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The Cocoon of the Fossorial Frog *Cyclorana australis* Functions Primarily as a Barrier to Water Exchange with the Substrate

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ABSTRACT

Studies of evaporative water loss using streams of dry air in the laboratory have demonstrated reduced rates in various taxa of cocooned frogs. However, because the cocoon is formed in subterranean burrows with humid microclimates and no air flow, loss of water by evaporation is likely to be negligible. In contrast, although potentially important, the influence of the cocoon on water exchange with the soil surface has not been characterized. In dry soils, there is a sizable water potential gradient between the frog and the soil; hence, we hypothesized that cocoons would play a role in reducing liquid water loss to dry substrates. Individuals of the burrowing frog *Cyclorana australis* (Hylidae: Pelodryadinae) were induced to form cocoons in the laboratory. On semisolid agar-solute substrates across a range of water potentials, the hygroscopic cocoon absorbed small but similar amounts of moisture. With the cocoon removed, the frogs gained or lost water, depending on the direction of the frog-substrate water potential difference. Plasma osmolality of cocooned frogs was significantly higher than in hydrated frogs. Because cocooned frogs did not exchange significant amounts of water at either high (wet) or low (dry) substrate water potentials, we conclude that the cocoon of fossorial frogs acts as a physical barrier that breaks the continuity between frog and substrate. We contend that the primary function of the cocoon is to prevent liquid water loss to drying clay and loam soils, rather than to prevent subterranean evaporative water loss.

Introduction

Cocoons composed of shed layers of squamous epithelia have been described in several lineages of fossorial anuran species from arid and seasonally dry environments (Lee and Mercer 1967; McClanahan et al. 1976; Loveridge and Withers 1981; Ruibal and Hillman 1981; McDiarmid and Foster 1987; Shoemaker 1988; Warburg 1997). In all species examined, the cocoon becomes thicker as new layers are formed and gradually hardens to encase the entire body except the external nares (McClanahan et al. 1983; Toledo and Jared 1993; Withers 1995, 1998; McMaster 2006). All species of the Australian hylid genus *Cyclorana* studied to date burrow and form a cocoon (van Beurden 1984; Withers 1995; Withers 1998; Withers and Thompson 2000; Booth 2006; McMaster 2006). *Cyclorana australis* (Gray 1842) is a large (60–100 g) member of the genus from monsoonal northern Australia that forms a cocoon during the annual dry season (Tracy et al. 2007).

The frog cocoon has a recognized role in reducing evaporative water loss (EWL) from the skin surface, when compared with individuals without a cocoon (McClanahan et al. 1983; Withers and Richards 1995; Christian and Parry 1997; Withers 1998). Resistance to water (vapor) loss increases with time and is associated with the number of layers formed (McClanahan et al. 1983; Withers 1998); *Cyclorana australis* is similar to other species in this respect (Christian and Parry 1997; Withers and Thompson 2000; Tracy et al. 2007). EWL measurements are carried out in laboratory chambers using flows of dry air. However, burrowing frogs find refuge underground, where humidity is high and there is no air flow (Seymour and Lee 1974; Tracy et al. 1978; Ackerman 1991; Warburg 1997). While EWL measurements are pertinent to terrestrial species exposed to subaerial conditions, they may be less relevant to fossorial anurans. Several authors have speculated that the presence of a relatively impermeable cocoon may reduce contact between the skin and the soil, thereby preventing or reducing liquid water loss to dry soils (Seymour and Lee 1974; Ruibal and Hillman 1981; Shoemaker et al. 1992; Booth 2006). Although Reno et al. (1972) tested segments of cocoon of the aquatic salamander *Siren intermedia* and suggested that the cocoon impeded diffusion of water, we are not aware of any study that has tested this hypothesis in intact animals or in cocooned anurans.

