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The Crab-Eating Frog, *Rana cancrivora*, Up-Regulates Hepatic Carbamoyl Phosphate Synthetase I Activity and Tissue Osmolyte Levels in Response to Increased Salinity

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ABSTRACT The crab-eating frog *Rana cancrivora* is one of only a handful of amphibians worldwide that tolerate saline waters. They typically inhabit brackish water of mangrove forests of Southeast Asia, but live happily in freshwater and can be acclimated to 75% seawater (25 ppt) or higher. We report here that after transfer of juvenile *R. cancrivora* from freshwater (1 ppt) to brackish water (10 →20 or 20 →25 ppt; 4–8 d) there was a significant increase in the specific activity of the key hepatic ornithine urea cycle enzyme (OUC), carbamoyl phosphate synthetase I (CPSase I). At 20 ppt, plasma, liver and muscle urea levels increased by 22-, 21-, and 11-fold, respectively. As well, muscle total amino acid levels were significantly elevated by 6-fold, with the largest changes occurring in glycine and β-alanine levels. In liver, taurine levels were 5-fold higher in frogs acclimated to 20 ppt. There were no significant changes in urea or ammonia excretion rates to the environment. As well, the rate of urea influx (J^{in}_{urea}) and efflux (J^{out}_{urea}) across the ventral pelvic skin did not differ between frogs acclimated to 1 versus 20 ppt. Taken together, these findings suggest that acclimation to saline water involves the up-regulation of hepatic urea synthesis, which in turn contributes to the dramatic rise in tissue urea levels. The lack of change in urea excretion rates, despite the large increase in tissue-to-water gradients further indicates that mechanisms must be in place to prevent excessive loss of urea in saline waters, but these mechanisms do not include cutaneous urea uptake. Also, amino acid accumulation may contribute to an overall rise in the osmolarity of the muscle tissue, but relative to urea, the contribution is small. *J. Exp. Zool. 301A:559–568, 2004.*

INTRODUCTION

Most amphibians do not survive well in saline waters. In contrast, *Rana cancrivora* Gravenhorst (1829) are abundant in coastal areas and mangroves of south east Asia. Their common name, the crab-eating frog, is derived from their preference for small invertebrates (including crabs) and vertebrates (Lim and Lim, '92). *R. cancrivora* are highly tolerant of brackish water, but survive well over a range of salinities (0–39 ppt; Gordon et al., '61). Salinity tolerance is not unique to *R. cancrivora* (*Xenopus laevis* and *Bufo viridis* are known to withstand 20 and 26 ppt, respectively), but they are probably the most tolerant of known amphibian species (Balinsky, '81).

It was first reported over 40 years ago, that *R. cancrivora* accumulate urea in body fluids when acclimated to saline water (Gordon et al., '61), a strategy similar to marine elasmobranchs where high internal urea balances the osmotic stress of the external environment (Smith, '36). Unlike elasmobranchs, however, *R. cancrivora* do not efficiently reabsorb urea from the renal tubules (Schmidt-Nielsen and Lee, '62), but there is some evidence for reabsorption across the urinary

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bladder wall (Chew et al., '72). Renal urea excretion rates decline considerably in saline water (after 2 d or more), mostly due to the sharp decrease in urine flow (Schmidt-Nielsen and Lee, '62). Gordon and Tucker ('68) measured total urea excretion rates (across the skin and via urine), but they added toluene to the water which may explain the very high rates reported. From an energetic standpoint, it would be futile to synthesize urea at relatively high rates only to lose it to the environment. Thus, further study is necessary to determine whether total urea excretion changes with salinity exposure.

There is evidence for active urea transport across the skin of several amphibians. Urea influx (mucosal-to-serosal) across the pelvic region of the ventral skin is dramatically enhanced in salt-acclimated amphibians and saturation kinetics have been demonstrated (Garcia-Romeu et al., '81; Rapoport et al., '88; Lacoste et al., '91; Dykto et al., '93). In other animals and tissues, changes in urea permeability have been linked to urea transport proteins (for reviews see, Sands et al., '97; Bankir et al., '98; Walsh and Smith, 2001). The cDNA for a facilitated urea transporter has been isolated and sequenced from the urinary bladder of the saline tolerant toad, *Rana esculenta* (Couriaud et al., '99). An active urea transporter in amphibian skin, however, has not been isolated. If an active skin urea transporter is induced with salinity acclimation in *R. cancrivora*, then an enhanced rate of urea influx across the skin would facilitate whole body urea retention. Hence, measurements of skin urea transport in frogs acclimated to saline waters would be useful to further characterize the mechanisms of urea retention.

The increase in tissue urea levels in *R. cancrivora* acclimated to saline water may be due, in part, to an increase in hepatic urea synthesis. Balinsky et al. ('72) reported an increasing trend in urea cycle enzyme activities with salinity acclimation (4 wk), although the sample size was small (n=2). Short-term exposure (2–3 d) to $\approx$14 ppt had no significant effect on the key urea cycle enzyme, carbamoyl phosphate synthetase I (CPSase I), nor the other enzymes (ornithine transcarbamylase, argininosuccinate synthetase, and arginase; Colley et al., '72). In both studies, enzyme measurements were expressed as μmoles of product formed per min per g wet weight liver. Progressive water depletion in muscle and liver was observed on exposure to the hyperosmotic environment (Colley et al., '72). Therefore, any changes in enzyme activity detected may be simply due to an overall concentration of cellular constituents. Thus, enzyme activities expressed per mg protein may give a more accurate assessment of true enzyme induction, if it occurs.

