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Water balance of field-excavated aestivating Australian desert frogs, the cocoon-forming *Neobatrachus aquilonius* and the non-cocooning *Notaden nichollsi* (Amphibia: Myobatrachidae)

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Summary

Burrowed aestivating frogs of the cocoon-forming species *Neobatrachus aquilonius* and the non-cocooning species *Notaden nichollsi* were excavated in the Gibson Desert of central Australia. Their hydration state (osmotic pressure of the plasma and urine) was compared to the moisture content and water potential of the surrounding soil. The non-cocooning *N. nichollsi* was consistently found in sand dunes. While this sand had favourable water potential properties for buried frogs, the considerable spatial and temporal variation in sand moisture meant that frogs were not always in positive water balance with respect to the surrounding soil. The cocoon-forming *N. aquilonius* was excavated from two distinct habitat types, a claypan in which frogs had a well-formed cocoon and a dune swale where frogs did not have a cocoon. Cocoons of excavated frogs ranged in thickness from 19.4 μm to 55.61 μm and consisted of 81–229 layers. Cocooned claypan *N. aquilonius* were nearing exhaustion of their bladder water reserves and had a urine osmolality approaching that of the plasma. By contrast, non-cocooned *N. aquilonius* from the dune swale were fully hydrated, although soil moisture levels were not as high as calculated to be necessary to maintain water balance. Both species had similar plasma arginine vasotocin (AVT) concentrations ranging from 9.4 to 164 pg ml^{-1}, except for one cocooned *N. aquilonius* with a higher concentration of 394 pg ml^{-1}. For both species, AVT showed no relationship with plasma osmolality over the lower range of plasma osmolalities but was appreciably increased at the highest osmolality recorded. This study provides the first evidence that cocoon formation following burrowing is not obligatory in species that are capable of doing so, but that cocoon formation occurs when soil water conditions are more desiccating than for non-cocooned frogs.

Key words: arid, cocoon, dehydration, desert frog, water balance, water potential.

Introduction

A number of studies have addressed the ability of arid-adapted frogs to maintain water balance in the laboratory, but very little is known of the water balance status of frogs aestivating in the field. This reflects the difficulty of finding frogs burrowed in remote arid areas. For Australian frogs, the cocoon-forming *Cyclorana platycephala* has been excavated in the field at intervals of up to 100 days after the commencement of aestivation, and it is estimated that they can aestivate continuously for periods of 2–3 years, utilising their urine volume as a water reserve (van Beurden, 1977; van Beurden, 1982). The North American spadefoot toads *Scaphiopus couchii* and *S. hammondii* have been excavated in the field soon after burrowing, and after seven and 10 months of aestivation (Shoemaker et al., 1969). Plasma osmolality increases from 300 mOsm kg^{-1} (when soil moisture content is high) to 420 mOsm kg^{-1} as the soil dries to a water potential equivalent to 600 mOsm kg^{-1}. In the laboratory, plasma osmolality increased from 300 mOsm kg^{-1} to 460 mOsm kg^{-1} for *S. couchii* kept in soil with a water potential equivalent to 450 mOsm kg^{-1}, so maintaining a favourable 10 mOsm kg^{-1} osmotic gradient with the soil (McClanahan, 1972). Under laboratory simulated natural conditions, the terrestrial toad *Bufo viridis* increases plasma osmolality from 400 mOsm kg^{-1} to 752 mOsm kg^{-1} following 82 days in soil (frogs were either partially or fully burrowed) (Katz and Gabbay, 1986).

Cocoon-forming frog species are relatively common in the arid interior of Australia (Cogger, 2000). Cocoon formation

was first described for a number of Australian species (Lee and Mercer, 1967), and it now appears that probably all members of the genera *Neobatrachus* and *Cyclorana* form a cocoon (Withers, 1995). Cocoon formation has also been described for seven other frog species in North America (Ruibal and Hillman, 1981), Central America (McDiarmid and Foster, 1987), South America (McClanahan et al., 1976) and Africa (Loveridge and Crayé, 1979; Grafe, 2000). Cocoon structure is similar among all species; the cocoon is formed by the accumulation of layers of shed epidermis that are normally eaten when the frog is active (McClanahan et al., 1976; Ruibal and Hillman, 1981; Withers, 1995). Frog cocoon is an effective barrier to evaporative water loss; the continuous addition of skin layers to the cocoon causes an exponential reduction in water loss for *Lepidobatrachus llanensis* (McClanahan et al., 1976) and *Neobatrachus* spp. and *Cyclorana* spp. (Withers, 1998a). Evaporative water loss (EWL) of cocooned frogs is reduced to 6.5–32% of non-cocooned rates for *Neobatrachus* spp. and 0.8–38% for *Cyclorana* spp. (Withers, 1998a). While the cocoon reduces water loss, it also presumably impedes water uptake from soil, so cocooned frogs would be more reliant than non-cocooned frogs on stored body water.

The water balance of burrowing frogs that do not form a cocoon is more linked to the hygric properties of the surrounding soil than for cocoon-forming species. The water potential of this soil affects the frog's capacity both to absorb water and to reduce transcutaneous loss of body water. Non-cocooning Australian species mostly burrow in sandy soils, e.g. *Heleioporus* spp., *Notaden nichollsi*, *Arenophryne rotunda*, *Myobatrachus gouldii* (Bentley et al., 1958; Packer, 1963; Slater and Main, 1963; Tyler et al., 1980; Tyler et al., 2000; Paltridge and Nano, 2001; Thompson et al., 2005; Cartledge et al., 2006). As a burrowing medium, sand has the advantage for non-cocooning species of high water potential at relatively low moisture content, facilitating water absorption by a burrowed frog. For example, the sandhill frog *Arenophryne rotunda* can maintain water balance in sand with a gravimetric water content of only 1–2% (Cartledge et al., 2006). *Scaphiopus couchii* burrow in soil consisting of a higher proportion of fine particles, which correspondingly requires higher moistures to generate water potentials favourable for water uptake by the frog (McClanahan, 1972). In soil moistures as high as 4–5%, frogs cannot maintain water balance in this soil type and produce urea to increase the water potential of the body fluids and maintain a favourable osmotic gradient with the surrounding soil (McClanahan, 1972; Jones, 1980). Cocoon-forming species have been reported to burrow in clay (Bentley et al., 1958; Lee and Mercer, 1967; van Beurden, 1982) but non-cocooning frogs appear to be excluded from this soil type, suggesting that they are not able to extract adequate moisture from the soil while burrowed.

The ability of frogs to maintain water balance is also under hormonal control; arginine vasotocin (AVT) is the principal antidiuretic hormone in lower vertebrates including frogs (Dantzler, 1967; Bakker and Bradshaw, 1977; Pang, 1977; Bradshaw and Rice, 1981). In anuran amphibians, the three major organs regulating water flux – the skin, bladder and kidneys – are all influenced by AVT (Pang, 1977). AVT increases the water permeability of both the skin and urinary bladder (Pang, 1977), reduces the glomerular filtration rate and increases tubular reabsorption in the kidney (Bentley, 2002). Aquaporins sensitive to AVT have been identified in the skin, bladder and kidney (Hasegawa et al., 2003). However, the stimulus for AVT release varies among species. For *Bufo marinus*, an increase in plasma osmolality is sufficient to increase plasma AVT (Konno et al., 2005), while for *Rana ridibunda* only an increase in osmolality accompanied by haemorrhage/hypovolaemia elicits increases in AVT (Nouwen and Kühn, 1985). There have been no measurements of AVT in aestivating frogs. For cocooned aestivating frogs, since maintaining water balance for an extended period is primarily a function of economical bladder water use, AVT's effects of reducing glomerular filtration rate and increasing bladder permeability could be beneficial and extend the period of water balance. For non-cocooning burrowing species, such as *N. nichollsi*, it is more difficult to predict a role for AVT. Dehydration by loss of water occurs when the soil water potential falls below the osmotic potential of the frog's body fluids; increasing cutaneous permeability in this situation would be of little benefit given that the water potential gradient does not favour water uptake.

