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Cocoon and Epidermis of Australian *Cyclorana* Frogs Differ in Composition of Lipid Classes That Affect Water Loss

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ABSTRACT

For amphibians to survive in environments that experience annual droughts, they must minimize evaporative water loss. One genus of Australian hylid frogs, *Cyclorana*, prevents desiccation by burrowing in the soil and forming cocoons composed of alternating layers of shed epidermis and glandular secretions. Previous data are inconclusive about the role that lipids play in reducing evaporative water loss through skin (cutaneous water loss [CWL]) when *Cyclorana* spp. are within cocoons. In this study, we measured CWL and lipids in the epidermis and in cocoons of five species of *Cyclorana*. CWL was significantly lower in frogs within cocoons than in frogs without cocoons. Surface-area-specific CWL for the three small species was significantly higher than that of the two larger species of *Cyclorana*, but this difference was not apparent in frogs within cocoons. Although lipids were responsible for more of the dry mass of the epidermis (approximately 20%) than of the cocoons (approximately 7%) we found that cerebroside and ceramides, two polar lipid classes, were almost exclusively found in cocoons. This suggests that these lipid classes are in the glandular secretions rather than in the epidermis. Because these polar lipids are the types that reduce water loss in birds (cerebrosides and ceramides) and mammals (ceramides), we conclude that they are important not only for holding together

the shed layers of skin but also for contributing to the barrier against water loss.

Introduction

Although many species of amphibians are aquatic or semi-aquatic, and therefore have access to water, others occupy terrestrial habitats in which water is present only sporadically; the latter species are particularly vulnerable to the threat of desiccation (Lillywhite 2006). Rates of total evaporative water loss (TEWL), the sum of cutaneous (CWL) and respiratory (RWL) water losses, vary widely among amphibians (Bentley 1966; Spotila and Berman 1976; Withers et al. 1984; Wygoda 1988), but in studies so far, CWL accounts for approximately 99% of TEWL (Spotila and Berman 1976; Bentley and Yorio 1979; Wygoda 1981). Adaptations employed by terrestrial amphibians to reduce CWL can be behavioral, such as seeking moist microclimates or burrowing in soil during drought periods; physiological, such as development of a barrier in their skin to prevent excessive water loss (Heatwole 1960; Shoemaker et al. 1972; McClanahan et al. 1976; Christian and Parry 1997; Tracy et al. 2007); or a combination of these.

Some amphibian species have low rates of CWL, suggesting that their integument forms a partial barrier to water vapor diffusion (Blaylock et al. 1976; Withers et al. 1984; Christian and Parry 1997; Young et al. 2005). Amphibian skin consists of a dermis and an epidermis, the outermost layer of which is the stratum corneum (SC), a thin layer of keratinized cells approximately 1 μm thick (Heatwole 1994; Lillywhite 2006). Anuran SC is loosely attached to the outer living layer of epidermis, the stratum granulosum, which sits on two subsequent layers, the stratum spinosum and stratum germinativum, the lower of which is attached to the basement membrane of the epidermis (Heatwole 1994; Lillywhite 2006). It is thought that an intercellular lipid layer within the epidermis reduces water vapor diffusion through the integument in some species (Lillywhite and Maderson 1988). Most species with reduced rates of CWL have a lipid layer in the skin, but the location of this layer varies among species. In the skin of members of *Litoria*, a genus of Australian hylid frogs with a wide range of rates of CWL, the more drought-tolerant species were found to have a lipid layer just below the stratum granulosum, whereas frogs that were prone to high rates of CWL had a lipid layer in the dermis (Amey and Grigg 1995). The dermis is below the epidermis, where the lipid, mucosal, and poison glands of frogs are located (Amey and Grigg 1995; Lillywhite 2006). Another

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method used by some amphibians to reduce water loss is the deposition and subsequent wiping of lipids onto the surface of the skin from glands within the dermis, as in the Phyllomedusidae (Shoemaker et al. 1972; Blaylock et al. 1976; Gomez et al. 2006).

