ADAPTATIONS TO A TERRESTRIAL EXISTENCE IN THE ROBBER CRAB *BIRGUS LATRO* L.

IX. HORMONAL CONTROL OF POST-RENAL URINE REPROCESSING AND SALT BALANCE IN THE BRANCHIAL CHAMBER

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Summary

The terrestrial robber crab *Birgus latro* L. regulates the composition of its final excretory product (termed P) depending on the availability of dietary salt by reabsorbing ions from urine passed over the gills. Laboratory and field-based studies investigated the nature of the mechanisms of control of this branchial ion uptake. *B. latro* were prepared such that their branchial chambers could be perfused with artificial urine, and the rate of ion transport from the artificial urine was determined. For *B. latro* acclimated to drinking fresh water, the rates of Na^+ and Cl^- uptake were more than four times those of crabs drinking 70 % sea water. Crabs were injected with either saline carrier or the same solution containing either dopamine or dibutyryl cyclic AMP (db-cAMP) (final concentration $8.7\times10^{-7}\,mol\,l^{-1}$ haemolymph). Dopamine and db-cAMP inhibited Na^+ and Cl^- uptake in animals acclimated to fresh water and markedly reduced their gill Na^+/K^+-ATPase activity. Dopamine stimulated the production of cyclic AMP within the branchial epithelial cells. Dopamine, released from the pericardial organs, acts as a primary messenger, and cyclic AMP acts as a second messenger most likely promoting phosphorylation of membrane proteins. In contrast to aquatic brachyuran crabs, ion transport in *B. latro*, an anomuran, is controlled *via* an inhibitory effect. Terrestrial crabs normally have access to fresh water and must salvage salt from their urine, and a mechanism to down-regulate a normally active uptake system seems more appropriate to their ecology. Whether the control is stimulatory or inhibitory in the various air-breathing crabs may depend on the osmoregulatory abilities of their aquatic ancestors, but in either case has significant implications for the evolution of crustaceans to life on land. Further work must establish whether terrestrial brachyuran crabs are similar to *B. latro* and whether this crab is unique amongst the anomuran crabs.

Key words: *Birgus latro*, land crab, salt, osmoregulation, dopamine, cyclic AMP, gill.

Introduction

The osmoregulatory problems of terrestrial crabs differ somewhat from those of aquatic species because external water is not available to provide a source of ions for osmoregulation. Behavioural osmoregulation may be used by crabs living close to the ocean shore (the crab elects to drink either saline or fresh water; e.g. Combs et al., 1992), but most must manage with only fresh water to drink. Terrestrial crustaceans produce a primary urine that is iso-osmotic with their haemolymph, which thus represents a potential route for major salt loss (for a review, see Greenaway, 1988). The diet is the primary source of salt intake for terrestrial animals and, since feeding is regulated by factors other than salt availability, salt homeostasis must be maintained by regulating salt loss.

Terrestrial crabs, including the anomuran land crab *Birgus latro*, release their primary urine from the antennal organs, and it is then either reingested or passed to the branchial chambers. The gills are universally retained by land crabs and play a key role in regulation of salt and water content (for a review, see Wolcott, 1992). In *B. latro* and brachyuran land crabs, ion-transporting tissue in the gills reabsorb salts, as required to maintain salt balance, to form a dilute final excretory product 'P' (Greenaway and Morris, 1989; Greenaway et al., 1990; Morris et al., 1991; Taylor et al., 1993). *B. latro* can rapidly vary the composition of the excretory product in response to the salinity of the drinking water, and the Na^+ content, for example, can be increased

from 20 to 500 mmol l^{-1} in less than 1 day (Taylor et al., 1993). The mechanisms by which salt reabsorption and the composition of P are controlled in terrestrial crabs have yet to be elucidated.

Aquatic brachyuran crabs employ hormones to control branchial ion uptake from the water. Bioamines such as dopamine and serotonin are released from modified axons of the pericardial organs into the haemolymph and increase in concentration during osmoregulatory challenges (e.g. Zatta, 1989). The gills from the euryhaline crab *Callinectes sapidus* respond to extracts of pericardial organs by increasing gill tissue cyclic AMP levels and Na$^+$ uptake rate (Kamemoto and Oyama, 1985), and the role of cyclic AMP in stimulating the osmoregulatory response has been confirmed in *C. sapidus* (Lohrmann and Kamemoto, 1987). The monoamine dopamine and cyclic AMP act similarly in *Carcinus maenas* to stimulate Na$^+$/K$^+$-ATPase activity (Sommer and Mantel, 1988), and dopamine promotes increased cyclic AMP levels (Sommer and Mantel, 1991). *Eriocheir sinensis* also employs a dopamine-stimulated, cyclic-AMP-mediated activation of Na$^+$ pumps (Trausch et al., 1989; Bianchini and Gilles, 1990; Detaille et al., 1992; Mo et al., 1998). A cyclic-AMP-dependent protein kinase and phosphorylation of a gill membrane protein have been implicated in activating branchial Na$^+$/K$^+$-ATPase (Trausch et al., 1989; Asselbourg et al., 1991; Mo et al., 1998). A similar hormonal regulation of branchial ion uptake might provide for the control of P composition in terrestrial crabs.