This study describes the water balance strategies of two desert frog species when burrowed in the field: the cocoon-forming *Neobatrachus aquilonius* and the non-cocooning *N. nichollsi.* Specific aims were to (1) assess the hydration state of burrowed frogs, (2) determine if frogs were in positive or negative water balance based on the hygric properties of the soil, (3) investigate the relationship between AVT and plasma osmolality for excavated frogs and (4) examine the number of layers comprising the cocoons of field-excavated *N. aquilonius* to investigate whether layer number can predict the time period of aestivation (Withers, 1995).

Materials and methods

Field excavations

We were fortunate to have the assistance of Aboriginal elders with extensive knowledge of where frogs were burrowed in the Gibson Desert and who could indicate the location of frog tunnels from surface features. Detailed information on excavation and ecology of burrowed frogs for this study is provided by Thompson et al. (Thompson et al., 2005). We visited Kiwirrkurra, in the Gibson Desert (22°49′S, 127°47′E) in late June 2003 and excavated six cocooned *Neobatrachus aquilonius* (Tyler, Davies and Martin 1981) from a single claypan. Each excavated tunnel contained one *N. aquilonius*. Eight *Notaden nichollsi* (Parker 1940) were also excavated from four sand dune locations surrounding the Kiwirrkurra community. Tunnels were located at the base, face and top of sand ridges. During a return trip in September 2004, one *N. aquilonius* with a thin cocoon was uncovered from a claypan site whilst eight *N. aquilonius* were excavated

from a site between two dunes (termed 'swale' throughout). In one instance, a *N. aquilonius* and a *N. nichollsi* were located side by side in a single burrow at the swale site. The swale site was located approximately 200 m from the nearest dune. None of the *N. aquilonius* from this swale site had a cocoon. During this second trip, 10 *N. nichollsi* were also excavated from seven separate burrows in sand dunes. Both species formed a loose-filled tunnel from the surface but no obvious chamber was found where the frog was located; however, the term 'burrow' will be used to denote the location of the frog in the soil.

Generally, the field site was very flat, consisting of red sand plains and dunes (Thompson et al., 2005). The three sites from which frogs were excavated (swale, claypan and dune) were all within 2.5–3.8 km of each other so we assume that all sites should have received similar rainfall during recent large rainfall events. Meteorological information from surrounding weather stations (Balgo ~300 km to the north and Giles ~200 km to the south) was used to determine when it was likely to have last rained. These data indicate that it probably rained substantially (60–100 mm) at the field site in February 2002, approximately 1.5 years prior to the excavation of frogs in late June 2003, and December 2003/January 2004 (90–120 mm), approximately 8–9 months prior to excavations during the second trip in September 2004. The claypan areas probably support pooled water for longer following rainfall due to the soil type and being lower in the landscape whereas dunes would quickly absorb rainfall. The swale site would probably be intermediate with respect to water holding.

Plasma and urine osmolality were measured for *N. nichollsi* and *N. aquilonius* caught active in the field at other sites in Western Australia following rainfall and allowed to fully hydrate in tapwater or moist soil overnight to represent control hydrated frogs. Burrowed frogs were grouped according to the habitat type and year in which they were excavated, and water balance comparisons were made between these groups, i.e. *N. nichollsi*: (i) controls, (ii) excavated from dunes in 2003 (Dune 2003), (iii) excavated from dunes in 2004 (Dune 2004), (iv) excavated from a swale alongside *N. aquilonius* (Swale 2004). *N. aquilonius*: (i) controls, (ii) excavated from claypan in 2003 (Claypan 2003), (iii) excavated from dune swale in 2004 (Swale 2004), (iv) excavated from claypan in 2004 (Claypan 2004). Plasma and urine osmolality were also measured for excavated frogs.

Soil analysis

Soil samples were collected from adjacent to burrowed frogs, and additional samples were taken at various intervals in the soil profile down to the burrowed frog. Soil moisture data are expressed as percent gravimetric water content (i.e. the difference between the wet and dry soil masses divided by the dry soil mass and expressed as a percentage). Dry soil masses were obtained by placing weighed soil samples in a 105°C oven overnight and reweighing.

Water potential of soil samples collected during 2004 was determined in the field using a Decagon Devices WP4 Dewpoint PotentiaMeter (Pullman, WA, USA). A water potential curve (relationship between soil moisture content and soil water potential) was also determined for soil samples collected from each of the habitat types where frogs were excavated in both 2003 and 2004 using the ceramic plate extraction technique (Slatyer, 1967). To do this, saturated soil samples were placed on a porous ceramic plate and exposed to pressure inside a sealed pressure chamber. Pressure chambers at 10, 100 and 1500 kPa (100 kPa=1 bar=0.99 atm) were used, and the moisture content of saturated soil was also measured at atmospheric pressure (0 kPa). Soil samples remained in chambers until no additional water could be forced from them (i.e. an equilibrium between forces retaining water in the soil sample and that applied by pressure was reached). Soil samples were then removed and the gravimetric water content was determined by drying and reweighing. A water potential curve was then plotted as the moisture retained within the soil against pressure.

Frog–soil water relationships

The moisture content necessary for excavated frogs to be in osmotic balance (i.e. neither gaining nor losing water) was calculated based on the plasma osmotic concentration. The plasma osmotic concentration (C, mOsm kg^{-1}) was converted to an equivalent osmotic pressure using the van't Hoff equation P=RTC (van't Hoff, 1887; Nobel, 1983) where P is osmotic pressure (MPa), R is the gas constant (8.314×10^{-6} m^{-3} MPa mol^{-1} K^{-1}), T is temperature (298K). P represents the hydraulic pulling force of the frog's body fluids on the water in the surrounding soil. This equivalent osmotic pressure (MPa), when substituted into the equation describing the water retention curve, gives the theoretical gravimetric water content of the soil necessary for frogs to be in water balance. Here, we are assuming that the frog's water potential is directly proportional to the osmotic concentration of the body fluids. This assumption has been tested previously for *A. rotunda*, and the calculated theoretical water content necessary for water balance was found to be in good agreement with the actual moisture threshold for absorption in this species (Cartledge et al., 2006).

Cocoon morphology

Intact cocoons were removed from the six *N. aquilonius* excavated from a claypan in 2003 and placed in resealable plastic bags for return to the laboratory. Triangular pieces of cocoon, approximately 2×4 mm, were cut and fixed in 2.5% gluteraldehyde overnight. Specimens were fixed in 0.1% osmium tetroxide following rinsing in 0.1 mol l^{-1} phosphate buffer (pH 7.2). Specimens were rinsed in deionised water, then dehydrated by a graded series of ethanol prior to being embedded in araldite/procure. Transverse gold sections (80–90 nm) were cut using a Diatome diamond knife (Hatfield, PA, USA) and an LKB ultramicrotome (LKB Instruments, Bromma, Sweden) and were then mounted on copper electron microscope grids. Sections were stained with uranyl acetate (acid and aqueous) and lead citrate. Cocoon sections were

viewed and photographed using a Philips 410 transmission electron microscope (Philips, Amsterdam, The Netherlands). Plate film negatives were scanned at 600 dpi. Images of cocoons were measured for overall cocoon thickness, individual layer thickness and total number of layers using ImageJ version 1.33u (Wayne Rasband, MA, USA). Individual layers were counted and measured along a single pixel line, bisecting the cocoon section, drawn at approximately 90° to the innermost cocoon layers. An individual layer was measured to include the epithelial cell layer and its underlying sub-corneal mucous layer. Between two and seven micrographs were examined for each frog cocoon, and from these the maximum number of cocoon layers counted was taken to be the best estimate of layers present, whereas layer width and total cocoon width were averaged across samples.

