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NITROGENOUS EXCRETION IN THE TERRESTRIAL CARNIVOROUS CRAB *GEOGRAPSUS GRAYI*: SITE AND MECHANISM OF EXCRETION

D. G. VARLEY AND PETER GREENAWAY

School of Biological Science, University of New South Wales, PO Box 1, Kensington, New South Wales, Australia 2033

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Summary

The rate and mechanism of nitrogen excretion were examined in *Geograpsus grayi*. This species excretes waste nitrogen as gaseous NH_3 in periodic bursts. The mean concentration of total ammonia ($[NH_3]+[NH_4^+]$) in the primary urine during bursts of excretion (1.72 mmol l^{-1}) was similar to that of haemolymph (2.07 mmol l^{-1}) but was significantly lower ($P<0.005$) than that of branchial fluid (80.6 mmol l^{-1}).

The effects of ion exchange inhibitors on the apical membrane of the gill epithelium in *Geograpsus grayi* were examined. The presence of an amiloride-sensitive Na^+/NH_4^+ exchanger was confirmed and a SITS-sensitive Cl^- influx suggested Cl^-/HCO_3^- exchange.

Thus, the site of nitrogenous excretion in this species is the branchial chamber, which is also the site of reprocessing of urine for ion regulation in other terrestrial crabs. Gaseous ammonia excretion is achieved by volatilisation of NH_3 from the branchial fluid. High partial pressures of ammonia in the branchial fluid are produced by apical Na^+/NH_4^+ exchange and elevation of the pH.

Introduction

Aquatic crustaceans are predominantly ammoniotelic and excrete waste nitrogen across the gills (Regnault, 1987; Greenaway, 1991). Various possible mechanisms have been suggested for the elimination of ammonia, notably outward diffusion of molecular NH_3 down its partial pressure gradient, outward diffusion of ionic NH_4^+ down its electrochemical gradient and exchange of NH_4^+ for Na^+ at the apical and/or basolateral membrane of the gill epithelium (reviewed by Greenaway, 1991). The contribution of each of these mechanisms to nitrogen excretion is controversial and may well vary between species. Thus, whilst *Callinectes sapidus* is believed to excrete molecular ammonia solely by diffusion (Kormanik and Cameron, 1981*b*; Cameron, 1986), NH_4^+/Na^+ exchange has also been reported in this species (Mangum *et al.* 1976; Towle *et al.* 1976; Pressley *et al.* 1981) and in other decapods (e.g. *Eriocheir sinensis*, Pequeux and Gilles, 1981, and *Macrobrachium rosenbergii*, Armstrong *et al.* 1981). The

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importance of ionic exchange as a means of ammonia excretion in decapods is generally unclear, but it seems likely to be present in many species even if utilised primarily for ionic regulation rather than for nitrogenous excretion.

Amiloride inhibits Na^+/H^+ exchange but may also affect Na^+/NH_4^+ exchange in crustaceans, although the evidence for this is controversial. In isolated perfused gills of the marine crab *Carcinus maenas*, amiloride reduced ammonium efflux by 29 % (Lucu *et al.* 1989), whilst in intact *Callinectes sapidus* the effect was variable: ammonia efflux was unaffected in seawater crabs (Kormanik and Cameron, 1981*b*) but showed considerable reduction in crabs maintained at reduced salinities (Pressley *et al.* 1981). In fresh water, ammonium efflux in *Procambarus* spp. and *Callinectes sapidus* was considerably reduced by amiloride treatment (Kirschner *et al.* 1973; Kormanik and Cameron, 1981*b*). It is possible that amiloride sensitivity alone is not an unequivocal indicator of apical Na^+/NH_4^+ exchange (Evans and Cameron, 1986) as a decrease in ammonia excretion in the presence of amiloride can also be explained by inhibition of Na^+/H^+ exchange and by the subsequent effect of this on the NH_3/NH_4^+ equilibrium.

Other nitrogenous excretory products are not produced to any extent by aquatic crustaceans, probably because of the lack of any selective advantage when water is available as an excretory sink and because of the additional metabolic cost of their synthesis. It has also been suggested that aquatic crustaceans lack the metabolic pathways needed to synthesize urea and purines (Claybrook, 1983), but recent work has revealed a *de novo* synthetic capacity for purines in the brine shrimp *Artemia salina* (Liras *et al.* 1992).

The terrestrial crustaceans face different conditions of water availability from those faced by their aquatic ancestors and might be expected to have followed other terrestrial arthropods in the production of nitrogenous waste products better suited to drier habitats. In fact, most terrestrial crustaceans remain predominantly ammoniotelic, the notable exception to date being the anomuran crab *Birgus latro*, which excretes urates in the faeces with minimal accompanying loss of water (Greenaway and Morris, 1989). Gecarcinid land crabs excrete ammonium, which is concentrated in excretory fluid in the branchial chamber prior to its release (Greenaway and Nakamura, 1991; Wolcott, 1991), while ocypodid land crabs concentrate ammonium in the urine before it is passed to the branchial chamber (De Vries and Wolcott, 1991). The terrestrial grapsid crab *Geograpsus grayi* and the terrestrial isopods excrete gaseous ammonia in periodic bouts of excretion (Greenaway and Nakamura, 1991; Dresel and Moyle, 1950; Wieser and Schweiser, 1970; Hartenstein, 1968, 1970). In the isopod *Porcellio scaber*, volatilisation of NH_3 periodically occurs from the pleon fluid and is associated with a large rise in the ammonia concentration of the haemolymph and pleon fluid (Wright and O'Donnell, 1993). It has been suggested that transport of ammonium from blood to pleon fluid occurs by apical Na^+/NH_4^+ exchange across the pleon epithelium. High levels of glutaminase in the body wall of terrestrial isopods suggest the use of glutamine as a detoxifying and storage substrate for ammonia between excretory bouts (Wieser, 1972).

