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DO AUSTRALIAN DESERT FROGS CO-ACCUMULATE COUNTERACTING SOLUTES WITH UREA DURING AESTIVATION?

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Summary

Australian desert frogs of the genera *Neobatrachus*, *Cyclorana* and *Heleioporus* experience significant dehydration, and iono- and osmoconcentration, during aestivation in the laboratory and accumulate substantial amounts of urea (100–200 mmol l^{-1}). We expected *a priori* that aestivating frogs probably would not need to accumulate balancing osmolytes but would accumulate trimethylamine oxide (TMAO) or betaine as counteracting solutes to urea. These aestivating frogs did not co-accumulate a substantial quantity of any particular balancing osmolyte or counteracting solute, such as a methylamine [TMAO, trimethylamine amine (TMA), betaine, sarcosine, glycerophosphorylcholine (GPC)] or polyol (inositol, mannitol, sorbitol) in plasma or muscle relative to urea accumulation. However, for aestivating frogs, the total concentration of all measured methylamines and polyols (TMAO + TMA + betaine + sarcosine + GPC + inositol) in muscle was approximately 35–45 mmol kg^{-1}, and so it is possible that all of these solutes have a combined counteracting osmolyte role in aestivating frogs at a ratio to urea of approximately 1:2.5, as has been described for elasmobranch fishes. Alternatively, the absence of substantial co-accumulation with urea of any particular solute suggests that aestivating frogs might not require any major extracellular or intracellular counteracting solutes (TMAO, betaine, GPC). The enzyme systems of these aestivating frogs may be insensitive to the perturbing effects of urea, or the perturbing effects of accumulated urea may be a mechanism for metabolic depression, during aestivation.

Key words: frog, *Cyclorana*, *Neobatrachus*, *Heleioporus*, aestivate, urea, methylamine, trimethylamine oxide, betaine, sarcosine, glycerophosphorylcholine, polyol.

Introduction

Urea is the primary nitrogenous waste product of most semi-terrestrial and terrestrial amphibians (Shoemaker *et al.* 1992). The body fluid urea concentration is generally 5–10 mmol l^{-1} for hydrated frogs, but sometimes is as high as 30 or 50 mmol l^{-1} (Table 1). In contrast, aestivating amphibians accumulate urea to a high concentration (McClanahan, 1972; Jones, 1980; Loveridge and Withers, 1981; Etheridge, 1990) because urea that is synthesised for ammonium detoxification cannot be excreted, as dehydrated or aestivating frogs are essentially anuric (Yokota *et al.* 1985; Shoemaker *et al.* 1992). The urea concentration is often higher than 100 mmol l^{-1} in these aestivating amphibians and may even exceed 300 mmol l^{-1} (Table 1).

Urea can assume physiological roles other than as a nitrogenous waste product. For example, it is a major osmolyte for marine elasmobranch fishes (approximately 350 mmol l^{-1}; Holmes and Donaldson, 1969; Withers *et al.* 1994*a*,*b*). Some frogs also accumulate large amounts of urea when acclimated to a high external salinity (e.g. *Xenopus laevis*, *Rana cancrivora*; Goldstein, 1972; Funkhouser and Goldstein, 1973; Romspert, 1976; Katz and Hanke, 1993) or during aestivation (e.g. *Scaphiopus couchii*; McClanahan, 1972) by an accelerated rate of urea synthesis (McBean and Goldstein, 1970; Jones, 1980). The high concentration of urea in these frogs promotes a favourable osmotic gradient for water transfer between the frog and its environment, and so the urea functions as a balancing osmolyte.

Solutes other than urea can also be important osmolytes in various animals, plants and procaryote organisms (Yancey *et al.* 1982; Anthoni *et al.* 1989, 1991). These solutes include inorganic ions such as Na^+ and K^+, amino acids (and their derivatives), methylamines (trimethylamine oxide, trimethylamine, betaine, sarcosine, glycerophosphorylcholine) and polyols (inositol, mannitol, sorbitol). For example, trimethylamine oxide (TMAO) and betaine are significant extracellular or intracellular osmolytes in elasmobranch fishes (see Holmes and Donaldson, 1969; Withers *et al.* 1994*a*,*b*). Various small nitrogenous solutes are significant osmolytes in the polychaete annelid *Nereis succinea* (Clark and Zounes, 1977). Betaine, glycerophosphorylcholine (GPC), sorbitol and inositol are significant osmolytes in the renal medulla of mammals (Yancey, 1988; Yancey and Burg, 1989, 1990;