Plasma and urine osmolytes

Plasma and urine samples were collected from frogs following their excavation in the field; in all cases, frogs were held in a sealed plastic resealable bag to prevent evaporative water loss until blood and urine were collected. Urine volume was obtained by weighing frogs, removing urine by inserting a cannula into the cloaca and then reweighing. Frogs were then double-pithed (cranial and spinal), and blood was collected from the ventricle of the heart into a series of heparinized capillary tubes. Blood was emptied from haematocrit tubes into a centrifuge tube, and plasma and cells were separated using a desktop micro-centrifuge (Tomy HF120; Tomy Seico Co., Tokyo, Japan). All frogs were dissected within a few hours of their excavation. Blood and urine samples were frozen at –20°C and returned to the laboratory for assay.

Total osmotic concentration was determined by freezing point depression for 15 μl samples of plasma and urine using a Gonotec Osmomat 030 freezing point osmometer (Berlin, Germany). Concentrations of sodium and potassium ions were measured by flame photometry for 5 μl samples using a Varian model 475 atomic absorption spectrophotometer (Palo Alto, CA, USA), and the concentration of chloride ions was determined for 5 μl (plasma) or greater (urine) samples by amperometric titration with a Buchler-Cotlove 4-2000 automatic titrating chloridometer (Buchler Instruments, Kansas, MO, USA). Urea and ammonia were measured for 5 μl samples by the method of Fawcett and Scott (Fawcett and Scott, 1960) using a Varian DMC80 spectrophotometer.

Arginine vasotocin

The radioimmunoassay for AVT was modified from Rosenbloom and Fisher (Rosenbloom and Fisher, 1974) by Rice (Rice, 1980) and is described in detail elsewhere (Rice, 1982). Briefly, [Arg^8]-Vasotocin (Auspep Pty Ltd, Parkville, Victoria, Australia) was radioactively labelled with ^{125}I (Amersham Biosciences, UK) using the chloramine-T method (Hunter and Greenwood, 1962). AVT was extracted from 50 μl plasma samples by distribution on C18 Sep-Pak cartridges (Waters division of Millipore, Billerica, MA, USA) and elution with 75% aqueous acetonitrile containing 4% acetic acid. Previous use of this protocol in our laboratory has demonstrated a recovery efficiency of 85% (Fergusson and Bradshaw, 1991). Plasma AVT concentrations were measured using a late-addition, double-antibody assay. Samples were incubated with antibody for 24 h, radioactivity added and incubated for a further 72 h. The second antibody (donkey-anti-rabbit; Abacus Diagnostics, Brisbane, Queensland, Australia) was then added to tubes to precipitate the bound fraction. Following a 24 h incubation, tubes were centrifuged, the supernatant was aspirated and the radioactivity in the pellet was counted on a Prias Autogamma (Packard) or Cobra II Autogamma counter (Packard). Intra-assay variation was assessed as the coefficient of variation in duplicate samples (Chard, 1987) and was found to be 5.5% for 237 duplicate samples run over 14 assays. Repeat assay of duplicate 50 μl plasma samples from a *Bufo marinus* pool indicated an inter-assay coefficient of variation of 8.9%. A plot of standard deviations against means indicated that the AVT data were heteroscedastic, and log transformation was applied to stabilise variances prior to statistical analysis.

Results

Soil analysis

The dune soil was classified as sand, soil from the claypan as sandy loam clay, and soil from the swale as loamy sand, according to their fractions of sand, silt and clay (Table 1) (categories after Marshall, 1947) (cited in McDonald and Isbell, 1990). Soils from the claypan and swale sites were similar in their combined percentages of fine particles (clay and silt), although the fine particle fraction in the claypan soil was largely composed of clay, while the swale soil had an equal amount of silt and clay making up the fine particle quotient.

The sand dunes where *N. nichollsi* were burrowed were

Table 1. *Particle size composition (% of total) of soil from burrows of Neobatrachus aquilonius (swale and claypan) and Notaden nichollsi (dune and swale)*

Particle size class	Dune sites (sand, *N*=3)	Claypan site (sandy loam clay, *N*=2)	Swale site (loamy sand, *N*=2)
Sand (0.02–2.0 mm)	97.0±0.75	84.6±2.45	86.6±0.88
Silt (0.002–0.02 mm)	0.5±0.12	2.0±0.10	7.1±1.10
Clay (<0.002 mm)	2.7±0.92	13.5±2.35	6.3±0.25

Data are means ± s.e.m.

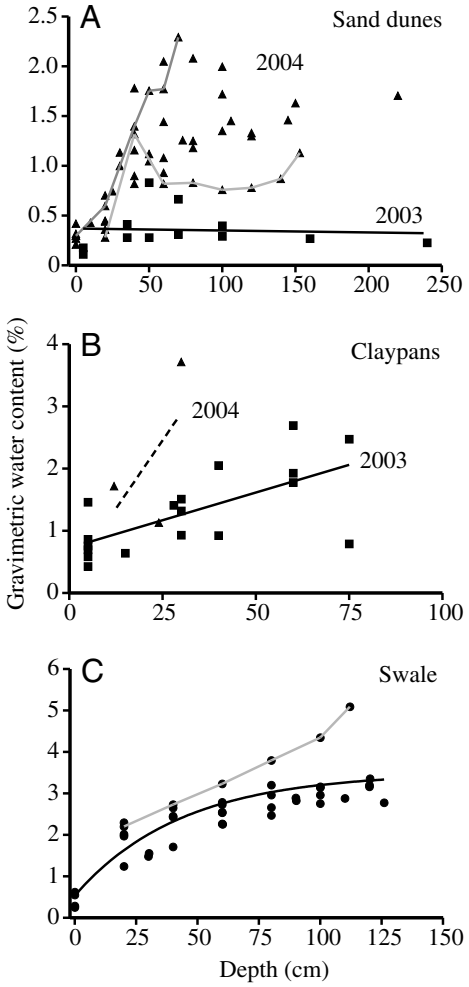

Fig. 1. Soil gravimetric water content (%) profiles of frog tunnels of (A) *Notaden nichollsi* in sand dunes in 2003 (squares) and 2004 (triangles); (B) cocooned *Neobatrachus aquilonius* in claypans in 2003 (squares) and 2004 (triangles); and (C) cocoonless *N. aquilonius* in a dune swale in 2004 (circles). Examples of individual burrow moisture profiles shown as grey lines.

much drier in 2003 than in 2004 (Fig. 1A). There was no relationship between soil moisture content and depth in 2003, but in 2004 there was a significant positive relationship between depth and gravimetric water ($F_{1,48}$=22.6, P<0.0001). However, there was substantial spatial variation in the relationship, with some burrows having a very steep increase in moisture with depth and others showing almost no increase in moisture with depth (two extremes with grey lines in Fig. 1A). Data from the claypan excavations of cocooned *N. aquilonius* in 2003 indicated a generally linear increase in moisture with depth (Fig. 1B). Only one frog was located at the claypan site in 2004 and so it was not possible to statistically analyse these data; however, the moisture content of the soil where this frog was located was generally higher than the soil moisture content recorded in 2003 (Fig. 1B). Soil samples collected during the excavation of cocoonless *N. aquilonius* from the swale site in 2004 indicated a soil moisture plateau between 2.7 and 3.4%, with only one of six excavations having a continued linear increase in moisture beyond this point to reach a maximum of 5.1% (Fig. 1C).