Terrestrial crabs derived from marine ancestors would probably share a common mechanism for the control of branchial ion transport. This is demonstrably the case in the amphibious crab *Leptograpsus variegatus*, in which dopamine and cyclic AMP also activate Na$^+$/K$^+$-ATPase (Morris and Edwards, 1995). This crab does not modify the primary urine after release, and the gills can only be used when the crab has access to water (Cooper and Morris, 1997). Nevertheless, it is clear that the mechanism is still present and active in crabs at early stages of independence from sea water. No data are available on the control of branchial transport in the more terrestrial species from either of the terrestrial crab lineages, the brachyurans and also the anomurans such as the terrestrial hermit crabs and *Birgus latro*. Since terrestrial crabs such as *B. latro* have routine access only to fresh water, the expectation would be that branchial uptake of salts would normally be activated, and short-term modifications would be *via* down-regulation to allow excess salt to be voided, a situation more akin to that faced by freshwater animals than to that described above for marine hyperosmotic regulators.

The present study of *Birgus latro* progressively determined the role of dopamine and cyclic AMP in the regulation of post-renal urine modification and the composition of P, first under laboratory conditions and then in crabs under field conditions. The relationships between these mechanisms and the terrestrial habit of *B. latro* and the availability of water on Christmas Island are discussed.

Materials and methods

Animal collection and maintenance

Robber crabs *Birgus latro* L. were collected from rainforest on Christmas Island, Indian Ocean, under permit from Parks Australia and transported by air under AQIS (Australian Quarantine Inspection Service) permit to Sydney. In the laboratory, the crabs were held in individual terraria at 25 °C with a 12 h:12 h light:dark photoperiod and fresh water to drink. The animals were fed weekly on a diet of mixed fruit, nuts and cat biscuit.

Pilot study of controls on branchial ion reprocessing

To determine whether branchial ion uptake was stimulated or inhibited by a hormonal control system, two groups of crabs (382±27 g, mean ± S.E.M., N=5) were tested. One group was supplied with deionised water (FW) for drinking, and the sole source of salt for these crabs was a cat biscuit supplied to each crab on every second day. The second group was supplied with 70 % sea water (70 % SW) for drinking and also received cat biscuits. Both groups were acclimated for 3 weeks, after which ion reclamation from the urine by the crabs was measured. The acclimation process was monitored by measuring the Cl$^-$ concentration of the released excretory fluid as described by Greenaway et al. (1990). A new steady state was reached after 2 weeks. Changes in the rate of branchial salt resorption from artificial urine in response to either saline or drug injections were measured using a perfusion method.

Determination of branchial salt uptake and the effect of drugs

Crabs from both the freshwater and 70 % seawater groups were prepared for branchial perfusion experiments as described by Morris et al. (1991). The perfusion experiments were carried out at 25 °C in humidified glass chambers containing a stainless-steel mesh upon which the animal rested above a collecting funnel. Briefly, at least 24 h prior to experimentation, polyethylene cannulae (0.97 mm i.d., 1.27 mm o.d.) were inserted through the anterior branchiostegite on each side, near the junction between the anterior and posterior gills. Saline solution of ionic composition close to that of the urine (=artificial urine; Table 1) was pumped to both branchial chambers at 1.5 ml min^{-1} using a peristaltic pump. After a delay while the branchial chambers filled, the fluid leaked out of the branchial chambers and was collected in the funnel and recirculated to the branchial chambers. Glass-fibre filters prevented debris from occluding the cannulae, and post-branchial samples could be withdrawn *via* a three-way tap in the line draining the collecting funnel.

Crabs were placed in the chambers, connected to the peristaltic pump and allowed to settle. To initiate the perfusion experiments, for each animal a volume of artificial urine of approximately 30 ml was circulated for 15 min, to fill the interstices of the branchial chambers, and then drained to waste. At the same time as the initial perfusate was supplied, the crab was injected either with 0.5 ml of the appropriate sterile saline (Table 1) as a sham treatment or with saline containing either dopamine at 2×10^{-4} mol l^{-1} or dibutyryl

Table 1. *The composition of the artificial urine solutions used for the perfusion of the branchial chambers of* Birgus latro *and of saline solutions used for saline injections and as carriers for test compounds*

	Artificial urine solutions (mmol l^{-1})		Saline injection solutions (mmol l^{-1})	
	70 % SW	FW	70 % SW	FW
NaCl	430	300	430	355
KCl	15	8	9	9
CaCl$_2$	10	10	17	15
MgCl$_2$	27	15	27	18

Birgus latro had been acclimated to drinking either fresh water (FW) or 70 % sea water (70 % SW). The composition of the solutions were based on data from Greenaway et al. (1990).

cyclic AMP (db-cAMP; a membrane permeable derivative of cyclic AMP) at 6.1×10^{-4} mol l^{-1}. After the 15 min preliminary perfusion, a second measured volume was supplied and recirculated (approximately 35 ml). Examination of the fluid volume in the circulation system revealed that less than 2 ml of fluid was resident in the branchial chambers. Samples of 0.3 ml were taken at the initial 15 min time and every 15 min thereafter up to 90 min. Samples were assayed in triplicate for Cl, Na and K concentrations (see below). The net rate of uptake of each was calculated as described previously (Morris et al., 1991) taking into account the progressively decreasing volume of the perfusate. For this pilot study, a repeated-measures design was employed such that each animal in either the FW or 70 % SW group received all three treatments with a week between each treatment.