The carnivorous land crab *Geograpsus grayi* excretes gaseous ammonia at a relatively high rate and alternates between periods of maximal and minimal excretion. This situation offers unique opportunities to study and identify the mechanisms involved in the

production of gaseous ammonia. *Geograpsus grayi*, in common with other land crabs, passes urine to the branchial chambers where salts are reabsorbed (Wolcott and Wolcott, 1985; Greenaway and Nakamura, 1991). The availability of salt-containing fluid, ion transport tissue in the gills (Farrelly and Greenaway, 1992) and a respiratory air stream to carry away NH_3 all indicate the branchial chambers as the probable site of ammonia excretion. In this study, the mechanism and site of excretion of gaseous ammonia in *Geograpsus grayi* were investigated. Measurements of pH, ammonia concentrations and total carbon dioxide concentrations in haemolymph, urine, branchial fluid and final excretory product (P) during periods of peak and of minimal excretion established the site of ammonia excretion as the branchial chambers. A perfusion technique was then used to follow the ionic and acid–base changes that occur in branchial fluid during gaseous excretion. The introduction of transport inhibitors into the perfusate allowed assessment of the role of ion exchange mechanisms in the excretory process.

Materials and methods

Geograpsus grayi Milne Edwards weighing 16–42 g were collected from rainforest on Christmas Island (Indian Ocean) and air-freighted to Sydney, where they were maintained in a constant temperature room at 25 °C and 80 % relative humidity on a 12 h:12 h light:dark cycle. Crabs were kept in individual plastic containers containing leaf litter and were fed raw prawn and dry cat biscuits *ad libitum* once a week. Deionized water was provided for drinking and once a month this was replaced by 5 % sea water for 1 week to ensure against ion depletion.

Rate of excretion

To measure gaseous ammonia excretion rates, each crab was placed on a stainless-steel mesh platform in a glass desiccator through which air was pumped. Any ammonia in the incoming air was removed by scrubbing in a gas washing bottle containing 100 mmol l^{-1} HCl and the air was humidified in a second gas washing bottle containing distilled water. Air leaving the desiccator was passed into the bottom of a burette containing 10 mmol l^{-1} HCl, *via* 2.08 mm o.d. polyethylene tubing, and any ammonia was trapped as NH_4^+. Total ammonia concentration, C_{NH_3} ($[NH_3+NH_4^+]$), in the acid was measured using an NH_4^+ electrode (Ingold, Urdorf, Switzerland) and an Orion 940 specific ion meter, and excretion rates of gaseous ammonia were calculated from these values.

During measurement of ammonia excretion, neither food nor water was placed in the desiccators in order to prevent the possibility of ammonia production by bacterial growth in the food or the release of final excretory product into drinking water. Every 48 h, however, the crabs were transferred to plastic containers and fed. Water was also given every 48 h by placing on the cheliped 50 μl droplets of deionized water which the crabs immediately drank. This process was repeated until the animal refused to drink.

Composition of haemolymph, urine, branchial fluid and P

In *Geograpsus grayi*, as in the other land crabs investigated, the antennal organ produces urine similar in ionic composition and concentration to the haemolymph

(Wolcott, 1991). Some of this is passed to the branchial chambers (branchial fluid) where its composition is modified before it is released as excretory product (P). The remainder of the urine is reingested by drinking (Greenaway and Nakamura, 1991).

To collect P, crabs were placed in glass desiccators, as described above, and excretory product then released passed through the mesh platform into a stainless-steel funnel and was collected in a small container of mineral oil. Branchial fluid was collected *via* catheters inserted through pre-drilled holes in the carapace above the gills. Urine samples were taken directly from the nephropores by mouth suction using a drawn-out glass capillary attached to a length of vinyl tubing. Samples of prebranchial haemolymph were taken from the infrabranchial sinus *via* the membrane at the base of the second and third walking legs using 22 gauge needles and glass syringes pre-chilled on ice to prevent clotting.

Samples of haemolymph, urine, branchial fluid and P were taken during periods of minimal gaseous excretion (measured rate <10 μmol kg^{-1} h^{-1}) and during periodic bouts of excretion (crabs were considered to be in an excretory state after NH_3 production reached 50 μmol kg^{-1} h^{-1}). This value was selected because periodic bouts of excretion exhibited by animals did not occur below this value. All samples were analysed for C_{NH_3}, total carbon dioxide concentration, (C_{CO_2}) ($[CO_2]+[HCO_3^-]+[CO_3^{2-}]$), and pH, with the exception of urine, of which there was always insufficient for pH analysis. Data were analyzed using unpaired *t*-tests to compare the above variables during times of excretion and non-excretion.