Some land-dwelling amphibians, including members of the Hylidae and Ranidae, survive dry periods by burrowing into the ground and forming a relatively water-impermeable structure known as a cocoon, which lies over the epidermis and is composed of alternating layers of exfoliated skin and of secretions from glands within the dermis (Lee and Mercer 1967; McClanahan et al. 1976; Loveridge and Crayle 1979; Ruibal and Hillman 1981; Christian and Parry 1997). Cocoons form a physical barrier that breaks the continuity between the frog and dry soils, thus preventing water loss to the soil (Reynolds et al. 2010). Although less important in humid burrows, cocoons also greatly reduce CWL, and this would be important in extremely dry burrows. The efficacy of cocoons in reducing CWL is well established. Individual *Cyclorana australis* lost 7.8 mg H₂O g⁻¹ h⁻¹ when not within a cocoon, but after they had formed a cocoon, CWL averaged 0.5 mg H₂O g⁻¹ h⁻¹, a 15-fold reduction (Christian and Parry 1997). Although cocoons are used by a variety of amphibians to survive dry periods, the biochemical constituents of cocoons and how they restrict water loss have received little attention (Smith 1931; Reno et al. 1972; Christian and Parry 1997).

Whether the barrier to water loss present in the skin of some terrestrial amphibian species is formed by proteins, lipids, or a combination of the two, is a subject of considerable discussion. In an analysis of cocoons and mucous secretions in *Litoria caerulea* and *C. australis*, mucous secretions and cocoons were composed primarily of proteins (Christian and Parry 1997), leading to the suggestion that proteins may play a role in the barrier to water diffusion. In contrast, after McMaster (2007) extracted the lipids from the cocoons of *Cyclorana* and re-measured water loss, CWL increased nearly sevenfold, suggesting that lipids within the skin were important in reducing CWL.

The manner in which lipid molecules interact and pack together affects the permeability of the skin to water diffusion (Potts and Francoeur 1990). In the mammalian SC, the outer layer of epidermis responsible for the water barrier, various classes of ceramides along with cholesterol and free fatty acids are thought to pack tightly together in lamellar layers forming a relatively impervious barrier to water loss. Polar head groups of ceramides associate tightly with one another, whereas the long, nonpolar fatty acid tails form a lattice structure (Bouwstra et al. 2000; Gu et al. 2008; Muñoz-Garcia et al. 2008b). In the avian SC, ceramides together with cerebrosides, the latter a ceramide with a hexose sugar attached, are the primary lipids involved in formation of a barrier to water vapor diffusion (Williams and Tieleman 2005). The mechanism of lipid packing between ceramides and cerebrosides within the avian SC remains unresolved. In mammals and birds, cholesterol seems to influence the fluidity of the lipid layer and thus increase water loss through the skin (Bouwstra et al. 2000; Wertz 2000;

Muñoz-Garcia et al. 2008b). The effectiveness of cocoons as water barriers in amphibians may involve lipid molecules, or it may be a consequence of the insulating effect of multiple layers of shed skin. We explored the role of lipids in cocoons by measuring lipids in both the epidermis and in the components of the cocoon, which includes shed skin and glandular secretions.

If cocoons are effective barriers to water loss simply because of the insulating effects of multiple layers of shed skin, we would predict that the lipids would be similar in the epidermis and in cocoons. If, on the other hand, the lipid components differ, then this would suggest that glandular secretions found between the layers of shed skin contain lipids. Furthermore, if these glandular lipids in the cocoon consist of cerebrosides and ceramides, we would conclude that they have a role in the cocoon's barrier to water loss because these polar lipids are the types that reduce water loss in the integuments birds and mammals. To explore these relationships, we measured CWL in five species of Australian hylid frogs (*Cyclorana* spp.), with and without a cocoon, and then measured the lipid composition of their cocoons and epidermis.