Laboratory perfusion studies of Birgus latro *acclimated to fresh water*

A second series of experiments was carried out on *B. latro* acclimated to fresh water for at least 3 weeks. In these experiments, a fully independent design was employed to assess the effects of injected dopamine, db-cAMP or saline. Twelve different crabs were used for each treatment (mass 412±6.3 g). The samples were analysed for Cl, Na, K, Ca and Mg (see below), and net uptake rates of each were calculated. The crabs used in these experiments were also injected with ^{51}Cr-labelled EDTA to provide a specific activity of 2000 cts min^{-1} 100 µl^{-1} haemolymph. The radioactivities of the artificial urine samples taken during the perfusion were measured using an LKB CliniGamma counter to detect whether the crabs urinated. Only two showed any evidence of urine contamination, and they were excluded from the data set and replaced with two other crabs.

The effects of drugs on branchial ion resorption under field conditions

A third set of perfusion studies was conducted on Christmas Island during the wet season between 24 January and 13 February 1997. Mean daily rainfall in the animal collection area was 22.8 mm, and rainfall occurred every day. The temperature in the forest ranged between 23 and 26.5 °C. Three groups of 12 crabs each (mass 448.6±8.98 g) were used in the experiments.

B. latro were collected from the forest on the day prior to experimentation. The branchial chambers of these animals were cannulated as described above. The crabs were then returned to the forest, where they were held in vertically oriented, open-ended drums for 24 h with fresh water to drink. The perfusion experiments were conducted in opaque plastic buckets the bases of which had been replaced with stainless-steel mesh and were sealed to a funnel that collected the recirculating artificial urine. Methods were otherwise similar to those described above. In addition, the effects of injections of saline, dopamine and db-cAMP on the activity of branchial Na$^+$/K$^+$-ATPase were determined as described by Morris et al. (1991), and the response of cyclic AMP concentrations within the gill epithelial cells to injected dopamine was measured.

Determination of branchial Na$^+$/K$^+$-ATPase activity and the effects of drugs

Thirty minutes after the injection, the crabs were cooled until completely torpid, the gills were removed from one side of the animal, and the animals were exposed to cold (4 °C) until they were dead. The gills were weighed and homogenised in 25 mmol l^{-1} Tris/acetate buffer containing phenylmethylsulphonyl fluoride (0.2 mmol l^{-1}), dithiothreitol (0.1 mmol l^{-1}) and aprotinin (100 units ml^{-1}). ATPase activity was determined in (i) a buffer of the following composition in mmol l^{-1}: MgCl$_2$, 6; NaCl, 100; KCl, 10; Tris, 25; adjusted to pH 7.4 with acetic acid and (ii) in the same buffer without KCl but containing 3.5 mmol l^{-1} ouabain which specifically inhibits Na$^+$/K$^+$-ATPase. The difference between the ATPase activity in the two buffers could then be attributed to Na$^+$/K$^+$-ATPase activity. The reaction was started by the addition of vanadium-free ATP optimised at a final assay concentration of 1.73 mmol l^{-1} and stopped after 20 min at 25 °C by the addition of trichloracetic acid (0.6 mol l^{-1}). After centrifugation at 10 000 *g* for 10 min, the inorganic phosphate (P$_i$) concentration was determined in the supernatant using the method of Fiske and Subbarow (Sigma kits 661-11 and 661-8).

Determination of cyclic AMP levels in the branchial epithelia and the effects of dopamine

The cyclic AMP within the gill tissue was assayed in two groups of six crabs (355–483 g) that had been acclimated to drinking fresh water and injected either with saline or with dopamine in saline (2×10^{-4} mol l^{-1} at 150 µl 100 g^{-1}). Thirty minutes after the injection, the crabs were cooled at 4 °C until torpid, the most posterior gill was removed and the wound was cauterised. The gill tissue was weighed (mean 0.443±0.004 g) and placed in a pre-weighed micro-centrifuge tube containing 500 µl of acidified ethanol solution (1:60, 0.1 mol l^{-1} hydrochloric acid:ethanol). The gill samples were crushed inside the tubes immediately to ensure complete contact of the

tissue with the acidified ethanol solution. The tubes of tissue in acid ethanol were stored at −20 °C for subsequent analysis of cyclic AMP concentration. One day prior to the assay, the acidified ethanol was evaporated from samples using a vacuum centrifuge. Concentrations of cyclic AMP in gill tissue were determined using an Amersham test kit (TRK-432) which uses a radioimmunoassay method. The radioactivity was measured using a liquid scintillation counter (Packard Tri Carb; model 1600 TR), which allowed the calculation of cyclic AMP concentration.