Perfusion experiments

Perfusions were carried out in glass desiccators, similar to those described above, using the method of Morris *et al.* (1991). Catheters were sealed into pre-drilled holes in the carapace with cyanoacrylate glue and connected to a peristaltic pump (Ismatec VP-MS/CA8). Artificial urine was pumped into the branchial chambers at 180 ml h^{-1} and leaked out around the margins of the branchiostegites. Leaked fluid passed through the mesh platform and was collected in a stainless-steel funnel and sump, from whence it returned to the pump. Fluid could be added to the system *via* the sump or drained by pumping to waste. Small samples of circulating fluid were taken for analyses through a three-way tap.

Artificial urine solutions were of a similar composition to urine collected from the nephropores (concentrations in mmol l^{-1}): Na^+, 439; K^+, 9.9; Ca^{2+}, 10.3; Mg^{2+}, 13; Cl^-, 460; C_{NH_3} 1.7; C_{CO_2}, 13.9. Artificial urine for inhibitor studies additionally contained amiloride (100 μmol l^{-1}) or SITS (3 mmol l^{-1}). For determinations of unidirectional sodium flux, ^{22}Na (Radiochemical Centre, Amersham) was added. During the latter perfusions, the fluid was circulated through a 5 ml plastic container mounted in the well of a solid scintillation detector and radioactivity was monitored using an Ortec modular counting system with a log/linear rate meter; output was displayed on a chart recorder.

A standard protocol for perfusion experiments was adopted as follows. Crabs in the excretory state were placed in the chamber and their branchial chambers were flushed by perfusion for 15 min with 5 ml of artificial urine. This fluid was drained off and then replaced with either 15 ml of fresh perfusate (control group) or 15 ml of artificial urine

containing either amiloride or SITS. This was circulated for 1 h and samples of the infusate were taken every 30 min for analysis of pH, C_{CO_2}, C_{NH_3} [Cl^-], [Na^+], [K^+], [Ca^{2+}] and [Mg^{2+}]. Net fluxes of these substances between animal and perfusate were calculated using the differences in concentrations in the perfusate and allowing for removal of 200 μl samples. Sodium flux, measured with ^{22}Na, was calculated using the general equation of Shaw (1963).

Analyses

C_{NH_3} in artificial urine, haemolymph, branchial fluid and excretory product was measured using a urea test kit (Boehringer Mannheim GmbH, catalogue no. 124788), which uses the Solorzano colour reaction. C_{CO_2} was measured using the method of Cameron (1971). pH was determined using the capillary pH electrode of a Radiometer BMS 3 Mark 2 blood microsystem and a PHM 73 pH/blood gas monitor (Radiometer, Copenhagen).

Cation concentrations were measured with a GBC 906 atomic absorption spectrometer (GBC, Melbourne) and Cl^- concentrations with a Radiometer CMT 10 chloride titrator. Differences between mean values for net fluxes of control and experimental groups were tested statistically using one-way analysis of variance and the Tukey HSD test from the Systat package. Means are expressed ± S.E.M. unless otherwise stated.

Results

Rate of excretion

The rate of total ammonia excretion (gaseous NH_3+NH_4^+ in the P) averaged over excretory and non-excretory periods was 94.9±14.7 μmol kg^{-1} h^{-1} (N=19). Of this, gaseous ammonia excretion contributed 78.3 % (74.3±12.5 μmol kg^{-1} h^{-1}) and the final excretory product 21.7 % (20.6±4.4 μmol kg^{-1} h^{-1}). Maximal excretion rates of gaseous ammonia during excretory bursts were much higher (220.3±20.5 μmol kg^{-1} h^{-1}; N=12). Bursts of excretion lasted from 3 h to about 3 days. A typical record of excretion is shown in Fig. 1.

Composition of haemolymph, urine, branchial fluid and P

The C_{NH_3} of the urine was low both during excretory (1.72±0.42 mmol l^{-1}; N=11) and non-excretory periods (0.96±0.31 mmol l^{-1}; N=5) (Fig. 2). Whilst C_{NH_3} in haemolymph also remained low, it was significantly greater (P<0.05) in excreting (2.07±0.28 mmol l^{-1}; N=15) than in non-excreting crabs (1.18±0.07 mmol l^{-1}; N=8). The mean values for C_{CO_2} in the urine of excreting (13.9±2.7 mmol l^{-1}; N=6) and non-excreting animals (11.0±3.8 mmol l^{-1}; N=5) (Fig. 3) were not statistically distinguishable from the equivalent levels in the haemolymph.