While underground during the 3-mo period before the cocoon has formed (Tracy et al. 2007), the ventral surface of *C. australis* is in intimate contact with the soil substrate. Liquid water exchange with the soil is dependent primarily on the water potential (WP) of the frog, because of the osmolality of the body fluids, and (in nonsaline soils and at shallow depths where osmotic and gravitational potentials are negligible) the affinity of the soil matrix for water, the matric potential (Seymour and Lee 1974; Hillyard 1976; Shoemaker 1988; Shoemaker et al. 1992). If the free energy of water in the frog (ψ_f) is greater than in the soil or substrate (ψ_s), then the WP dif-

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ference will favor water movement out of the frog. The rate of water flux will depend on the size of the difference, the permeability of the (ventral) skin, and the surface area in contact with the substrate (Tracy 1976; Spotila et al. 1992; Hillman et al. 2009), mediated in part by the action of neurohypophysial hormones (Hays 1990; Bentley 2002). In drying soils, ψ_s can drop rapidly at the drying front (Ackerman 1991) to levels lower (more negative) than ψ_f. In this situation, water is lost by isolated skin preparations (Hillyard 1976) and by intact frogs to artificial substrates (Tracy and Rubink 1978; Tracy et al. 2007) and drying soil (Booth 2006). During the second half of the 6-mo period that *C. australis* are underground (Tracy et al. 2007), the frogs form a cocoon so that the ventral surface of the skin is no longer in direct contact with the soil. We hypothesized that the cocoon acts as a barrier to water exchange (gain or loss) with the soil, breaking the continuity between the skin and the substrate.

We therefore tested the influence of the cocoon of *C. australis* on liquid water exchange with the substrate in cocooned animals, in comparison with frogs without cocoons. We used a semisolid agar osmoticum to tightly control WP and emulate the soil surface for the following reasons: (1) because of the drying characteristics of soil, it is difficult to maintain soil at a constant WP in the range equivalent to frog body fluids; (2) in liquid osmotic solutions, the integrity of the cocoon is lost, whereas on agar, the cocoon remains intact. We measured the osmolality of the body fluids in cocooned and hydrated frogs to examine the influence of the difference between ψ_s and ψ_f. We also investigated the rate of EWL over time in cocooning frogs to determine the time required for development of substantial resistance to water flux.

Material and Methods

Animals and Cocoons

Animals were obtained during the wet season from sites in the vicinity of Darwin (12°24′S, 130°50′E), Northern Territory, Australia, and were maintained in the laboratory on a diet of insects and with free access to water. Immediately before forming a cocoon, the frogs were fully hydrated; the frogs were encouraged to store dilute fluid in their bladders by supplying them with water at one end of a tilted container. During cocoon formation, the frogs were maintained in the laboratory in darkened, ventilated containers at 24° ± 1°C and a relative humidity of 45%–60%. After 2–3 wk, the cocooning frogs were placed on nonstick baking paper so that the cocoon did not adhere to the bottom of the container and peel away from the ventral surface when the animals were removed. All frogs formed cocoons for 8 or more weeks before trials to ensure that the cocoon had sufficient thickness, associated in previous studies with high resistance to EWL (Christian and Parry 1997; Tracy et al. 2007). All experimental procedures were approved by the Charles Darwin University Animal Ethics Committee.

Water Exchange Trials

A 1% solution of agar (1 g agar per 100 mL water) was prepared with varying concentrations of sucrose or polyethylene glycol (PEG) 1000 (Sigma-Aldrich 81188). PEG is an inert, nonionic, long-chain polymer (Steuter et al. 1981) that is manufactured at various average molecular weights. PEG 1000 was chosen because it is soluble in water but has a relatively high molecular weight, so that it should have a large reflection coefficient for biological membranes and therefore is unlikely to be permeable to frog skin. Sucrose is highly soluble and with a lower molecular weight (342 Da) but has more predictable osmotic effects. Two hundred milliliters of agar-solute solution was poured into snap lock containers (15 cm × 10 cm × 7 cm) and allowed to cool and solidify overnight. This volume of agar-solute mixture was sufficient so that the uptake of water by the animal would not greatly influence the WP of the substrate over the course of a trial, and it also provided a sufficient surface area for the frogs to move around. The containers were fully enclosed to minimize EWL during trials and were covered with cloth bags to reduce disturbance to the frogs. Condensed moisture, formed overnight in the humid environment of the container, was removed before the trial. All trials were performed in the laboratory at 24° ± 1°C.

