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Osmoregulation in the terrestrial Christmas Island red crab *Gecarcoidea natalis* (Brachyura: Gecarcinidae): modulation of branchial chloride uptake from the urine

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Summary

Crabs generally produce urine iso-osmotic to their haemolymph, but terrestrial crabs are able to vary the composition of their final excretory fluid (termed P) post-renally, in the branchial chambers. Regulatory aspects of branchial urine processing were investigated in the Christmas Island red crab *Gecarcoidea natalis* acclimated to drinking either freshwater (FW crabs) or 70% seawater (SW crabs). FW crabs released dilute P (mean $[Cl^-]$ 8.8 mmol l^{-1}). Drinking 70% seawater caused the mean $[Cl^-]$ of the P to rise to 376 mmol l^{-1} over 5 days, approaching the haemolymph $[Cl^-]$. FW crabs with saline-perfused branchial chambers absorbed chloride at a high rate (10 mmol kg^{-1} h^{-1}), and haemolymph $[Cl^-]$ increased at approximately 20 mmol l^{-1} h^{-1}. SW crabs exhibited elevated haemolymph osmolalities and ion concentrations and zero branchial chloride uptake. FW crabs that were salt-loaded by branchial chamber perfusion over several hours downregulated, and eventually ceased, chloride uptake. The rate of downregulation, but not the initial chloride flux, was dependent on initial haemolymph $[Cl^-]$. Intravascular infusion of NaCl caused immediate reduction in branchial $[Cl^-]$ of 80%. Crabs ingested and regurgitated the perfusion saline, supporting suggestions that reingestion of urine could conserve water and ions. Dopamine upregulated branchial chloride transport in *G. natalis*. This is consistent with the ion-regulatory effects of dopamine in euryhaline marine brachyurans but contrasts with its inhibitory effects in the terrestrial anomuran *Birgus latro*. Dopamine increased the rate of urine release in FW crabs. Urine composition appears to be unimportant in ionic regulation, except in the case of magnesium, levels of which were elevated in the urine of SW crabs.

Key words: *Gecarcoidea natalis*, land crab, osmoregulation, chloride reabsorption, excretion, dopamine, cyclic AMP, gill.

Introduction

Although crabs invariably produce urine that is nearly iso-osmotic to their haemolymph, both brachyuran and anomuran terrestrial crabs are capable of conserving salts by post-renal modification of the urine. Urine released from the nephropores is directed into the branchial chambers to form a modified final excretory fluid (termed P), which emerges at the bases of the legs (*Gecarcinus lateralis*, *Ocypode quadrata*, Wolcott and Wolcott, 1985, 1991; *Birgus latro*, Greenaway et al., 1990; Morris et al., 1991; Taylor et al., 1993; *Geograpsus grayi*, Varley and Greenaway, 1994). Branchial ion reabsorption is suggested by a fall in the osmolality of salines recirculated through the branchial chambers of *B. latro* (Morris et al., 1991) and *G. grayi* (Varley and Greenaway, 1994).

In terrestrial crabs, gas exchange takes place primarily across branchiostegal lungs (Taylor and Taylor, 1992) but all species retain gills, implicating them in ion reabsorption. However, contributions to ionoregulation from other epithelia have not been excluded. Mantel (1968) demonstrated uptake of water and salts by the foregut of *G. lateralis* and considered this organ to be important in osmoregulation. In *Gecarcoidea natalis*, the posterior gills possess ultrastructural features indicative of active ion transport (Farrelly and Greenaway, 1992) but in *B. latro*, all gills, and also the branchiostegites, demonstrate high activities of transport ATPases (Morris et al., 1991).

Intravascular infusions of water and salines, and experimental manipulation of the drinking water salinity, indicate that crabs adjust the composition of P according to the requirements of ionic homeostasis (Wolcott and Wolcott, 1985, 1991; Taylor et al., 1993). However, our understanding of urine reprocessing as a homeostatic response to altered salt and water status is incomplete, being based on partial data for only a few species. More information is needed on the timing, rates and capacity for adjustment of urine reprocessing following a

perturbation. Also, it is unclear how adjustments in P composition are achieved *in vivo*. In principle, crabs could vary the residence time of the urine within the branchial chambers, its route to the exterior, the perfusion of the osmoregulatory surfaces with haemolymph, epithelial salt transport rate or a combination of these. In addition, in *B. latro*, variations in the volume of P produced appear to be achieved by adjustments to the filtration rate and by reingestion of the urine (Greenaway et al., 1990; Taylor et al., 1993).

There is evidence that urine reprocessing is under endocrine control, but this is incompletely understood. It is not known whether urine processing is modulated in response to internal (blood osmolytes, volume) or to extrinsic (e.g. drinking water composition) stimuli. The pericardial hormone dopamine inhibits branchial salt reabsorption in the anomuran *B. latro* (Morris et al., 2000). However, this role contrasts with that in hyper-regulating aquatic brachyurans, in which abundant evidence indicates that dopamine increases salt uptake by activation of a branchial epithelial Na^+/K^+-ATPase (Zatta, 1987; Sommer and Mantel, 1988, 1991; Morris and Edwards, 1995; Mo et al., 1998). Morris et al. (2000) suggested that a state of continuous salt uptake, turned off hormonally, was a better adaptation to terrestrial life than the converse strategy of switching on salt uptake as required. However, it is yet to be shown whether a reversed role for dopamine is a general feature of terrestrial crabs.

This paper examines homeostatic aspects of post-renal reabsorption of chloride in a terrestrial brachyuran, *G. natalis*. This crab produces a dilute final excretory fluid when maintained on a freshwater drinking regimen (Greenaway and Nakamura, 1991). We measured the steady rates of branchial chloride uptake attainable by crabs supplied with freshwater and determined the time course of downregulation of chloride uptake in salt-loaded crabs. Crabs were salt-loaded: (i) by acclimation to saline drinking water over a period of weeks, (ii) by self-loading during prolonged perfusion of the branchial chambers with saline over a period of hours, and (iii) by direct NaCl injection over a period of minutes. A possible role for dopamine in switching the uptake system on or off was examined in crabs acclimated to saline and to fresh drinking water.