During non-excretory periods, fluid was rarely present in the branchial chamber and only one sample was obtained in thirty attempts. The C_{NH_3} of this sample (0.77 mmol l^{-1}) was similar to that of urine, while the C_{CO_2} (3.38 mmol l^{-1}) was low in comparison with values for branchial fluid from excreting crabs but within the range of urine from non-excreting crabs. Thus, the general composition of this sample was similar to that of urine

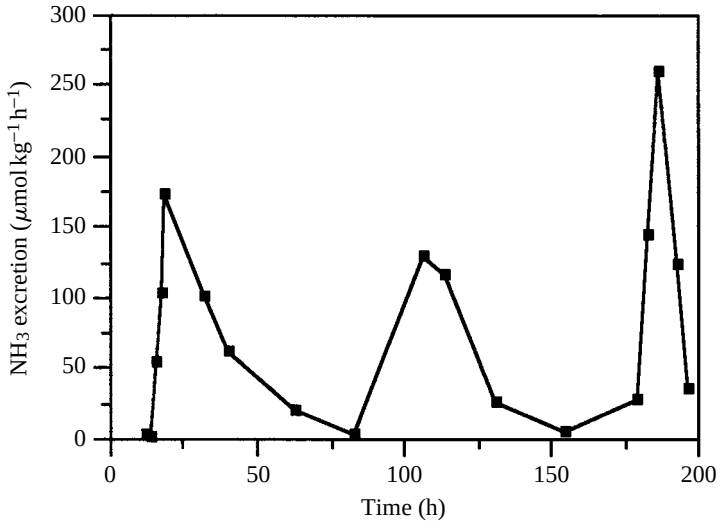

Fig. 1. Periodic excretion of gaseous ammonia by an individual of *Geograpsus grayi*.

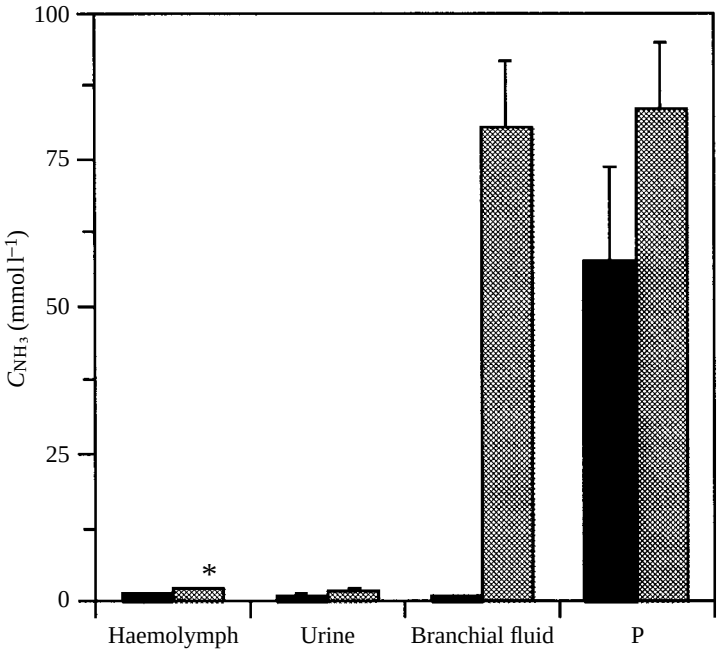

Fig. 2. The total ammonia concentration C_{NH_3} (mean + S.E.M.) of haemolymph, urine, branchial fluid and final excretory product (P) of *Geograpsus grayi* during periods of gaseous ammonia excretion (cross-hatched bars) and during non-excretory periods (filled bars). An asterisk indicates a significant difference (P<0.05) between means of excreting and non-excreting crabs. Note that N=1 for branchial fluid of non-excreting crabs. See the text for other values of N.

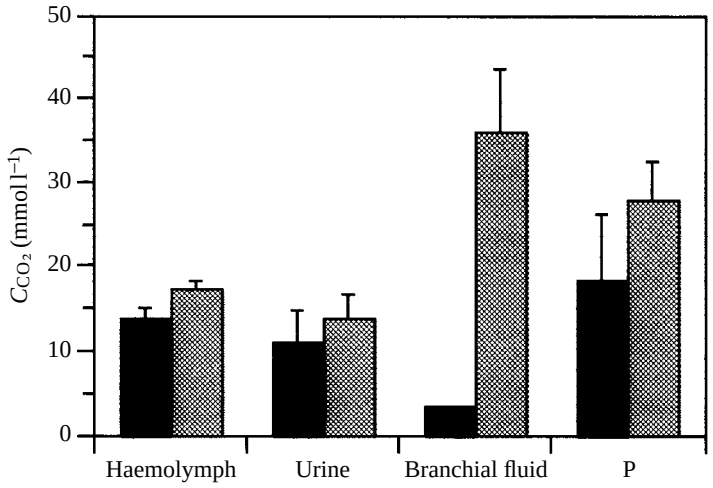

Fig. 3. The C_{CO_2} (mean + S.E.M.) of haemolymph, urine, branchial fluid and final excretory product (P) of *Geograpsus grayi* during periods of gaseous ammonia excretion (cross-hatched bars) and during non-excretory periods (filled bars). The means for excreting and non-excreting crabs were not significantly different. See legend to Fig. 2 and the text for values of *N*.