Methods

Study Animals

We collected individuals of the *Cyclorana* genus—*C. australis*, *C. longipes*, *C. maculosa*, and *C. platycephala*—from sites throughout the Northern Territory, Australia: Larrimah (15°34'13"S, 133°12'23"E), North Daly Waters (15°56'23"S, 133°26'7"E), Daly Waters (16°15'12"S, 133°22'09"E), Dunmarra (16°42'56"S, 133°25'10"E), Newcastle Creek (17°15'28"S, 133°27'15"E), and Newcastle Waters (17°22'19"S, 133°24'47"E), then transported them to our lab in Darwin. Frogs were induced to form cocoons during May through June, 2009, by placing each individual in a plastic container at 23°C and 50%–60% relative humidity for 1 to 2 wk. When cocoon formation was initiated by frogs, we placed their containers in an incubator that controlled temperature at 23°C and relative humidity at 90%. Frogs remained within cocoons in a state of estivation for 50–70 d before measurements of CWL.

CWL Measurements

We measured CWL of frogs using an open-flow system (Young et al. 2005). We placed frogs in a circular plexiglas chamber with an internal diameter of 57 mm and a rubber stopper on each end. We measured two individuals simultaneously in separate chambers. Air was pumped at 380 mL/min through a column of Drierite, and then into the chamber. We controlled air flow into each chamber using Sierra mass-flow controllers calibrated daily against a bubble flow calibrator (Gilibrator 2 primary flow calibrator; Sensidyne, Clearwater, FL). Air exiting each chamber was routed into a plexiglas chamber containing a Vaisala HMP130Y humidity and temperature sensor (Vaisala, Helsinki). Probes for each chamber were calibrated at the beginning and end of the experiment against a Vaisala HM11

calibrator (Vaisala) using standardized saturated salt solutions. The chambers and sensors were placed inside a Sanyo incubator (MIR 253; Osaka) maintained at 25°C. We allowed the temperature and humidity of the chambers to remain stable for a minimum of 1 h before frogs were placed inside. Once in the chamber, most frogs initiated a water-conserving posture (WCP), in which they tucked their legs under their bodies and lowered their bodies and heads against the substrate, thus reducing the exposed surface area (Heatwole 1963). We measured water loss from a frog only if it adopted a WCP. Outputs of humidity and temperature sensors of the air downstream from the chamber were monitored with a PowerLab recording system (AD Instruments, Bella Vista, New South Wales). Baseline relative humidity without a frog in the chamber was recorded before and after trials with a frog in the chamber. In all cases, relative humidity after the test runs returned to pretrial levels. The lowest average humidity that was stable for at least 20 min with and without a frog in the airstream was used in the calculation for each trial.

Rates of CWL were measured for frogs when they were in and out of a cocoon; the latter measurement was made after frogs had rehydrated on a wet paper towel for 24 h. Cocoons were removed by gently teasing the cocoon away from the epidermis using forceps. Then we cut along the sides of the cocoon with scissors and removed the remainder with forceps. We allowed frogs to rehydrate, blotted them dry with a paper towel, and weighed them to the nearest 0.001 g (Shimadzu BX 420H balance). We then placed the individual into the middle of the chamber and sealed it with a rubber stopper. We placed a cloth over the chamber to minimize illumination within the chamber during measurements. During each trial, we continuously monitored humidity within the chamber. When relative humidity values were consistently low, we verified that the frog was in a WCP and then recorded relative humidity for each trial. After the trial, we recorded mass of the frog and measured skin temperature using a Raytek noncontact infrared thermometer. For frogs in a moving airstream, skin temperature and cloacal temperature are not significantly different (Wygoda 1984), so skin temperature can be used for body temperature.

