Hydrated body-fluid osmolality values for species of *Cyclorana*

S J REYNOLDS

School of Environmental and Life Sciences, Charles Darwin University, Darwin, NT 0909, Australia ✉ *steve.reynolds@cdu.edu.au*

A hydration procedure for frogs is described that involves two episodes of bladder emptying and rehydration prior to determining hydrated body-fluid osmolality values. The mean osmolality of hydrated *Cyclorana* species was similar for *C. australis* (lymph 225 mOsm/kg, urine 31 mOsm/kg), *C. longipes* (lymph 211 mOsm/kg, urine 40 mOsm/kg), *C. cultripes* (lymph 213 mOsm/kg, urine 14 mOsm/kg)and *C. platycephala* (lymph 214 mOsm/kg, urine 21 mOsm/kg). These standardised values provide a baseline for comparison with dehydrated and active frogs. Using this method the investigator can accurately determine standard mass (the mass of a hydrated frog with an empty bladder), which requires two or more weight measurements.

KEYWORDS: burrowing frog, *Cyclorana*, hydration, lymph, osmolality, plasma, urea, urine.

INTRODUCTION

Determining the hydrated state of a frog is important in providing a baseline or comparison value in studies relating to hydration state. This is essential in studies of the effect of dehydration on the concentration of osmolytes (Shoemaker 1964; Hillman 1978), locomotor performance (Beuchat *et al*. 1984; Preest & Pough 1989) and dehydration tolerance (Thorson 1955; Hillman 1980). Various authors have compared hydration of frogs in response to salinity (Gordon *et al.* 1961; Ruibal 1962a), periods of underground aestivation (McClanahan 1972; Katz 1989; Cartledge *et al.* 2006, 2008; Reynolds *et al.* 2011) and seasonal availability of moisture (Reynolds & Christian 2009).

The genus *Cyclorana* includes 13 species of fossorial hylids (subfamily Pelodryadinae) in northern and arid Australia (Tyler & Knight 2009). Species of *Cyclorana* undergo seasonal dormancy, have capacious bladders and develop relatively impermeable cocoons (Withers 1993, 1995; Christian & Parry 1997; Tracy *et al*. 2007; Reynolds 2011). This study details a standard hydration procedure and the resultant body-fluid osmolality values obtained for four species of *Cyclorana* from monsoonal northern Australia. The procedure also allows the investigator to measure standard mass (SM), i.e. the weight of a hydrated frog with an empty bladder (Ruibal 1962b).

MATERIALS AND METHODS

All animals were obtained from the northern monsoonal region of the Northern Territory of Australia: *Cyclorana australis* from within a 50 km radius of Darwin (12°27'50"S, 130°50'30"E), *C. longipes* from Mickett Creek (12°24'28"S, 130°56'43"E) east of Darwin, *C. cultripes* from between Daly Waters (16°15'15"S, 133°22'12"E) and Newcastle Creek (17°15'45"S, 133°27'10"E), and C. *platycephala* from 5–15 km south of Dunmarra (16°40'46"S, 133°24'46"E). Animals were returned to the laboratory and maintained in ventilated containers (18 x 12 x 8 cm) and supplied with moist paper towels. They were fed 1–2 times a week when active. The last feed was at least five days before the procedure. Two groups of *C. australis* were used. Members of the first group (n = 12; sampled February 2008) were kept in the laboratory for 3–4 weeks and the second group for 3–4 months (n = 6; sampled July 2009) before the hydration procedure. *Cyclorana longipes* (n = 5; sampled March2009) were maintained in the laboratory for 1–2 months, *C. cultripes* (n = 4; sampled March 2008) for 4–5 days, and *C. platycephala* (n = 9; sampled April2009) for 1–2 months.

The hydration procedure required three consecutive days. At each weighing, excess fluid on the skin was removed by blotting with paper towels and the animals were weighed to the nearest 0.01 g on an electronic balance. On the first day each frog was weighed, the bladder fluid was drained, and then the frog was reweighed (SM0). The frogs were placed in a tilted container with aged tap water at one end (water access) and were left overnight. The following morning they were weighed, and the first standard mass measurement (SM1) was made by applying pressure on the area overlying the bladder, and by cannulating the cloaca with a piece of thin flexible tubing, or in smaller frogs (*C. longipes* and *C. cultripes*) with a pipette tip. After removing the bladder fluid, the frog was weighed and placed in shallow water (~5 mm depth) in a level container so that the ventral surface was in contact with the water. The next day SM was measured a second time (SM2), and lymph and urine were collected as the samples for analysis. The lowest mass recorded was used as the SM. Lymph was extracted from the femoral lymph sac with a fine gauge needle (Terumo U-100 1.0 mL 27G) as described by Reynolds *et al.* (2009). Osmolality was measured with an Advanced Instruments (freezing-point) Micro-Osmometer (Model 3300) and the BUN reagent (Thermo Electron TR12015) spectrophotometric assay was used to measure urea concentrations. One-way ANOVA was used to test the equality of means.