and the fluid had probably just been released from the antennal organs. In excreting crabs, samples of branchial fluid could be obtained with a 50 % success rate, although the volumes were often very small (10–30 μl). The C_{NH_3} of the branchial fluid was significantly (P<0.005) higher than that of the haemolymph during periods of excretion (80.6±11.3 mmol l^{-1}; N=33) (Fig. 2), and the highest value recorded was a remarkable 219 mmol l^{-1}. In excreting crabs, the pH (8.065±0.05; N=22) and C_{CO_2} (36.0±7.3 mmol l^{-1}; N=9) of the branchial fluid were consistently and significantly (P<0.01) higher than values for the haemolymph of excreting [pH=7.659±0.02 (N=15); C_{CO_2}=17.2±1.1 mmol l^{-1} (N=15)] and non-excreting crabs [pH=7.588±0.03 (N=6); C_{CO_2}=13.7±1.4 mmol l^{-1} (N=7)] (Figs 3, 4).

The mean C_{NH_3} (83.5±11.4 mmol l^{-1}; N=28) and C_{CO_2} (27.6±4.8 mmol l^{-1}; N=26) of P from excreting crabs were very similar to those of fluid sampled directly from the branchial chamber of excreting crabs. However, the pH (7.697±0.24; N=7) was closer to haemolymph levels, possibly due to the loss of some CO_2 as the P sits in the collection funnel. The C_{NH_3} (57.9±15.5 mmol l^{-1}; N=10), C_{CO_2} (18.3±7.8 mmol l^{-1}; N=10) and pH (7.429±0.25; N=5) of final excretory product from non-excreting crabs did not differ significantly from values for excreting crabs. P was released more regularly from crabs which were excreting; 48 % of crabs excreting ammonia released P on the same day as excretion occurred compared with 22 % of non-excreting crabs.

Perfusion experiments

During perfusion of the branchial chambers of control crabs, several consistent changes were observed. The [Na^+] and [Cl^-] in the perfusate fell steadily whilst,

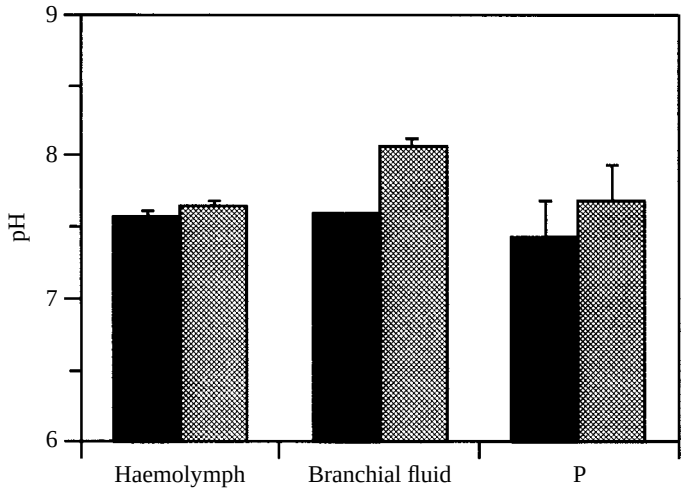

Fig. 4. The pH (mean + S.E.M.) of haemolymph, branchial fluid and final excretory product (P) of *Geograpsus grayi* during periods of gaseous ammonia excretion (cross-hatched bars) and during non-excretory periods (filled bars). The means for excreting and non-excreting crabs were not significantly different. See legend to Fig. 2 and the text for values of *N*.

concomitantly, the C_{NH_3}, C_{CO_2} and pH rose towards the values measured in the branchial fluids of excreting crabs presented above although, given the relatively large volume of fluid in circulation (15 ml), these were never attained.

The addition of 100 μmol l^{-1} amiloride to the perfusate significantly reduced (P<0.05) the net NH_4^+ efflux (animal to perfusate) by 83% (efflux=0.19±0.29 mmol kg^{-1} h^{-1}; N=8) compared with the efflux in the control group (1.13±0.29 mmol kg^{-1} h^{-1}; N=15). The presence of 3 mmol l^{-1} SITS in the perfusate did not significantly reduce net efflux (0.64±0.31 mmol kg^{-1} h^{-1}; N=8) compared with controls (P>0.3) (Fig. 5). Net Na^+ influxes (perfusate to haemolymph) in groups of crabs perfused with artificial urine containing amiloride or SITS did not differ significantly from the mean for the control group (6.383±2.7 mmol kg^{-1} h^{-1}; N=11). However, unidirectional Na^+ influx, measured using ^{22}Na, was significantly higher (P<0.05) in the control group (18.5±2.4 mmol kg^{-1} h^{-1}, N=4) than in crabs perfused with artificial urine containing amiloride (7.42±2.4 mmol kg^{-1} h^{-1}; N=4). In crabs perfused with SITS, influx of Na^+ (16.4±2.4 mmol kg^{-1} h^{-1}; N=4) was similar to control levels. Net Cl^- influx was almost totally eliminated by the presence of SITS in the perfusate (from 6.18±1.3 mmol kg^{-1} h^{-1} (N=8) to 0.023±1.3 mmol kg^{-1} h^{-1}, N=4) but not by amiloride.