The WP of agar was estimated by preparing solutions of 1% agar plus solute (PEG 1000 or sucrose) across a range of concentrations and then measuring the WP of the agar osmoticum using a Psypro dew point psychrometer with a C-52 sample chamber (Wescor, Logan, UT). The average difference in WP between solutions and preparations of solutions in agar was used as the WP attributed to agar. The osmotic concentration of PEG 1000 was calculated on the basis of average molecular weight and then measured in prepared solutions using an Advanced Instruments micro-osmometer model 3300 (Norwood, MA). Because PEG 1000 behaved as a nonideal nonelectrolyte (Steuter et al. 1981; Sweeney and Beuchat 1993; Wood et al. 1993), the estimate of concentration (and hence osmotic contribution to WP; Janacek and Sigler 1996) was based on the measurements from the osmometer. The freezing point osmometry method is accurate for relatively dilute mixtures of osmolytes in water (Sweeney and Beuchat 1993). Solutions of sucrose in water closely approximate ideal behavior, at least at low concentrations (Michel 1972), so osmotic WP was calculated on the basis of the amount of sucrose dissolved in water at 24°C. The total WP of the agar + solute substrate was calculated as the combined matric (due to agar; estimated as −210 kPa) and osmotic (due to dissolved solute) WPs.

Each frog was used in three water exchange trials using the same substrate WP. The frog was placed in a container with an agar + PEG or agar + sucrose substrate. The mass of the frog placed on the substrate for 10 s was taken as the initial mass, then the trial was begun. Mass was measured to 0.01 g at 15-min intervals over a 90-min period. The time spent weighing the frog was minimized so as to reduce the possibility of excess water loss while outside of the trial container. Water exchange was the total mass gain or loss over the 90 min,

converted to hourly rates. Before all weighings, frogs were removed from the agar substrate and placed on a dry paper towel to absorb excess moisture on the ventral surface.

In each instance, the first trial was of cocooned frogs, obtained from the darkened containers in which they had been forming cocoons. Cocooned frogs were placed gently on the agar-solute substrate so as to minimize disturbance to the animal and preserve cocoon integrity. At the end of the 90-min trial, the frog was removed from the substrate and the cocoon was detached by making an incision on the dorsal surface and then gently peeling the dried skin away from the frog. The cocoons were weighed and then oven dried at 60°C for 5 d, at which point they attained constant weight (measured to 0.01 g).

Frogs with cocoons removed were then placed on a newly prepared substrate at the same WP and following the same weighing procedures. At the end of this trial, they were provided with moistened paper towels. On the following day, the frogs were given access to water and allowed to rehydrate. After 5 d with access to water, the bladders were drained to give a measure of standard mass (bladder empty weight of a hydrated frog; Ruibal 1962). The following day, a second measure of standard mass was made. To ensure full hydration, the frogs were then placed in shallow water overnight. Trials of the hydrated frogs were carried out the following morning, after emptying the bladder. This final mass was used as the standard mass for surface area specific calculations; surface area was estimated using the equation of McClanahan and Baldwin (1969). These values were multiplied by one-third to account for the ventral surface in contact with the substrate.