The three site soil types had similar saturation points at 24–28% moisture but different propensities to liberate water under increasing pressure (Fig. 2A). Claypan and swale soil, which had a higher proportion of fine particles (Table 1), held more water under increasing pressure. Dune sand lost water most readily, to a minimum of 1.2% at –1500 kPa, while both the claypan and swale held water more strongly, with moisture contents of 3.8% and 3.2%, respectively, at 1500 kPa. The water potential of soil samples collected at each site during excavations in 2004 and measured with the WP4 Dewpoint PotentiaMeter also indicated similar substantial differences in the propensities of each soil type to liberate water in the range of moistures found in the field (Fig. 2B). In general agreement with the laboratory-derived water potential data (Fig. 2A), dune sand had a higher water potential (i.e. a higher propensity to liberate water) at a given moisture than either swale or claypan soil. Three samples taken from a single tunnel at the swale site had water potential values not discernible from 0 kPa (beyond

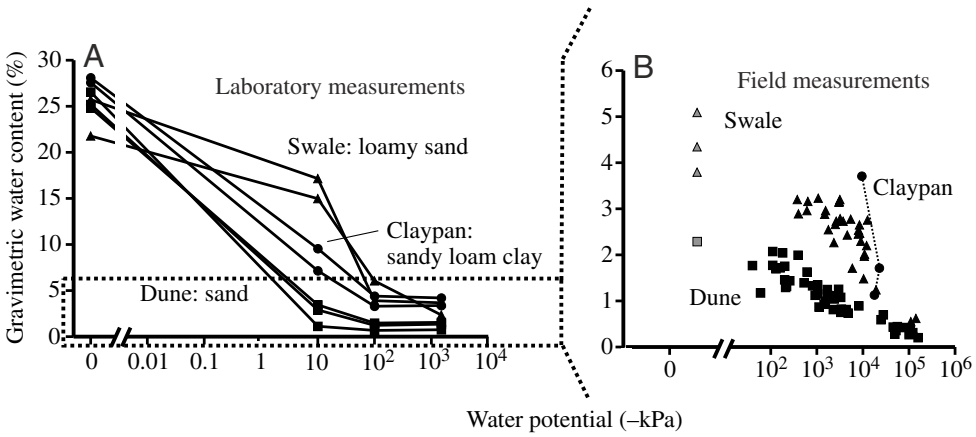

Fig. 2. (A) Water retention curves for soil from *Notaden nichollsi* (dune and swale) and *Neobatrachus aquilonius* (claypan and swale) burrows. Each line represents a curve for a soil sample collected at a different excavation point within each site. (B) Relationship between soil gravimetric water content and water potential (Decagon Dewpoint PotentiaMeter) for dune, swale and claypan soil. Data are for soil samples taken at intervals from the soil surface down to the depth of the burrowed frog, during excavations in 2004. Grey symbols in B are beyond the resolution of the Decagon analyser and are between 0 and –100 kPa.

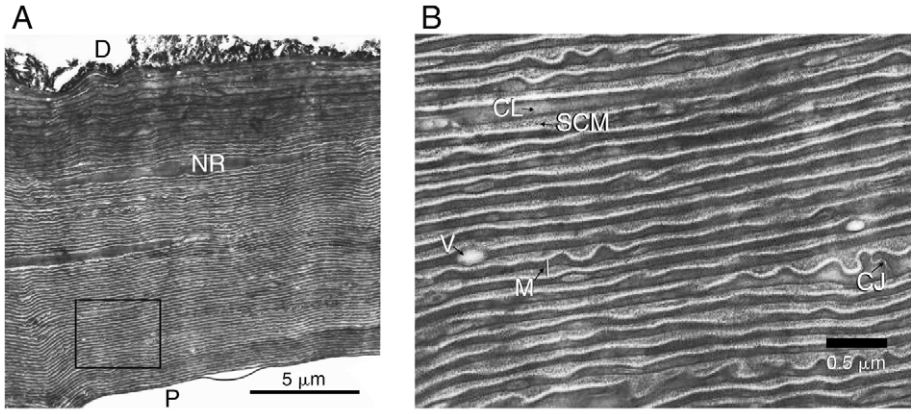

Fig. 3. (A) Electron micrograph of a transverse section through the cocoon of a *Neobatrachus aquilonius* excavated from a claypan in 2003. D, distal; P, proximal; NR, nuclear remnant. (B) Enlarged section of the box shown in A, showing individual cocoon layers. CL, cell layer composed of the remnants of dead epithelial cells; SCM, granular sub-corneal mucous layer; M, example of typical layer width measurement taken to include the cell layer and its underlying mucous layer; V, section through a cell vacuole; CJ, cell junction.

the detection limits of the Decagon Dewpoint PotentiaMeter); however, this tunnel had unusually high moisture contents (see Fig. 1C).

Cocoon morphology

Electron micrographs of the cocoons of *N. aquilonius* showed an organised, laminar structure, consisting of layers of electron-dense squamous epithelial cells with less dense layers of granular sub-corneal mucous between them (Fig. 3). The layers of stratum corneum cells had remnants of nuclei, vacuoles and other organelles. The outer (distal) surface of each cell layer had numerous protrusions, while the inner surface was relatively smooth.

Five of the six frogs were found to be encased in cocoons ranging from 81 to 106 layers thick, while one individual had a cocoon at least twice as thick with 229 layers (Table 2). Total cocoon thickness from the first five frogs ranged from 19.40±2.02 μm to 24.65±1.79 μm thick, and the sixth individual had a cocoon 55.61±0.18 μm thick (Table 2). Individual layer thickness varied from 0.04 to 2.55 μm, with the thickest layers typically occurring in the first 5–10 outer layers of each cocoon. Overall, the cocoons of *N. aquilonius* had a mean layer thickness of 0.22±0.008 μm. This is an overestimate, however, being positively skewed by a small number of thicker layers observed in the outermost parts of the cocoon and some layers which were sectioned through the thicker parts of nuclear remnants. Therefore, the median layer thickness of 0.18 μm may be a more representative measure of layer width.

Rates of cocoon formation were estimated for five other *Neobatrachus* species (Withers, 1995); while *N. aquilonius* was not included in this study, the minimum (0.2 layers day^{-1}) and maximum (0.35 layers day^{-1}) rates of layer shedding were used as a guide to the likely rate of layer formation for *N. aquilonius* excavated in the current study. Extrapolating from the number of layers in the cocoons of excavated frogs and the formation rates previously described (Withers, 1995), the excavated *N. aquilonius* had been burrowed for between 338±64.3 and 590±112.5 days (range, 232–530 days for five individuals, 655–1145 days for one remaining individual).

Plasma and urine osmolytes

Control *N. nichollsi* had a plasma osmolality of 266±7.7 mOsm kg^{-1} (*N*=7) and a urine osmolality of 76±10.5 mOsm kg^{-1} (*N*=5). The hydration state of burrowed *N. nichollsi* differed significantly between years. In 2003 (Fig. 4A), the plasma (348±11.5 mOsm kg^{-1}, *N*=7, *P*<0.001)

Table 2. *Layer thickness, number of layers and total thickness for cocoons removed from six N. aquilonius excavated from a claypan in the Gibson Desert*

Frog (*N* cocoon sections measured)	1 (7)	2 (4)	3 (2)	4 (7)	5 (5)	6 (2)	Means ± s.e.m. (*N*=6 frogs)
Number of cocoon layers	106	99	102	81	91	229	118±22.5
Total cocoon thickness (μm)	22.3	24.6	21.4	19.4	24.5	55.6	28.0±5.58
Maximum layer thickness (μm)	2.55	0.94	1.12	1.65	1.28	1.46	1.5±0.23
Minimum layer thickness (μm)	0.06	0.04	0.06	0.07	0.08	0.08	0.07±0.006
Mean layer thickness (μm)	0.20	0.23	0.22	0.24	0.26	0.22	0.23±0.008
Median layer thickness (μm)	0.15	0.19	0.19	0.18	0.20	0.18	0.18±0.007
Days aestivating (minimum rate)*	303	283	292	232	260	655	337.5±64.3
Days aestivating (maximum rate)*	530	495	510	405	455	1145	590±112.5

*Estimate based on rates of layer formation determined for *Neobatrachus* spp. (Withers, 1995) at a minimum rate of 0.2 layers day^{-1} and a maximum rate of 0.35 layers day^{-1}.