RESULTS

The osmolality of plasma and urine for the two groups of *C. australis* were similar (plasma osmolality $F_{1,16} = 0.32$, P = 0.58; urine osmolality $F_{1.16} = 1.63$, P = 0.22) so the values

were pooled for further analyses. Body fluid osmolality was similar for the four species of *Cyclorana* when hydrated (Table 1). Differences between species for the measured parameters were not significant with the exception of urine osmolality (plasma osmolality $F_{3,31}$ = 2.77, P = 0.058; plasma urea $F_{3,19}$ = 1.16, P = 0.35; urine osmolality $F_{3,32}$ = 3.37, P = 0.03; urine urea $F_{3,20}$ = 2.35, P = 0.10). Paired comparisons of means (Tukey HSD) for the significant result failed to detect a difference between species. Across all individuals of all species urea contributed 0.5–4.8% of plasma osmolality and 29–72% of urine osmolality.

Standard mass (range) was 26.5–60.1 g for *C. australis,* 5.4–9.7 g for *C. longipes,* 7.7–11.2 g for *C. cultripes* and 14.9–56 g for *C. platycephala.* Generally the mass after the bladder was drained the first time (SM0) was greater than the SM1 and SM2 measurements (Table 2). The exception was *C. cultripes* where SM0 was the lowest standard mass measurement. The SM2 measurement was noticeably lower in *C. platycephala.* The standard mass obtained after an overnight period in water (SM2) was the lowest mass for 14 of 18 *C. australis,* 4 of 5 *C. longipes,* 0 of 4 *C. cultripes* and 8 of 9 *C. platycephala.* Between measurements there was an increase in mass due to storage of fluid (water) in the bladder.

Table 1 Body fluid osmolality (mOsm/kg) and urea concentrations (mmol/L) for species of *Cyclorana* following the hydration procedure.

Species		Lymph	Urine
C. australis	Osmolality	225.2±15.2 (18)	31.3±13.7 (18)
	Urea	6.3±3.7 (6)	22.3±14.7 (6)
C. longipes	Osmolality	211±7.5 (5)	40±28.3 (5)
	Urea	4.4±1.8 (5)	25.2±21 (5)
C. cultripes	Osmolality	212.8±3.7 (4)	14±2.2 (4)
	Urea	3.7±0.8 (4)	7.6±3.3 (4)
C. platycephala	Osmolality	214.1±11.2 (8)	21.3±5.4 (9)
	Urea	5.8±2.5 (8)	12.1±4.4 (9)

Values are mean ± standard deviation (sample size in brackets).

Table 2 Variability in initial bladder empty mass (SM0), standard mass with water access (SM1) and standard mass for frogs in water overnight (SM2) on consecutive days for species of *Cyclorana.*

Species	*n*		SM0	SM1	SM2
C. australis	18	Mean	1.26	-0.28	-0.98
		SD	1.25	0.98	0.69
C. longipes	5	Mean	0.22	0.09	-0.31
		SD	0.14	0.10	0.21
C. cultripes	4	Mean	-0.32	0.18	0.15
		SD	0.33	0.23	0.22
C. platycephala	9	Mean	2.00	0.54	-2.54
		SD	1.85	1.14	2.11

The values are the average of the measurement (in grams) minus the average of the three values for each individual frog. SD, standard deviation.

DISCUSSION

Amphibians attain a fully hydrated state when water is readily available, generally from moist soil or directly from surface water. The mean plasma osmolality for *Cyclorana* species in this study varied from 211 to 225 mOsm/kg, and urine osmolality was from 14 to 40 mOsm/kg. Urea comprised <5% of the solute concentration of plasma and up to 72% of that of urine. In comparison, body-fluid osmolality values for hydrated frogs from the literature are generally higher, with some exceptions (Table 3). The methodology used to hydrate frogs will affect the osmolality values obtained, so that placing frogs on moist soil overnight (Cartledge *et al.* 2008) may be insufficient to reduce the osmolality of the body fluids to fully hydrated levels. Konno *et al.* (2005) also found that plasma osmolality was higher (241 mOsm) on moist soil with water access than when frogs were hydrated in water (222 mOsm). Similar hydrated osmolality values were obtained for *Rhinella marina* by Reynolds & Christian (2009). Hence, I conclude that it is necessary to place frogs in water overnight to ensure full hydration.