In initial experiments, it was noted that there were intermittent periods of urine output and of the ingestion and regurgitation of the perfusion saline. It was necessary to account for these accurately in terms of volume and chloride flux to determine the true branchial flux. A radioactive filtration marker was used to estimate urine flow, and flow-through branchial chamber perfusion was employed instead of the recirculating method used previously (Morris et al., 1991). The flow-through system permitted observation of temporal changes in chloride flux and in the movements of urine and oral fluids.

Materials and methods

Animals and maintenance

Crabs *Gecarcoidea natalis* (Pocock, 1888) of both sexes (mass 120–200 g) were collected under permit from Christmas Island, Indian Ocean, and maintained at the Universities of Canterbury and New South Wales at 25°C, approximately 80% relative humidity. They were provided with food (dry fallen leaves and occasional cat biscuits) and fresh water *ad libitum*. Experiments were carried out at 25°C.

Effects of the salinity of the drinking water on the [Cl^-] of the final excretory fluid

Crabs were transferred from the freshwater culture to individual chambers constructed from 10 l plastic buckets fitted with lids and stainless-steel mesh bottoms. A conical plastic bag, containing 10 ml of paraffin oil, attached below the bucket, trapped excreted fluids (P), which were removed with a syringe and cannula *via* a small hole. Crabs were provided with 20 ml of drinking water (dyed to reveal spillage), which was changed daily, and a leached cottonwood leaf. The drinking water was initially tapwater and was switched to 70% seawater ([Cl^-]=360 mmol l^{-1}) on day 4; P was collected for a further 5 days, and the volume and [Cl^-] of the samples were recorded. At the final collection time, a haemolymph sample (approximately 200 µl) was removed for [Cl^-] analysis, using a syringe and 21-gauge needle *via* a predrilled hole in the carapace above the pericardial sinus.

Measurement of chloride uptake from saline-perfused branchial chambers

At least 1 day before perfusion, the carapace was predrilled for pericardial blood-sampling and tracer injection, and holes approximately 1 mm in diameter were drilled bilaterally through the dorsal carapace over the roof of each branchial chamber and cauterized to prevent blood loss. A polyethylene cannula (5 cm; 1 mm o.d.) was inserted approximately 1 mm into each chamber, glued (with cyanoacrylate) into place and temporarily plugged. At least 1 h before the first haemolymph sample, approximately 10 MBq 100 g^{-1} body mass of a filtration marker (^{51}Cr-EDTA; Amersham, UK) in saline (50 µl 100 g^{-1} body mass) was slowly injected into the pericardial sinus and allowed to circulate. A haemolymph sample was taken 30 min before perfusion commenced, and the total [Cl^-] of the perfusion saline was adjusted to that of the haemolymph by addition of NaCl (other cations as chlorides, in mmol l^{-1}: K^+, 6; Ca^{2+}, 14; Mg^{2+}, 7). At the start of the experiment, the cannulae were connected to the perfusion lines, and the crab was placed into a covered plastic box and allowed to settle for approximately 1 h. Saline was then infused into both branchial chambers using two channels of a peristaltic pump at a total flow rate of approximately 0.6 ml min^{-1} (calibrated at the start and end of each run). After a delay of several minutes, fluid emerged at the leg bases and collection commenced. This fluid drained into a funnel, from where it was continuously removed and distributed into timed fractions (manually or using a Gilson fraction collector). A second haemolymph sample was taken at the end of the infusion. The volume, [Cl^-] and radioactivity of each outflow fraction and haemolymph sample were measured.

Calculation of branchial, urinary and oral chloride fluxes

The rate of branchial net uptake of chloride ($J_{Cl,Gill}$) was estimated for each collected fraction as: $J_{Cl,Gill}$ = chloride input in saline + chloride input in urine – chloride in outflow or:

$$J_{Cl,Gill} = \{(V - U)[Cl^-]_i + U[Cl^-]_u - V[Cl^-]_o\}/tm, \quad (1)$$

where $[Cl^-]_i$ and $[Cl^-]_o$ are the chloride concentrations in the perfusion saline and the collected fluid, respectively, V is the volume of perfusate collected in each time interval (t), $[Cl^-]_u$ is the chloride concentration of the urine and m is the mass of the crab. The volume of urine, U, in each fraction:

$$U = A_u/SA_h, \quad (2)$$

where A_u is the total radioactivity of the sample and SA_h is the specific activity of the haemolymph at the sampling time, estimated by linear interpolation from the specific activities of the initial and final haemolymph samples. The chloride concentration of the urine was calculated from:

$$[Cl^-]_u = 1.09[Cl^-]_h, \quad (3)$$

where $[Cl^-]_h$ is the chloride concentration of the haemolymph. The factor 1.09 was obtained from paired analyses of haemolymph and urine for crabs maintained under conditions similar to those used in these experiments (see Table 1). The chloride concentration of the haemolymph, $[Cl^-]_h$, at each collection time, was estimated by linear interpolation between the initial and final haemolymph samples.

The volume of saline retained R by the crab in each sampling interval was:

$$R = P + U - V, \quad (4)$$

where P is the input from the peristaltic pump. R varied as a result of irregular drainage from the branchial chambers. However, large or sustained positive values were interpreted as ingestion of the saline and large negative values as regurgitation. In control runs without animals, evaporation was negligible. Net uptake of chloride by drinking of the saline, $J_{Cl,Drinking}$, was:

$$J_{Cl,Drinking} = R[Cl^-]_i/tm, \quad (5)$$

negative values indicate regurgitation, and net flux of chloride in the urine, $J_{Cl,Urine}$, was:

$$J_{Cl,Urine} = -U[Cl^-]_u/tm, \quad (6)$$

a negative value indicating efflux.

In preliminary experiments, the filtration marker was not used and the branchial chambers were perfused with a hypo-osmotic saline ($[Cl^-]$=200 mmol l^{-1}, other ion concentrations as given above), simulating partially processed urine. The rate of branchial chloride uptake (uncorrected for urine flow) was estimated from the volume of fluid collected in each interval and the difference in the $[Cl^-]$ of the input and output fluids. This method generally gave results similar to those obtained using the marker. However, an apparent inhibition of branchial chloride uptake following certain treatments was shown to be caused by the release of urine hyperosmotic to the perfusate.

