

High Ammonia and Low pH in the Urine of the Ghost Crab, *Ocypode quadrata*

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Abstract. Nitrogen excreted into the urine (<1 mM) has generally been considered a negligible component of total nitrogen output of crustaceans. But concentrations of ammonia >100 mM were found in the urine of laboratory-held *Ocypode quadrata*, suggesting that this notion might not be applicable to all crustaceans. To address this issue, hemolymph and urine were removed from freshly captured *O. quadrata* and analyzed for nitrogenous catabolites and major ions. Hemolymph composition was similar to that of other crustaceans, but the urine was acidic (\bar{X} pH = 5.50) and contained ammonia, often at >100 mM. Other nitrogenous catabolites in the urine (urea, amino acids, and uric acid) were much less concentrated: totaling <12 mM on average. The ionic composition of the urine was similar to that of other crustaceans, with the exception that Na was much less concentrated than Cl^- . Total osmolality of hemolymph and urine was similar. The Na^+/K^+ ATPase activity was greater in the antennal glands than in the posterior gills of *O. quadrata*, suggesting that this enzyme is important for ammonia concentration and Na resorption. This pattern of enzyme activity was not present in two terrestrial brachyurans whose urine contains little ammonia. The evolutionary significance of high ammonia concentrations in the urine of ghost crabs is unclear.

Introduction

Previous research has demonstrated that the gills and digestive tract are the major organs of nitrogen excretion in decapod crustaceans (Horne, 1968; Gifford, 1968;

Cameron and Batterton, 1978; Harris and Andrews, 1985; Graszynski and Bigalke, 1987; Greenaway and Morris, 1989; Greenaway and Nakamura, 1991; Wolcott, 1991). The renal organ of decapods is important for water balance (review, Mantel and Farmer, 1983) and acid-base balance (Wheatly, 1985), and has been thought to be unimportant in nitrogen excretion. Urine contained nitrogen concentrations generally totaling less than 1 mM in the species studied (Horne, 1968; Mangum *et al.*, 1976; Cameron and Batterton, 1978; Harris and Andrews, 1985; Greenaway and Morris, 1989; Greenaway and Nakamura, 1991; Wolcott, 1991), and was neutral or of slightly alkaline pH (Cameron and Batterton, 1978; Truchot, 1979; Wolcott, 1991).

Aquatic decapods primarily excrete ammonia at the gills. Most terrestrial crabs also employ the gills for nitrogen excretion; however, in this case the fluid bathing the gills is not water from the environment, but urine. Urine is passed from the nephropores to the branchial chambers, where salts are resorbed, typically in association with ammonia excretion (Greenaway and Nakamura, 1991; Wolcott and Wolcott, 1984, 1985, 1991; Wolcott, 1991). The nitrogen content and acid-base status of urine before and after reprocessing by the gills have been measured only in herbivorous (low nitrogen input) species. The relatively small volume of urine passed to the branchial chambers can apparently accommodate the amounts of ammonia excreted by herbivores (Greenaway and Nakamura, 1991; Wolcott, 1991). Terrestrial crabs that are carnivorous must eliminate greater amounts of nitrogen, but because the amount of fluid into which ammonia can be excreted is limited, the risk of ammonia toxicity is increased.

The terrestrial brachyuran *Ocypode quadrata*, which inhabits exposed beaches on the Atlantic coast of the Americas, is primarily carnivorous (Wolcott, 1978) and

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reprocesses urine (Wolcott and Wolcott, 1985). Our preliminary data from laboratory-held *Ocypode quadrata* showed high concentrations (>100 mM) of ammonia in acidic ($\text{pH} < 6$) urine, a pattern atypical of crustaceans but typical of vertebrates. Deamination of dietary protein in the liver of vertebrates decreases urinary pH and increases urinary ammonia output (Minnich, 1972; Scott, 1972; Pitts, 1974; Long and Skadhauge, 1983; Halperin *et al.*, 1985; Dantzler, 1989). Moreover, carnivorous and omnivorous mammals have more acidic urine than herbivores (Long and Giebisch, 1979). Comparable data are scant for invertebrates.