Skeletal muscle tissue urea levels increase with increased external salinity in *R. cancrivora*, although not to the same degree as plasma urea levels (Gordon and Tucker, '68). One possibility is that urea is not the only osmolyte that increases in muscle tissue under these conditions. Withers and Guppy ('96) reported that six desert frog species (covering three genera) from Western Australia did not accumulate any specific organic osmolyte (eg., trimethylamine oxide, betaine, sacrosine) in plasma or muscle tissue, besides urea, when naturally aestivating. This is distinctly different from marine elasmobranchs, where intracellular compartments co-accumulate organic osmolytes, primarily trimethylamine oxide and urea (Yancey et al., '82). Interestingly, the ratio of urea:amino acids is $\approx$2:1 in muscle tissue of the marine skate, *Raja erinacea* and total amino acid levels are significantly changed with environmental salinity stress (Forster and Goldstein, '76). When two *R. cancrivora* were acclimated to 26 ppt, there was no clear increase in the levels of six different amino acids in skeletal muscle tissue (Gordon and Tucker, '68). Two other partial amino acid surveys of muscle tissue found an increasing trend in the levels of some individual amino acids, but key compounds were unchanged or not measured (eg., β-alanine, taurine; Balinsky et al., '72; Colley et al., '72). A complete analysis of all the individual amino acids has not been performed on *R. cancrivora* tissues, to our knowledge.

The aim of the present study was to further examine the osmoregulatory response of *R. cancrivora* to clarify the following questions. 1) Does the rate of total urea excretion change with increased salinity? 2) Does skin urea permeability change with increased salinity? 3) Is there an induction of hepatic CPSase I specific activity? 4) Do amino acids co-accumulate with urea in intracellular compartments? To answer these questions, juvenile *R. cancrivora* were exposed in the laboratory to waters of increasing salinity (up to 25 ppt) and ammonia and urea excretion rates, urea flux rates across the ventral pelvic skin, the concentrations of urea in plasma, liver and muscle, the levels of individual amino acids in liver and muscle, and liver CPSase I activity were measured.

MATERIALS AND METHODS

Animals and experimental protocol

Rana cancrivora were purchased from a local market in Singapore. The majority of the experiments were conducted on frogs that were 0.61 g ± 0.02 and were 1–2 months passed stage XXV (metamorphosis completed, following the guidelines of Uchiyama et al., '90). To obtain sufficient blood to measure plasma Na^+ and Cl^- concentrations, slightly larger frogs were used (1.59 g ± 0.13g). Full grown *Rana cancrivora* have a mass of 10–15g, and therefore the frogs used in the present study were juveniles. They were held for at least 1 month prior to experimentation in 20-L plastic chambers containing a small volume (≈200 ml) of water (1 ppt, 27–28°C) with access to dry platforms. Separate groups of frogs were acclimated to either 10 or 20 ppt for one month. Water was changed regularly. During this period, frogs were fed red worms (*Tubifex* sp.). The photoperiod was 12 h dark: 12 h light.

Short-term exposure to higher salinities was performed as follows. At the start of the experiment, those at 10 ppt were transferred to 20 ppt for 4 days and those at 20 ppt were transferred to 25 ppt for 4 days. This protocol was designed to acutely stimulate the frogs with an osmotic shock that was well within their physiological range. Frogs were not fed during this period of time. At the end of 4 days all animals were sacrificed (double pithed or blow to head), blood samples were collected in capillary tubes and stored on ice prior to centrifugation (4°C, 5 min, 5000 g). Due to the small mass of frogs, blood from 5–7 frogs was pooled for a single measurement. Plasma was collected and either stored (−80°C) until later analysis of urea concentration (within 1 week) or measurements of Na^+ and Cl^- concentrations were performed immediately. Livers were removed, individually weighed and used immediately in enzyme assays or frozen and stored at −80°C until later analysis of urea concentration (within 1 week). In order to determine tissue water content, the thigh muscles from 5–7 frogs were pooled as one sample, the wet mass was recorded, and the tissue was dried in an oven (90°C) to constant weight (24 h) before the dry weight was recorded. The water content (%) was calculated as (wet tissue mass − dry tissue mass) ÷ (wet tissue mass) × 100.