Ion assay methods

The concentration of Cl was measured using a CMT 10 chloride titrator (Radiometer, Copenhagen, Denmark) and those of Na, K, Ca and Mg were measured using a GBC 906 atomic absorption spectrophotometer (GBC, Melbourne, Australia).

Statistical analyses

Except for the pilot study (see previously), initial analysis was by analysis of variance (ANOVA) and *post hoc* testing by Tukey's HSD and contrast analysis. Results from the pilot study were analysed using repeated-measures ANOVA. One-sample *t*-tests were used to discriminate some uptake rates from zero uptake. A value of $P<0.05$ was taken as significant, and data are presented as means ± S.E.M.

Results

Pilot laboratory data on the mechanism of salt regulation

In the pilot study, the rates of Na and Cl uptake from artificial urine circulated through the branchial chamber of *B. latro* were 4.1- and 8.2-fold greater, respectively, in crabs acclimated to drinking fresh water than in crabs drinking 70 % SW (Table 2). The low rates of K movement made analysis difficult, but comparison of the pooled K uptake rates by *B. latro* on 70 % SW with those of crabs on fresh water showed small differences in K uptake rate (Table 2). *B. latro* on 70 % SW took up some K from the corresponding artificial urine (containing K at 15 mmol l⁻¹), but those on fresh water and supplied with artificial urine (with K at 8 mmol l⁻¹) had K uptake rates indistinguishable from zero flux (one-sample *t*-test). There was no discernible effect of any drug treatment on the Na and Cl uptake rates of *B. latro* acclimated to 70 % SW. For crabs drinking fresh water, dopamine depressed the rate of Na uptake (Table 2), but the variability between animals and the variance of the Cl data made it impossible to determine any significant effect on Cl uptake. The size and nature of the effects of the treatments on crabs drinking fresh water in the pilot study were best appreciated by examining results for individual animals (Fig. 1). Since the effects of the treatments were apparently to depress the rate of salt uptake by crabs acclimated to fresh water and because the rate in 70 % SW acclimated animals was relatively low, all subsequent determinations were made on animals that had been drinking fresh water.

Table 2. *Net rates of uptake of salts from the branchial chamber of laboratory-maintained* Birgus latro *that had been provided with either fresh water (FW) or 70 % sea water (70 % SW) to drink for 3 weeks previous to the experiments*

| | | Net uptake (µmol g⁻¹ h⁻¹) | | |
		Na	Cl	K
70 % SW	Saline	0.488±0.162	0.374±0.162	0.025±0.017
	Dopamine	−2.11±1.972	0.496±0.372	0.053±0.014
	db-cAMP	0.745±0.286	−0.333±0.654	0.033±0.026
FW	Saline	1.977±0.038‡	3.065±1.179‡	−0.084±0.179
	Dopamine	−0.72±0.878*	1.462±0.721	−0.191±0.166
	db-cAMP	0.995±0.769	0.793±0.560	−0.096±0.212

Treatments were applied in the order shown, with 1 week in between each.

‡Rates for saline-injected *Birgus latro* drinking FW significantly greater than for saline-injected crabs drinking 70 % SW.

*Uptake rate significantly reduced compared with that of saline-injected *Birgus latro.*

db-cAMP, dibutyryl cyclic AMP.

Values are means ± S.E.M. (*N*=5).

Salt uptake by Birgus latro *acclimated to drinking fresh water in the laboratory*

The more extensive laboratory perfusion studies of *B. latro* provided with fresh water to drink revealed the rates of uptake of Na and Cl from circulating artificial urine to be essentially the same, between 4.1 and 4.2 µmol g⁻¹ h⁻¹ (Table 3), and similar to rates obtained from the pilot study. The rates of Ca, Mg and K uptake were close to 0.1 µmol g⁻¹ h⁻¹ or less, and that for K was not significantly different from a rate of zero.
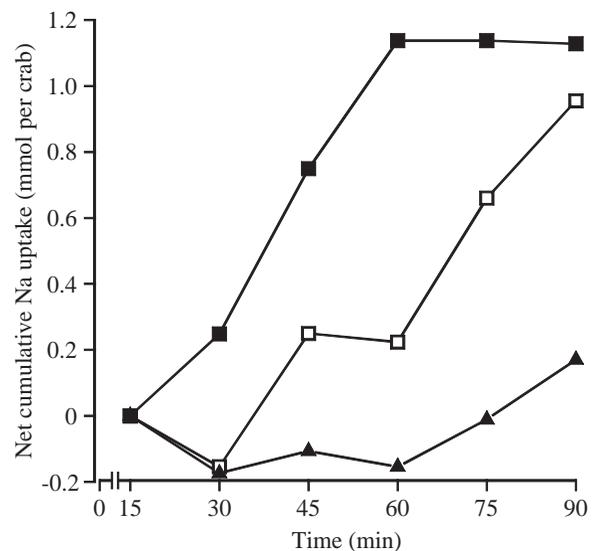

Fig. 1. The cumulative uptake of Na by a typical *Birgus latro* (crab no. 4) acclimated for 3 weeks to drinking fresh water and given a 0.5 ml injection of saline (■), dopamine (□) or dibutyryl cyclic AMP (▲) to give a circulating concentration of approximately $8.7×10^{-7}$ mol l⁻¹. Each injection was separated by a period of 1 week.