Effects of salt loading on chloride fluxes

Chloride fluxes in crabs acclimated to fresh drinking water during prolonged saline perfusion of the branchial chambers

The branchial chambers of crabs taken from the freshwater cultures were perfused with saline for 5–8 h. This established the time course of the onset of chloride uptake, and the effects of prolonged self-loading with salt on chloride transport, urine flow and the ingestion of branchial fluid. The relationship between the branchial chloride fluxes and the $[Cl^-]$ of the haemolymph was also examined.

Chloride fluxes in crabs acclimated to saline drinking water

These experiments were designed to determine the effect of chronic salt-loading on branchial chloride transport. Crabs were maintained in cultures supplied with 70% seawater ($[Cl^-]$=360 mmol l^{-1}) for drinking for 7–9 days before commencing perfusion.

The effects of NaCl injection on chloride fluxes in freshwater-acclimated crabs

This series examined responses to acute elevation of haemolymph [NaCl]. The branchial chambers of crabs from the freshwater culture were perfused for approximately 1 h to establish steady uptake. Branchial chamber perfusion was then stopped for approximately 5 min while 5 mol l^{-1} NaCl (800 µl 100 g^{-1} body mass) was infused *via* a pre-drilled hole into the pericardial sinus. Branchial chamber perfusion was then restored, and the outflow was collected for a further 2 h. The $[Cl^-]$ of the perfusate (mean 424 mmol l^{-1}) was adjusted to be approximately midway between the measured initial $[Cl^-]_h$ and the estimated $[Cl^-]_h$ after the injection. The distribution of injected chloride and elevation of $[Cl^-]_h$ were similar to those in perfused crabs absorbing chloride over several hours (see Results), indicating that circulation was maintained. Mild impairment of locomotor function was evident in two individuals, and only four crabs were subjected to this treatment. The response to injection of NaCl was compared statistically with that of control crabs injected with iso-ionic saline (next section).

Effects of dopamine and cyclic AMP on chloride fluxes

Freshwater-acclimated crabs

Crabs were taken from the freshwater culture and their branchial chambers were perfused for 0.5–1 h until a steady uptake had been established. Without interruption of perfusion and fluid collection, perfusion saline containing 1.0 mmol l^{-1} dopamine (250 µl 100 g^{-1} body mass), was injected into the pericardial sinus. The concentration of dopamine, distributed throughout the extracellular space (24.3±1.0 ml 100 g^{-1} body mass) (Greenaway, 1994) was therefore approximately 10 µmol l^{-1}. The injection took approximately 1 min, and a further 1 min elapsed before removing the needle and sealing the hole. To reduce oxidation, dopamine was bubbled with nitrogen until use. In a similar series, crabs were injected with saline (250 µl 100 g^{-1} body mass) containing 6 mmol l^{-1} dibutyryl

cyclic AMP (dbc-AMP, final haemolymph concentration approximately 60 μmol l^{-1}). Control crabs were injected with a similar volume of perfusion saline.

Saline-acclimated crabs

Branchial chambers of crabs from cultures supplied with saline drinking water (70% seawater) for 30–50 days were perfused for 60 min before injecting dopamine as above. Other conditions were as for the freshwater-acclimated crabs injected with dopamine.

Chemical analyses

Samples of haemolymph, P and the afferent and efferent branchial chamber perfusion salines were analyzed for [Cl^-] within 24 h using an electrometric chloride titrator (Radiometer CMT10). Haemolymph samples were diluted immediately with distilled water (1:3) to reduce coagulation in the titration vessel. The radioactivity of fluid samples was measured using a gamma counter (Packard or LKB-Wallac Minigamma). Samples were adjusted to a standard volume with distilled water and corrected for background radioactivity. Urine and haemolymph samples were diluted and analyzed for metal atoms by flame atomic absorption spectroscopy (GBC, Avanta; Na and K, air–acetylene; Ca and Mg, N_2O–acetylene; CsCl used to suppress ionization). Osmolalities were determined by vapour pressure osmometry (Wescor 5520).

Data analyses

Values in the text, table and graphs are means ± S.E.M.; see figure legends for *N* values. Urinary and oral chloride fluxes are converted to approximate flow rates on the right-hand axes of graphs, which divide the chloride flux by the mean [Cl^-] of the perfusion saline. Flows given in the text were individually calculated for each crab and sample time.

Time courses of flux changes are shown at a resolution of 0.25–0.5 h, for several hours, but for statistical analysis of the effects of injected substances, the mean flux rate for each crab during 1 h following the injection was compared with a baseline rate established during 1 h prior to the injection (repeated-measures ANOVA; Statistica software, Statsoft, Tulsa, OK, USA). As there were significant time-dependent changes in the control FW crabs, individual 'responses' were calculated (mean rate after treatment minus mean rate before) and compared among treatments by one-way analysis of variance (ANOVA). Tukey's HSD test was used for multiple *post-hoc* contrasts. Repeated-measures ANOVA was used for analysis of changes in P concentration and production rate and for comparison of haemolymph and urine ion concentrations in FW and SW crabs. A probability P of <0.05 was taken as statistically significant unless stated otherwise.

Results

Composition of haemolymph and urine from crabs maintained on freshwater and saline regimens

In crabs from cultures supplied with freshwater, the haemolymph and urine were iso-osmotic (approximately 800 mosmol kg^{-1}) and of similar ionic composition (Table 1). The haemolymph and urine of crabs supplied with 70% seawater (approximately 700 mosmol kg^{-1}) for 30–50 days were hyperosmotic (approximately 1150 mosmol kg^{-1}) and hyperionic to these fluids in the freshwater group but remained iso-osmotic to each other. Urinary [Na^+] and [K^+] were not significantly different from haemolymph values in either the freshwater or the saline crabs, but [Ca^{2+}] was depleted and [Cl^-] elevated in the urine of both groups. Urinary [Mg^{2+}] was more than double that of the haemolymph in the saline group. The ratio of urinary [Cl^-]:haemolymph [Cl^-] was similar in the freshwater and saline groups.