Body Fluid Osmolality

Body fluids were not obtained from cocooned frogs, since this would have affected the integrity of the cocoon and thus its permeability and the rate of water uptake or loss. Therefore, at the end of trials with the cocoon removed (the second trial), urine was obtained by inserting a flexible plastic cannula into the cloaca and applying pressure to the abdomen in the area overlying the bladder. Plasma samples were obtained from the femoral lymph sac using a fine-gauge needle (Terumo U-100 1.0-mL 27-gauge insulin needle), as described by Reynolds et al. (2009); blood plasma and lymph in this species are essentially equivalent. In hydrated animals, urine was obtained as the bladder was being emptied before trials, and plasma was obtained at the end of the trial. Osmolality of body fluids was measured with an Advanced Instruments (freezing point) micro-osmometer (Model 3300) calibrated with standards supplied by the manufacturer. Frog WP is related to plasma osmolality (osmotic concentration, C) by the equation $\psi_f = -CRT$, where R is the gas constant and T is the absolute temperature (see Shoemaker et al. 1992). Hence, at 20°C, $\psi_f = -C \times 2.436$ (osmolality in mOsm and WP in kPa). Plasma and urine osmolality were not significantly different in the sucrose and the PEG groups (Mann-Whitney U-test, $P > 0.1$ in all cases) for cocooned and hydrated animals, so these values were pooled.

Water Loss during Cocoon Formation

Mass loss rates of cocooned frogs maintained in dark, ventilated containers were measured by periodically weighing *Cyclorana australis* in the laboratory over a period of 10 wk. Mass was determined for frogs without access to water, and the first measurement was made after 1 wk. Withers (1998) termed this evaporative water loss and assumed that reductions in water loss were due primarily to the development of cocoon layers. There would likely be a minor component of mass loss due to metabolism over this period, although in the case of lipid catabolism this is negligible where metabolic water is retained in the body.

Results

Cocoons of frogs used in the water exchange trials were well formed, dry to the touch, and parchment like. Frogs in cocoons were relatively immobile, and the cocoon generally remained intact. Toward the end of the trials, the cocoon was moist to the touch over most of the ventral surface and was rendered quite supple, and in some cases, it began to pull apart along the midline of the belly, whereas the dorsal cocoon remained dry and relatively inflexible. Noncocooned animals on substrates with high (less negative) WPs tended to press their pelvic patch area against the substrate, in some cases forming an indentation on the surface of the agar. In contrast, hydrated frogs on substrates of very low WP frequently made scraping movements on the surface of the agar with their hind limbs and, in some cases, lifted their fingers and toes above the substrate.

Cocooned frogs showed a relatively low and constant increase in mass (range 5.5–14.6 mg cm^{-2} h^{-1}), irrespective of the WP (Fig. 1). None of the cocooned frogs lost water to the substrate. In contrast, when cocoons were removed, water uptake was rapid on pure agar (137 mg cm^{-2} h^{-1}) but decreased on substrates (agar + sucrose and agar + PEG) with low WP (Fig. 1). Water was lost by two of the decocooned frogs at very low WPs where the gradient favored water movement in this direction, but most decocooned frogs had relatively high plasma osmolality and, as a consequence, absorbed water. In rehydrated individuals, uptake was possible on substrates with WPs greater than approximately −600 kPa (equivalent to 250 mOsm kg^{-1}), but for more negative values all frogs lost water (Fig. 1). In all cases, water loss or gain in frogs without cocoons corresponded with the calculated difference between ψ_f (based on plasma osmolality) and ψ_s (based on a WP for agar of −210 kPa in addition to WP due to concentration of solute). Osmolality of the plasma and urine of cocooned frogs was significantly higher than in hydrated frogs (t-test, $P < 0.001$ in both cases; Table 1).