On the basis of this study and others where frogs were placed in water rather than on moist soil or with access to water, the plasma osmolality of anurans when hydrated is ~210–230 mOsm (Table 3). Reported values for *Bufo viridis* are consistently high and this species may be an exception. McClanahan (1972) did not provide details of his hydration procedure other than to indicate that he followed the method of Ruibal (1962b) in determining standard weights, hence I cannot comment on the value for *S. couchii*. Osmolality is useful as an index of hydration state when compared with hydrated values (Minnich 1982), and frogs that become dehydrated in the dry season attain osmolality values that are clearly elevated (plasma >300 mOsm) when compared to hydrated frogs (Reynolds & Christian 2009; Reynolds *et al.* 2011). Similarly, plasma osmolality of the aquatic frog *Xenopus laevis* is ~230 mOsm when hydrated and exceeds 400 mOsm when dehydrated (Hillman 1978).

Species of *Cyclorana* in monsoonal northern Australia are largely terrestrial in the wet season and fossorial in the dry season (Tyler *et al.* 1983; Tracy *et al.* 2007; Reynolds 2011). *Cyclorana australis* and *C. longipes* spend extended periods in the water or on saturated soils during the breeding season (S J Reynolds unpubl. data),

Table 3 Plasma osmolality (mOsm/kg) and urea concentrations (mmol/L) reported for a selection of hydrated anuran species.

Species	Osmolality	Urea	Reference
Scaphiopus couchii	301	33	McClanahan (1972)
Bufo viridis	332	22	Shpun *et al.* (1992)
Notaden nichollsi	266	20	Cartledge *et al.* (2006)
Neobatrachus aquilonius	220	7	Cartledge *et al.* (2006)
Cyclorana platycephala	250	4	Cartledge *et al.* (2008)
Cyclorana australis	222	12	Reynolds (2011)
Rhinella marina	228	15	Reynolds & Christian (2009)
Rhinella marina	222	9	Konno *et al.* (2005)

and *C. platycephala* is largely aquatic during wet weather (Robinson 1989; McMaster 2006; S J Reynolds unpubl. data). These periods in wet environments are likely to result in full hydration as outlined in this study. In addition, during the early stages of burrowing, mean plasma (214 mOsm) and urine (35 mOsm) osmolality of *C. australis* were similar to hydrated values reported here (Reynolds *et al*. 2011). Fossorial frogs are fully hydrated and have full bladders in these initial stages of burrowing.

The osmolality and solute composition of anuran lymph is generally equivalent to blood-plasma (Reynolds *et al*. 2009) and various authors (Ruibal 1962a; McClanahan 1972) have used plasma or lymph interchangeably. This is due to high endothelial permeability and comparatively rapid fluid exchange in frogs (Hillman *et al.* 2004). Thus, the hydrated values reported here are comparable with previous studies.

The procedure described here involves emptying the bladder twice prior to determining body-fluid osmolality. However, larger frogs (>100g) may require an additional bladder emptying episode to ensure that excess waste products (principally urea) are voided. Some consideration must be given to the condition of the animal prior to the procedure, as dehydrated frogs will accumulate osmolytes in the plasma (Shoemaker 1964; Hillman 1978). The condition of the body fluids prior to the hydration procedure will differ in the wet and dry season, and the osmolality of frogs obtained from burrows will vary with duration underground (Cartledge *et al.* 2008; Reynolds *et al.* 2011). Feeding prior to the hydration procedure may also influence solute concentrations, because urea will accumulate in the body fluids after a high protein meal. By allowing a period of five days following feeding and emptying the bladder twice, the frogs become fully hydrated. The two groups of *C. australis* tested had similar plasma and urine osmolalities, which suggests that the results obtained using this method are repeatable.

In addition to hydrating the frogs this procedure provides an accurate estimate of standard mass, which requires two or more measurements (Ruibal 1962b). Species of *Cyclorana* accumulated fluid in the bladder overnight, and excess solutes were removed when the bladder was emptied. In general the SM2 measurement was the lowest, suggesting that the frogs were fully hydrated at this time.

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REFERENCES

Beuchat C A, Pough F H & Stewart M M 1984. Response to simultaneous dehydration and thermal stress in three species of Puerto Rican frogs. *Journal of Comparative Physiology B***154**, 579–585.

Cartledge V A, Withers P C & Bradshaw S D 2008. Water balance and arginine vasotocin in the cocooning frog *Cyclorana platycephala* (Hylidae). *Physiological and Biochemical Zoology* **81**, 43–53.

Cartledge V A, Withers P C, McMaster K A, Thompson G G & Bradshaw S D 2006. Water balance of field-excavated aestivating Australian desert frogs, the cocoon-forming *Neobatrachus aquilonius* and the non-cocooning *Notadennichollsi* (Amphibia: Myobatrachidae). *Journal of Experimental Biology* **209**, 3309–3321.

Christian K & Parry D 1997. Reduced rates of water loss and chemical properties of skin secretions of the frogs *Litoriacaerulea* and *Cycloranaaustralis*. *Australian Journal of Zoology* **45**, 13–20.