To measure nitrogen excretion rates, individual frogs were weighed, transferred to chambers containing ≈5 ml of water, and fasted for 72 h before the start of the experiment to eliminate the potential problem of waste material in the flux chambers. This period of time also enabled the animals to adapt to their experimental chamber and thereby minimize stress. At the start of the experiment on day 1, chamber water was replaced with 6 ml of autoclaved water (1 ppt control or 20 ppt saline). After 4 and 8 h, water samples were removed (1.2 ml), and frozen (−80°C) until later analysis of ammonia and urea concentrations (within 1 week). There were no significant differences between the 0–4 h flux and the 4–8 h flux and therefore, data were pooled. To determine if microbial activity in the water altered the levels of water ammonia and /or urea over time, the remaining chamber water was left for an additional 2 h, then collected, frozen and later analyzed. Control chambers without frogs were also assessed for any changes in water ammonia or urea levels over the 8-h flux period. There was no evidence that bacterial contamination altered the concentrations of ammonia or urea in the water samples. An alternative protocol was used to collect tissue samples for the measurement of muscle and liver amino acids, ammonia and urea concentrations. Frogs held at 1 ppt for ≈ 2 months were transferred, in sequence, to 10 ppt for 2 d, 13 ppt for 1 d, 17 ppt for 2 d, and finally to 20 ppt for 2 days. Thus, the total time of exposure for frogs in 10 ppt was 2 d and in water 13–20 ppt was 5 d. A sub-sample of frogs at 1, 10 and 20 ppt were sacrificed (double pithed). Skeletal muscle tissue was excised from the thigh region and livers were removed, frozen in liquid nitrogen and stored at −80°C until later analysis of amino acids (within 1 month).

Water and tissue ammonia, urea and ion analyses

Water samples were analyzed for ammonia and urea levels using the enzymatic assay described by Kun and Kearney ('71) and a colorimetric assay described by Rahmatullah and Boyde ('80), respectively. Plasma samples were first deproteinized in 1 volume of 6% tricholoroacetic acid, centrifuged (5000 g, 3 min, 4°C) and the urea concentration of the supernatant was determined. For tissue determinations, frozen tissue was weighed, ground to a fine powder in liquid nitrogen and homogenized in 1–5 vol (w/v) of 6% $HClO_4$. After centrifugation at 10 000 g for 15 min, the supernatant was neutralized with 2 $mol \cdot l^{-1}$

KH_2CO_3. The supernatant ammonia and urea concentrations were determined. The concentrations of Na^+ and Cl^- in the plasma were analyzed by a flame photometer (Corning 410, Corning Ltd, Halstead, U.K.) and a chloride analyzer (Corning 925, Corning Ltd.), respectively.

Analysis of free amino acids (FAA)

Samples for FAA analysis were weighed, ground to a fine powder in liquid nitrogen, and homogenized (Ultra-Turrax homogenizer, Germany) in 5 vol (w/v) of 6% tricholoroacetic acid three times (20 s each with 10-s intervals) at 24 000 rev.min^{-1}. The supernatant was obtained after centrifugation (4°C, 15 min, 10,000g), adjusted to pH 2.2 with 4 mol.l^{-1} lithium hydroxide and diluted with 0.2 mol.L^{-1} lithium citrate buffer (pH 2.2). The concentration of individual amino acids was determined using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. Results were expressed as μmol.g^{-1} wet mass tissue.

Enzyme analysis

Liver tissue was homogenized with 2 ml of extract buffer (0.05mmol L^{-1}) 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES), pH 7.7, 0.5 mmol L^{-1} ethylenedinitrilo tetra acetic acid (EDTA), 0.05mmol L^{-1} KCl, and 2 mmol L^{-1} dithiothreitol (DTT)) at 4°C, subjected to brief sonication, and centrifuged at 14000 g for 10 min. The resulting supernatant was passed through a Sephadex G-25 column equilibrated with extract buffer before measuring enzyme activities (Korte et al., '97). CPSase was measured as previously described (Korte et al., '97) except that 0.05mmol L^{-1} NH_4Cl was present as the N-donating substrate. Glutamine synthetase (GSase) activity was measured as described by Shankar and Anderson ('85). Enzyme activities are expressed as units g^{-1} liver tissue and units mg^{-1} protein, where unit represents μmol min^{-1}. Protein was measured by the dye-binding method of Bradford ('76) using Bio-Rad Laboratories reagents and bovine serum albumin as a standard.

Urea flux across isolated skin patches

Urea flux rates across isolated pelvic skin patches were measured on frogs acclimated to 1 and 20 ppt for ≈1 month. Frogs were sacrificed (double pithed) and the skin from the ventral pelvic region was mounted between two halves of a lucite Ussing-type chamber (surface area 0.126 cm^2, volume of hemichamber 4.3 mL). Ringer solution of the following composition (mmol.L^{-1}) (110 NaCl, 5 Na_2HPO_4, 4 KCl, 0.5 $CaCl_2$, 2 urea; pH 7.4) was then added to both sides of the skin and allowed to acclimate for 30 min. Following the acclimation period, urea influx (J^{in}_{urea}) was measured by replacing Ringer solution on the mucosal side with Ringer solution containing 1, 2, 5, 100, 200 or 300 mmol.L^{-1} urea and 2 μCi-ml^{-1} [^{14}C] urea (57 Ci.M^{-1} Amersham) and on the serosal side with Ringer solution containing isosmolar mannitol solutions (compared to the urea concentration). Thus, the rate of urea transport was measured under an imposed urea gradient. In preliminary experiments, we attempted to measure the rate of urea flux in the absence of an imposed urea gradient, that is, the urea concentration on the mucosal side was equal to that on the serosal side. The data were very inconsistent and the values were low, leading to the conclusion that urea was not transported in the absence of an imposed gradient. The ''cold side'' was replaced every 20 min over an 80 min sampling period. The 4 flux rates measured over each 20 min period were then averaged and taken as n = 1. In a preliminary experiment, urea flux rates were measured with a 2 or 200 mmol.L^{-1} urea gradient over a 6 hr period to ensure flux rates were not altered over an extended experimental period. Urea efflux (J^{out}_{urea}) was measured as described for J^{in}_{urea} except that the serosal solution contained urea and the mucosal solution, mannitol. To ensure proper mixing, each hemichamber contained a stir bar. Activity of the [^{14}C] urea was determined using a liquid scintillation counter and fluxes were calculated and expressed in μmol.h^{-1}.cm^{2-1}.