Table 3. *Net rates of uptake of salts from the branchial chamber of laboratory-maintained* Birgus latro *that had been provided with fresh water to drink for 3 weeks prior to perfusion experiments*

| | Net rate of uptake ($\mu mol\, g^{-1}\, h^{-1}$) | | | | |
	Na	Cl$^-$	Ca	Mg	K
Saline	4.13±1.22	4.19±0.47	0.12±0.02	0.09±0.02	0.09±0.10
db-cAMP	2.83±0.80	2.29±0.64*	0.10±0.02	0.04±0.02	−0.04±0.06
Dopamine	0.45±0.88*	1.83±0.48*	0.05±0.02*	0.04±0.02	−0.09±0.09

*Rates significantly lower than those for saline-injected *Birgus latro*.
For each treatment, $N=12$, and 12 different crabs were used in each treatment ($N=36$ in total).
Db-cAMP, dibutyryl cyclic AMP.
Values are means ± S.E.M.

Once again, the effect of dopamine was to depress the uptake of Na and also of Cl$^-$ (Table 3). The rate of Na uptake in dopamine-treated *B. latro* was reduced to 11 % of that of saline-injected crabs, while the rate of uptake of Cl$^-$ was reduced to 44 %. Crabs injected with db-cAMP had similarly reduced uptake rates (Table 3).

The injection of dopamine also significantly reduced the rate of uptake of Ca from artificial urine, but there was no significant effect on the rate of Mg uptake

(0.04–0.09 $\mu mol\, g^{-1}\, h^{-1}$) (Table 3). The rate of uptake of K by *B. latro* from the circulating artificial urine was not altered significantly by either db-cAMP or dopamine and remained close to zero (Table 3).

Branchial salt uptake, ATPase activity and drug effects in Birgus latro *in the field*

The Na and Cl uptake rates in *B. latro* taken from the rainforest of Christmas Island were similar to those for crabs acclimated to drinking fresh water in the laboratory (Fig. 2). The rate of Na uptake from the artificial urine within the branchial chambers was not significantly different from that for Cl. Injection of dopamine depressed both Na and Cl uptake rates, while db-cAMP depressed Cl uptake by 45 % (Fig. 2). The rates of uptake of K, Ca and Mg by *B. latro* taken from the field (Fig. 3) were indistinguishable from those measured in the laboratory for crabs acclimated to fresh water, and again the rate of K uptake was effectively zero. As was found in the laboratory determinations, the injection of dopamine reduced the rate of Ca uptake from artificial urine perfusing the branchial chambers (Fig. 3). Dopamine and db-cAMP promoted a net loss of K in *B. latro* collected from the rainforest (one-sample t-test), but had no detectable effect on the low rate of Mg uptake (Fig. 3).

The activity of Na$^+$/K$^+$-ATPase in the gills of *B. latro* freshly collected from the rainforest and then injected with dopamine was 54 % of that in crabs injected with saline but was reduced significantly (by 80 %) only after injection with db-cAMP (Fig. 4A). The injection of dopamine into *B. latro* taken from the field increased the concentration of cyclic AMP in the gills to levels 2.3 times greater than those of saline-injected animals (Fig. 4B).
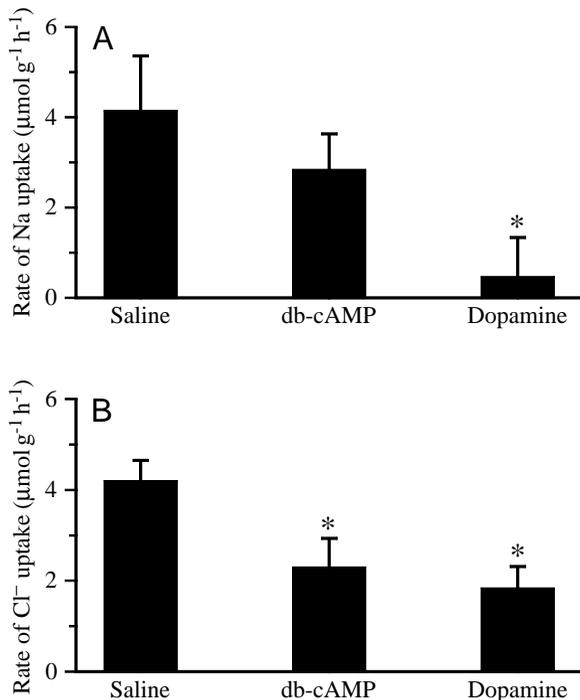