Net CO_2 efflux (2.59±0.38 mmol kg^{-1} h^{-1}, N=7) into artificial urine did not change significantly with the addition of either amiloride or SITS to the perfusate. The small effluxes of calcium and magnesium measured in control groups [0.154±0.12 mmol kg^{-1} h^{-1} (N=7) and 0.129±0.06 mmol kg^{-1} h^{-1} (N=7) respectively] were also unaffected by amiloride or SITS (Fig. 6). Similarly, the net efflux of K^+ measured in control perfusions (0.659±0.13 mmol kg^{-1} h^{-1}, N=7) was not significantly affected by either inhibitor.

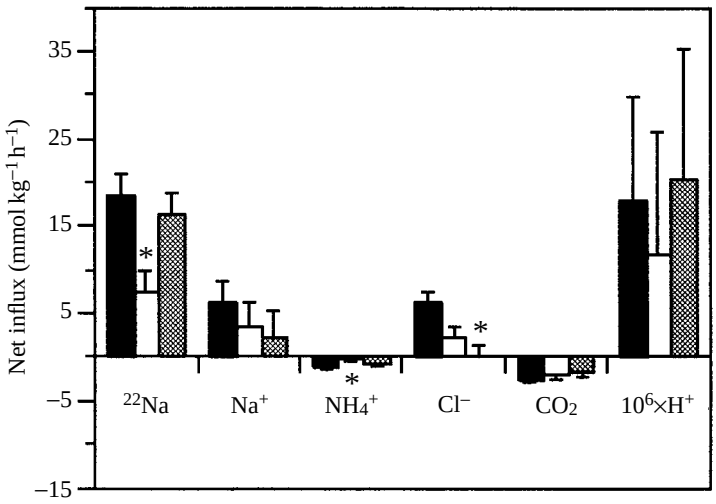

Fig. 5. The unidirectional flux of ^{22}Na and net influxes of Na^+, total ammonia (NH_4^++NH_3), Cl^-, total CO_2 and acid equivalents (mean + S.E.M.) from artificial urine (filled bars) and artificial urine containing 100 μmol l^{-1} amiloride (open bars) or 3 mmol l^{-1} SITS (cross-hatched bars) into *Geograpsus grayi* during a 1 h perfusion of the branchial chambers. Asterisks indicate a significant difference (P<0.05) between mean values for inhibitor treatments and controls. Values of N are given in the text.

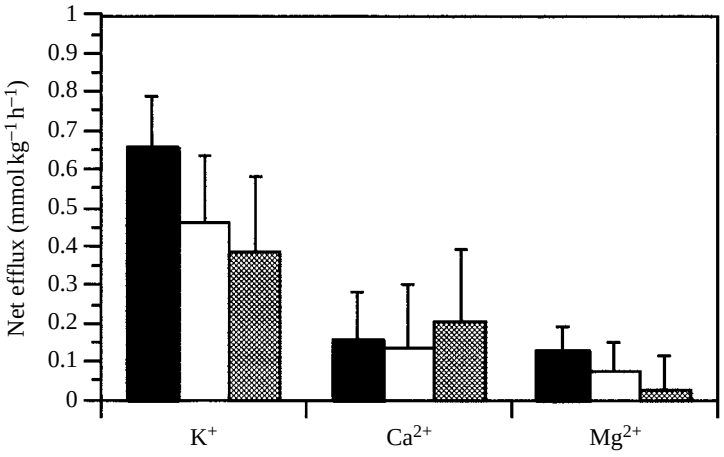

Fig. 6. The net efflux of K^+, Ca^{2+} and Mg^{2+} (mean + S.E.M.) from *Geograpsus grayi* into artificial urine (filled bars) and artificial urine containing 100 μmol l^{-1} amiloride (open bars) or 3 mmol l^{-1} SITS (cross-hatched bars) during a 1 h perfusion of the branchial chambers. No significant differences were found between mean values for inhibitor treatments and controls. Values of N are given in the text.

The pH of the perfusate consistently became more alkaline during perfusion. The shift was typically by 0.3–0.4 pH units from the starting pH of 7.5 within 1 h. Analysis of apparent net proton influx indicated no significant change to this pattern in the presence of either SITS or amiloride (Fig. 5). However, the buffering capacity of the perfusate was unknown and samples were too small for the measurement of titratable alkalinity.

Discussion

Ammonia is toxic to animals although crustaceans are generally more tolerant to ammonia than typical vertebrates, some species in particular exhibiting high blood concentrations (reviewed by Greenaway, 1991). The C_{NH_3} values in blood and urine of *Geograpsus grayi* were typical of other terrestrial ammoniotelic crustaceans, whilst the level in the branchial fluid and P were approximately two orders of magnitude higher than those of the blood and urine. This implicates the branchial chambers, specifically the branchial fluid, as the site of nitrogen excretion, as suggested previously (Greenaway and Nakamura, 1991). The gills are the most likely source of ammonia as they are bathed by branchial fluid; indeed, samples of fluid could only be collected by placing the catheter on the anterior floor of the branchial chamber in the hypobranchial and epibranchial region. The walls of the branchial chamber are not in contact with the branchial fluid.