Statistics

Data between exposure groups (i.e., 1, 10, 20, 25 ppt) were compared by a single factor ANOVA followed by Student-Neuman-Keul's multiple range test. To evaluate differences between specific means (e.g. urea flux rates across the skin, 1 *vs* 20 ppt) two sample t tests were used. Differences were regarded as statistically significant at $P < 0.05$. Data are presented as means ± SE (N).

RESULTS

There was a significant increase in CPSase I activity when frogs were exposed to saline waters,

either 20 ppt (1.7-fold) or 25 ppt (1.6-fold) for 4 d, compared to control frogs (1 ppt). Likewise, CPSase I specific activity was significantly higher in the 20 ppt (1.4-fold) and 25 ppt groups (1.5-fold) relative to the frogs in 1 ppt (Table 1). Liver GSase activity was very low, near the limit of detection (data not shown).

Liver urea levels were 21-fold higher at 20 ppt and 29-fold higher at 25 ppt relative to frogs in 1 ppt (Fig 1). Likewise, plasma urea levels were significantly higher (22-fold) at 20 ppt (Fig. 1, Table 2). Plasma Na^+ and Cl^- concentrations were significantly elevated in frogs at 20 and 25 ppt over control concentrations (Table 2). The changes in plasma ion levels were linear between 1 and 25 ppt, with values at 25 ppt significantly higher relative values at 20 ppt. There were no significant differences in skeletal muscle water content between frogs in 1 ppt (72.3% ± 0.7) and 20 ppt (74.8% ± 1.4) or 25 ppt (72.5% ± 0.9; n=5 for all groups).

There was no significant difference between urea excretion rates at 1 ppt (0.48 ± 0.01 μmol. $g^{-1}.h^{-1}$ (N=7)) and 20 ppt (1.11 ± 0.44 μmol.g^{-1}. h^{-1}(N=7)). Likewise, there was no significant difference between ammonia excretion rates at 1 ppt (0.23 ± 0.02 μmol.$g^{-1}.h^{-1}$ (N=7)) and 20 ppt (0.39 ± 0.15 μmol.$g^{-1}.h^{-1}$ (N=7)). The percentage of nitrogen wastes excreted as urea-N was 81–85% (urea-N excretion/(urea-N excretion + ammonia-N excretion)).

There was a linear relationship between J_{urea} and the urea concentration for both urea influx and efflux at either 1 or 20 ppt. Data are shown for only J^{in}_{urea} at 20 ppt (Fig. 2). There were no significant differences between J^{in}_{urea} at 1 and 20 ppt nor J^{out}_{urea} at 1 and 20 ppt at any urea concentrations from 1 to 300 mmol.L^{-1} (Fig. 3). J^{in}_{urea} was higher than J^{out}_{urea} at 1, 2, 5 (20 ppt only) and 300 mmol.L^{-1} urea (Fig. 3).

When frogs were exposed to 10 ppt or 20 ppt, the total amino acid content of the liver was significantly higher (36–136%) compared to control frogs at 1 ppt. Interestingly, taurine alone was 5-fold higher in liver tissue from frogs at 20 ppt relative to 1 ppt (Table 3). A more dramatic change was observed in the amino acid content of muscle tissue. Total amino acid levels were 180% and 540% higher in frogs exposed to 10 ppt and 20 ppt, respectively, relative to 1 ppt (Table 4). β-alanine and glycine represented the largest contribution to this total pool of amino acids and both increased considerably from 1 to 20 ppt (6- to 11-fold, respectively) (Table 4). Several other amino acids were elevated by 5- to 6-fold in the 20 ppt group

TABLE 1. Effects of salinity (20, 25 ppt) on liver CPSase I activities in Rana cancrivora relative to control values (1 ppt)

Activity	Condition		
	1 ppt	20 ppt	25 ppt
CPSase I units.gm^{-1} liver	3.84 ± 0.32	6.61 ± 0.46[a]	6.29 ± 0.47[a]
CPSase I units.mg^{-1} protein	0.0267 ± 0.0014	0.0383 ± 0.0027[a]	0.0393 ± 0.0079[a]

[1]Results represent means ± S.E. (N=7).
[a]Significantly different from control value $P < 0.05$.