In cocooned frogs, there was a relatively rapid initial decrease in EWL, followed by a gradual reduction over the first month (Fig. 2). The frogs had sticky or dry skin (indicative of incipient cocoon formation) at the time of the first measurement, and thereafter the dorsal skin (cocoon) was dry. There was a transient increase in EWL at around 55 d associated with tempo-

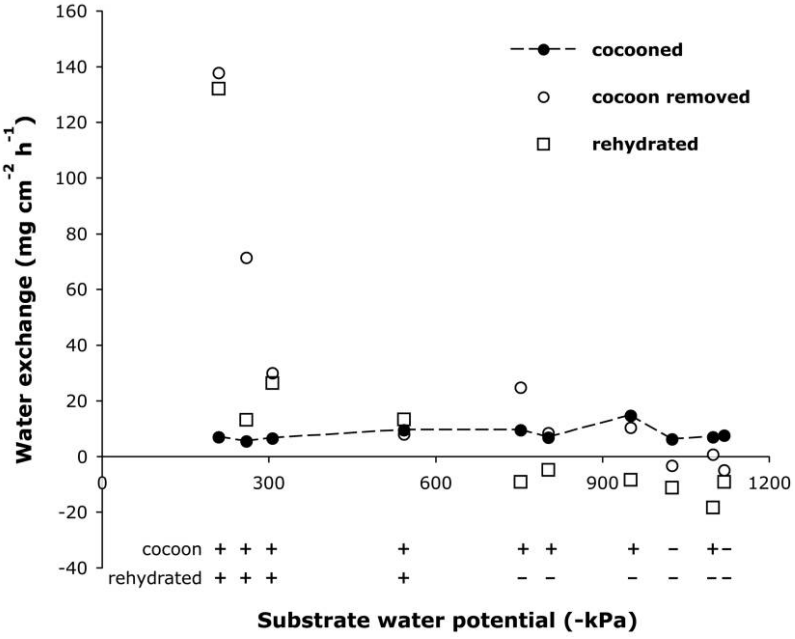

Figure 1. Water exchange of *Cyclorana australis* ($n = 10$) on semisolid substrates of agar + solute at various water potentials. Experimental procedures were carried out in the laboratory at 24° ± 1°C. The frog cocoon (*filled circles*) absorbed consistently low quantities of water, regardless of substrate water potential. Water exchange in frogs without cocoons was dependent on the water potential difference between frog and substrate. For frogs with the cocoon removed, a plus sign indicates that the water potential difference ($\psi_f - \psi_s$, where ψ_f is calculated from plasma osmolality and ψ_s is based on agar + solute) favored movement of liquid water from the substrate to the frog, whereas a minus sign indicates that the gradient was in the opposite direction.

rarily warm conditions in the laboratory. After this period, mass loss was relatively constant at ~0.2 mg g^{-1} h^{-1}.

Discussion

A small amount of mass was gained by *Cyclorana australis* with a well-developed cocoon, regardless of the WP of the substrate. The cocoon was moistened over much of the ventral surface, in contrast to the dry condition of the dorsal cocoon. The difference in mass between a freshly removed and an oven-dried cocoon ($n = 7$) was between 59% and 88% of the increase in mass of the frog while on the substrate, thus accounting for most of the water absorbed by the cocooned frogs. This suggests that moisture was absorbed by the multiple epidermal layers of the cocoon, infiltrating the fibrous matrix and causing expansion of the layers (Withers 1995). The observed absorption or adsorption of liquid water may be due to the hygroscopic nature of the dry epithelial layers (Withers 1998); since the cocoon absorbs moisture in humid air (Withers 1998), via capillary action it should absorb moisture from an agar substrate. It is significant that in cocooned frogs no water was lost to the substrate even at very low WPs. In contrast, two of the frogs on substrates of low WP lost water when the cocoon was removed, and several of the hydrated frogs lost water to the substrate. We infer from these findings that the cocoon forms a barrier to water loss from the frog to substrates of low WP (Seymour and Lee 1974; Shoemaker et al. 1992; McNab 2002; Booth 2006). The physical separation of the surface of the frog skin from the substrate means that regardless of the direction and magnitude of the WP difference, there is no physiological exchange. This is supported by the lack of osmotic water uptake, where there was a large difference in WP between the frog and the substrate (Fig. 1). Thus, the cocoon acts as a physical barrier that breaks the continuity between the frog and the soil, decoupling the frog from the osmotic gradient. The intact cocoon forms an effective barrier on dry substrates, whereas on wet substrates the cocoon would eventually soften and split open.