Table 1. *Plasma urea concentration for a variety of anuran amphibians when normally hydrated (control) and during osmotic stress resulting from acclimation to high ambient salinity, dehydration or aestivation*

	Urea concentration ($mmol\,l^{-1}$)	
Species	Control	Osmotic stress
Bufo bufo[1]	12	10
Rana temporaria[2]	3	13
Thoropa miliaris[3]	8	13
Rana pipiens[4]	12	20
Rana esculenta[5]	2	22
*Siren intermedia**,[6]	8	32
*Siren lacertina**,[7]	8	49
*Cyclorana platycephala**,[8]	–	76
*Pyxicephalus adspersus**,[9]	13	81
Bufo woodhousei[10]	13	120
*Neobatrachus kunapalari**,[8]	8	130
Xenopus laevis[11]	7	155
*Heleioporus albopunctatus**,[8]	0	163
*Neobatrachus pelobatoides**,[8]	3	163
Ambystoma tigrinum[12]	15	182
*Neobatrachus sutor**,[8]	–	195
*Scaphiopus couchii**,[13]	33	200
Batrachoseps spp.[14]	48	218
*Cyclorana maini**,[8]	–	224
Bufo viridis[15]	32	272
Rana cancrivora (adult)[16]	40	350

Aestivating species are indicated with an asterisk.

[1]Schoffeniels and Tercafs (1965); [2]Ackrill *et al.* (1969); [3]Abe and Bicudo (1991); [4]Jungreis (1971); [5]Garcia-Romeu *et al.* (1981); [6]Asquith and Altig (1986); [7]Etheridge (1990); [8]this study; [9]Loveridge and Withers (1981); [10]Jones (1982); [11]Romspert (1976); Schlisio *et al.* (1973); [12]Romspert and McClanahan (1981); [13]McClanahan (1972); [14]Jones and Hillman (1978); [15]Katz and Gabbay (1986); [16]Gordon and Tucker (1968).

Balaban and Burg, 1987; Wolff *et al.* 1989; Bedford *et al.* 1995), while inositol, betaine and taurine are significant osmolytes in the renal medulla of birds (Lien *et al.* 1993). Trimethylamine amine (TMA) is an important buoyancy solute in a pelagic deep-sea crustacean *Notostomus gibbosus* (Sanders and Childress, 1988).

Many osmolytes have no, or negligible, effects on macromolecular function; they are compatible solutes. For example, the amino acids glycine and proline have little effect on the K_m or V_{max} of some enzymes of marine invertebrates (Bowlus and Somero, 1979). However, many osmolytes have physiological effects in addition to their role in osmotic balance. Perturbing osmolytes have deleterious effects on macromolecular structure and function. For example, urea adversely affects the catalytic action of some enzymes in some elasmobranch fishes (Rajagopalan *et al.* 1961; Yancey and Somero, 1978, 1979, 1980; Hochachka and Somero, 1984; Somero, 1986). Na^+ and K^+ adversely affect lactate dehydrogenase (LDH) activity in the polychaete worm *Nereis succinea* (Clark and Zounes, 1977). In contrast, counteracting osmolytes have stabilising effects at physiological concentrations on macromolecular structure and function, and counteract the deleterious effects of perturbing solutes. For example, methylamines (TMAO, betaine, sarcosine, GPC) and polyols (inositol, sorbitol, mannitol) are proposed to be compatible solutes. TMAO and betaine often counteract the effects of urea on enzyme V_{max} or K_m in elasmobranch fishes, at a ratio to urea of approximately 1:2 (Yancey and Somero, 1978, 1979, 1980; Hochachka and Somero, 1984; Somero, 1986). It has been suggested that small nitrogenous solutes have a counteracting role for deleterious ionic effects in a polychaete annelid worm (Clark and Zounes, 1977).

Whether any solutes other than urea, Na^+ and Cl^- are significant extracellular osmolytes in amphibians is unclear. The total osmotic concentration of plasma can be accounted for by the sum of the major inorganic ions (Na^+, Cl^-, K^+) and urea for some amphibians (*Thoropa miliaris* in the laboratory, Abe and Bicudo, 1991; *Rana cancrivora* tadpoles, Gordon and Tucker, 1965; hydrated and aestivating *Siren lacertina*, Etheridge, 1990; control and aestivating *Scaphiopus couchii*, McClanahan, 1972; salinity-acclimated *Batrachoseps* spp, Jones and Hillman, 1978). However, there is a substantial difference of more than $50\,mosmol\,l^{-1}$ for other amphibians (adult *Rana cancrivora*, Gordon and Tucker, 1968; *Bufo viridis*, Katz and Gabbay, 1986; Katz and Hoffman, 1990; *Xenopus laevis*, Romspert, 1976; *Thoropa miliaris* in the field, Abe and Bicudo, 1991), although it is not clear whether any particular solute fills a major portion of this extracellular solute gap. Little is known of the balance or role of intracellular osmolytes in amphibians.