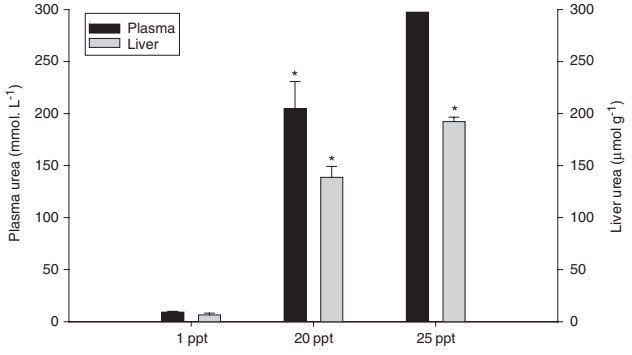

Fig. 1. Plasma (mmol.l^{-1}) and liver (μmol.gm^{-1}) urea concentrations in *R. cancrivora* exposed to control water (1 ppt) or saline waters (20 ppt or 25 ppt). Means ± S.E. [Liver, 1 ppt (N=7), 20 ppt (N=7), 25 ppt (N=6); Plasma, 1 ppt (N=5), 20 ppt (N=7), 25 ppt (N=1)]. An asterisk indicates significant difference to respective value at 1 ppt.

TABLE 2. Effects of salinity (20, 25 ppt) on plasma Na^+, Cl^- and urea concentrations in Rana cancrivora relative to control values (1 ppt)

Activity	Condition		
	1 ppt	20 ppt	25 ppt
Na^+mequiv.l^{-1}	126.6 ± 1.6	209.6 ± 2.7[a]	233.2 ± 3.4[a,b]
Cl^-mequiv.l^{-1}	95 ± 2.1	180.0 ± 2.7[a]	200.6 ± 3.8[a,b]
Urea mmol.l^{-1}	9.2 ± 0.8	204.8 ± 26.0[a]	297.4[c]

[1]Results represent means ± S.E. (N=5–7).
[a]Significantly different from 1 ppt.
[b]Significantly different from 20 ppt.
[c]n=1.

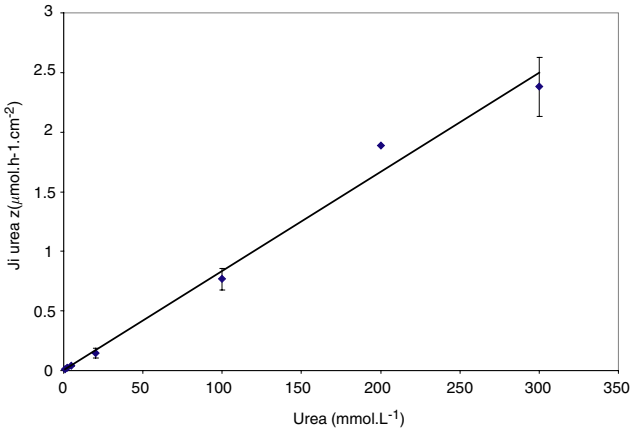

Fig. 2. The *in vitro* rate of urea influx (J^{in}_{urea}) across the pelvic region of the ventral isolated skin in *B. cancrivora* measured over a range of urea concentrations acclimated to saline water (20 ppt). Means ± S.E. (n = 3–5, except at 200 mmol L^{-1} where n = 1).

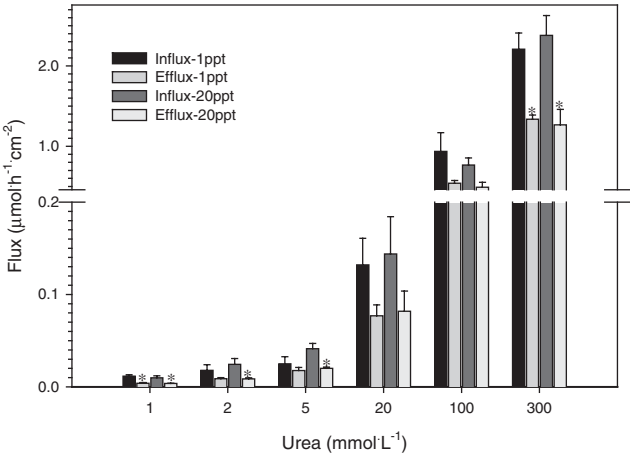

Fig. 3. The rate of urea influx (J^{in}_{urea}) and efflux (J^{out}_{urea}) across the pelvic region of the ventral isolated skin of *R. cancrivora* acclimated to control water (1 ppt) or saline water (20 ppt) at different urea concentration gradients. An asterisk indicates a significant difference between (J^{in}_{urea}) and (J^{out}_{urea}) measured under the same conditions (i.e., salinity and urea concentration). No significant differences were found between frogs acclimated to 1 ppt and 20 ppt external salinity. Means ± S.E. (n = 3–5).

(alanine, asparagine, aspartate, serine, and threonine), however, many others remained unchanged. Of those amino acids that did significantly change, there was a step-wise increase in concentration between 1, 10, and 20 ppt in most cases. Urea concentration in muscle tissue at 10 ppt (38.1 ± 2.3 μmol.gm^{-1}) and 20 ppt (130.4 ± 2.3 μmol.gm^{-1}) was 3- and 11-fold higher, respectively, over control levels (1 ppt 11.5 ± 0.7 μmol.gm^{-1}). There were no significant changes in ammonia levels in muscle tissue with changing salinity (1 ppt 4.9 ± 0.5 μmol.gm^{-1}; 10 ppt 3.8 ± 0.5 μmol.gm^{-1} ; 20 ppt 4.0 ± 0.1 μmol.gm^{-1}).