Gordon M S, Schmidt-Nielsen K & Kelly H M 1961. Osmotic regulation in the crab-eating frog (*Rana cancrivora*). *Journal of Experimental Biology* **38**, 659–678.

Hillman S S 1978. Some effects of dehydration on internal distributions of water and solutes in *Xenopus laevis*. *Comparative Biochemistry and Physiology A* **61**, 303–307.

Hillman S S 1980. Physiological correlates of differential dehydration tolerance in anuran amphibians. *Copeia* **1980**, 125–129.

Hillman S S, Hedrick M S, Withers P C & Drewes R C 2004. Lymph pools in the basement, sump pumps in the attic: the anuran dilemma for lymph movement. *Physiological and Biochemical Zoology* **77**, 161–173.

Katz U 1989. Strategies of adaptation to osmotic stress in anuran amphibians under salt and burrowing conditions. *Comparative Biochemistry and Physiology A* **93**, 499–503.

Konno N, Hyodo S, Takei Y, Matsuda K & Uchiyama M 2005. Plasma aldosterone, angiotensin II, and arginine vasotocin concentrations in the toad, *Bufo marinus*, following osmotic treatments. *General and Comparative Endocrinology* **140**, 86–93.

McClanahan L Jr 1972. Changes in body fluids of burrowed spadefoot toads as a function of soil water potential. *Copeia* **1972**, 209–216.

McMaster K A 2006. Ecophysiology of Australian cocooning and non-cocooning, burrowing, desert frogs. PhD thesis, University of Western Australia, Perth (unpubl.).

Minnich J E 1982. The use of water. *In:* Gans C ed. *Biology of the Reptilia. Vol. 12*, pp. 325–395. Academic Press, London.

Preest M R & Pough F H 1989. Interaction of temperature and hydration on locomotion of toads. *Functional Ecology* **3** 693–699.

Reynolds S J 2011. Body fluid osmolality and hydric relations of frogs from monsoonal northern Australia. PhD thesis, Charles Darwin University, Darwin (unpubl.).

Reynolds S J & Christian K A 2009. Environmental moisture availability and body fluid osmolality in introduced toads, *Rhinellamarina*, in monsoonal northern Australia. *Journal of Herpetology* **43**, 326–331.

Reynolds S J, Christian K A & Tracy C R 2009. Application of a method for obtaining lymph from anuran amphibianns. *Journal of Herpetology* **43**, 148–154.

Reynolds S J, Christian K A & Tracy C R 2011. Changes in body fluids of the cocooning fossorial frog *Cyclorana australis* in a seasonally dry environment. *Comparative Biochemistry and Physiology A* **160**, 348–354.

Robinson M 1989. Comparison of feeding behaviours of the aquatic Australian hylid frogs *Litoria dahlii* (Boulenger, 1896) and *Cyclorana platycephalus* (Gunther, 1873) and the terrestrial hylid frog *Cyclorana novaehollandiae* (Steindachner, 1867). *Herpetofauna* **19**, 8–25.

Ruibal R 1962a. Osmoregulation in amphibians from heterosaline habitats. *Physiological Zoology* **35**, 133–147.

Ruibal R 1962b. The adaptive value of bladder water in the toad, *Bufo cognatus*. *Physiological Zoology* **35**, 218–223.

Shoemaker V H 1964. The effects of dehydration on electrolyte concentrations in a toad, *Bufo marinus*. *Comparative Biochemistry and Physiology* **13**, 261–271.

Shpun S, Hoffman J & Katz U 1992. Anuran amphibia which are not acclimable to high salt, tolerate high plasma urea. *Comparative Biochemistry and Physiology A* **103**, 473–477.

Thorson T B 1955. The relationship of water economy to terrestrialism in amphibians. *Ecology* **36**, 100–116.

Tracy C R, Reynolds S J, McArthur L J, Tracy C R & Christian K A 2007. Ecology of aestivation in a cocoon-forming frog, *Cyclorana australis* (Hylidae). *Copeia* **2007**, 901–912.

Tyler M J, Crook G A & Davies M 1983. Reproductive biology of the frogs of the Magela Creek System, Northern Territory. *Records of the South Australian Museum* **18**, 415–440.

Tyler M J & Knight F 2009. *Field guide to the frogs of Australia*. CSIRO Publishing, Collingwood.

Withers P C 1993. Metabolic depression during aestivation in the Australian frogs, *Neobatrachus* and *Cyclorana*. *Australian Journal of Zoology* **41**, 467–473.

Withers P C 1995. Cocoon formation and structure in the aestivating Australian desert frogs, *Neobatrachus* and *Cyclorana*. *Australian Journal of Zoology* **43**, 429–441.

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