Effects of drinking saline on [Cl^-] and rate of release of final excretory fluid (P)

Individual daily P production by crabs supplied with fresh water varied between 0 and 17.5 ml. The mean rate of P

Table 1. *The ionic composition of the haemolymph and the urine of* Gecarcoidea natalis *supplied with either freshwater or saline (70% seawater) for drinking for 30–50 days*

	Drinking freshwater (*N*=8)		Drinking saline (*N*=7)	
	Haemolymph	Urine	Haemolymph	Urine
Osmolality (mosmol kg^{-1})	799±29^a	794±29^a	1158±28^b	1147±23^b
[Na^+] (mmol l^{-1})	374±12^a	387±15^a	542±12^b	526±8^b
[K^+] (mmol l^{-1})	7.5±0.4^{a,b}	7.1±0.5^a	9.6±0.5^b	8.7±0.7^{a,b}
[Ca^{2+}] (mmol l^{-1})	15.4±0.7^a	9.4±1.5^b	22.0±0.7^c	13.0±0.7^{a,b}
[Mg^{2+}] (mmol l^{-1})	9.9±1.2^a	12.0±3.3^a	21.7±1.3^b	51.6±5.1^c
[Cl^-] (mmol l^{-1})	382±11^a	413±14^b	561±17^c	619±15^d
$[Cl^-]_u/[Cl^-]_h$	1.08±0.02^a		1.11±0.02^a	
Overall $[Cl^-]_u/[Cl]_h$	1.09±0.01 (*N*=15)			

Values are means ± S.E.M.
Haemolymph and urine values are paired within the freshwater (*N*=8) and the seawater (*N*=7) groups.
Means that bear the same superscript are not significantly different (repeated-measures ANOVA, Tukey's HSD test, $P<0.05$).

production during the first day was 5.11±1.77 ml day^{-1} (approximately 3.4% of body mass per day) and fell to 1.61±0.47 ml day^{-1} (approximately 1.1% of body mass per day) over 4 days (Fig. 1). On switching the drinking water to 70% seawater, P production increased again to approximately 4.5 ml day^{-1} (repeated-measures ANOVA; days 5 and 6 significantly greater than days 3 and 4).

Crabs supplied with freshwater produced dilute P, mean [Cl$^-$] decreasing from 12.46±2.51 mmol l^{-1} on day 1 to 8.83±0.95 mmol l^{-1} on day 4. On switching to seawater, the [Cl$^-$] of the P increased to 376±18 mmol l^{-1} on day 9 (Fig. 1; mean values on days 6–9 were all significantly greater than those on days 1–5; repeated-measures ANOVA). On day 9, the [Cl$^-$] of the P was not significantly different from the [Cl$^-$] of the drinking water (360 mmol l^{-1}) but was still hypo-ionic to the haemolymph (436±36 mmol l^{-1} for the six crabs that produced P on day 9; paired *t*-test, $P<0.01$). Crabs differed greatly in the rate of adjustment of the [Cl$^-$] of the P, which increased to half the blood concentration in less than 1 day in some crabs to more than 5 days in others.

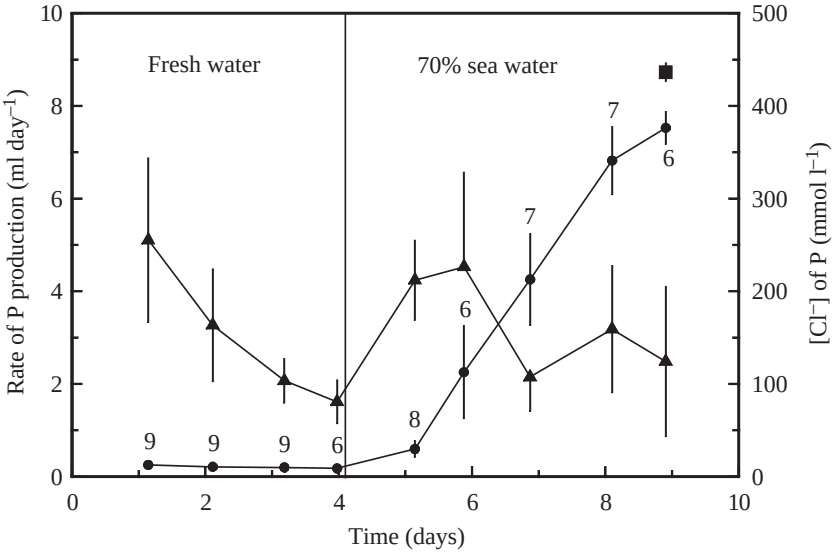

Fig. 1. Changes in the daily rate of release (triangles) and the chloride concentration (circles) of the final excretory fluid (P) of *Gecarcoidea natalis* before and after switching from fresh drinking water to 70% seawater ([Cl$^-$]=360 mmol l^{-1}). Square, chloride concentration of the haemolymph. Values are means ± S.E.M.; where error bars are absent, they are smaller than the symbol. Numbers next to symbols are the numbers of crabs that produced P on that day and included in the mean [Cl$^-$]. For all other means, $N=9$.

Time course of chloride uptake from the branchial chamber perfusate in crabs acclimated to fresh drinking water

On commencing perfusion with saline of the branchial chambers of crabs from the freshwater culture, branchial chloride uptake was initially low but increased to a maximum rate over approximately 1 h (Fig. 2) and then slowly declined. In some crabs, chloride uptake ceased completely within 5 h, but others maintained chloride uptake for more than 8 h. The mean rate of branchial chloride uptake ($J_{Cl,Gill}$) over the period 0.5–1.5 h was 8.68±0.60 mmol kg^{-1} h^{-1}; between 4.5 and 5.5 h, it was 4.42±1.19 mmol kg^{-1} h^{-1} (significantly different; paired *t*-test, $P=0.005$). The difference in [Cl$^-$] between the perfusion inflow and outflow was 20–50 mmol l^{-1} at a perfusion rate of approximately 0.6 ml min^{-1}.