The present study determines the extent of urea accumulation during aestivation and whether methylamines co-accumulate with urea as counteracting osmolytes for three genera of Western Australian anuran amphibians, *Cyclorana*, *Neobatrachus* and *Heleioporus*. Two of the genera (*Cyclorana*, *Neobatrachus*) are common semi-arid- and arid-zone frogs which aestivate and form a cocoon (Withers, 1993, 1995), whereas the other (*Heleioporus*) is more mesic and aestivates but apparently does not form a cocoon (Withers, 1995).

Materials and methods

We studied *Cyclorana maini* (Tyler and Martin), *Cyclorana platycephala* (Günther), *Neobatrachus pelobatoides* (Werner), *Neobatrachus kunapalari* (Mahony and Roberts), *Neobatrachus sutor* (Main) and *Heleioporus albopunctatus* (Gray). Frogs were collected opportunistically, by hand in breeding choruses or on roads, from various localities in Western Australia. They were taken to the laboratory, washed in tap water, and individually housed in plastic containers. Blood and tissues were obtained from control frogs within a few days of capture. Other frogs were induced to aestivate by maintaining them in plastic containers, without access to free water and in constant darkness. These specimens were sampled opportunistically after varying periods (range about 20–100

days) of aestivation. In addition, some specimens of *N. pelobatoides*, *N. kunapalari* and *H. albopunctatus* were allowed to aestivate for 67 days prior to blood and tissue sampling.

Blood samples were obtained from doubly pithed frogs directly from the ventricle into heparinised microhaematocrit tubes. Each sample was centrifuged to determine the haematocrit, and the plasma was separated and stored at –80 °C. A urine sample was occasionally obtained directly from the bladder of doubly pithed frogs and stored at –80 °C. Tissues, usually skeletal muscle (from the hindlimbs) but occasionally also liver, kidney, ventricle, tongue and skin, were dissected from the doubly pithed frogs, frozen immediately in liquid nitrogen and stored at –80 °C.

We also analysed plasma and muscle samples from two elasmobranch fishes, the epaulette shark (*Hemiscyllium ocellatum* Bonnaterre) and the shovel-nose ray (*Rhinobatos typus* Bennett), for comparative purposes and to verify solute analyses for the frogs.

Plasma (5 µl) was analysed for sodium and potassium concentration using a Varian 475 atomic absorption spectrophotometer, using caesium as an internal standard. Osmotic concentration was determined for a 15 µl plasma sample using a Gonotek Osmomat 030 cryoscopic osmometer. Plasma urea concentration was determined for 5 µl samples of plasma, using the hypochlorite/phenol-nitroprusside method (modified from Fawcett and Scott, 1960).

Plasma and tissues were prepared for analysis by high-performance liquid chromatography (HPLC) as follows. Tissue samples were thawed, weighed to ±0.001 g, homogenised in 2–4 ml of chilled 6 % perchloric acid (PCA) using an Ultra Turrax T25 (Junke and Kunkel), then centrifuged for 5 min at 2500 ***g***. The supernatant was removed and stored at –80 °C until analysed. A 25 or 50 µl sample of plasma or urine was added to 1 ml of chilled 6 % PCA and treated as for tissue samples. Solute concentrations were calculated for tissues as millimoles per kilogram of wet tissue mass, and for plasma and urine as millimoles per litre. Tissue solute concentrations are calculated for homogenised muscle and are not corrected to intracellular concentrations. For urea, which distributes freely across cell membranes, the intracellular and extracellular concentrations are similar. For other solutes, such as TMAO, betaine, sarcosine and GPC, which are sequestered within cells, the intracellular concentration would be approximately 15 % higher than the muscle concentration, assuming an extracellular space of approximately 15 % for muscle (e.g. Gordon, 1965; Flanigan *et al.* 1993).