The mechanism by which NH_3 is volatilised from the branchial fluid is fairly clear. At the high pH of the branchial fluid (8.065), approximately 8 % of C_{NH_3} was present as NH_3, creating a large partial pressure gradient favouring diffusion of NH_3 from the branchial fluid into the air convected through the branchial chamber. The P_{NH_3} of branchial fluid of excreting crabs was calculated to be approximately 3.33 Pa using the values for the solubility and pK′ of ammonia in 0.5 mol l^{-1} NaCl solution given by Kormanik and Cameron (1981*b*). Thus, the rate of volatilisation will depend on the pH and C_{NH_3} of the branchial fluid as these two factors determine P_{NH_3}. The P_{NH_3} of air will remain low and constant because it is continually renewed by the action of the scaphognathites.

The mechanisms involved in the concentration of ammonia in the branchial fluid and the elevation of the pH must also be considered. The outward movement of ammonia across the gill could occur by a variety of routes, and the available data allow the possible contributions of these to be assessed. Clearly, efflux across the gills cannot depend on passive diffusion of NH_3 from the haemolymph to the branchial fluid since the partial pressure gradient for NH_3 actually favours movement in the opposite direction. It is also unlikely that the electrochemical gradient for NH_4^+ is directed outwards. The calculated equilibrium potential for NH_4^+ across the gill epithelium (using mean values for $[NH_4^+]$ of the haemolymph and branchial fluid) is 94 mV (haemolymph positive) and facilitation of passive efflux would require a higher potential. Since transepithelial potentials in crustaceans are usually negative and much smaller, passive transport is unlikely (although the potential difference has not been measured in *Geograpsus grayi*). A favourable gradient for excretion could only be set up by elevation of total ammonia concentrations in the cytosol of the gill epithelial cells. Such concentrations could be produced by intracellular deamination of amino acids or catabolism of purines or other

nitrogenous molecules transferred into the cells from the haemolymph. This could permit outward diffusion of NH_3, but the charged NH_4^+ would not readily cross cell membranes unaided. There would also be a large gradient for diffusion of ammonia from the cell back into the haemolymph and, unless the basal membrane was considerably less permeable to ammonia than the apical membrane, as much ammonia might diffuse back to the haemolymph as would pass into the branchial fluid. Passive diffusion is unlikely to contribute significantly to efflux of ammonia in *Geograpsus grayi*.

By default, concentration of ammonia in branchial fluid is likely to be facilitated by an ion exchange mechanism on the apical membrane of the gill epithelium. This is consistent with a large body of evidence (reviewed by Regnault, 1987) indicating that aquatic crustaceans use ion exchange mechanisms for ammonium excretion across the gill, although it is at variance with data for the marine crabs *Callinectes sapidus* (Kormanik and Cameron, 1981*a*,*b*) and *Cancer irroratus* (Kormanik and Evans, 1984), which appear to excrete NH_3 by diffusion across the gills. As volatilisation of ammonia in *Geograpsus grayi* relies on an alkaline pH in the branchial fluid, clearly Na^+/H^+ exchange plus acid-trapping of NH_3 cannot be the basis for ammonia excretion in this species. Therefore Na^+/NH_4^+ exchange is the most likely mechanism for concentrating ammonia in the branchial fluid.

Amiloride, which is known to inhibit Na^+/H^+ exchange in *Carcinus maenas* (Shetlar and Towle, 1989) and movement of Na^+ through channels (Siebers *et al.* 1989), substantially decreased unidirectional sodium influx from the perfusion fluid to the haemolymph in *Geograpsus grayi*, although the net influx of Na^+ was not reduced significantly. Amiloride simultaneously reduced net ammonium movement into the perfusate and these two factors strongly suggest the presence of an apical Na^+/NH_4^+ exchange system in the gill epithelia, responsible for more than 80 % of ammonium transport. As sodium transport was not completely inhibited, it is possible that virtually all ammonium efflux could occur by this mechanism. The basolateral Na^+/K^+-ATPase in crustaceans is known to substitute NH_4^+ transport for K^+ (Towle and Holleland, 1987; Towle, 1993) and may provide the driving gradient for Na^+/NH_4^+ exchange, both supplying NH_4^+ for exchange and providing the inward gradient for sodium. The epithelial cells of the gills of *Geograpsus grayi* possess the basal infolding, amplification of apical membranes and abundant mitochondria characteristic of transporting tissue (Farrelly and Greenaway, 1992). It is possible that the mechanism in *Geograpsus grayi* represents an intensification of an existing ion transport process present in aquatic ancestors rather than the independent evolution of a new mechanism during adaptation to the terrestrial habitat.