Fig. 2. The rate of (A) Na uptake and (B) Cl uptake by the gills of *Birgus latro* perfused with artificial urine appropriate for crabs drinking fresh water (see Table 1). The crabs were given either saline injections or injections of dopamine (2×10^{-4} mol l^{-1}) or dibutyryl cyclic AMP (db-cAMP; 6.1×10^{-4} mol l^{-1}). $N=12$ for each treatment. An asterisk indicates a significant reduction in rate compared with saline-injected animals. Experiments were performed on crabs freshly collected from the rainforest of Christmas Island during the wet season. Values are means + S.E.M.

Discussion
Control of post-renal modification of urine and the formation of the excretory product

In brachyuran crabs, the activity of branchial ion-transport mechanisms is thought to be regulated by a bioamine/cyclic AMP system. Thus, dopamine is a primary messenger that stimulates branchial ATPase and Na$^+$ uptake (Sommer and Mantel, 1988; Trausch et al., 1989; Mo et al., 1998; Detaille et
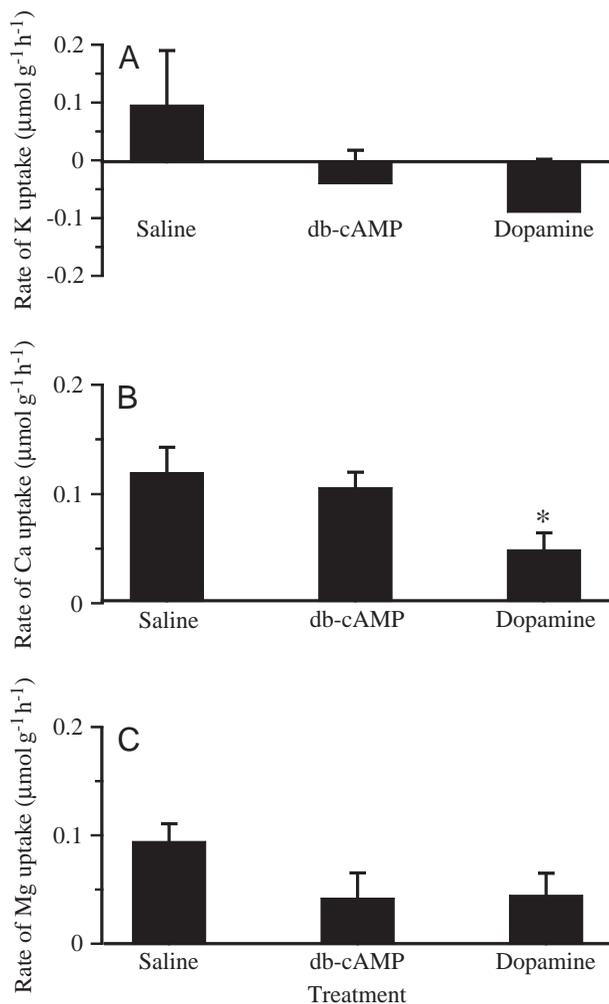
Fig. 3. The net rate of (A) K uptake, (B) Ca uptake and (C) Mg uptake from the branchial chambers of *Birgus latro* taken directly from the rainforest of Christmas Island during the wet season and perfused with artificial urine appropriate for crabs acclimated to drinking fresh water (see Table 1). The crabs were given either saline injections or injections of dopamine (2×10^{-4} mol l^{-1}) or dibutyryl cyclic AMP (db-cAMP; 6.1×10^{-4} mol l^{-1}). $N=12$ for each treatment. An asterisk indicates indicates a significant reduction in rate compared with saline-injected animals. Values are means + S.E.M.

al., 1992), a situation similar to that reported in mussels *Lignmia subrostrata* (Dietz and Scheide, 1982; Scheide and Dietz, 1983, 1986) and in the gills of *Aplysia californica* (Weiss and Drummond, 1981). Administration of membrane-permeable cyclic AMP to aquatic brachyuran crabs, or to their perfused gills, can also stimulate branchial Na$^+$ transport and Na$^+$/K$^+$-ATPase activity (Lohrmann and Kamemoto, 1987; Sommer and Mantel, 1988; Bianchini and Gilles, 1990; Detaille et al., 1992; Morris and Edwards, 1995; Mo et al., 1998). The effect of cyclic AMP in the osmoregulatory response of aquatic brachyurans appears to be mediated *via* activation of protein kinase (Asselbourg et al., 1991). Phosphorylation of basolateral membrane gill Na$^+$/K$^+$-ATPase, or of an associated membrane

protein, has been strongly inferred (Trausch et al., 1989; Asselbourg et al., 1991).

