, 1981). Greenaway (1981) suggested that Mg regulation during hypersaline exposure might be achieved by the high urinary [Mg]. While *P. warreni* has a three- to fourfold greater urinary flow than *H. transversa* (Table 6) and Mg is also concentrated in the urine, this difference persisted in air-breathing animals and is not a specific response to hypersaline exposure.

The water loss rate in the urine of *P. warreni* at 0.7 μl g^{-1} h^{-1} was only 5 % of the total water turnover and within the range for the Potamoidea (Table 6). Water permeability λ varies from 0.46 in the crayfish *Procambarus clarkii* to 0.12–0.24 for freshwater crabs, and the value of 0.20 for *P. warreni* was thus typically low (for comparative values and the definition and derivation of λ for crabs, see Greenaway, 1980). Greenaway (1980) pointed out that osmotic water influx should be in the region of 1.1 μl g^{-1} h^{-1}, which is almost double the eventual urinary flow in *P. warreni* and so, like other freshwater species, *P. warreni* requires extra-renal water excretion when submerged (Shaw, 1959*b*; Harris, 1975; Greenaway, 1980).

The low K'_{Na} value for *P. warreni* is also typical of freshwater crabs (Table 6) and implies that the Potamonidea as well as the Parathelphusoidea have a low Na permeability (see Table 6 and citations provided there). The K'_{Ca} of *P. warreni* was unexceptional, but was 17 times greater than K'_{Na} (Table 6). The 'hard water' of the Mooi River may provide *P. warreni* with sufficient Ca so that an extraordinarily low permeability is not required to reduce Ca loss rates (Table 6). The minimum equilibrium concentration of Na attained by *P. warreni* (0.12 mmol l^{-1}; Table 6) was lower than that measured for *Potamon niloticus* by Shaw (1959*b*), who suggested that the minimum equilibrium concentration might influence the species distribution of African potamonid crabs. The K_m for Na uptake was also typical of that for potamid species (Table 6) and is clearly important in maintaining the low equilibrium Na concentration. In contrast, the maximum Na uptake rate for *P. warreni* from Mooi River water was relatively low (Table 6), but the [Na] in the Mooi River was 8.8 times greater than the minimum equilibrium concentration

and 5.6 times the K_m. Thus, the low equilibrium concentration was due to exceptionally low loss rates. However, simulated migration of *P. warreni* into 'soft water' stimulated maximum Na uptake by nearly threefold, similar to the sixfold increase in *H. transversa* (Greenaway, 1981) and to a rate more typical of other species (Table 6). It would be interesting to obtain data from a separate population inhabiting water with naturally lower Na and Ca concentrations.

P. warreni produces a near-iso-osmotic urine but prevents major losses by reabsorbing 94 % of the water and most of the ions in the primary filtrate. This water conservation mechanism is typical of the freshwater crabs (e.g. 94 % for *H. transversa*, Greenaway, 1981; and 54 % *P. edulis*, Harris, 1975) and can be correlated with Na reabsorption. The salt reabsorption in the antennal gland becomes increasingly important in soft water. In Mooi River water, Na uptake by resorption in the antennal organ of *P. warreni* was 1.53 μmol g^{-1} h^{-1} and 73 % of the total uptake since 0.56 μmol g^{-1} h^{-1} (derived from Fig. 2) entered over the body. For Na-depleted crabs in soft water, the uptake at the body surface was only 0.09 μmol g^{-1} h^{-1} (see Fig. 2), and thus the antennal organ accounted for more than 90 % of Na uptake. This mechanism may be of some specific importance since *P. edulis* produced a hypernatric urine but relied almost entirely on trans-integumental Na uptake to compensate (Harris and Micallef, 1971; Harris, 1975).

Water and ion balance limitations to terrestrial excursion

The primary strategy for air-breathing and terrestrial activity appears to be an extreme minimisation of losses. For example, only 7 ml of water is lost by a 60 g *P. warreni* daily, and this is readily replaced, while the daily urinary Na loss is only 5.4 mmol.