Respiratory Water Loss

We calculated respiratory water loss (RWL) as the product of breathing rate and tidal volume for active frogs and for frogs in cocoons. The allometric equation for tidal volume in an amphibian is $TV = 0.4W^{0.73}$ where TV is tidal volume (mL air breath⁻¹) and W is mass (g; Hutchison et al. 1968; Spotila and Berman 1976). We counted the number of breaths taken by a frog during 10 min when it was within a cocoon and 10 min when it was not within a cocoon to calculate breaths per minute. We then multiplied tidal volume by breathing rate to get milliliters of air exhaled per minute. Assuming exhaled air was saturated with water vapor, we determined RWL as RWL (g H₂O min⁻¹ cm⁻²) = $TV \times BR [s_d(T_i) - RH_s d_a(T_a)]/SA$, where BR is breathing rate in breaths per minute, $s_d(T_i)$ is saturation vapor density of water at lung temperature, $s_d(T_a)$ is saturation

vapor density of water at air temperature (both in g mL⁻¹ min⁻¹), RH is relative humidity expressed as a decimal fraction, and SA is total surface area of the animal in square centimeters. Saturation vapor densities were determined using the Smithsonian meteorological tables (List 1971).

Mean RWL equaled 1.2% ± 0.18% of TEWL in frogs within cocoons ($n = 26$) and 0.053% ± 0.006% of TEWL in frogs without cocoons ($n = 25$). These values agree with research on the leopard frog *Rana pipiens* (Spotila and Berman 1976) and on *Bufo bufo*, *Xenopus laevis*, *Agalychnis danicolor*, and *Scaphiopus holbrooki* (Bentley and Yorio 1979; Wygoda 1981). Because RWL appears to be a small component of TEWL, we have used TEWL as a proxy for CWL.

Cocoon Samples

We estimated the effect of lipids on water loss through the cocoon by measuring water loss through cocoon sections before and after lipid extraction. We removed the cocoon from each frog and then cut a disk 0.83 cm in diameter out of the dorsal surface of the cocoon. We used a silicone sealant to affix the disk onto a 2-mL glass vial containing a known mass of water. We allowed the silicone to dry for 24 h, and during this time the vial was placed within a closed container to which was added 1 cm of deionized water. Because it was inside a container with high humidity, the cocoon disk also became hydrated. We then placed the vial covered by the cocoon disk into our chamber and measured water loss as we had done for frogs. We freeze-dried the remainder of the cocoon and weighed it on a balance to the nearest 0.00001 g (Sartorius CP225D, Goettingen). We extracted lipids from the cocoon (see "Lipid Extraction" below), freeze-dried the sample again, and weighed the sample to the nearest 0.01 mg. We determined the dry mass of lipids of the cocoon by subtracting the mass of the freeze-dried cocoon with lipids extracted from the mass of the freeze-dried cocoon before extraction. We then rehydrated the cocoon in a glass vial in the closed container with 1 mL of deionized water for 24 h and cut out a second 0.83-cm disk from the dorsal surface and attached this sample to a 2-mL glass vial as before and measured water loss through the cocoon disk with lipids extracted.

Calculations

We calculated rates of CWL from the equations of Bernstein et al. (1977) for an open-flow system, in conjunction with tables of saturation vapor density (List 1971): $CWL = V_e \rho_e - V_i \rho_i$, where V_e and ρ_e are the flow rate and the water vapor density of the excurrent air, respectively, and V_i and ρ_i are the corresponding incurrent values. Water vapor density was determined by measuring the temperature of the incurrent or excurrent air, and the saturation vapor density was obtained from List (1971).

We determined surface area of frogs using the empirical equation based on mass from McClanahan and Baldwin (1969), an equation validated for Australian hylid species by Buttemer (1990). Frogs, when in WCP, expose only two-thirds of their

surface area to the air (Withers et al. 1982; Young et al. 2005; Tracy et al. 2008), thus we estimated the effective surface area as two-thirds of the total surface area of the frog. We then divided CWL rate by the effective surface area to calculate surface-specific CWL (ssCWL). Total resistance to water loss (R_t) was calculated by dividing the vapor density difference between the skin of the frog and the air in the chamber by ssCWL (Spotila and Berman 1976), assuming that vapor density at the frog skin was the saturation vapor density at skin temperature.