The individual maximum rate of chloride uptake (10.12±0.45 mmol kg^{-1} h^{-1}, averaged over 0.5 h) occurred at times between 0.5 and 2.5 h in different individuals. At the start of perfusion, the haemolymph chloride concentration, $[Cl^-]_h$, of these crabs ranged from 307 to 408 mmol l^{-1} (mean 365±37 mmol l^{-1}). The peak uptake rate was not correlated with the initial $[Cl^-]_h$ and was quite constant (Fig. 3). However, after 4.5 h of perfusion, a significant negative correlation ($r^2=0.68$, $P<0.01$) existed between $J_{Cl,Gill}$ and the initial $[Cl^-]_h$; i.e. branchial chloride uptake was more rapidly attenuated in crabs with high haemolymph chloride concentrations. Haemolymph chloride concentration rose by a mean of 81 mmol l^{-1} to 446±31 mmol l^{-1} (range 412–494 mmol l^{-1}) during the 5–8 h of perfusion.

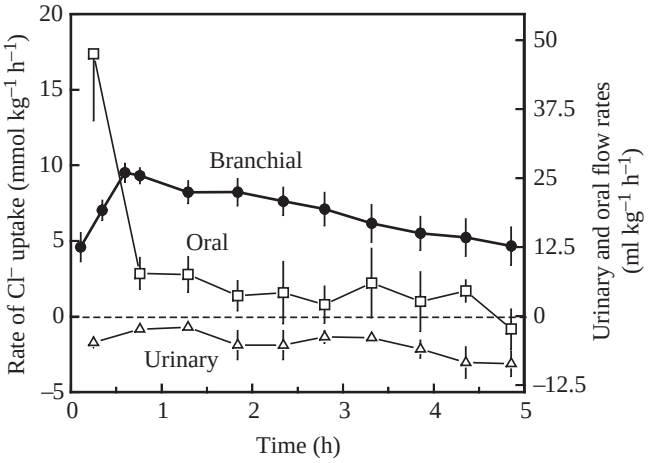

Fig. 2. Chloride fluxes of crabs acclimated to fresh drinking water during bilateral perfusion of the branchial chambers with saline, partitioned into branchial uptake (red symbols), urinary output (green symbols) and ingestion of perfusate (oral). Values for urine flow and fluid ingestion may also be read as flow rates on the right-hand axis. Negative values indicate a loss from the crab. Values are means ± S.E.M. ($N=8$).

Urine flow increased steadily from a mean rate of 1.54±0.46 ml kg^{-1} h^{-1} in the period 0.5–1.5 h to 7.69±1.56 ml kg^{-1} h^{-1} between 4.5 and 5.5 h (Fig. 2; the initial high value is probably an artefact caused by wash-out of residual tracer from the branchial chambers) ($P<0.01$, paired *t*-test).

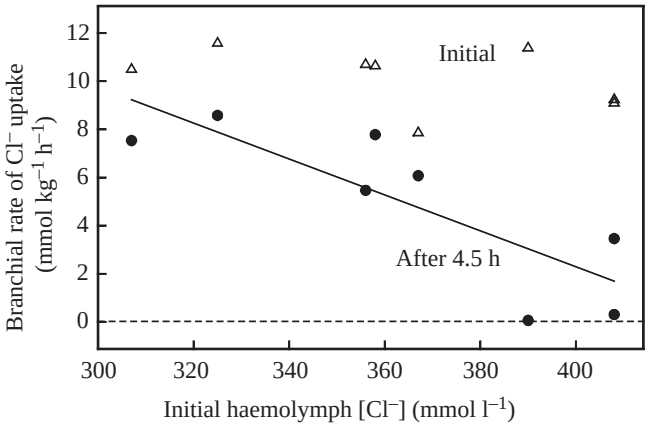

Fig. 3. The relationship between the branchial rate of chloride uptake and the initial chloride concentration of the haemolymph for crabs acclimated to fresh drinking water and with the branchial chambers bilaterally perfused with saline. Triangles, initial (peak) rates (averaged over 0.5 h); circles, rates after 4.5 h of perfusion (averaged over 1 h). r^2=0.68, P<0.01.

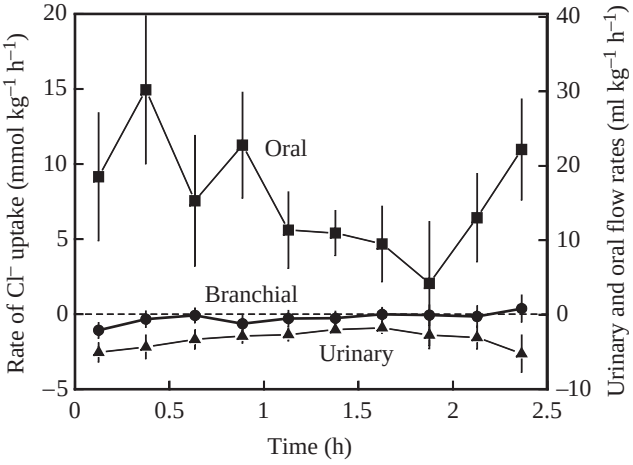

Fig. 4. Chloride fluxes and fluid movements in crabs acclimated to saline drinking water (70% seawater), showing branchial uptake, urinary output and ingestion of perfusate (oral). Values are means ± S.E.M. (N=6). Other details as for Fig. 2.

Crabs typically retained a large volume of the saline during the first hour or so, partly because of initial filling of the branchial chambers and partly because of ingestion, and continued to ingest fluid at a decreasing rate thereafter (oral flow rates, Fig. 2).

Effects of acclimation to saline drinking water on chloride fluxes

Crabs that had been drinking 70% seawater for 7–9 days showed approximately zero branchial chloride uptake during 2.5 h of branchial perfusion (Fig. 4; most individuals showed a small overall net loss). The initial $[Cl^-]_h$ of these crabs was higher than that of crabs on the freshwater regimen (497.5±8.7 mmol l^{-1}) but did not rise significantly during perfusion (final $[Cl^-]_h$=502.4±8.9 mmol l^{-1}, paired t-test). The rate of urine output in the saline-acclimated crabs was initially higher than in the freshwater group but it did not increase during 2.5 h of perfusion. Ingestion (and occasional regurgitation) of the perfusate occurred throughout the period of perfusion (Fig. 4; periodic negative oral flows, representing regurgitations, are obscured in the mean values). Fluid was retained at approximately double the rate observed in the freshwater-acclimated crabs and exceeded urine output. This was also evident from swelling of the arthrodial membranes and pericardial sacs in some crabs.