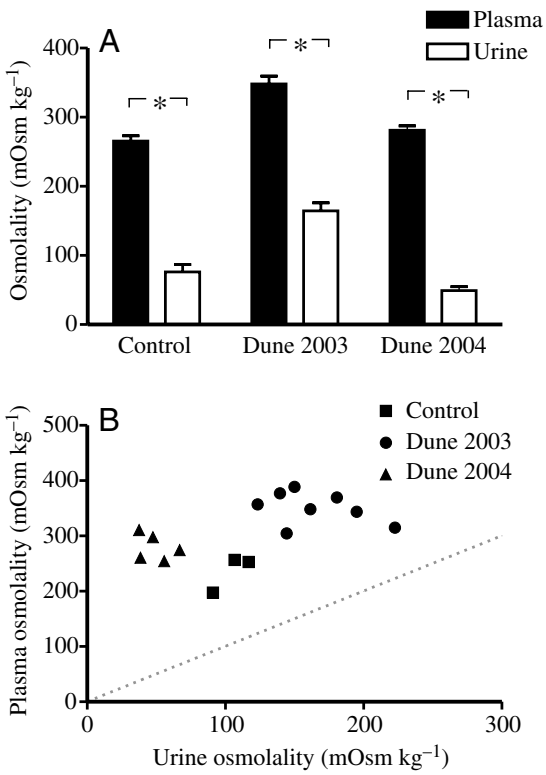

Fig. 4. *Notaden nichollsi*. (A) Total osmotic concentration of the plasma and urine of control (plasma *N*=7, urine *N*=5) and aestivating individuals excavated from sand dunes in 2003 (plasma *N*=7, urine *N*=8) and 2004 (plasma *N*=10, urine *N*=5). * indicates significant difference between plasma and urine osmolalities (one-tail *t*-test, $P<0.05$). (B) Relationship between plasma and urine osmolality for controls and frogs excavated from sand dunes in 2003 and 2004. The broken line represents isosmolality.

and urine (165±11.6 mOsm kg^{-1}, *N*=8, $P<0.001$) concentrations of excavated *N. nichollsi* were significantly higher than controls. However, in 2004, plasma (281±6.4 mOsm kg^{-1}, *N*=10) and urine (49±5.5 mOsm kg^{-1}, *N*=5) were not significantly different from controls. The urine of excavated *N. nichollsi* was always significantly more dilute than the plasma (Fig. 4B), and frogs excavated in 2004 had urine even more dilute than controls.

Table 3 presents concentrations of the major osmolytes in the plasma and urine of control *N. nichollsi*, frogs excavated from sand dunes in 2003 and 2004, and the single individual found at the swale site in 2004. The higher total osmotic concentration of the plasma of *N. nichollsi* excavated in 2003 is due to significantly increased concentrations of sodium, chloride and urea compared with controls. The significantly higher total urine osmotic concentration is explained largely by the significant increase in urea. The single *N. nichollsi* found at the swale site had osmolyte concentrations similar to *N. nichollsi* excavated in 2004. Osmolyte concentrations of *N. nichollsi* excavated in 2004 were either not different from or otherwise significantly lower than seen in control frogs.

Neobatrachus aquilonius excavated from the claypan site had significantly higher plasma (324±22.6 mOsm kg^{-1}, *N*=6) and urine (286±40.1 mOsm kg^{-1}, *N*=5) osmolalities than controls ($P<0.001$) and frogs excavated from the swale site ($P<0.001$) (Fig. 5A). Frogs excavated from the swale site had a plasma osmolality of 195±8.0 mOsm kg^{-1} (*N*=7) and urine osmolality of 35.5±5.52 mOsm kg^{-1} (*N*=8), similar to control frogs (plasma=220±6.8 mOsm kg^{-1}, *N*=8; urine=48±7.3 mOsm kg^{-1}, *N*=10). *N. aquilonius* excavated without cocoons from the swale in 2004 had urine osmotic concentrations lower than the plasma and similar to control frogs (Fig. 5A). By contrast, cocooned *N. aquilonius* excavated from the claypan site had urine osmotic concentrations approaching that of the plasma, and one individual had isosmotic plasma and urine concentrations (Fig. 5B).

Table 4 summarises the concentrations of major osmolytes contributing to the total osmolality of the plasma and urine for control *N. aquilonius*, cocooned frogs excavated from claypans in 2003 and 2004 (one individual only) and cocoonless frogs excavated from the swale site in 2004. The increased total plasma osmolality of cocooned *N. aquilonius* excavated from the claypan site in 2003 is due to significant increases in sodium, chloride and urea concentrations. The significant increase in urine osmotic concentration, however, appears to be explained to a large extent by the increase in sodium

Table 3. *Total osmotic concentration and concentrations of contributing osmolytes in the plasma and urine of control and aestivating N. nichollsi excavated from sand dunes during 2003 and 2004 and from the swale in 2004*

	Control		Aestivating 2003 (dune)		Aestivating 2004 (dune)		Aestivating 2004 (swale)	
	Plasma (*N*=8)	Urine (*N*=5)	Plasma (*N*=7)	Urine (*N*=8)	Plasma (*N*=10)	Urine (*N*=5)	Plasma (*N*=1)	Urine (*N*=1)
Na^+ (mmol l^{-1})	127±4.5	19.1±6.06	154±4.8**	43.2±4.93	114±3.3	6.5±0.02*	80.2	6.4
Cl^- (mmol l^{-1})	92±5.8	1.8±1.80	118±5.25*	2.4±1.14	98±4.5	2.7±1.31	99	3.5
K^+ (mmol l^{-1})	11.3±0.29	3.9±0.95	11.1±0.41	10.2±1.11	6.7±0.45**	2.8±0.25*	5.0	3.2
Urea (mmol l^{-1})	20.2±2.05	37.2±8.8	56±8.2**	105±10.9*	34.1±4.09	20.9±1.42	16.4	22.6
Ammonia (mmol l^{-1})	3.2±0.28	3.4±0.59	3.4±0.25	13.0±0.13*	5.2±0.37**	2.0±0.53*	3.9	3.6
Total (mOsm kg^{-1})	266±7.7	76±10.5	351±10.3**	165±11.6**	271±6.4	49±5.5	249	56

Values are means ± s.e.m.

Significant differences between aestivating and control values are indicated; Dunnett's *post-hoc* multiple comparisons test: *$P<0.05$, **$P<0.001$.