The supernatant of plasma, urine or homogenised tissue was neutralised with 2.5 mol l^{-1} KOH and filtered using a Sep-Pak C_{18} cartridge. Then, 30 µl of 1 mol l^{-1} $CaCl_2$ was added to the filtrate to precipitate phosphate, which was removed using a Millipore HV 0.45 µm filter. The resultant filtrate was either analysed immediately using HPLC or stored at –80 °C for subsequent HPLC analysis.

Standard solutions (40 µmol l^{-1}) of urea, TMAO, TMA, betaine, sarcosine, inositol, glucose, lactate, mannitol and GPC were prepared in double-deionised water for HPLC analysis (all reagents from Sigma Chemicals).

HPLC analysis of the tissue samples was accomplished using a Waters 6000A solvent delivery system, U6K injector, temperature-controlled column system, 410 differential refractive index detector and data module. The mobile phase was heated, and degassed with 100 % helium. A Bio-Rad Aminex HPX-72-0 organic strong-base analysis column was used for determination of TMAO, betaine, TMA and urea, using a mobile phase of 0.15 mol l^{-1} NaOH at a flow rate of 0.6 ml min^{-1} (G. N. Somero, personal communication; Yancey, 1988). A Waters Sugar Pak I column (heated to 84 °C) was used for analysis of inositol, mannitol, GPC, urea, betaine and sarcosine, using a mobile phase of 50 mg l^{-1} calcium disodium EDTA at a flow rate of 0.3 ml min^{-1} (see Wolff *et al.* 1989).

The mininum detectable limit of solutes was about 5 picomoles, which corresponds to about 0.04 mmol l^{-1} or mmol kg^{-1}, depending on the particular solute and the sample mass.

Results

All species of *Neobatrachus* and *Cyclorana* studied were found to aestivate readily and to form a cocoon in the laboratory; in contrast, *Heleioporus albopunctatus* became less active but did not assume a water-conserving posture or form a cocoon, even after 10 weeks of restriction from water (see Withers, 1995).

Effects of aestivation on body fluid solutes

The effects of 67 days of aestivation on body fluid composition were most marked for the smallest species, *Neobatrachus pelobatoides* (Table 2), for which there was a significant (*t*-test, $P<0.05$) decline in body mass and a significant increase in haematocrit, plasma concentrations of sodium, potassium and urea, and osmotic concentration. Body mass expressed as a percentage of initial mass declined significantly for *N. kunapalari*; haematocrit did not change significantly, but the plasma concentrations of sodium and urea, and osmotic concentration, increased significantly with aestivation. Body mass expressed as percentage of initial mass declined significantly for *Heleioporus albopunctatus*, while the haematocrit, plasma concentrations of sodium and urea, and osmotic concentration, increased markedly during aestivation.

The elevation in urea concentration was much greater for all three species during aestivation than would be expected simply from dehydration, i.e. relative to the change in osmotic and ionic concentrations. Most of the increase in urea concentration with aestivation can therefore be ascribed to continued synthesis and retention.

HPLC analyses

The Bio-Rad Aminex column enabled the identification of TMAO and betaine as fast ‘sister’ peaks, and TMA, urea and inositol as progressively slower peaks. Unfortunately, the betaine and TMAO peaks were sometimes partially obscured by a large and apparently non-specific earlier peak; this was a

Table 2. *Blood haematocrit, plasma sodium, potassium, urea and osmotic concentrations and body mass for control (normally hydrated) and aestivating (after 67 days)* Neobatrachus pelobatoides, N. kunapalari *and* Heleioporus albopunctatus

	Neobatrachus pelobatoides		*Neobatrachus kunapalari*		*Heleioporus albopunctatus*	
	Control	Aestivating	Control	Aestivating	Control	Aestivating
Haematocrit (%)	33±2 (6)	45±5 (5)*	28±4 (4)	29±5 (4)^{NS}	33 (1)	39±4 (3)
Sodium (mmol l^{-1})	106±2 (6)	191±7 (6)*	119±6 (6)	149±4 (4)*	69 (1)	199±21 (3)
Potassium (mmol l^{-1})	4.2±0.4 (6)	13.9±1.3 (6)*	4.8±0.9 (6)	5.8±0.6 (4)^{NS}	–	6.5±1.4 (3)
Osmotic concentration (mosmol l^{-1})	236±8 (6)	683±31 (6)*	296±6 (6)	504±25 (4)*	230 (1)	633±15 (3)
Urea (mmol l^{-1})	2.6±0.3 (6)	163±9 (5)*	8.3±0.9 (6)	130±8 (4)*	0 (1)	163±14 (3)
Mass (g)	11.8±1.7 (6)	4.7±0.5 (6)*	16.2±2.4 (6)	13.4±1.7 (4)^{NS}	44.6 (1)	40.0±2.9 (3)
Mass (% of initial hydrated body mass)	100	67.3±1.1 (6)*	100	83.5±1.3 (5)*	100	78.1±4.4 (3)*

Values are mean ± S.E.M., with the number of observations in parentheses.
*Significant difference between control and aestivating value at $P<0.05$; NS, not significantly different.