We therefore characterized the ionic and nitrogenous composition of urine and hemolymph from *O. quadrata* freshly captured from the field. Results suggested possible mechanisms of ammonia concentration in the renal organ. Consequently, we also measured the activities of the Na^+/K^+ ATPases in the antennal glands and other ion-transporting tissues of *O. quadrata*, and compared them to the activities in two species of herbivorous terrestrial crabs, *Cardisoma guanhumi* and *Gecarcinus lateralis*, for which previous research had demonstrated low urinary nitrogen concentrations (Wolcott, 1991). Na^+/K^+ ATPases are important in excretion of NH_4^+ across gills of aquatic crabs and fishes (Towle *et al.*, 1976; review, Evans and Cameron, 1986; Towle and Hølleland, 1987), and may play a role in ammonia transport by other organs.

Materials and Methods

Collection of animals and samples

Ghost crabs, *Ocypode quadrata* (Fabricius), were captured after dusk on Bogue Bank, North Carolina, and immediately brought back to the laboratory where the urine was collected. Animals were restrained by being strapped to a board; the nephropore cover was deflected, and the tip of a fire-drawn pipette was inserted just inside the nephropore. Urine flow usually began immediately upon nephropore deflection; when this did not occur, gentle suction was applied by mouth tube. To minimize air exposure of the sample and possible pH change, the pH was immediately measured with a Microelectrodes, Inc., Model 710 electrode. pH measurements (of blood samples) obtained using a completely anaerobic procedure for sample withdrawal agreed to within 1% of values from the minimal air exposure method described above. The remaining urine was frozen for later analyses.

The crabs were kept overnight in plastic containers with a small amount of moist sand from their habitat. The following morning, a fire-drawn pipette was inserted into the arthrodial membrane of a leg and hemolymph was withdrawn. According to previous research on *O. quadrata*, the hemolymph ion concentrations for an individual can increase or decrease by as much as 15% over an 8-h

period (Hall, 1982; Wolcott and Wolcott, 1985), although the average change is considerably less. For this reason, we considered the 8 h elapsed time between urine and hemolymph collection to be unimportant for urine to hemolymph comparisons.

Chloride concentration, osmolality, and pH were measured immediately on whole blood. After a clot had formed in the remaining sample, it was disrupted and the sample was centrifuged (10 min at 10,000 rpm, 4°C); the serum was drawn off and frozen for later analyses. Urine and serum for atomic absorption spectrophotometry were frozen in sealed hematocrit tubes.

Analytical methods

Osmolality was measured with a Wescor osmometer (Model 5100B). Cl^- concentrations were measured on 2- μl samples with a Buchler-Cotlove direct readout automatic titrator. For blood we first placed the sample into 0.5 ml of deionized water to free any Cl^- that might be trapped within the clot. Ammonia was measured by the Berthelot method (Sigma Technical Bulletin No. 640). Urea was hydrolyzed by urease (Sigma Chemical Co.), and the urea concentration estimated by difference in ammonia between hydrolyzed and unhydrolyzed samples of urine and hemolymph. Amino acids were measured in urine (only) as ninhydrin positive substances (method of Lee and Takahashi, 1966) after precipitation of the proteins with trichloroacetic acid. Uric acid was measured by the difference in absorbance (292 nm) of samples hydrolyzed and unhydrolyzed by uricase (Sigma Kit No. 292). Total nitrogen was measured on urine (only) by Nesslerization (Fisher Scientific) after micro-Kjeldahl digestion and distillation. Concentrations of Na, Mg, K, and Ca were measured by atomic absorption spectrophotometry (AAS; Perkin Elmer Model 2400). AAS measures the concentrations of both the ionized and elemental forms of these species, but most of these elements exist as ions in blood and urine.

The amount of water absorbed or secreted into the primary urine during modification was not estimated with marker studies. Thus urine/hemolymph ratios may not reflect simple reabsorption or secretion of a molecule. Rather, they may be considered estimates of relative absorption or secretion.