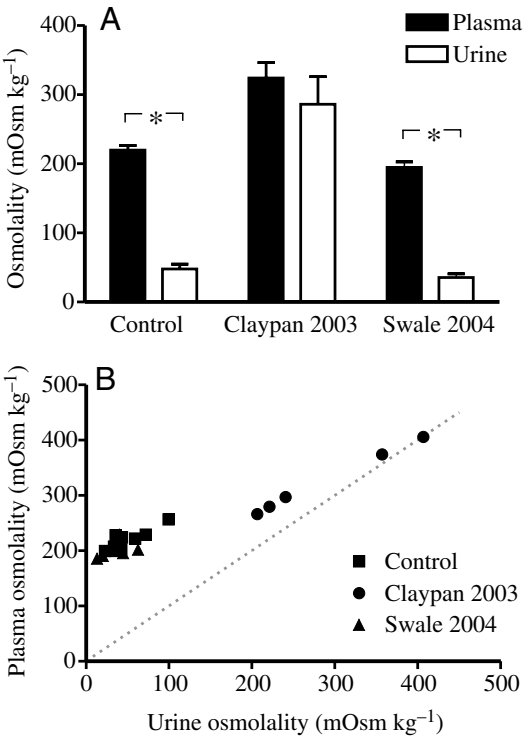

Fig. 5. *Neobatrachus aquilonius*. (A) Total osmotic concentration of the plasma and urine of control (plasma *N*=8, urine *N*=10) and cocooned aestivating frogs excavated from a claypan in 2003 (plasma *N*=6, urine *N*=5) and non-cocooned aestivating frogs from a dune swale in 2004 (plasma *N*=7, urine *N*=8). * indicates significant difference between plasma and urine osmolalities (one-tail *t*-test, $P<0.05$). (B) Relationship between plasma and urine osmolality for control and excavated cocooned frogs from a claypan in 2003 and excavated without cocoons from a swale in 2004. The broken line represents isosmolality.

concentration, with urea making the only other significant contribution. Osmolyte concentrations of cocoonless frogs excavated from the swale site were very similar to those of control frogs. The single cocooned *N. aquilonius* found at a claypan in 2004 had an osmotic concentration lower than cocooned frogs excavated in 2003, largely due to lower concentrations of sodium and chloride.

Frog–soil water relationships

N. nichollsi excavated from dunes in 2003 experienced soil moistures in deficit of water balance requirements, while in 2004 the soil moisture was in excess of requirements for water balance (Table 5). *N. aquilonius* at the claypan site in 2003 would have been losing water to the surrounding soil with moisture levels one half of that necessary for osmotic balance; however, water losses would have been reduced by the presence of their cocoon. The *N. aquilonius* excavated from the moister claypan in 2004 was at a soil moisture approaching that necessary for osmotic balance. While the average soil moisture level at the swale site was high (3.0%) relative to the sand dunes, frogs here were still in deficit to the theoretical level necessary to maintain water balance in this soil. Only one burrow at the swale site had a moisture level high enough for a favourable osmotic gradient for the frog (shaded grey in Fig. 1C). This was the burrow found with a *N. nichollsi* together with a *N. aquilonius*.

Arginine vasotocin

There was a significant but weak positive relationship between plasma AVT concentration and plasma osmolality for both *N. nichollsi* ($F_{1,17}$=5.6, $P<0.05$, r^2=0.25) and *N. aquilonius* ($F_{1,12}$=8.6, $P<0.05$, r^2=0.42). However, the linear relationship for *N. aquilonius* is biased by a large increase in AVT for the individual with the highest osmolality (394 pg ml^{-1}; Fig. 6B); this frog had urine isosmotic with the plasma (data point on the isosmotic line in Fig. 5B). All other AVT concentrations were measured for frogs with plasma osmolalities in the range of 186–297 mOsm kg^{-1}, and within this range there was no relationship with AVT. Similarly, for *N. nichollsi*, the significant relationship of plasma osmolality with AVT was due to the individual with the highest osmolality (389 mOsm kg^{-1}) having a higher osmolality than all other individuals (98.8 pg ml^{-1} *versus* 8.0–57.5 pg ml^{-1}), and amongst all other individuals there was no relationship between plasma osmolality and AVT concentration.

Table 4. *Total osmotic concentration and concentrations of contributing osmolytes in the plasma and urine of controls and aestivating N. aquilonius excavated from a claypan during 2003, from a swale site in 2004 and claypan in 2004*

	Control		Aestivating 2003 (claypan)		Aestivating 2004 (swale)		Aestivating 2004 (claypan)	
	Plasma (*N*=8)	Urine (*N*=10)	Plasma (*N*=6)	Urine (*N*=4)	Plasma (*N*=7)	Urine (*N*=8)	Plasma (*N*=1)	Urine (*N*=0)
Na^+ (mmol l^{-1})	73±4.0	2.7±0.55	144±7.6**	172±1.0**	79±4.0	6.6±0.12	107	–
Cl^- (mmol l^{-1})	63±3.0	1.7±0.50	98±7.3**	9.0±5.20*	67±6.8	3.4±1.69	91.8	–
K^+ (mmol l^{-1})	8.3±1.62	0.9±0.20	13.3±0.34*	13.0±0.33**	5.6±0.37	4.4±0.48**	7.9	–
Urea (mmol l^{-1})	7.3±0.89	24.8±7.15	66±6.3**	84±11.5**	8.1±1.61	11.6±2.82	75	–
Ammonia (mmol l^{-1})	0.8±0.11	0.80±0.11	3.7±0.44*	11.4±2.73**	4.9±1.16**	2.1±0.30	3.5	–
Total (mOsm kg^{-1})	220±6.82	47.7±7.26	324±22.6**	286±40.1**	195±8.0	35.5±5.52	211	–

Values are means ± s.e.m.

Significant differences between aestivating and control values are indicated; Dunnett's *post-hoc* multiple comparisons test: *$P<0.05$, **$P<0.001$.

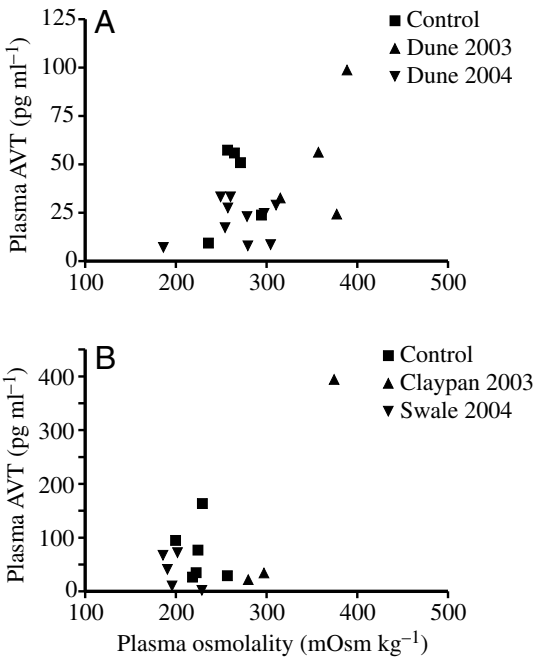

Fig. 6. Relationship between plasma AVT concentration and plasma osmolality for control and field-excavated (A) *Notaden nichollsi* and (B) *Neobatrachus aquilonius*.

Discussion

Frogs of the cocoon-forming species *N. aquilonius* and the non-cocooning species *N. nichollsi* were excavated in the Gibson Desert of central Australia. Cocooned *N. aquilonius* were found burrowed in claypans while *N. nichollsi* were burrowed in sand dunes, consistent with general descriptions of their habitats (Tyler et al., 2000). However, *N. aquilonius* without a cocoon were also excavated from a swale site, which provides the first evidence that cocoon formation while burrowed is not obligatory in species that have this capacity. Meteorological information from surrounding weather stations (Balgo ~300 km north and Giles ~200 km south) was used to determine when it was likely to have last rained at the study location. These data indicate that it may have rained substantially in February 2002, approximately 1.5 years prior to the excavation of frogs in late June 2003, and December 2003/January 2004, approximately 8–9 months prior to excavations during the second trip of September 2004. This compares well with the pattern of hydration state of burrowed frogs between 2003 and 2004. Frog–soil water relations were examined for frogs in each of the habitat types (sand dune, claypan and swale), indicating that *N. nichollsi* were in positive water balance in sand dunes in 2004 while all other frogs excavated were in soil with a theoretical deficit to moisture requirements.