Effects of dopamine, cyclic AMP and NaCl injection on chloride fluxes in freshwater-acclimated crabs

Dopamine and dbc-AMP did not change the rate of branchial chloride uptake by FW crabs. Controls injected with iso-ionic saline decreased $J_{Cl,Gill}$ from 7.23±0.67 mmol kg^{-1} h^{-1} in the hour before injection to 6.24±0.61 mmol kg^{-1} h^{-1} in the hour following (Fig. 5A). Similar small decreases in $J_{Cl,Gill}$ followed pericardial infusion of dopamine (Fig. 5B; from 6.86±0.89 to

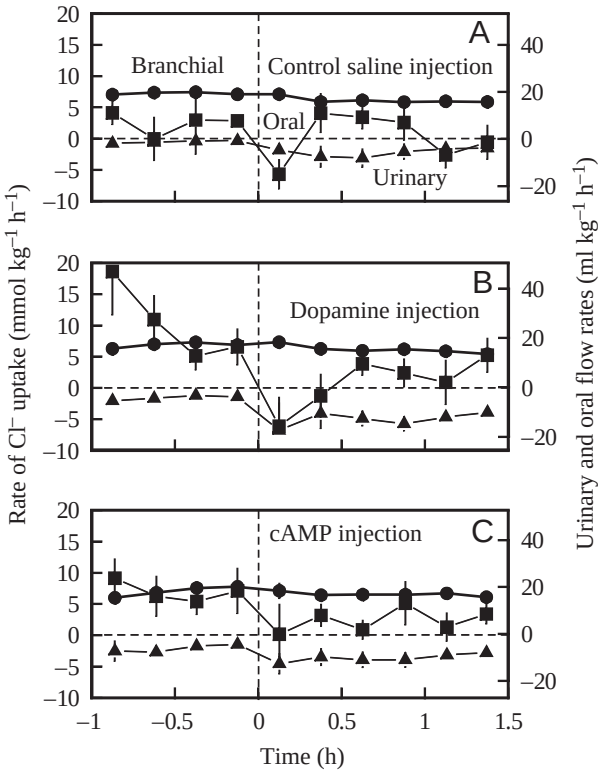

Fig. 5. The effects of dopamine and cyclic AMP on chloride fluxes and fluid movements in crabs acclimated to fresh drinking water showing branchial, urinary and oral fluxes. At time zero, 250 µl 100 g^{-1} total body mass of saline (A) or 1.0 mmol l^{-1} dopamine (B) or 250 µl 100 g^{-1} total body mass of 6 mmol l^{-1} dibutyryl cyclic AMP (C) was injected pericardially. Values are means ± S.E.M. (N=5, 9 and 8, respectively). Other details as for Fig. 2.

6.43±0.88 mmol kg^{-1} h^{-1}) and of dbc-AMP (Fig. 5C; from 7.37±0.79 to 6.64±0.91 mmol kg^{-1} h^{-1}). Although statistically significant overall (P<0.0001; repeated-measures ANOVA,

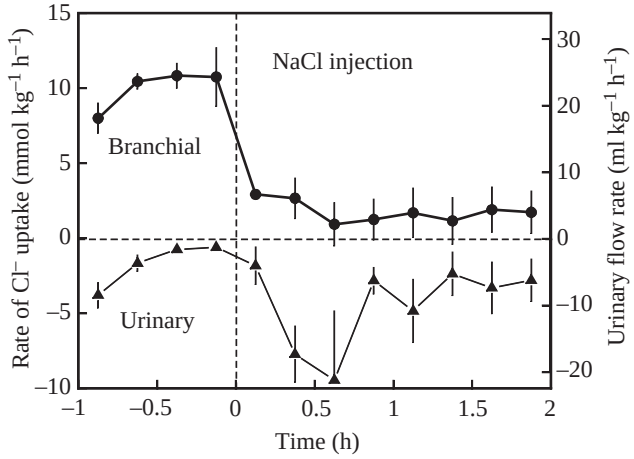

Fig. 6. The effects of acute NaCl-loading on the branchial uptake of chloride and urinary chloride flux for crabs acclimated to fresh drinking water. At time zero, 800 µl 100 g^{-1} total body mass of 5 mol l^{-1} NaCl was injected pericardially over 5 min, raising haemolymph [Cl$^-$] by 78 mmol l^{-1}. Values are means ± S.E.M. (N=4). Other details as for Fig. 2.

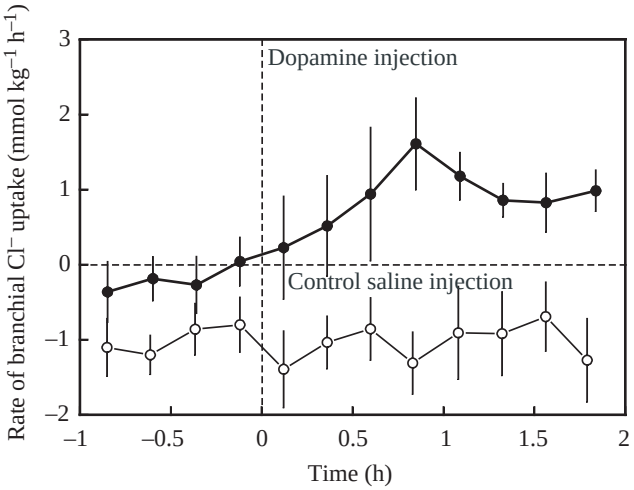

Fig. 7. The effects of dopamine on branchial chloride fluxes in crabs acclimated to saline drinking water (70% seawater). Crabs were injected at time zero with 250 µl 100 g^{-1} total body mass of saline (open circles; N=8) or 1.0 mmol l^{-1} dopamine (filled circles; N=10). Values are means ± S.E.M.

contrast analysis) and of similar magnitude to the decline in $J_{Cl,Gill}$ observed in non-injected crabs (Fig. 2), these responses were not significantly different from each other (ANOVA, Tukey).