Dopamine is also a primary messenger in the regulation of branchial ion uptake by *B. latro*, and again this message is conveyed in the haemolymph to branchial epithelial cells and transduced to cyclic AMP. However, in *B. latro*, the increase in cyclic AMP levels promoted a marked decrease in transport of Na and Cl and a reduction in the activity of Na$^+$/K$^+$-ATPase, not an activation as in aquatic species. The dopaminergic promotion of elevated cyclic AMP levels in *B. latro* is probably through stimulation of adenylate cyclase, which normally leads to activation of protein kinase and a protein phosphorylation step. In *B. latro*, this depresses Na$^+$/K$^+$-ATPase activity. Further studies are needed to determine whether this is a phylogenetic difference in the regulatory mechanisms employed by anomuran compared with brachyuran crabs or a peculiarity of salt regulation by *B. latro*.

The mechanisms concerned with the regulation of the branchial transport of ions other than Na$^+$ have been less well studied, but it appears that Cl$^-$ levels may also be directly regulated by monoamines and not driven by changes in Na$^+$ uptake rate. Detaille et al. (1992), with reference to the work of Bianchini and Gilles (1990), suggested that cyclic AMP and/or dopamine activated Cl$^-$ channels on the apical membrane of gill epithelial cells separately from their effects on membrane ATPase. Dopamine and db-cAMP increased Na$^+$ influx across isolated gills of *Eriocheir sinensis* perfused in Cl$^-$-free saline; the mechanisms of regulation of Cl$^-$ and Na$^+$ levels are therefore probably independent (Mo et al., 1998), although the same primary and second messengers are involved. Studies using preparations of isolated perfused posterior gills from *Eriocheir sinensis* showed that the inclusion of cyclic AMP in the perfusate had considerably greater effect on net Cl$^-$ uptake, which increased by 2.8-fold compared with an increase of only 0.68-fold for Na$^+$ uptake (Bianchini and Gilles, 1990), again suggesting that regulation of Cl$^-$ uptake is not inextricably linked to that of Na$^+$. Morris et al. (1991) determined that the gills of *B. latro* have similar permeability to Na$^+$ and Cl$^-$. Thus, if cyclic AMP acts to regulate both the basolateral membrane Na$^+$/K$^+$-ATPase and the apical membrane Cl$^-$ channels in *B. latro*, then this is carefully integrated to provide for a matched down-regulation of Cl$^-$ and Na$^+$ uptake.

When acclimated to drinking fresh water, *B. latro* will withdraw significant amounts of Ca from the urine passing through the branchial chambers (Greenaway and Morris, 1989; Taylor et al., 1993). The anterior and posterior gills of *B. latro* are rich in basolateral Ca^{2+}-ATPase, which is probably important in extruding Ca^{2+} from epithelial cells into the haemolymph (Morris et al., 1991), but Na$^+$/Ca^{2+} exchange channels with a higher transport capacity could become important sites for regulating Ca^{2+} uptake if the cytosolic Ca concentration ever became elevated into the micromolar range (e.g. Flik et al., 1994). *B. latro* can vary the rate of Ca uptake from the urine; it is down-regulated in salt-replete crabs, in concert with Na$^+$ and Cl$^-$ uptake (Taylor et al., 1993). While dopamine strongly inhibited Ca^{2+} uptake in *B. latro*, cyclic AMP had no significant

Fig. 4. (A) Na^+/K^+-ATPase activity of the gills *Birgus latro* expressed per unit mass of wet tissue (N=6 for each treatment) and (B) the cyclic AMP (cAMP) concentration in the gills. ATPase activity was determined in animals injected with a saline solution or with dopamine or dibutyryl cyclic AMP (db-cAMP; see Materials and methods for dosages). The concentration of cyclic AMP in the gills was measured in crabs (N=6 for each treatment) that had received either a saline injection or a dopamine injection ($2\times10^{-4}\,mol\,l^{-1}$ at $1.5\,\mu l\,g^{-1}$). An asterisk indicates a significant difference compared with the value obtained for saline-injected crabs. Values are means + S.E.M.
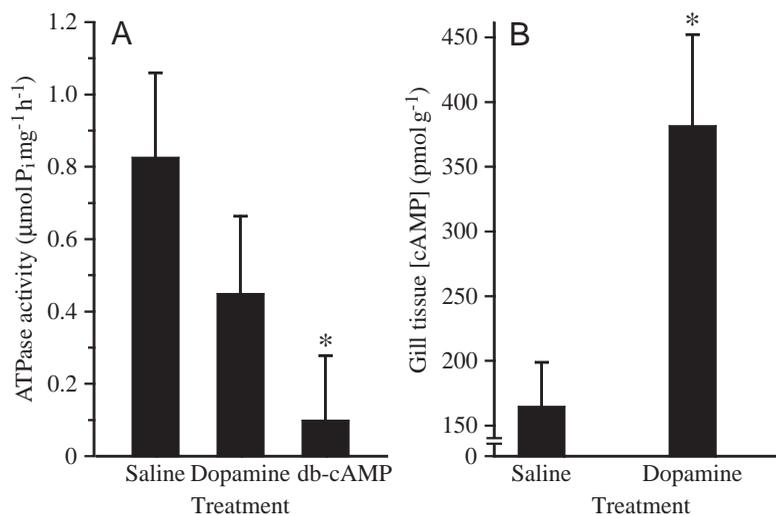
effect, revealing a control mechanism quite different from that regulating Na^+ uptake. Further work is needed to determine whether cyclic AMP or some other second messenger is involved in the regulation of Ca^{2+} uptake or whether dopamine is perhaps able to inhibit Na^+/Ca^{2+} exchange directly.