Water balance of cocooned N. aquilonius

In 2003, the six *N. aquilonius* excavated from a claypan all had a well-formed thick cocoon, and the moisture in the claypan soil was only half that necessary to balance the osmotic pressure of the plasma. Therefore, these *N. aquilonius* would have been losing water to the surrounding soil although their cocoons would have considerably decreased the rate of loss (Withers, 1998a). Presumably, these frogs had burrowed into the claypan while the clay was soft with moisture and had garnered water from the soil until the soil had dried enough to limit water gain and the frogs then formed a cocoon. Our study provides some evidence that this may also be the case for *N. aquilonius* in 2004. The single individual excavated from a claypan in 2004 had only a very thin cocoon, which was too fragile to prepare a section for layer counting. Moisture levels in the soil surrounding this frog (3.7%) were only slightly less than calculated to be necessary for the frog to maintain water balance (3.8%). Although rainfall had not occurred for several months prior to the excavation of this frog, it appears that *N. aquilonius* in claypans do not form a cocoon until they are in negative water balance. This is also consistent with the findings for cocoonless *N. aquilonius* excavated from the swale (see below). Once the cocoon has formed, frogs are almost a closed system, not voiding urine and relying on stored water to maintain hydration; water loss/gain is limited by the cocoon and respiratory water loss is low because of the decreased ventilation requirements of metabolic depression (Withers, 1993). Similarly, *Cyclorana platycephala* commonly do not form a cocoon until two weeks (van Beurden, 1984) or longer (McMaster, personal observation) after burrowing, which may

Table 5. *Plasma osmolality and calculated equivalent water potential for excavated N. nichollsi and N. aquilonius, soil moisture content corresponding to this pressure (balancing soil moisture) and the actual soil moisture found at the site of excavation (field soil moisture)*

		Plasma osmolality (mOsm kg^{-1})	Equivalent water potential (kPa)	Balancing soil moisture (%)	Field soil moisture (%)	Moisture surplus (%)
Notaden nichollsi	2003 Dune	351	–869	1.2	0.4	–0.8
	2004 Dune	271	–671	1.2	1.5	+0.3
	2004 Swale	249	–618	4.3	5.1	+0.8
Neobatrachus aquilonius	2003 Claypan	324	–803	3.8	1.9	–1.9
	2004 Swale	195	–483	4.4	3.0	–1.4
	2004 Claypan	211	–522	3.8	3.7	–0.1

A negative moisture surplus indicates a deficit of water to osmotic balance requirements.

be because soil moisture is high enough to maintain hydration during this initial period.

The cocoons of *N. aquilonius* excavated in this study comprised the most layers in a frog cocoon yet recorded (81–229 layers), and the cocoon is thus likely to have been formed over a significantly longer period of time than for any previous studies of cocoon-forming species. *L. llanensis* cocoon formed over 150 days had up to 60 layers, and a *Cyclorana cultripes* cocoon formed over 85 days had 51 layers (McClanahan et al., 1976; Withers and Thompson, 2000). In general, cocoon thickness should reflect the number of layers. Our measurements of individual cocoon layer thicknesses (0.20–0.26 μm) are smaller than previously recorded for *Neobatrachus* spp. (*N. sutor*, 0.62 μm; *N. kunapalari*, 0.57 μm; *N. pelabatoides*, 0.57 μm) (Withers, 1995) but are similar to the layer thickness of 0.2 μm described for *L. llanensis* (McClanahan et al., 1976).

Based on meteorological data, which indicate the last significant rainfall in the region occurred in February 2002 (approximately 1.5 years or ~548 days before our 2003 study), rates of cocoon formation for five of the six excavated *N. aquilonius* are similar to the minimum rate reported by Withers of 0.2–0.22 layers per day (Withers, 1995). However, one individual was anomalous and appeared to have either formed a cocoon at approximately twice the rate of the other five individuals examined or formed a cocoon over almost twice the time. This frog was excavated from within metres of the other five frogs excavated so it is unlikely that the burrow of this individual failed to be penetrated by rainfall in February 2002 when all other nearby burrows apparently were. If water penetrated the burrow, the frog's presumed existing cocoon would have been soaked and its integrity compromised. Hence, it is unlikely the extra layers of this individual's cocoon indicate a longer period of burrowing and aestivation and rather that this *N. aquilonius* formed layers at almost twice the rate of the other *N. aquilonius* in the vicinity. The rate of shedding in this frog was 0.42 layers day^{-1}, which is faster than the fastest rate of 0.35 layers day^{-1} observed for *Neobatrachus* spp. (Withers, 1995).

The current study appears to be one of a very small number to investigate the ability of cocooned frogs to maintain water balance during aestivation in the field. In the laboratory, the South American cocoon-forming *L. llanensis* has a hydrated plasma osmolality of 212 mOsm kg^{-1}, which increases to 363 mOsm kg^{-1} after 44 days of cocooned aestivation when induced to aestivate with an empty bladder (McClanahan et al., 1976). The lack of an initial bladder water reserve explains the relatively rapid increase in osmolality as the frogs were without a urinary buffer to increasing osmotic concentration. *Cyclorana platycephala* has been reported to store on average 57% of its standard mass as dilute urine, which, based on rates of water loss of burrowed cocooned frogs, was calculated capable of countering water losses for 2–3 years (van Beurden, 1982). However, in this study, plasma and urine osmolality were not measured and so it remains unknown if the urine would become isosmotic with the plasma in advance of 2–3 years, as was found to be the case in the current study for *N. aquilonius*, at which point further water must be withdrawn by active solute transport mechanisms. The osmolality of the plasma and urine of cocooned *N. aquilonius* excavated from the claypan site in 2003 was significantly higher than both that of control frogs and aestivating but cocoonless *N. aquilonius* burrowed at the swale site. The concentration of the urine of the cocooned frogs was nearing isosmotic and so, in the absence of a favourable osmotic gradient, these frogs would have to actively withdraw water from the bladder to maintain hydration. In addition, three of these frogs had virtually no urine volume remaining (these frogs had the highest plasma osmotic concentrations). The osmotic data would seem to indicate that frogs were becoming water limited by 1.5 years, and we speculate that cocooned frogs of this species might not aestivate for periods greater than 2 years.

Neobatrachus aquilonius without a cocoon

The soil of the swale site where *N. aquilonius* were found burrowed without cocoons was a sandy loam. The high fine-particle component caused this soil type to hold water strongly in the range of pressures equivalent to the osmolality of frog plasma and therefore the soil must have high moisture content for burrowed frogs to maintain water balance. The field moisture at the swale site where cocoonless *N. aquilonius* were excavated was lower than calculated to be necessary for water balance based on the water potential curve. However, the osmolality of the plasma of frogs burrowed at this site was not significantly different from controls, indicating that they were not yet experiencing dehydration, which probably explains their lack of cocoon formation. As moisture levels at this site were already below the theoretical water balance requisite and moisture levels appeared to have reached a plateau, we suggest that any further drying of the swale soil would induce the formation of a cocoon by the frogs burrowed there. These frogs were found burrowed within a narrower range of depths than at any other site (range, 100–126 cm; mean, 113±3.7 cm). The moisture *versus* depth data indicated that moisture had generally reached a plateau at or before the frogs' depth of burrowing (Fig. 1C).

The concentration of sodium in the plasma of hydrated *N. aquilonius* (control, 73 mmol l^{-1}; swale 2004, 79 mmol l^{-1}) was considerably lower than hydrated *N. nichollsi* (control, 127 mmol l^{-1}; dune 2004, 114 mmol l^{-1}), and previously reported hydrated plasma sodium concentrations are generally in the range of 100–125 mmol l^{-1} (Shoemaker, 1964; Shoemaker et al., 1969; Hillman, 1978; Degani, 1985; Katz and Hanke, 1993; Hoffman and Katz, 1997). These low sodium values suggest that *N. aquilonius* 'over-hydrates' when water is available, which may be an adaptation for maximal water storage in anticipation of cocoon formation where only body water is available to maintain hydration. However, such low sodium values have not been reported for hydrated frogs of other cocoon-forming species, such as *N. pelobatoides* (106 mmol l^{-1}), *N. kunapalari* (119 mmol l^{-1}) and *L. llanensis* (134 mmol l^{-1}) (McClanahan et al., 1976; Withers and Guppy, 1996).