In the agar-solute substrate, there is a matric component from agar and an osmotic component from dissolved solute (sucrose or PEG), whereas in most soils WP is primarily due to the matric potential (influenced by the properties of the soil matrix). Nonetheless, the total WP drives the chemical energy balance, resulting in mass water movement (Slatyer 1967; Hillel 1971). The substrate used in the experiments most closely resembles a clay soil, in which water is retained even at low WPs, and there is (in addition to matric forces) an osmotic potential due to interlayer swelling associated with solvation forces between exchangeable cations and water molecules (Yong 1999). The conductivity of sand soils falls rapidly once the bulk water has drained from the soil (Ackerman 1991), whereas agar maintains hydraulic conductivity because water movement is unrestricted within the interstices of the gel matrix (Wiggins 1990). This type of substrate is thus of greatest relevance to studies of terrestrial or fossorial species that exchange water

Table 1: Osmolality of body fluids of cocooned and rehydrated *Cyclorana australis* used in water exchange trials

Group	Plasma Osmolality (mOsm kg^{-1})	*n*	Equivalent Mean Frog WP (kPa)[a]	Urine Osmolality (mOsm kg^{-1})	*n*
Cocooned	427.3 ± 73.6	9	−1,040	439.2 ± 95.6[b]	10
Rehydrated	234.4 ± 12.2	10	−570	45.0 ± 31.6	10

Note. Osmolality values are means ± SD. *n*, sample size; WP, water potential.

[a] Calculated from mean plasma osmolality at 20°C.

[b] The reported mean osmolality for urine is higher than that for plasma because there was insufficient plasma for measurement in one of the cocooned frogs, and this individual had an elevated urine osmolality (with this individual excluded, $n = 9$ and mean urine osmolality = 421.9 mOsm kg^{-1}). The urine and plasma means are not significantly different, and in all cases the urine of an individual frog was hyposmotic to or isosmotic with plasma.

with clay or loam soils, including species of *Cyclorana*, which use a wide variety of soil types, including clay soils (Booth 2006; McMaster 2006; S. J. Reynolds, K. A. Christian, and C. R. Tracy, personal observation). In contrast, experiments that examine water exchange using solutions or distilled water (e.g., Adolph 1933; Tracy 1976) may be more appropriate for aquatic frogs. An advantage of the agar osmoticum is that WP can be tightly controlled. It is suitable for assessing behavior associated with water uptake, such as the water absorption response (Stille 1958; Hillyard et al. 1998). In this study, for example, some frogs adopted a distinctive posture where the digits were lifted off the substrate. This may enable the frog to rest on the less permeable keratinized skin at the base of the feet and hands.

As a consequence of more than 8 wk without fluid intake in a relatively desiccating environment, the osmolality of the plasma and urine of cocooned *C. australis* was significantly higher than in hydrated frogs, in some cases exceeding 500 mOsm kg^{-1} (equivalent to −1,220 kPa). On high-WP substrates, the high osmolality of the plasma favored rapid water uptake as a result of the strong gradient, and even on substrates with WPs as low as −800 kPa (=330 mOsm kg^{-1}), uptake was possible in dehydrated frogs. In contrast, hydrated frogs with low plasma osmolality lost water to the substrate at this WP. The WP gradient drives the direction of water flow; however, water movement out of the frog was relatively slow, even where there was a large WP difference. This difference between relatively rapid uptake rates and slower loss rates (Fig. 1) is concordant with previous findings (Hillyard 1976; Tracy and Rubink 1978; Tracy et al. 2007) and is indicative of differential permeability depending on the direction of water flux. Hormonal influence on blood flow and skin permeability and recruitment of aquaporins to apical membranes (Willumsen et al. 2007) may enhance rates of uptake, whereas on dry substrates the permeability of the integument is reduced to minimize losses. The accumulated cocoon layers retard the passage of water molecules evaporating from the (saturated) skin surface to the air inside the burrow chamber. Because the cocoon forms while the animal is in a burrow (Tracy et al. 2007), evaporative losses are presumably primarily by diffusion rather than convection, since there is no air flow. With an increase in the number of epithelial layers, the cocoon slows rates of vapor diffusion; hence, resistance to EWL increases. Similarly, the cocoon may serve to reduce losses of liquid water to the soil by retarding movement of water from the skin to the substrate, effectively isolating the frog from the soil.