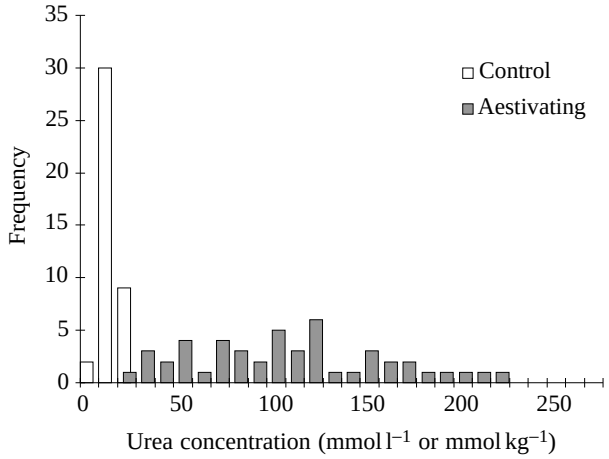

Fig. 1. Frequency distribution of plasma (mmol l^{-1}) and muscle (mmol kg^{-1}) urea concentrations measured for the various *Neobatrachus* species, *Cyclorana* species and *Heleioporus albopunctatus* frogs used in this study. The range of aestivation time was about 20–100 days.

greater problem for tissue samples than for plasma or urine. The Sugar Pak I column enabled the determination of levels of glucose, inositol, mannitol, GPC, urea, betaine and sarcosine.

Solutes accumulated by frogs

For the muscle samples of the various species examined, the urea concentration was low (<30 mmol kg^{-1}) for control, hydrated frogs whereas it often exceeded 100 mmol kg^{-1} and occasionally 200 mmol kg^{-1} for aestivating frogs (Fig. 1). The wide range of urea concentrations measured for aestivating frogs presumably reflects different periods in aestivation, about 20–100 days, as well as possible differences due to species and body mass.

Muscle of control *Cyclorana* spp had a moderate concentration of GPC (14.2 mmol kg^{-1}), low concentrations of urea (5.4 mmol kg^{-1}) and betaine (5.9 mmol kg^{-1}), and very low concentrations (<1 mmol kg^{-1}) of inositol and TMAO; neither sarcosine nor TMA was detected in any samples (Table 3). Muscle of aestivating *Cyclorana* spp had a markedly and significantly higher concentration of urea (99.2 mmol kg^{-1}), a significantly higher concentration of GPC (20.7 mmol kg^{-1}) and slightly, but not significantly, higher concentrations of inositol and TMAO; the concentration of betaine was unchanged (4.5 mmol kg^{-1}); sarcosine and TMA remained undetectable. Muscle analyses for *Neobatrachus* spp yielded similar results to those for *Cyclorana* spp, with a moderate concentration for control individuals of GPC (14.8 mmol kg^{-1}), low concentrations of urea (5.1 mmol kg^{-1}), betaine (2.1 mmol kg^{-1}), sarcosine (1.6 mmol kg^{-1}) and TMAO (0.6 mmol l^{-1}), and no detectable TMA. For aestivating *Neobatrachus* spp, the

Table 3. *Results from solute analysis of muscle from pooled specimens of* Cyclorana *and* Neobatrachus *when normally hydrated (control) and aestivating (about 20–100 days)*

	Control	Aestivating
Cyclorana		
Inositol	0.39 (2)	1.7±1.0 (10)^{NS}
GPC	14.2 (2)	20.7±4.4 (13)*
Sarcosine	0 (2)	0 (10)
TMAO	0.7 (2)	3.4±1.5 (9)^{NS}
Betaine	5.9 (2)	4.5±1.6 (9)^{NS}
TMA	0 (2)	0 (8)
Urea	5.4 (2)	99.2±18.7 (13)*
Neobatrachus		
Inositol	0.74±0.18 (12)	10.4±7.0 (7)^{NS}
GPC	14.8±3.7 (12)	14.2±4.9 (7)^{NS}
Sarcosine	1.6±0.9 (12)	11.5±4.6 (7)*
TMAO	0.62±0.44 (12)	0 (9)^{NS}
Betaine	2.1±1.3 (12)	3.3±1.0 (7)^{NS}
TMA	0 (12)	0 (9)^{NS}
Urea	5.1±1.6 (12)	118.5±9.8 (9)*

Results are given as mmol kg^{-1}.
Values are mean ± S.E.M., with the number of observations in parentheses.
NS indicates no significant difference between control and aestivating values, and an asterisk indicates a significant difference at $P<0.05$, *t*-test.