By contrast, infusion of hyperionic NaCl over 5 min caused an abrupt 80% depression of branchial chloride uptake (Fig. 6; from 10.01±0.49 to 1.95±0.80 mmol kg^{-1} h^{-1}). This was significantly greater than the control response (P<0.0001, ANOVA, Tukey) and persisted for more than 2 h. During NaCl infusion, $[Cl^-]_h$ increased by 78 mmol l^{-1} (from 380.0±34.6 to 458.5±20.3 mmol l^{-1}), a change similar to that observed in perfused crabs freely absorbing chloride over 5–8 h (see above), corresponding to a chloride distribution volume of 0.510 l kg^{-1}.

The rate of urine production increased steadily in all groups of injected FW crabs (Figs 5, 6). In saline-injected controls, the increase, from 1.05±0.20 ml kg^{-1} h ^{1} before injection to 2.26±1.05 ml kg^{-1} h^{-1} after the injection, was similar to the change over the corresponding period in non-injected crabs (from 1.85±0.67 to 2.85±0.90 ml kg^{-1} h^{-1}). Larger increases in urine production followed injection of dopamine (from 1.58±0.47 to 10.58±1.96 ml kg^{-1} h^{-1}), dbc-AMP (from 3.39±0.91 to 9.53±2.53 ml kg^{-1} h^{-1}) and hyperionic NaCl (from 1.32±0.27 to 10.11±2.5 ml kg^{-1} h^{-1}). Within all three experimental groups, the increases were statistically significant (P<0.001, P<0.05, P<0.05, respectively; repeated-measures ANOVA, Tukey), but only the dopamine response differed significantly from the control response (P<0.05, one-way ANOVA, Tukey).

An increase in urine output also followed injection of dopamine in the preliminary series, which did not employ the filtration marker. This was inferred from an apparent reduction in the branchial chloride uptake as the more concentrated urine entered the perfusion flow [see Materials and methods: dopamine reduced the uncorrected uptake from 6.83±0.67 to 3.56±1.16 mmol kg^{-1} h^{-1} (mean ± S.E.M., N=9)], which was significantly different (t-test) from the control response (from 6.26±0.93 to 6.27±1.18 mmol kg^{-1} h^{-1}, mean ± S.E.M., N=6).

The crabs ingested perfusion fluid throughout the experiment. Both the dopamine group and the saline controls regurgitated fluid immediately following the injection (Fig. 5).

Effects of dopamine on branchial chloride fluxes in saline-acclimated crabs

In crabs acclimated to saline drinking water for 30–50 days, mean branchial chloride flux $J_{Cl,Gill}$ was negative, indicating a small net loss (Fig. 7). Dopamine caused a highly significant switch from net loss in the hour before to net uptake in the hour following the injection (from –0.11±0.32 to 1.12±0.63 mmol kg^{-1} h^{-1}), but there was no change in the saline-injected control crabs (from –0.56±0.27 to –0.65±0.35 mmol kg^{-1} h^{-1}) (P<0.01, P>0.99 respectively; repeated-measures ANOVA, Tukey). $J_{Cl,Gill}$ peaked at 1.72±0.81 mmol kg^{-1} h^{-1} approximately 1 h after dopamine injection and was sustained for more than 2 h. An increase in urine output was observed in both dopamine-injected and control-injected crabs, but the responses were not statistically different from each other.

Relationship between the rate of chloride uptake and the increase in haemolymph chloride concentration

The haemolymph chloride concentration increased in perfused crabs that showed net uptake of chloride and decreased in those showing a net loss. The ratio of the branchial chloride flux (corrected for urinary contribution) to the rate of change of haemolymph [Cl$^-$] gives an estimate of the mean volume of distribution of the absorbed chloride (‘chloride space’). There were no significant differences in the

chloride space among freshwater-acclimated crabs either uninjected or injected with saline, dopamine or cAMP, for which the pooled mean was 0.501±0.026 l kg^{-1} (N=25).

Discussion

Red crabs provided with fresh drinking water absorbed chloride from their branchial chamber fluid at approximately 10 mmol kg^{-1} h^{-1}, double the rate reported for *B. latro* (Morris et al., 1991, 2000), and perhaps reflecting the greater gill area in *G. natalis* (Greenaway, 1999). Haemolymph [Cl$^-$] increased at approximately 20 mmol l^{-1} h^{-1} and was distributed into a chloride space of 0.501 l kg^{-1}. This is greater than the haemolymph volume in this crab (0.243 l kg^{-1}) (Greenaway, 1994), suggesting that absorbed chloride was also distributed intracellularly.

Branchial processing of urine was clearly adjusted to the requirements of ionic homeostasis. Switching the drinking water of non-perfused crabs from freshwater to saline, caused the [Cl$^-$] of the P to increase 40-fold. Haemolymph osmolality and ion concentrations were elevated in SW crabs and may provide a signal to switch off branchial salt uptake. This is supported by zero net uptake of chloride observed in SW crabs, by abrupt inhibition of chloride uptake on infusion of NaCl into FW crabs and by downregulation of branchial chloride uptake during prolonged saline perfusion of FW crabs. Similar negative feedback control of sodium and chloride fluxes has been proposed for freshwater crayfish (Shaw, 1964; Mo and Greenaway, 2001). However, the observation that the rate of down-regulation of $J_{Cl,Gill}$, rather than initial $J_{Cl,Gill}$, was dependent on $[Cl^-]_h$ merits further investigation. The relative importance of endocrine mediation (as discussed below) *versus* 'autoregulatory' modulation of ion fluxes in response to internal osmotic change (Onken, 1996) must also be considered.

Downregulation of chloride uptake prevented swamping of the haemolymph with salt during experimental saline irrigation of the gills, but its importance for crabs in the rainforest is less clear. Normally, the quantity of chloride reclaimed would be limited by the volume of urine entering the branchial chambers and by the increasing gradient from haemolymph to P. However, such a mechanism would be advantageous during breeding migrations when red crabs immerse themselves in seawater for many hours (Hicks et al., 1990). This 'dipping' behaviour facilitates rehydration and replenishment of salts lost during the migration (Greenaway, 1994).