The regulation of K^+ levels by *B. latro* acclimated to drinking fresh water seems to be different from that of the other ions measured since there appears to be little or no K^+ uptake from the urine, and in *B. latro* taken from the field dopamine and cyclic AMP promoted a small net K^+ loss. Taylor et al. (1993) showed that *B. latro* had K^+ concentrations of $9.1\,mmol\,l^{-1}$ in the haemolymph but of $16.5\,mmol\,l^{-1}$ in the excretory product P after 9 days of drinking fresh water. On Christmas Island, *B. latro* have a varied diet, and fruits are an important component so that excess dietary K^+ is quite likely. Mg^{2+} is the least well-regulated haemolymph ion in *B. latro* (Taylor et al., 1993) and, although Mg^{2+} is reclaimed from urine passing through the branchial chamber, conclusions regarding the control of this process cannot yet be made.

Modulation versus *acclimation of branchial ion uptake*

Acclimation of *B. latro* to drinking saline water both minimised branchial salt uptake (Taylor et al., 1993) and removed the sensitivity of the ion transport to dopamine or cyclic AMP. In *Carcinus maenas*, transfer to 40 % sea water initially increased endogenous cyclic AMP levels to the same extent as an injection of dopamine, but cyclic AMP levels declined after acclimation to saline water (Sommer and Mantel, 1991). The effective half-lives of bioamines in crustaceans are measured in minutes (Wilkens et al., 1985). Sommer and Mantel (1991) and Trausch et al. (1989) propose that a dopamine/cyclic AMP activation of protein kinase and activation of membrane exchangers and pumps represents a rapid, short-term effect on pre-existing proteins. Longer-term acclimation might include the synthesis of more of the membrane proteins or of similar proteins with different activities (e.g. Siebers et al., 1982; Holliday, 1985) as the cyclic AMP signal declines. In *B. latro*, the marked reduction in both ion pumping and Na^+/K^+-ATPase

activity in response to drinking saline water is concomitant with the loss of response to dopamine and cyclic AMP, and could be similarly accounted for either by synthesis of a different ATPase and ion exchangers or by the removal of the putative phosphorylation mechanisms. Given the lack of response to cyclic AMP, it seems unlikely that dopamine receptor down-regulation alone would be adequate. Hormonal modulation of ion transport must then be of primary importance in *B. latro* acclimated to drinking fresh water when presented with an acute surfeit of salt.

Significance of inhibition over stimulation of branchial ion uptake

B. latro on Christmas Island have routine access only to fresh water, and their 'normal' circumstance will therefore be to limit urinary salt loss by active resorption. Sommer and Mantel (1991) conclude that utilising a dopamine/cyclic AMP control mechanism for anything other than rapid, short-term stimulation of ion pumping does not make sense in *Carcinus maenas*. The inhibition of ion uptake by dopamine in *B. latro* is opposite from the stimulatory effect of the same messenger in aquatic brachyuran crabs but, using the argument of Sommer and Mantel (1991), it would be energetically conservative to employ hormonal controls to modify the basic condition rather than to maintain it. Thus, on the rare occasions when *B. latro* become salt-replete, a dopamine/cyclic AMP system can down-regulate not only rates of Na^+ and Cl^- uptake but also that of Ca^{2+} and possibly effect net K^+ excretion. Those populations of *B. latro* that do live close to the shore and avail themselves of the sea water can afford to have lower-activity ion-uptake mechanisms and employ some form of behavioural osmoregulation in place of variable post-renal urine modification.

If such a model of salt regulation should hold generally true for terrestrial crabs, and logically it should, then terrestrial brachyuran crabs should also employ an inhibitory control mechanism rather than the stimulatory mechanism exhibited by their marine relatives. However, terrestrial species have appeared in several disparate brachyuran families, and the

mechanism of salt regulation may in each case depend on the osmoregulatory abilities of their aquatic ancestors. In this case, terrestrial species arising from osmoconforming ancestors would not *a priori* be constrained preferentially to adopt either up- or down-regulation as a strategy. Therefore, down-regulation of ion transport may have arisen uniquely in *B. latro* since intertidal hermit crabs have a weak osmoregulatory capacity (e.g. Sabourin and Stickle, 1980) and terrestrial hermit crabs continue to utilise shell water. Examination of species from terrestrial brachyuran families, for example the gecarcinids, is now important to establish whether this is a unique feature or whether phylogenetically distinct terrestrial decapods have all adopted a 'reverse' control mechanism.

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