TABLE 3. Effects of salinity on the concentrations (μmol gm^{-1} wet mass) of various free amino acids (FAA) and total FAA (TFAA) in the liver of Rana cancrivora

	Condition		
FAA	1 ppt	10 ppt	20 ppt
Ala	0.80 ± 0.09	0.83 ± 0.06	0.50 ± 0.06[a,b]
β-Ala	0.29 ± 0.03	0.47 ± 0.03	0.78 ± 0.22[a]
Asn	0.15 ± 0.02	0.30 ± 0.02[a]	0.16 ± 0.02[b]
Asp	0.26 ± 0.04	0.49 ± 0.04[a]	0.32 ± 0.04[b]
Arg	0.05 ± 0.00	0.07 ± 0.00[a]	0.04 ± 0.00[b]
Cit	0.13 ± 0.02	0.20 ± 0.02	0.96 ± 0.32[a,b]
Cys	0.18 ± 0.02	0.17 ± 0.02	0.15 ± 0.02
Gln	0.37 ± 0.05	0.42 ± 0.03	0.21 ± 0.04[a,b]
Glu	1.1 ± 0.1	1.3 ± 0.0	1.4 ± 0.1
Gly	0.77 ± 0.06	1.6 ± 0.1[a]	1.9 ± 0.2[a,b]
His	0.21 ± 0.01	0.39 ± 0.05[a]	0.27 ± 0.03[b]
Hy-Pro	0.44 ± 0.09	0.38 ± 0.04	0.51 ± 0.03
Ile	0.09 ± 0.01	0.09 ± 0.00	0.06 ± 0.01
Leu	0.24 ± 0.03	0.27 ± 0.01	0.18 ± 0.02[b]
Lys	0.30 ± 0.03	0.28 ± 0.02	0.21 ± 0.01[a]
Met	0.05 ± 0.01	0.06 ± 0.00	0.02 ± 0.00[a,b]
Orn	0.09 ± 0.01	0.17 ± 0.01	0.37 ± 0.04[a,b]
Phe	0.08 ± 0.01	0.10 ± 0.00	0.07 ± 0.01[b]
Pro	0.72 ± 0.12	0.67 ± 0.06	0.79 ± 0.06
Ser	0.28 ± 0.03	0.38 ± 0.01[a]	0.22 ± 0.02[b]
Tau	3.2 ± 0.3	5.8 ± 0.3[a]	16 ± 1[a,b]
Thr	0.29 ± 0.03	0.20 ± 0.00[a]	0.33 ± 0.02[b]
Try	0.02 ± 0.01	0.03 ± 0.00	0.03 ± 0.01[a,b]
Tyr	0.07 ± 0.01	0.07 ± 0.00	0.05 ± 0.00[a,b]
Val	0.29 ± 0.03	0.27 ± 0.00	0.19 ± 0.00[a,b]
TFAA	11 ± 1	15 ± 0	26 ± 1[a,b]

Results represent means ± S.E. (N=5).
[a]Significantly different from the corresponding 1 ppt salinity, $P < 0.05$.
[b]Significantly different from the corresponding 10 ppt salinity, $P < 0.05$.

DISCUSSION

The dramatic elevation of plasma and tissue urea levels (11- to 28-fold) after exposure to 20 or 25 ppt for 4 d (Fig. 1) may be the result of an increase in the rate of urea synthesis and/or a decrease in the rate of urea excretion. Our results indicate that the key urea cycle enzyme, CPSase I, is induced in juvenile *R. cancrivora* following several days in a hyperosmotic environment, suggesting a stimulation of the rate of urea synthesis (Table 1). There was no significant change, however, in the total rate of urea loss to the environment. Under these experimental

TABLE 4. Effects of salinity on the concentrations (μmol gm^{-1} wet mass) of various free amino acids (FAA) and total FAA (TFAA) in the muscle of Rana cancrivora

	Condition		
FAA	1 ppt	10 ppt	20 ppt
Ala	0.42±0.01	1.2±0.1[a]	2.1±0.4[ab]
β- Ala	3.8±0.2	5.8±0.5[a]	22±1[a,b]
Asn	0.09±0.01	0.29±0.06[a]	0.50±0.07[a,b]
Asp	0.16±0.02	0.22±0.02	0.77±0.06[a,b]
Arg	0.06±0.01	0.02±0.00[a]	0.03±0.00[a]
Cit	0.08±0.02	0.07±0.01	0.15±0.02[a,b]
Cys	0.02±0.00	0.08±0.01[a]	0.06±0.00[a,b]
Gln	0.26±0.02	1.1±0.2[a]	0.81±0.05[a]
Glu	0.28±0.02	0.50±0.06[a]	0.54±0.05[a]
Gly	1.5±0.1	4.6±0.5[a]	16±1[a,b]
His	0.05±0.00	0.09±0.00	0.04±0.00[b]
Hy-Pro	0.07±0.02	0.05±0.00	0.23±0.03[a,b]
Ile	0.03±0.00	0.04±0.00	0.04±0.00
Leu	0.06±0.00	0.08±0.01	0.10±0.01[b]
Lys	0.15±0.01	0.08±0.02[a]	0.14±0.02[b]
Met	ND	ND	ND
Orn	0.05±0.01	0.06±0.00	0.07±0.01
Phe	0.04±0.00	0.05±0.00[a]	0.04±0.00
Pro	0.15±0.01	0.19±0.02	0.55±0.08[a,b]
Ser	0.22±0.02	0.50±0.09	1.3±0.2[a,b]
Tau	1.4±0.1	1.7±0.2	3.2±0.4[a,b]
Thr	0.20±0.01	0.24±0.03	0.98±0.20[a,b]
Try	ND	ND	ND
Tyr	0.03±0.00	0.04±0.00	0.03±0.00
Val	0.06±0.00	0.08±0.01	0.11±0.01[a,b]
TFAA	9.2±0.3	17±1[a]	50±3[a,b]