A burrow of *C. australis* during the dry season experiences a gradual drying of the soil from the surface downward due to evaporation. This results in a drying front (Ackerman 1991), with moist soil below the front and relatively dry soil nearer the surface (from which it is not possible for a frog to obtain water). This sharp gradient in WP may be the cue for cocoon formation; Tracy et al. (2007) noted that in the field the cocoon does not form until the soil has reached relatively low WPs, several months after the frogs bury. Similarly, Booth (2006) found that cocoons were not formed until the third month of estivation, corresponding with drying of the soil. The inducement to form a cocoon in sand may be vapor loss to the dry soil. However, clay soils retain moisture at low WPs so that cocoon formation may coincide with development of a WP gradient, which is potentially more desiccating because water exchange is more rapid by hydraulic conduction than vapor diffusion (Hillman et al. 2009).

The observed drop in EWL during the early cocooning phase (McClanahan et al. 1983; Withers 1998; Tracy et al. 2007; Fig.

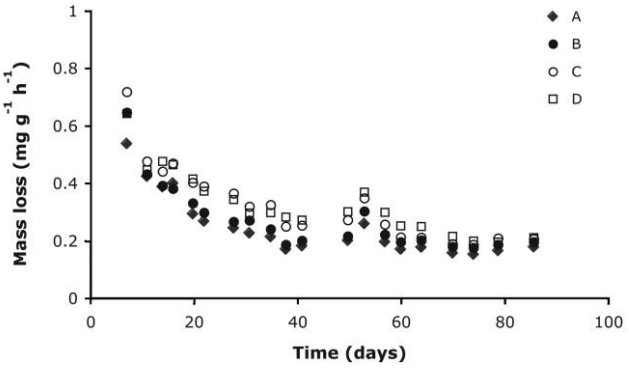

Figure 2. Mass loss over time during cocoon formation in *Cyclorana australis* ($n = 4$). Individual animals (*A–D*) without access to water were maintained in the laboratory and weighed periodically. Note that the first measurement was taken after 7 d, when cocoon formation had already begun. A color version of this figure is available in the online edition of *Physiological and Biochemical Zoology*.

2) is evidence of a relatively rapid change in permeability. Rates of water loss in this study were as low as 0.2 mg g^{-1} h^{-1} after 60 d with a cocoon, compared with water loss of 1.2–1.7 mg g^{-1} h^{-1} for hydrated *C. australis* maintained under similar conditions (S. J. Reynolds, unpublished data). The measured loss is slightly lower than a previous value for *C. australis* (0.5 mg g^{-1} h^{-1} after 90 d) using an open flow system (Christian and Parry 1997). Cocooned EWL rates are similar to *Lepidobatrachus llanensis* after 40 d (~0.9 mg g^{-1} h^{-1}; McClanahan et al. 1983), *Cyclorana maini* at 80 d (Fig. 1 in Withers 1998), and *Pyxicephalus adspersus* during cocoon formation (Loveridge and Withers 1981). The ability to form a cocoon may eliminate the necessity of constructing deep burrows and allow fossorial frogs to use drier parts of the landscape. There is some evidence that noncocooning fossorial frogs remain in a moist layer of soil (Roberts 1984; Cartledge et al. 2006*a*, 2006*b*) from which they can absorb water or at least remain in equilibrium with the soil water.