P. warreni had a high water content of 74 % of body mass and a haemolymph volume of 38 % of body mass, which is very similar to the values of 74.7 % for water content and 35 % for haemolymph volume in *Holthuisana transversa* (Greenaway, 1980). This was interpreted (Greenaway, 1980) as a possible water reserve for use in a semi-arid habitat, but was unchanged in air-breathing *P. warreni*. It seems likely that the high water and haemolymph content is a feature of some Potamoidea rather than an adaptation to arid habitats. In addition, Dandy and Ewer (1961) determined a value of only 67.4 % body water for *P. warreni* and its congenerics, suggesting that gravimetric methods, and thus some values in the literature, underestimate body water content of freshwater crabs.

Water turnover was 30 times slower in emersed *P. warreni* than in those in water. *P. warreni* apparently lost water to 95 % saturated air at a rate of 5.09±0.78 μl g^{-1} h^{-1}, which was faster than for *H. transversa* in the laboratory but similar to *H. transversa* under burrow conditions, and also similar to field rates for the terrestrial gecarcinid *Gecarcoidea natalis* and anomuran *Birgus latro* (Greenaway, 1994). In humid air, evaporation accounted for more than 85 % of the water efflux from *P. warreni*, and it is noteworthy that there was no increase in either haemolymph or urinary salt concentrations in *P. warreni* as a consequence of 4 days in air. Clearly, no specific adjustment was made in ion reabsorption with respect to air-breathing, perhaps since efficiency is already near to or greater than 90 % in crabs with access to water.

Nitrogen excretion as a limitation to terrestrial excursion

Excretion of nitrogen waste is a potential limitation on the duration of terrestrial excursions by *P. warreni* since it excretes almost no nitrogen to air and the urine is not a vehicle for elevated urea concentrations. Linton and Greenaway (1995) suggested that a similar near-cessation of nitrogen excretion in *H. transversa* implied reduced nitrogen catabolism and temporary nitrogen storage.

The ammonia excretion rate of 70 μmol kg^{-1} h^{-1} in submerged *P. warreni* was unexceptional for freshwater amphibious Brachyura. For example, Linton and Greenaway (1995) measured rates of up to 36.4 μmol kg^{-1} h^{-1} for *H. transversa*, while the brackish-water *Cardisoma carnifex* gave rates of 125 μmol kg^{-1} h^{-1} (Wood *et al.* 1986) and the freshwater *C. hirtipes* rates of 61–88 μmol kg^{-1} h^{-1} (Dela-Cruz and Morris, 1998) under field conditions.

P. warreni held in 'amphibious' conditions showed a 5.3-fold increase in ammonia excretion during the initial 30 min of submersion. After 96 h of enforced air-breathing, this pulse of excretion was 70 times greater than the normal aquatic rate. The rapidity and brevity of this pulse clearly showed that nitrogen wastes stored during terrestrial forays are rapidly excreted on return to water. Air-breathing *H. transversa* also exhibited elevated rates of ammonia release to water immediately on re-immersion, possibly from the mobilisation of stored nitrogen (Linton and Greenaway, 1995). *P. warreni* differed from *Holthuisana transversa* in that, in the latter, elevated nitrogen clearance rates persisted for at least 3 h and not just a few minutes. Elevated rates of ammonia excretion to water were observed in dehydrated *C. carnifex* (Wood *et al.* 1986) and pulsatile production was observed in *C. hirtipes* (Dela-Cruz and Morris, 1998), suggesting that this might be a general limitation for freshwater amphibious crabs. The ability to excrete stored nitrogen waste rapidly may have ecological advantages for *P. warreni* since the requirement by this species for water for submersion is for a very brief period, suitable for diel or possibly more extended excursions onto land.

Branchial NH_4^+ release appears to be the only route for nitrogen excretion in *P. warreni*, and the branchial irrigation experiments showed that the pulsatile excretion was also *via* this route. Both amphibious crabs and those held in air for 24 h showed elevated branchial release of NH_4^+ that was almost dissipated after 5 min. It seems unlikely that this NH_4^+ represents accumulated NH_4^+/NH_3 within the gill epithelium since haemolymph levels remain low and because pH remains unchanged (Adamczewska *et al.* 1997); P_{NH_3} also remained low. Apical H^+/NH^+ exchange and V-ATPase-driven cell alkalisation have been suggested as likely mechanisms of transbranchial ammonia transport (Linton and Greenaway, 1995), but experimental evidence is required to confirm this. Further study is required to determine the storage product in

P. warreni, but an accessible intermediate such as glutamine rather than, for example, urate is implicated by the pulsatile nature of the excretion.

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