Results represent means±S.E. (N=5).
[a]Significantly different from the corresponding 1 ppt salinity, $P<0.05$.
[b]Significantly different from the corresponding 10 ppt salinity, $P<0.05$; ND, not detectable.

conditions, therefore, we conclude that urea levels increase because of an accelerated rate of production.

The lack of change in urea excretion rates, despite the large increase in tissue-to-water gradients, indicates that mechanisms must be in place to prevent excessive loss of urea in saline waters. The osmotic concentration of the urine is typically lower than that of the plasma in *R. cancrivora* (Dicker and Elliot, '70), although there appears to be no reabsorption of urea from the renal tubules (Schmidt-Nielsen and Lee, '62). There is evidence for urea reabsorption from the urinary bladder of *R. cancrivora* (Chew et al., '72), however, similar to observations in a few other species of amphibians (Levine et al., '73a,b; Martial et al., '91). Active urea transport across the skin may also help to replace lost urea in frogs and toads pre-adapted to saline waters (Garcia-Romeu et al., '81; Rapoport et al., '88; Lacoste et al., '91; Dykto et al., '93). Consequently, we expected to observe a higher rate of urea influx in frogs acclimated to 20 ppt versus control frogs (1 ppt). This was not the case (Fig. 3). Furthermore, if urea transport was wholly or partly dependent on a carrier-mediated mechanism, then we would expect a typical saturation curve, as has been observed in other amphibians (Garcia-Romeu et al., '81; Dytko et al., '93). Again, this was not observed (Fig. 2). It should be noted that at high urea concentrations there is probably an increase in paracellular conductance (Erlij and Martinez-Palomo, '71), thus a passive paracellular component at high urea concentrations would mask transcellular, active urea transport, if present. Given this rationale, we would have expected to observe a saturation curve only at very low urea concentrations, as reported in other tissues in other animals (Pilley and Wright, 2000; Fines et al., 2001; Morgan et al., 2003), but this was not the case in the present study (Fig. 2). Interestingly, there was a higher rate (at most 2-fold) of urea influx relative to efflux at 1, 2, 5 and 300 $mmol.L^{-1}$ urea (Fig. 3), but this asymmetry was very small compared to values reported for other salt-tolerant amphibians (13- to 46-fold; Garcia-Romeu et al., '81; Rapoport et al., '88; Lacoste et al., '91; Dytko et al., '93). It is possible that skin urea transporters are expressed later in development, but this seems unlikely. The switch from ammonotelism to ureotelism occurs prior to the completion of metmorphosis and the ureosmotic strategy is clearly in place in these post metamorphic juveniles. Thus, the evidence to date in juvenile *R. cancrivora* suggests that skin urea reabsorption is not an important mechanism to reduce urea loss in a hyperosmotic environment. The daily loss of urea on a whole-body basis can be estimated from the efflux data in Fig. 2 ($\sim$1 $\mu mol.h^{-1}.cm^{-2}$ efflux at 200 $mmol.L^{-1}$). The daily loss across the abdominal patch (<1 cm^2) in a juvenile frog (mean mass 0.6 g) would be $\sim$24 μmole per day. This value is close to the measured urea excretion rate in frogs exposed to 20 ppt (1.11 $\pm$0.44 $\mu mol.g^{-1}.h^{-1}$ or 16 μmole per animal per day). This daily urea loss represents 13% of tissue urea levels (Table 2, 20 ppt plasma urea, 205 $mmol\ L^{-1}$ or 123 μmole per frog), a moderate loss that would have to be replaced by new urea synthesis.

The increase in muscle tissue urea levels was accompanied by a significant accumulation of amino acids (Table 4). To estimate the total

osmotic contribution of urea versus the amino acids in muscle tissue, the molar changes between 1 and 20 ppt were calculated to be 119 mmol.L^{-1} for urea and 38 mmol.L^{-1} for the amino acids. Thus, urea contributes approx. 3 times more osmotically active particles to the intracellular muscle compartment compared to the amino acids. If the same analysis is done for liver tissue (note the slightly different experimental protocols to reach 20 ppt), it is immediately obvious that urea is a far more important osmolyte than amino acids in this tissue as well (urea, 132 mmol.L^{-1}; amino acids, 3 mmol.L^{-1}).