The high C_{CO_2} in the branchial fluid and its elevated pH must also be explained. The apical membranes in the gill epithelial cells of crustaceans are believed to house Cl^-/HCO_3^- antiporters (Lucu, 1989; Towle, 1993) and this system would allow concentration of HCO_3^- in the branchial fluid in exchange for Cl^-. The inhibitor of Cl^-/HCO_3^- exchange, SITS, effectively eliminated net chloride influx during perfusion of the branchial chambers, which lends strong support for the presence of this mechanism in *Geograpsus grayi*. SITS, however, did not decrease total CO_2 efflux significantly. The absence of this effect could be explained by a large diffusional contribution from

respiratory CO_2 to this efflux, thus masking the effect of SITS on the Cl^-/HCO_3^- exchange component of CO_2 efflux. Also, during perfusion experiments, CO_2 was continually lost from the perfusate into the air while passing from the crab to the collecting funnel, so the apparent fluxes presented probably heavily underestimated the real net flux. Measurement of the respiratory component of CO_2 loss and the error produced by CO_2 loss from the perfusate would require combined perfusion and respiration experiments.

The P_{CO_2} of the branchial fluid was calculated using measured values for C_{CO_2} and pH, and values for pK_1', pK_2' and αCO_2 for sea water and pure water at 25 °C (Skirrow, 1965), in the Henderson-Hasselbalch equation. Exact values cannot be given as the salt concentration of the branchial fluid varied from an initial composition similar to that of sea water (when it first enters the branchial chamber) to near that of fresh water by the time it is released as P. The P_{CO_2} calculated in this fashion lies between 933 and 1866 Pa (for initial and final urine, respectively). Clearly, the gradient for diffusion of CO_2 into the branchial air stream was as much as three orders of magnitude higher than that for ammonia at the mean pH of branchial fluid, and calculation of P_{CO_2} and P_{NH_3} of individual branchial fluid samples confirmed this. The partial pressure gradient for ammonia diffusion would only exceed that for carbon dioxide if the pH of the branchial fluid reached 9, at the measured values of C_{CO_2} and C_{NH_3}.

It is likely that the elevation of the branchial fluid pH, during periods of excretion, was also related to the activity of ion exchange mechanisms in the gill epithelium. From the present data it is not possible to determine whether H^+ or OH^- or their equivalents were being transported across this epithelium. However, the net effect of an apical Cl^-/HCO_3^- exchanger would be to increase the pH of the branchial fluid bathing the gills due to changes in strong ion difference (SID) (Stewart, 1978).

The various components of the excretory process together suggest the excretory mechanism described below. The first step is the release into the branchial chambers of fresh urine with low C_{NH_3} and C_{CO_2}, and high $[Na^+]$ and $[Cl^-]$. The pH of the urine is unknown at this stage, but is probably close to haemolymph levels (around 7.6). The activity of ion exchangers in the gill epithelium then raises the C_{NH_3}, C_{CO_2} and the pH of the fluid whilst lowering its $[Na^+]$ and $[Cl^-]$. As these levels rise, partial pressure gradients favouring loss of CO_2 and NH_3 to the air are set up and increase until an equilibrium is reached between the rates at which NH_4^+ and HCO_3^- are transported into the branchial fluid and the rates at which the two gases are lost. The reaction will be:

$$NH_4^+ + HCO_3^- \rightarrow H_2O + CO_2\uparrow + NH_3\uparrow .$$

At this point, the C_{NH_3} and C_{CO_2} will remain more or less constant whilst $[Na^+]$ and $[Cl^-]$ will continue to decline. Eventually the $[Na^+]$ and $[Cl^-]$ will become limiting and the process will slow and stop unless sodium and chloride are recycled or (more likely) the branchial fluid is discarded and replaced with fresh urine.

The measured rate of total ammonia excretion was very similar to that reported earlier (Greenaway and Nakamura, 1991). *Geograpsus grayi* is clearly ammoniotelic with a nitrogen output at the high end of the range of published values for terrestrial crabs (Wood and Boutilier, 1985; Wolcott and Wolcott, 1987; Greenaway and Morris, 1989).

However, in the present study, a higher proportion of ammonia excretion occurred *via* the final excretory product (21.7 % compared with 8.3 % of total excretion) than was found by Greenaway and Nakamura (1991). It is likely that the percentage of ammonia excreted by each route depends on fluid balance. With high water intake, there would be high P flow and excretion by that route would be enhanced.

The mechanism of excretion utilised by *Geograpsus grayi* allows a considerable increase in the amount of nitrogen that can be excreted per unit volume of water lost. The C_{NH_3} of P released is much higher than routine levels seen in gecarcinids (Greenaway and Nakamura, 1991; Wolcott, 1991) and similar to that reported for *Ocypode quadrata* (De Vries and Wolcott, 1991). Additionally, very large amounts of ammonia are lost by volatilisation. There is, in fact, no need for *Geograpsus grayi* to release any P and under dry conditions it may be retained by reingestion. The high ratio of nitrogen excreted to water used potentially allows the species greater flexibility either in the direction of increased utilisation of protein or in the penetration of drier habitats.

It is possible that the limiting factor for NH_3 excretion by *Geograpsus grayi* is the availability of Na^+ and Cl^- as it must be present in the branchial chamber for excretion to occur. This possibility will be examined in future research. As pointed out by Greenaway and Nakamura (1991), the periodicity of NH_3 release implies sequestering of amino nitrogen in some less toxic form in the haemolymph or tissues during non-excretory periods. This aspect will also be addressed in future manuscripts.

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