Table 4. *Plasma concentrations of solutes for* Neobatrachus *spp.*

	Control (*N*=5)	Aestivating (*N*=6)
Inositol	0.02±0.02	0.34±0.28^{NS}
GPC	0.09±0.07	0.86±0.82^{NS}
Sarcosine	0.02±0.02	0.01±0.01^{NS}
TMAO	0.86±0.86	0.10±0.10^{NS}
Betaine	0.94±0.75	0.22±0.22^{NS}
TMA	0	0
Urea	4.7±1.5	113±16*

Results are presented as mmol l^{-1}.

Values are mean ± S.E.M., with the number of observations in parentheses. NS indicates no significant difference between control and aestivating values, and an asterisk indicates a significant difference at $P<0.05$, *t*-test.

concentration of GPC did not change significantly (14.2 mmol kg^{-1}), urea concentration increased markedly and significantly (118.5 mmol kg^{-1}), betaine remained low at 3.3 mmol kg^{-1}, inositol increased but not significantly to 10.4 mmol kg^{-1}, sarcosine concentration increased but not significantly to 11.5 mmol kg^{-1}, and TMAO and TMA were not detected. A single control *Heleioporus albopunctatus* had no detectable levels of urea or other solutes, but urea concentration increased with aestivation to 94.3 mmol kg^{-1} and betaine concentration increased to 3.3 mmol kg^{-1}.

The concentrations of most solutes in plasma of *Neobatrachus* spp were much lower than those in muscle, although the sample sizes were also smaller (Table 4). For control *Neobatrachus* spp, all solutes were essentially absent (<1 mmol l^{-1}; TMA, inositol, GPC, sarcosine, TMAO, betaine), except urea (4.7 mmol l^{-1}). For aestivating *Neobatrachus* spp, all solutes were still essentially absent (<1 mmol l^{-1}), except for urea, which increased dramatically and significantly to 113 mmol l^{-1}. For aestivating *Cyclorana* spp, only urea (56±25 mmol l^{-1}, *N*=4) was detected in plasma. No plasma was analysed for control *Cyclorana* spp or *Heleioporus albopunctatus*.

Other tissues (liver, ventricle, skin, tongue) and urine samples were analysed for a few aestivating frogs. Urea, but not methylamines, was detected in liver, ventricle, skin, tongue and urine of *Cyclorana maini*, but 10–20 mmol l^{-1} betaine was detected in liver and ventricle of *C. platycephala*. Low concentrations (<20 mmol l^{-1}) of TMAO, sarcosine and betaine were detected in the liver, ventricle, tongue and skin of *Neobatrachus* spp, and urea was present at a similar concentration to those in muscle. None of these solutes, or urea, was detected in the tissues (liver, tongue, ventricle, skin) or urine of a control *H. albopunctatus*, and only urea was present in the tissues (liver, ventricle, skin) of aestivating *H. albopunctatus* (2 mmol l^{-1} betaine was detected in the urine of one *H. albopunctatus*).

The polyol mannitol was occasionally detected at low concentrations, and a peak probably corresponding to sorbitol (based on the standards of Wolff *et al.* 1989) was also occasionally detected, at low concentrations. Neither polyol was quantitatively analysed owing to its low and variable concentration.

Solutes accumulated by fishes

Plasma of the shovel-nosed ray *Rhinobatos typus* had substantial concentrations of TMAO (50±3 mmol l^{-1}, *N*=5, mean ± S.E.M.) and urea (317±33 mmol l^{-1}, *N*=5) but no detectable betaine or TMA. A single sample of muscle from a shovel-nosed ray contained TMAO (53 mmol kg^{-1}), betaine (77 mmol kg^{-1}) and urea (250 mmol kg^{-1}), but no detectable TMA. Similar results were obtained for plasma of the epaulette shark *Hemiscyllium ocellatum* (*N*=1), which contained substantial levels of TMAO (55 mmol l^{-1}) and urea (164 mmol l^{-1}), but no betaine or TMA. A muscle sample (*N*=1) from an epaulette shark contained TMAO (60.3 mmol kg^{-1}), betaine (63 mmol kg^{-1}) and urea (147 mmol kg^{-1}), but no detectable TMA.