To be certain that the high ammonia concentrations we measured with the Berthelot assay were not due to an interfering substance in the urine of *O. quadrata*, we used suppressed ion chromatography (CS3 columns) to analyze urine from laboratory-held crabs for ammonium. The eluant was 0.5 mM DL-2,3-diamino-propionic acid monohydrochloride in 12 mM HCl, and the regenerant was 0.05 M tetrabutylammonium hydroxide. The retention time of ammonium was 3.38 min with a precision of 3.5%

from six standard runs. These laboratory-held animals had urinary ammonia concentrations similar to those of the freshly captured animals (measured by the Berthelot assay).

For the purposes of this paper, ammonia refers to a combination of ionized (NH_4^+) and unionized (NH_3) forms of this molecule.

Na⁺/K⁺ ATPase assays

Cardisoma guanhumi were collected at Fort Pierce, Florida, and maintained in the laboratory in individual habitats that included a pool of dilute (<50%) seawater at the bottom of an artificial burrow. *Gecarcinus lateralis* were collected at Spittal Pond, Bermuda, and maintained in the laboratory in individual plastic mouse cages (8.5-l) containing a petri dish of dilute seawater. Water was renewed twice weekly, and crabs were fed leaf litter and lettuce *ad libitum*. Holding conditions for the herbivorous species support long-term survival (>4 years) and multiple successful moltings. *C. guanhumi* and *G. lateralis* had been in the laboratory for about 10 months, overwintering on a seasonally varying light-dark cycle, before sacrifice for enzyme assays.

Ocypode quadrata used for enzyme assays were collected as described previously for this species. *O. quadrata* were maintained in plastic mouse cages (8.5-l) containing acid-washed sand dampened (10% water by weight) with dilute seawater. This species was fed dried squid 2–3 times per week for at least 10 days before sacrifice. All species were given access to dilute medium to stimulate reclamation of ions by the gills through the induction of gill Na^+/K^+ ATPases, thus precluding the underestimation of enzyme activity.

Detailed procedures for measuring specific enzyme activity of Na^+/K^+ ATPases appear in Holliday (1985), but are briefly as follows. Animals were killed by disrupting the ventral nerve cord. Three tissues—antennal gland, the basal portion of gill 7, and claw muscle—were removed from each crab, rinsed, and homogenized by hand in cold homogenizing medium. The basal portions of posterior gills were selected for assay because they have been implicated in sodium transport in several species of osmoregulating crabs (reviewed by Towle, 1984). Claw muscle was included as a 'reference.' Though ions are transported within it, muscle is unimportant for the overall ion balance of the organism.

Enzyme activity was measured as the phosphate liberated from ATP by each homogenate in two reaction media, one containing optimum concentrations of all ions and the other lacking potassium and containing ouabain. After 15 min at 30°C, the reaction was stopped and phosphate measured colorimetrically. Enzyme activity was calculated as the difference in phosphate liberated by each

homogenate into the media, and is expressed as $\mu\text{moles PO}_4 \text{ liberated} \cdot \text{mg}^{-1} \text{ protein} \cdot \text{h}^{-1}$. Protein concentrations in the homogenates were measured colorimetrically with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA); bovine serum albumin was the standard.

Statistical analyses

Differences between mean concentrations of each variable measured in the hemolymph and urine were tested by paired *t* tests. Exceptions were pH and ammonia, which deviated from normality and so did not fit the assumptions of parametric statistics. A Wilcoxon test was used to test for differences between these variables. For simplicity, nonparametric Spearman correlations were used for all associations between variables tested. Measurements are expressed as means $\pm 95\%$ confidence intervals unless otherwise stated. For specific activity of Na^+/K^+ ATPases, differences among the means for each tissue were tested by one-way ANOVA followed by multiple comparison (Bonferroni) tests within each species.