The WP of natural substrates varies from 0 kPa for pure water to large negative values (<−10,000 kPa) in dry soils. In wet soil, the mass flow of water is in the direction of the frog (Tracy 1976), but there should be an intermediate WP (the absorptive threshold) where the frog and substrate are in equilibrium (Shoemaker et al. 1992; Hillman et al. 2009). Water uptake in hydrated frogs occurred on substrates with WPs greater than −300 kPa (=120 mOsm kg^{-1}), but water was lost to substrates with WPs lower than −600 kPa (=250 mOsm kg^{-1}). In one hydrated frog, some water uptake was possible at ~−540 kPa, close to the osmotic WP of frog plasma (226 mOsm kg^{-1} in this individual; equivalent to −550 kPa). Similarly, Sinsch (1987) found that water uptake in *Rana* spp., though greatly reduced, was possible on soils at −550 kPa, and *Cyclorana alboguttata* gained mass on soils at −400 kPa but lost mass on soils at −1,000 kPa (Booth 2006). In trials on substrates close to the equivalent WP of the frog, during some 15-min intervals the animal gained mass, but in others mass was lost, suggesting that they were close to the absorption threshold.

At relatively high WPs of >−100 kPa, water is held in the soil voids as a result of capillary forces (Hillel 1971; Ackerman 1991), and frogs are able to absorb water from the soil (Dole 1967; Walker and Whitford 1970). At lower WPs, when the bulk water has drained from the soil, liquid water is present as thin films (adsorbed water) on the surface of soil particles, and vapor is present in the interstices. In this situation, the skin surface is losing water to the vapor phase of the soil pores, which are at close to saturation vapor pressure, or to the soil particles themselves. In the latter case, it would be necessary for the soil particles to attract water to their surfaces in order to drive water movement in the direction of the soil. Liquid water moves between soils at different WPs along its potential gradient (Slatyer 1967; Hillel 1971). However, in the case of the movement of water at the skin-soil interface, it is unclear how the soil matrix interacts with the osmotic milieu of the body fluids, specifically the plasma in the area of the pelvic patch. In cocooned frogs, there may be a space between the skin and the cocoon (Withers 1998), which would prevent mass transfer of liquid water from skin to cocoon. Whether water loss is by liquid or vapor diffusion may depend on the hydraulic conductivity and vapor conductivity of the soil (Ackerman 1991) and is likely to involve a relatively abrupt transition from rapid liquid water exchange to lower evaporation rates due to vapor diffusion (Shokri et al. 2009).

Studies of EWL are carried out in the laboratory under conditions very different from those inside a burrow where the cocoon is formed. The relationship between WP and relative humidity (RH) is logarithmic (Papendick and Mulla 1986; Ackerman 1991), so that at WPs of −570 kPa, corresponding to the body fluids of a hydrated frog (~230 mOsm kg^{-1}; Table 1), equivalent RH is greater than 99%. Inside burrows, humidity is high even in relatively dry soils (Tracy et al. 1978, 2007); for example, at a WP of −10,000 kPa, RH exceeds 90%. Hence, the vapor gradient is relatively small, and in addition, EWL is not intensified by air movement. In contrast, loss of fluid to substrates of low WP has a direct bearing on conditions in a burrow, particularly in clay and clay loam soils. The accumulation of epithelial layers is similar in all cocooning anurans; hence, we anticipate that the findings for *C. australis* are applicable to other species. Cocoon formation has been documented in other burrowing organisms (Greenwood 1986; Pusey 1986; Etheridge 1990; Petersen et al. 2008), and although some cocoons are comprised solely of mucus, others are formed from multiple epithelial layers. Most, if not all, cocooning species live in arid or seasonally dry environments where water becomes unavailable (either for drinking or via osmotic uptake). It is our contention that the cocoon is an adaptation to a subterranean existence and that cocoons have evolved largely to reduce rates of water loss (liquid and vapor) to dry soils.

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