Experimentally perfused crabs were unable bypass the absorptive surfaces so that changes in the [Cl$^-$] of the perfusate must have been due to changes in branchial chloride transport. However, direct shedding of urine remains a possible means of increasing salt loss for crabs in the field.

On saline perfusion of FW crabs, the rate of chloride uptake rose to a maximum over approximately 1 h. This might reflect the time taken for fluid to percolate between the gill lamellae, although they are provided with elaborate spacing structures to facilitate this process (Farrelly and Greenaway, 1992). Or perhaps chloride transport is activated only when fluid enters the branchial chambers. Both *G. natalis* and *B. latro* detect the entry of fluid into the branchial chambers, adopting a characteristic stance at the onset of perfusion, apparently to assist retention of fluid during processing.

Stimulation of branchial chloride uptake by dopamine in saline-acclimated *G. natalis* is consistent with the osmoregulatory role of this pericardial neurohormone in aquatic brachyurans (Sommer and Mantel, 1988; Morris and Edwards, 1995; Mo et al., 1998; Morris, 2001). Thus, in the evolution of a terrestrial lifestyle, *G. natalis* has retained an endocrine mechanism present in its marine brachyuran ancestors. By contrast, dopamine has an inhibitory effect on the uptake of chloride and other ions in the terrestrial anomuran *B. latro* (Morris et al., 2000).

Dopamine did not restore chloride uptake rates of SW crabs to the level in FW crabs. However, dopamine appears to regulate the existing Na^+/K^+-ATPase (Sommer and Mantel, 1988; Morris and Edwards, 1995; Mo et al., 1998; Morris, 2001; Towle et al., 2001). Red crabs maintained on the saline regimen for several weeks completely shut down net chloride uptake. The small response may reflect dedifferentiation of ionocytes, associated with reduced membrane infoldings and levels of transport enzymes, as observed in seawater-acclimated euryhaline crabs (Towle et al., 1976; Péqueux et al., 1984; Compere et al., 1989). Such changes might be reversed slowly. Although dopamine had no effect on the rate of chloride uptake in FW crabs in the present study, another bioamine, serotonin stimulated sodium transport in freshwater-acclimated *G. natalis* (Morris, 2001). Interrelationships between these two bioamines and osmoregulation require clarification.

Besides regulating branchial ion transporters in decapods, dopamine and other biogenic amines have widespread vasomotor effects, influencing cardiac function (Wilkens and McMahon, 1992; Wilkens and Mercier, 1993; Wilkens, 1999), flow through the cardio-arterial valves (Kuramoto and Ebara, 1994; Kuramoto et al., 1992, 1995), arterial resistance (Wilkens, 1997) and branchial resistance (Taylor et al., 1995). Branchial resistance changes occur in 'efferent valves', which are located in the gill lamellae of aquatic crabs (Taylor, 1990; Taylor and Taylor, 1986, 1991, 1992) and are also observed in the gills of *G. natalis* (Farrelly and Greenaway, 1992). Thus, endocrine modulation of branchial ion transport *in vivo* might involve both direct effects on epithelial transport and changes in gill perfusion.

Dopamine acutely increased the rate of urine formation in FW crabs, and serotonin has been shown to have a similar effect when chronically injected into red crabs in the field (Morris, 2001). Whether these are components of an integrated response that regulates salt and water balance, or a more generalized stress reaction, remains to be established. The widespread effects of bioamines suggest they may not be primarily agents of osmoregulation in crabs. The eyestalks have been implicated in the control of osmoregulation in

decapods (Berlind and Kamemoto, 1977; Mantel, 1985; McNamara et al., 1990; Charmantier-Daures et al., 1994; Pierrot et al., 1994; Eckhardt et al., 1995; Onken et al., 2000; Spanings-Pierrot et al., 2000), including *Gecarcinus lateralis* (Mantel, 1968), and thus deserve more attention in other terrestrial crabs.

Red crabs consistently imbibed the perfusion saline and regurgitated intermittently, indicating that they could adjust P volume by reingestion of urine, as proposed for *B. latro* (Greenaway et al., 1990; Taylor et al., 1993). Mantel (1968) demonstrated iso-osmotic absorption of saline in the foregut of *G. lateralis*, and proposed an osmoregulatory role for this organ. However, the foregut did not contribute to dilution of the perfusate in the present study. Calculation of branchial chloride fluxes assumed that regurgitated fluids had the same [Cl^-] as the perfusate. Chloride uptake in the foregut would have been evident as pulses, synchronized with regurgitations. Examination of the time course of chloride fluxes in individual crabs indicated that this was not the case (data not shown). Presumably, in *G. natalis*, the site of reabsorption of ions is located in the posterior gills, in which ion-transporting epithelia predominate (Farrelly and Greenaway, 1992).

The compositions of the haemolymph and urine of FW and SW crabs confirm that the antennal organs are unimportant in osmotic and ionic regulation, as in *B. latro* (Taylor et al., 1993), emphasizing the importance of post-renal processes. Haemolymph osmolality, [Na^+], [Ca^{2+}] and [Cl^-] were all elevated by approximately 45% after several weeks of the 70% seawater regimen and were similarly increased in the urine. Haemolymph [K^+] was better regulated (28% rise), but this was not associated with an appropriate change in the urine. Renal handling of Mg^{2+} appears to be regulatory in *G. natalis*. Although haemolymph [Mg^{2+}] doubled in SW crabs, urinary [Mg^{2+}] increased fourfold. *G. natalis* resembles marine *Brachyura* in this respect (Robertson, 1949, 1953; Lockwood and Riegel, 1969) but differs from the anomuran *B. latro*, in which urinary and haemolymph [Mg^{2+}] are similar and which is unable to excrete magnesium during prolonged seawater exposure (Taylor et al., 1993).

Although *G. natalis* lacks a diluting segment its renal system, branchial and oral processing of the urine provide a versatile system for salt and water regulation. Despite their evolutionary divergence 160 million years ago, and independent emergence onto land, anomuran and brachyuran terrestrial crabs have acquired essentially similar osmoregulatory systems, although differing with respect to the role of dopamine. It remains to be investigated whether this difference is characteristic of the Anomura or of coenobitids or whether it is unique to coconut crabs.

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