Results

The pH of urine from freshly caught *Ocypode quadrata* was significantly lower and more variable ($\bar{X} = 5.50$; range = 4.68–7.56) than that of hemolymph ($\bar{X} = 7.58$; range = 7.42–7.68; Wilcoxon $Z = 4.78$, $P < 0.0001$). In addition to being more acidic, urine contained >100 times more ammonia than hemolymph (Table I). Ion chromatography confirmed that the high urine ammonia concentrations observed with the Berthelot assay were due to ammonia, rather than to a potentially interfering substance such as an amine. Ion chromatography yielded concentrations 23.7% higher on average than the Berthelot assay ($n = 4$). Urea concentrations in urine and hemolymph were similar and were low compared with urine ammonia (Table I). When concentrations of ammonia-, urea-, and amino-nitrogen are summed, they account for an average of 88% of the total Kjeldahl-nitrogen measured, when comparing individuals for which all of the nitrogenous variables were measured. Thus in Table I, we are accounting for a majority of the nitrogenous catabolites in the urine. Of the accountable nitrogen in the urine, ammonia-N = 90.9%, urea-N = 5.8%, amino-N = 3.3%, and uric acid-N = 0.1%.

Na and Cl^- are the major ionic constituents of both urine and hemolymph of *O. quadrata* (Table II). For all inorganic elements measured, and for total osmolality, there was a significant difference in concentration between the urine and the hemolymph (Table II). For total osmolality and Cl^- , this difference is fairly small but consistent. The concentrations of these elements in the urine appear to be strongly regulated. Mg and K appear to be

Table I

Nitrogenous components of urine and hemolymph from freshly captured *Ocypode quadrata*

Fluid	Nitrogen concentration (mM)				
	Ammonia-N	Urea-N	Amino-N	Uric Acid-N	Kjeldahl-N
Urine	116.31 ± 34.85 (n = 18)	7.43 ± 2.40 (n = 18)	4.16 ± 2.03 (n = 18)	0.12 ± 0.08 (n = 14)	179.6 ± 73.1 (n = 8)
Hemolymph	0.86 ± 0.39 (n = 17)	6.52 ± 1.02 (n = 18)	—	Undetectable (n = 10)	—
U/H	135.24	1.14	—	—	—
P	0.0001*	0.551			

P value is for Wilcoxon Test (ammonia) and paired *t* test (urea).

preferentially secreted and Na and Ca resorbed, probably as ions (Table II).

When comparing concentrations in the urine and hemolymph of each variable measured, a significant correlation was found only for total osmolality ($r = 0.557$, $P = 0.038$). This suggests that for all variables except total osmolality, the processes that regulate the concentration of the substance in the urine are not dependent simply upon the concentration of that substance in the hemolymph.

Urine ammonia concentrations were correlated with several variables measured, including urine pH, Cl^- , Na, and Mg ($r = -0.707$, $P = 0.001$; $r = 0.628$, $P = 0.005$; $r = -0.682$, $P = 0.005$; $r = 0.635$, $P = 0.015$, respectively). The two negative correlations (ammonia vs. pH and ammonia vs. Na) are suggestive of ion exchange processes that might result in high urinary ammonia.

For all species, Na^+/K^+ ATPase activity of claw muscle was low and significantly different from that of antennal gland (Table III). Specific enzyme activity of antennal gland was significantly lower than or not different from that of gill 7 for the two species (*C. guanhumi* and *G. lateralis*) with low urine ammonia. ATPase activity of

antennal gland from *O. quadrata* was significantly greater than activity of gill 7.

Discussion

Urine removed from the bladders of freshly captured *Ocypode quadrata* is acidic and the ammonia concentration is up to 134 times greater than that of the hemolymph. This urine to hemolymph ratio for ammonia is two orders of magnitude higher in *O. quadrata* than in previously studied crustaceans (1.0–3.8) (Green *et al.*, 1959; Cameron and Batterton, 1978; Harris and Andrews, 1985), though the hemolymph ammonia falls within published values (Gifford, 1968; Horne, 1968; Mangum *et al.*, 1976; Cameron and Batterton, 1978; Henry and Cameron, 1981; Wood and Boutilier, 1985; Greenaway and Morris, 1989; Greenaway, 1991). Other nitrogenous catabolites (urea, free amino acids, and uric acid) were present in low levels in the hemolymph and urine of ghost crabs, as in other decapods (Horne, 1968; Gifford, 1968; Henry and Cameron, 1981; Greenaway and Nakamura, 1991; Wolcott, 1991).