One important consideration in our study is whether the changes observed in tissue osmolytes were simply due to the loss of cell water that may occur in a hyperosmotic environment. Colley et al. ('72) reported a 9% decrease in water content of muscle tissue of adult *R. cancrivora* after 7 or more days at 14 ppt. In the present study, we found no significant change in muscle water content after 4 days at either 20 or 25 ppt. Thus, the muscle amino acid and urea data cannot be explained by a lower cell water content. Although we did not determine liver water content it is unlikely to have changed substantially given that there was no change in muscle water content. Moreover, the significant increase in liver CPSase I specific activity (μmol.min^{-1}.mg^{-1} protein; Table 1) indicates that cell water content changes do not explain the higher level of enzyme activity in saline waters. A previous study in adult *R. cancrivora* reported a larger increase ($\approx$3.5 fold) in liver CPSase I activity (specific activity was not measured) with salinity acclimation (Balinsky et al., '72). Differences in the level of enzyme induction may relate to differences in acclimation protocols, assay methods, development stage and size of the frogs. Interestingly, earlier studies of *Xenopus laevis* acclimated to hyperosmotic environment claimed that liver CPSase I activity increased more than 4-fold, (McBean and Goldstein, '70) but when this was revisited more recently, activities were no more than 2-fold higher under similar conditions (T. Lindley, K. Kingsley, and P.M. Anderson, unpublished data). Regardless, there are marked changes in tissue osmolytes and CPSase I activity that are not related to changes in cell water content.

Liver GSase activity was essentially non-detectable and there was no increase in activity when frogs were exposed to high salinity. These results are consistent with the presence of a CPSase I and not a CPSase III enzyme (Anderson, 2001).

A number of specific amino acids appear to play an important role in tissue osmoregulation. The most pronounced change in liver tissue was the 5-fold increase in taurine levels at 20 ppt relative to 1 ppt (Table 3). Taurine accumulates in osmotically stressed cells by up-regulation of a Na^+-coupled transporter (Lang et al., '98). In muscle tissue, taurine was again significantly higher (by 2.3 fold) in frogs exposed to 20 ppt compared to the control frogs, but the changes in β-alanine and glycine levels were more dramatic (6 and 11 fold) and taken together contribute 76% to the total of all amino acids (20 ppt, Table 4). Taurine, β-alanine, and glycine (and some others) are known to be compatible organic solutes, that is, elevated intracellular levels are ''compatible'' with macromolecule function and effective in stabilizing membranes (Yancey et al., '82). When *Bufo viridis* and *B. boreas* were acclimated to 13 ppt, tissue taurine levels were elevated (Balinsky, '81). In *R. cancrivora* skeletal muscle, glycine (and β-alanine) levels showed an increasing trend after salinity exposure, but taurine levels either did not change or were not determined (Gordon and Tucker, '68; Colley et al., '72; Balinsky et al., '72). Our study, therefore, is the first comprehensive analysis of individual amino acids in *R. cancrivora* and identifies taurine, β-alanine and glycine as the key amino acids involved in osmoregulation.

It should also be pointed out that besides urea and amino acids, there may be small changes in other intracellular organic osmolytes, such as methylamines and polyols as observed in aestivating desert frogs (Withers and Guppy, '96) or inorganic ions. As well, both Na^+ and Cl^- changes were significantly elevated in frogs exposed to 20 and 25 ppt relative to 1 ppt (Table 2). These values are very similar to published values for both adult and stage XXV *R. cancrivora* (Uchiyama et al. '90; Shoemaker et al., '92). It is unlikely that additional osmolytes contribute significantly to plasma osmolarity because the concentration of Na^+, Cl^- and urea added together (1 ppt 232 mmol.L^{-1}; 20 ppt 595 mmol.L^{-1}; 25 ppt 732 mmol.L^{-1} data from Table 2) are similar to published plasma osmolarity values (Shoemaker et al., '92) and our own preliminary analysis on the same animals (1 ppt 231 mOsm; 20 ppt 527mOsm; 25 ppt 732 mOsm (n=1)).

It was mentioned in the Introduction that accurate measurements of total urea excretion in *R. cancrivora* were missing from the literature.

Our values are considerably lower (by ≈ 50%) than those reported for adult frogs by Gordon and Tucker ('68), who added the solvent toluene to the external water to prevent microbial decomposition of urea. We are confident that our lower values are not due to such contamination, as technical control experiments were performed to ensure that urea was not lost or added to the water during the flux period. Developmental stage and mass differences will affect excretion rates, but typically smaller organisms have higher rates than larger organisms due to scaling effects. The difference between the urea excretion rates presented by Gordon and Tucker ('68) and the present study probably reflect the addition of toluene to the water which may have altered the properties of the skin. Moreover, the urea excretion rates determined in this study for *R. cancrivora* in 1 ppt water were very similar to those reported for the bullfrog, *R. catesbeiana*, accounting for differences in temperature (Wright and Wright, '96).

In conclusion, the crab-eating frog *R. cancrivora* is very tolerant of saline water, apparently in part because of their rapid accumulation of organic osmolytes, namely urea. This elevation of urea concentrations in body fluids is most likely due to an acceleration of urea synthesis via the induction of the key OUC enzyme, CPSase I, and possibly other enzymes. Amino acids, particularly β-alanine, glycine, and taurine, co-accumulate in tissues with urea. Even though urea is the predominate osmolyte, a marked increase in total amino acids in the muscle tissue also contributes to the overall increase in the osmotic concentration. The lack of change in skin urea influx after acclimation to 20 ppt sets *R. cancrivora* apart from other salt tolerant amphibian species and underlines the importance of further research on the ecophysiology of this fascinating species.

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