Discussion

Urea accumulates to high concentrations in the plasma and tissues of aestivating *Cyclorana* and *Neobatrachus* species (Tables 3, 4), as has been reported previously for other aestivating amphibians (see Table 1). The urea that accumulates during aestivation must be the result of continued urea synthesis and an absence of excretion (cocooned frogs cannot void urine), because the moderate dehydration that accompanies aestivation would not increase the urea concentration by even a factor of 2. The plasma sodium concentration of *Neobatrachus* and *Cyclorana* species increased slightly (about 1.3- to 1.8-fold) with aestivation (Table 2) owing to this minor concentrating effect of dehydration. The plasma potassium concentration tended to increase to a greater extent (1.2- to 3.3-fold) than the plasma sodium concentration, presumably because of some redistribution of K^+ from the intracellular to the extracellular space, in addition to the effects of dehydration.

Whether the urea that accumulates during aestivation has any beneficial or detrimental physiological role for these Western Australian frogs is not known. Urea has been shown to accumulate at an accelerated (not just control) rate in *Scaphiopus couchii* and influences the distribution of water between this aestivating frog and the soil (McClanahan, 1972; Jones, 1980). *Heleioporus albopunctatus*, which like *S. couchii* aestivates but does not form a cocoon, also accumulates urea, perhaps also to maintain a favourable water potential gradient. Whether urea accumulation contributes to a favourable distribution of water for cocoon-forming *Neobatrachus* or *Cyclorana* species (since the cocoon forms a considerable barrier to water exchange) and whether these frogs also accelerate urea synthesis during aestivation is not known.

The frogs investigated in this study accumulate a high concentration of urea (100–200 mmol l^{-1} or mmol kg^{-1}) during aestivation, but they do not appear to co-accumulate a substantial

concentration of any particular counteracting methylamine or polyol osmolyte (Tables 3, 4). Specifically, three methylamines that might be expected to accumulate as a counteracting solute (TMAO, betaine, sarcosine) are not present at appreciable concentrations during aestivation. Polyols are also apparently not significant osmolytes in these aestivating frogs. Inositol is present at low concentrations; low concentrations of mannitol, and presumably sorbitol, were occasionally detected.

The near absence of TMAO in aestivating frogs is significant. TMAO is present in chondrichthyean fishes at a higher concentration than the other methylamines (betaine, sarcosine; Holmes and Donaldson, 1969; Withers *et al.* 1994*a*,*b*; present study) and has a major role as a balancing osmolyte and a counteracting solute. TMAO is also present at high concentrations (10–200 mmol l^{-1}) in hagfish, teleost fishes (particularly the marine species) and some marine invertebrates (Dyer, 1952; Love, 1980; Yancey *et al.* 1982; Anthoni *et al.* 1989; present study), despite their low urea concentrations, and so TMAO presumably has a role as a balancing osmolyte and perhaps as a counteracting solute (to Na^+ and Cl^-) in these animals. However, TMAO clearly has no significant role either as a compatible or as a counteracting solute in aestivating frogs. TMAO might be the preferred methylamine of aquatic species because of its role in buoyancy (Withers *et al.* 1994*a*,*b*).

Similarly, the low concentration of betaine in aestivating frogs is perhaps even more surprising than the absence of TMAO. This absence of a significant role for betaine as either a balancing osmolyte or a counteracting intracellular osmolyte was unexpected, given the very common role of betaine as an important intracellular balancing osmolyte and counteracting solute in diverse animals as well as plants and procaryotic organisms (Holmes and Donaldson, 1969; Robertson, 1980; Yancey *et al.* 1982; Yancey, 1988; de Meis and Inesi, 1988; Yancey and Burg, 1989; Wolf *et al.* 1989; Anthoni *et al.* 1991; Dragolovich and Pierce, 1992; Yeong-Hau *et al.* 1993; Withers *et al.* 1994*a*,*b*; Coelho-Sampaio *et al.* 1994; Sola-Penna *et al.* 1995; Pierce *et al.* 1995; Shinagawa *et al.* 1995; Bedford *et al.* 1995; present study). Aestivating frogs do not need to accumulate betaine as a balancing osmolyte because they do not have to balance their body fluid osmotic concentration against an external fluid medium, in contrast to marine osmoconforming animals which require specific intracellular osmolytes (e.g. betaine, amino acids) to balance the high external osmotic concentration (due mainly to Na^+ and Cl^-). Betaine also appears to be an unimportant osmolyte in an aquatic amphibian during hypersaline stress; during hyperosmotic acclimation (to 300 mosmol l^{-1} NaCl), the aquatic frog *Xenopus laevis* shows only a minor increase in betaine levels (to about 5 mmol l^{-1}; Wray and Wilkie, 1995). Despite not being needed as a balancing osmolyte, betaine might still be expected to play a role as a major counteracting solute in aestivating frogs, but this is clearly not the case. Muscle homogenates of the aestivating frogs had a betaine concentration of only 2–6 mmol l^{-1} (Table 3). Aestivating snails also have only very low concentrations of methylamines, primarily betaine (Rees and Hand, 1993).