This extremely high concentration of urine ammonia has implications for the overall nitrogen balance of the

Table II

Ionic and elemental constituents of urine and hemolymph from freshly captured *Ocypode quadrata*

Fluid	Concentration (mM)*					Osmolality
	Cl^-	Mg	Na	K	Ca	
Urine (n = 13–18)	415 ± 13	43.8 ± 14.1	254.6 ± 50.5	19.5 ± 3.4	9.3 ± 1.2	788.2 ± 29.0
Hemolymph (n = 16–17)	378 ± 10	12.2 ± 1.6	413.1 ± 39.0	7.7 ± 1.1	13.8 ± 1.8	841.3 ± 17.1
U/H	1.09	3.59	0.62	2.53	0.72	0.94
P	0.0006	0.0024	0.0002	0.0005	0.001	0.0031

* mOsm/kg for osmolality.

P value is for paired *t* test.

Whole blood was analyzed for Cl^- and osmolality.

Table III

Specific enzyme activities of Na^+/K^+ ATPase ($\mu\text{mol PO}_4 \cdot \text{mg}^{-1}$ protein $\cdot \text{h}^{-1}$) from various tissues of brachyurans on dilute media

Taxon	Tissue		
	Antennal gland	Gill 7	Claw muscle
<i>Cardisoma guanhumi</i> (n = 6) F = 897.4 P < 0.0001	3.93 ± 0.48 A	13.25 ± 0.51 B	1.84 ± 0.58 C
<i>Gecarcinus lateralis</i> (n = 6) F = 10.59 P < 0.0016	8.83 ± 3.56 A	4.80 ± 3.34 A/B	2.16 ± 0.73 B
<i>Ocypode quadrata</i> (n = 14) F = 75.2 P < 0.0001	29.60 ± 5.2 A	17.01 ± 3.21 B	1.25 ± 0.49 C

Activity values are means ± 95% confidence intervals. Results of analysis of variance and groupings by Bonferroni tests (experimentwise $\alpha = 0.05$) appear below each species. Means with different letter designations are significantly different from one another (within each species).

organism. The rate of release of urine after reprocessing by the gills of *O. quadrata* (about 2% body weight/day; De Vries and Wolcott, 1993) can serve as a conservative estimate of the rate of urine production. This estimate falls within the range of urine production rates observed for other terrestrial crabs (Harris, 1977; Kormanik and Harris, 1981; Greenaway *et al.*, 1990). Use of this value and the data from Table I yields an output of ammonia of $2500 \mu\text{mol N} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ via urine, which is 1–2 orders of magnitude greater than the urinary nitrogen output rate of every crab studied, whether aquatic or terrestrial (Binns, 1969; Cameron and Batterton, 1978; Harris and Andrews, 1985; Greenaway and Nakamura, 1991). In addition, this estimate approximates the combined rates for all nitrogen output routes in *Geograpsus grayi* (also a carnivore), the species with the highest nitrogen output rate yet measured for terrestrial crabs (Wood and Boutilier, 1985; Wolcott and Wolcott, 1987; Greenaway and Morris, 1989; Greenaway and Nakamura, 1991; Wolcott, 1991). Other routes of nitrogen excretion in *O. quadrata* were insignificant (De Vries and Wolcott, 1993; Wolcott and DeVries, unpub. data). Urine production rates (before reprocessing by the gills) must be measured and a rigorous nitrogen budget must be constructed under controlled conditions in the laboratory if we are to better our understanding of this phenomenon.

The urine of *O. quadrata* is isosmotic with the hemolymph, but not isoionic. In this study, Mg and K appeared to be secreted, and Ca and especially Na resorbed. Gifford (1962) found similar patterns of secretion and reabsorption of the major cations during urine formation by freshly

collected *Ocypode albicans* (= *quadrata*), though the magnitude of the modification for each ion measured was less. The renal organs of other terrestrial and semiterrestrial decapods also appear capable of modifying the inorganic content of the urine to some degree. The urine and hemolymph of those species have concentrations of the major ions (except Na) that are similar to the concentrations found in *O. quadrata* (review, Mantel and Farmer, 1983; present study).