Water balance of non-cocooning N. nichollsi

The red dune sand, in which *N. nichollsi* were consistently found burrowed, released water readily, and moisture contents as low as 1.2% were calculated to be adequate to maintain hydration. However, there was considerable spatial and temporal variation in moisture content within the dunes. Frogs excavated in 2003 were in soil of an average moisture content of 0.4%, which represents a theoretical deficit of moisture for water balance, and the plasma osmotic concentration of frogs excavated during this year (351 mOsm kg^{-1}) was significantly higher than for controls (265.5 mOsm kg^{-1}), indicating dehydration. By contrast, moisture levels within the dunes in 2004 were on average 1.5% and in excess of the level necessary for water balance, which is consistent with the plasma osmotic concentration of these frogs (271.3 mOsm kg^{-1}) not being significantly different from controls. The spatial variation in the dunes likely explains the variability in burrowing depth of different *N. nichollsi* individuals described by Thompson et al. (Thompson et al., 2005).

The increased total osmolality of *N. nichollsi* burrowed in the drier sand dunes of 2003 indicates moderate dehydration. The plasma urea concentration of *N. nichollsi* in 2003 was similar to that of cocooned *N. aquilonius* in the same year, and presumably both groups of frogs had been aestivating for a similar length of time since the last significant rainfall event. The concentration of urea in the plasma of cocooned *N. aquilonius* represents the accumulation of this waste, albeit at a reduced metabolic rate (Withers, 1993). Therefore, the similar levels found in aestivating *N. nichollsi* suggests that urea is not being actively produced as a balancing osmolyte, as has been described for burrowed *Scaphiopus couchii* experiencing negative water balance (Jones, 1980). Given the large moisture deficit experienced by *N. nichollsi* excavated in 2003, if urea synthesis were an osmotic strategy employed by this species, then it would be expected that urea should have been making a greater contribution to the overall osmolality of these frogs in negative water balance. Instead, the increase in total osmolality of *N. nichollsi* in the drier sand of 2003 was mostly accounted for by increases in sodium and chloride and to a lesser extent urea. It appears that, like cocoon-forming species, *N. nichollsi* accumulate urea as a storage osmolyte, due to their lower water turnover when burrowed (Withers, 1998b).

Given that *N. nichollsi* do not use urea synthesis as a soil water-harvesting strategy during progressive dehydration, water stored in the bladder is the prime water source when experiencing unfavourable osmotic conditions. *N. nichollsi* can hold up to 50% of their standard mass in the bladder (Main and Bentley, 1964), which places them amongst species with the highest relative bladder storage capacity. Bladder capacity has been correlated with aridity of the environment; aquatic species have a bladder capacity of only 2–8%, while semi-terrestrial, terrestrial/semi-arid species have higher capacities of 2–21%, arid-adapted species store around 50% (for a review, see Heatwole, 1984) and in extreme cases *C. platycephala* (van Beurden, 1984) and *Bufo cognatus* (Shoemaker et al., 1969) have been recorded with bladder stores of over 100% of standard mass. While bladders of *N. nichollsi* were drained following their excavation, we suspect that some frogs may have emptied their bladder during excavation (*N. nichollsi* have been observed to urinate readily in response to handling in the laboratory). This would explain why many individuals were found to have urine volumes less than 10% of their standard mass.

Arginine vasotocin

To our knowledge, this is the first time that AVT has been measured in the plasma of aestivating desert frogs. Generally, the two species had similar plasma AVT concentrations (9.4–164 pg ml^{-1}) and most excavated individuals had mean concentrations not significantly different from control individuals. Concentrations were also similar to that of hydrated *Rana ridibunda* and *Bufo marinus* (Nouwen and Kühn, 1983; Nouwen and Kühn, 1985; Konno et al., 2005) but higher than hydrated *Rana catesbeiana* (Rosenbloom and Fisher, 1974; Sawyer and Pang, 1975; Pang, 1977). One cocooned *N. aquilonius* was found to have much higher AVT concentrations than any other frog (394 pg ml^{-1}). This frog had the highest osmolality of the excavated *N. aquilonius* and was the only frog in which the plasma and urine were isosmotic. As bladder reserves are the primary source of water available to a cocooned frog, it seems that the dramatic increase in AVT is indicative of impending osmotic and volumetric stress. However, a number of cocooned *N. aquilonius* had no urine reserves at all and yet did not have particularly high AVT concentrations. Further work is required to examine the role of AVT in cocoon-forming frog species. For *N. aquilonius*, there was no relationship between AVT and plasma osmolality over the range of 186–297 mOsm kg^{-1}, which encompassed the vast majority of all excavated (cocooned and non-cocooned) *N. aquilonius*. Similarly, excluding the individual with the highest osmolality, there was no relationship between plasma osmolality and AVT for *N. nichollsi* over the range of osmolalities exhibited. Konno et al. (Konno et al., 2005) appear to provide the only comparable study that has measured plasma AVT concentrations over a range of plasma osmolalities (~200–350 mOsm kg^{-1}) in a terrestrial amphibian (*B. marinus*). In this species, the linear relationship had a stronger correlation (r^2=0.68) than observed in our study but concentrations were variable at higher osmolalities. While there have been few studies to measure circulating AVT concentrations in relation to plasma osmolality, there has been a consistent finding of increased AVT with high osmolality in amphibians (e.g. Nouwen and Kühn, 1983; Nouwen and Kühn, 1985; Konno et al., 2005) and reptiles (Rice, 1982; Ford and Bradshaw, 2006; Ladyman et al., 2006). The increased AVT concentration of frogs with the greatest osmolalities suggests that a similar relationship is likely for the desert-aestivating species of this study; however, it appears that the majority of frogs had not yet been pushed to the threshold where AVT release was stimulated.

Summary

Our primary objective was to investigate the field water balance of a cocoon-forming species and a non-cocooning species as a function of the physical properties of the soil where they burrow. Our results support previous suggestions that non-cocooning species are largely confined to burrowing in friable sandy soil types that have high water potential at low soil moisture (Heatwole and Lim, 1961; Etheridge, 1990; Cartledge et al., 2006). However, we found that considerable temporal and spatial variability in moisture contents was experienced by the sand dune burrowing non-cocooning *N. nichollsi* and that frogs of this species were not always found in soil of a favourable osmotic gradient. The advantages of cocoon formation for reducing EWL have been well established in the laboratory but the ability of cocooned frogs to maintain water balance using reduced EWL and bladder reserves has largely not been addressed. Our findings for cocooned *N. aquilonius* indicate that urine may become isosmotic with the plasma and greatly depleted in volume in advance of the next rainfall. Half of the excavated individuals had exhausted bladders even though data from the other excavated cocooned frogs indicate that isosmoticity can be approached even when urine volume is still as high as 15.0–26.6% of standard mass. Both species had similar plasma AVT concentrations ranging from 9.4 to 164 pg ml^{-1}, except for one cocooned *N. aquilonius* with a higher concentration of 394 pg ml^{-1}. For both species, AVT showed no relationship with plasma osmolality over the lower range of plasma osmolalities but was appreciably increased at the highest osmolality recorded. We have found that cocoon formation is not obligatory in *N. aquilonius*. It appeared that frogs had not formed a cocoon at the swale site because they had burrowed in a different soil type that afforded them adequate water to maintain full hydration. Cocooned *N. aquilonius* were excavated from a claypan. Their cocoons had 81–229 layers and, using the maximum rate of layer formation observed by Withers for *Neobatrachus* spp. (Withers, 1995), the number of layers generally agrees with the estimated length of time frogs are thought to have been aestivating based on rainfall data.

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