The moderate concentration of GPC (14–20 mmol kg^{-1}) in muscle of both hydrated and aestivating frogs indicates a possible minor role as an intracellular osmolyte. GPC has a strong urea-counteracting role in mammalian renal medulla (MDCK) cells (Subramaniam and Jackson, 1992) and is a significant osmolyte (3–125 mmol kg^{-1}) in the renal medulla of mammals (Balaban and Burg, 1987; Yancey, 1988; Yancey and Burg, 1989; Bedford *et al.* 1995) but not of birds (1 mmol kg^{-1}; Lien *et al.* 1993).

Urea-accumulating aestivating frogs do not co-accumulate substantial concentrations of any particular methylamine (TMAO, betaine, sarcosine) or polyol (inositol, mannitol, sorbitol) as a compatible or counteracting solute. However, a combination of various non-urea organic osmolytes (e.g. methylamines and polyols) might provide a counteracting osmolyte role. For aestivating *Cyclorana* spp, the total concentration of inositol + GPC + sarcosine + TMAO + betaine + TMA is approximately 35 mmol kg^{-1} compared with approximately 21 mmol kg^{-1} for hydrated frogs, but the difference is not significant. For *Neobatrachus* spp, the corresponding values are 43 and 23 mmol kg^{-1} respectively, but again the difference is not significant. For muscle of aestivating frogs, the ratio of these summed osmolytes to urea is approximately 1:2.5 (if corrected to intracellular concentrations, the ratio would be approximately 1:2.2), which is close to the ratio of 1:2 for methylamines:urea that is reported to be optimal in many elasmobranch fishes and mammals (e.g. Yancey and Somero, 1979, 1980; Hochachka and Somero, 1984; Yancey, 1988). However, the ratio of summed osmolytes to urea is about 1:0.3 for muscle of hydrated *Cyclorana* spp and 1:0.2 for *Neobatrachus* spp, and so these solutes may either have other roles that preclude their levels being reduced to maintain the ‘optimal’ 1:2 ratio for non-aestivating frogs, or their concentrations may be kept high in preparation for aestivation if they do have a counteracting role. In any case, the high ratio of these presumed counteracting solutes to urea in non-aestivating frogs presumably has some perturbing effects on macromolecules in the absence of urea.

The low concentration of counteracting solutes in aestivating frogs, particularly of TMAO and betaine, might reflect a different urea-sensitivity of their enzymes compared with those of elasmobranch fishes (Rajagopalan *et al.* 1961; Yancey and Somero, 1978, 1979, 1980) or provide an intrinsic mechanism (Hand and Somero, 1982; Yancey *et al.* 1982; Hochachka and Somero, 1984) for the substantial metabolic depression that is observed in these frogs during aestivation (Withers, 1993). The absence of counteracting solutes would only provide an intrinsic mechanism for metabolic depression if the enzymes of these aestivating frogs experience inimical biochemical consequences even at moderate urea concentrations (since metabolic depression is essentially complete after 4 weeks from the onset of aestivation, whereas the urea concentration would presumably increase

progressively over time). Grundy and Storey (1994) reported that urea and TMAO have no, or only minor, effects on the kinetic properties and maximal enzymatic activities of two muscle enzymes (pyruvate kinase and phosphofructokinase) in the aestivating spadefoot toad *Scaphiopus couchii* and a semi-aquatic frog *Rana pipiens*, although spadefoot toads naturally accumulate a substantial concentration of urea during aestivation. Thus, the biochemical effects of urea on protein structure and function, and the role of methylamines as counteracting osmolytes, may differ fundamentally between urea-accumulating frogs and ureo-osmoconforming elasmobranch fishes.

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