The sodium in the urine of *O. quadrata* is about 150 mM lower than both the Na in the hemolymph and the Cl^- . Low urinary Na concentrations have been previously described for *O. quadrata* (Gifford, 1962) and *U. pugilator* (Green *et al.*, 1959), though this pattern is the exception among crustaceans (review, Mantel and Farmer, 1983). Nevertheless, for *O. quadrata*, like other crustaceans, the gills are the major organ of salt balance (review, Mantel and Farmer, 1983). Gills of *O. quadrata* can resorb 90% of the salt in the urine during reprocessing (Wolcott and Wolcott, 1985).

Low concentrations of Na and high concentrations of ammonia in the urine could result from action of Na^+/K^+ ATPases, with substitution of NH_4^+ for K^+ . This ionic substitution has been demonstrated at physiological pH in isolated gill membrane vesicles (Towle and Hølleland, 1987). Moreover, specific activity of Na^+/K^+ ATPases in the antennal gland is higher than that in the posterior gills of *O. quadrata*. Terrestrial crabs with little urinary nitrogen do not show this increased activity, suggesting that the enzyme is important for the transport of ammonia by the renal organ of *O. quadrata*. Similar ratios (gill: antennal gland) and absolute values of Na^+/K^+ ATPase activities were found in *Cardisoma carnifex* and *Gecarcinus lalandii*, congeners of the low urinary nitrogen species that we assayed (Towle, 1981).

The low urine pH and the negative correlation between urine pH and urine ammonia suggest acid-trapping as another possible mechanism of ammonia concentration within the urine. This mechanism entails the diffusion of NH_3 dissolved in the hemolymph, down its partial pressure gradient, and into the acidic urine; there it would immediately be ionized to NH_4^+ , which would maintain the gradient. Action of Na^+/H^+ antiporters may contribute to the lowering of urine pH by the renal organ, as well as to the reduction of Na concentration. Na^+/H^+ antiporters have been found in gills of crabs (Towle, 1985), but have not been studied in their renal organs. Other mechanisms of ammonia transport across membranes are also possible (reviews in Kormanik and Cameron, 1981; Evans and Cameron, 1986; Regnault, 1987).

Although *O. quadrata* was initially selected for study because of its high-nitrogen diet, the vertebrate-like pattern of low pH and high ammonia in the urine appears to have a taxonomic component within the Decapoda as well.

Every species within the family Ocypodidae that has been studied has a high-ammonia urine of acidic or neutral pH, despite gross differences in dietary nitrogen inputs. The ocypodid *Uca pugilator* is probably omnivorous (Miller, 1961; Robertson and Newell, 1982) and produces high-ammonia urine ($\bar{X} = 75 \text{ mM}$ [Green *et al.*, 1959]; $\bar{X} = 92 \text{ mM}$ [Wolcott and De Vries, unpub. data]) of neutral pH ($\bar{X} = 7.1$, [Wolcott and De Vries, unpub. data]). *Ucides cordatus*, another ocypodid, also produces high-ammonia, acidic urine (\bar{X} ammonia = 80 mM ; \bar{X} pH = 6.0 [Wolcott and De Vries, unpub. data]), although this species is primarily herbivorous (Garcia and Bonnelly de Calventi, 1983). One might speculate that ancestral Ocypodidae were carnivorous, and that the mechanisms for forming a high-ammonia, acidic urine were retained when herbivory evolved in some members of the family.

Like urinary composition, the primary mode of nitrogen excretion may prove to vary along taxonomic lines. Of the species studied, gecarcinids (three species) release waste nitrogen into the branchial fluid primarily as ammonia (Greenaway and Nakamura, 1991; Wolcott, 1991); a grapsid releases primarily NH_3 gas at the gills (Greenaway and Nakamura, 1991); a coenobitid releases uric acid into the feces (Greenaway and Morris, 1989); and ocypodids (three species) excrete ammonia directly into the urine (Green *et al.*, 1959; De Vries and Wolcott, 1993; present study). This plasticity in chemistry and mode of nitrogen excretion among terrestrial crabs may be related to the proposed independent colonization of the land by various families and genera (Little, 1983; Hartnoll, 1988). For the Ocypodidae at least, the previously held notion that the crustacean renal organ is unimportant for nitrogen excretion must be reconsidered. Whether the special urinary characteristics of this group serve functions in addition to the elimination of nitrogen is open for investigation.

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