To help understand potential advantages of cocoon formation, we used the biophysical model of Tracy et al. (2010) to calculate the length of time it would take a frog in a burrow to dehydrate to lethal levels, which we assumed to be when they evaporated water equivalent to 30% of their standard mass, defined by Ruibal (1962) as the mass of a fully hydrated frog with an empty bladder. We used the mean R_t with and without a cocoon for each species and the mean mass of the fully hydrated frogs (i.e., without cocoons) in calculations. Relative humidity in the burrow was calculated from the equations of Tracy et al. (1978): $\rho_a/\rho_s = e^{(\Psi/RT)}$, where ρ_a and ρ_s are, respectively, the vapor densities of air and of air saturated with water vapor at the same temperature, so their ratio is equivalent to relative humidity, Ψ is the water potential of the soil, R is the gas constant for water vapor (462 kPa K⁻¹), and T is soil temperature (K). Soil water potential was assumed to be -1.5 MPa, based on measurements of soil at burrow depth of *C. australis* late in their dormancy period (Tracy et al. 2007), so burrow relative humidity was 0.99. We assumed a constant burrow temperature of 25°C, which is the average soil temperature at burrow depth for *C. australis* over the duration of the dormancy period (C. R. Tracy, unpublished data). The appendix, in the online edition of *Physiological and Biochemical Zoology*, lists all model parameters and values.

Lipid Extraction

Following CWL measurements, we killed each frog by stunning and double-pithing. We removed the skin using scissors and forceps, making an incision down the ventral surface of the abdomen and of the limbs. We then separated the epidermis from the dermis by placing the skin in a hot-water bath at 60°C for 2 min. This method allows separation of the epidermis but retains the integrity of lipids within (Wertz et al. 1986; Gay et al. 1994; Muñoz-García et al. 2005). We freeze-dried the cocoon and epidermis (Dynavac FD3 freeze-dryer, Sydney) and weighed them on a balance to the nearest 0.01 mg. To minimize water uptake by the freeze-dried cocoons or epidermis during weighing, we taped a mesh bag filled with Drierite inside the balance.

We extracted lipids from both cocoons and epidermis using 2 : 1, 1 : 1, and 1 : 2 v/v mixtures of chloroform : methanol with 50 mg/L BHT added as an antioxidant (Law et al. 1995; Muñoz-García and Williams 2005). We dried the lipid mixtures by placing the glass vial into a hot-water bath at 50°C with a constant stream of nitrogen over the opening. We then freeze-

dried the epidermis and cocoons again and reweighed them, again with Drierite inside the balance. We subtracted the weight of the freeze-dried cocoons with lipids extracted from the original weight to determine the percentage of dry mass that can be attributed to lipids in each epidermis and each cocoon. We transported lipids to Columbus, Ohio, in glass vials on ice.

In Ohio, we recorded the minimum volume of 2 : 1 chloroform : methanol needed to dissolve the lipids and then dried the samples using a stream of nitrogen with a gas manifold (N-EVAP, model 11155-O; Organomation, Berlin, MA). We removed any proteins and/or salts from the lipid extracts by adding 4 parts 2 : 1 chloroform : methanol to 1 part nanopure water and allowed the aqueous phase, which contains any contaminants that might have been present in the lipid extract, to separate from the chloroform : methanol phase for 1 h. We removed the aqueous phase from the vial, dried the sample, and redissolved the lipids with 2 : 1 chloroform : methanol.

Thin-Layer Chromatography

To identify lipid classes, we used analytical thin-layer chromatography (TLC) and quantified the lipid classes using photodensitometry (Muñoz-García and Williams 2007; Muñoz-García et al. 2008a, 2008b). We estimated known quantities of lipid standards on each plate and used only the plates in which the error was less than 10%.

To remove contaminants, we developed 20 × 20-cm silica gel G plates (250 μm thick; Analtech, Newark, NJ) with chloroform : methanol (2 : 1) to the top and then activated plates in an oven at 110°C for 30 min to remove any water. We scored the plate into lanes: seven lanes used for lipid standards and eight used for our samples, each loaded in duplicate. We pipetted 5 μL of each lipid extract and each standard in the preadsorbent area of the plate using a Teflon-tipped 10-μL Hamilton syringe.

To identify and quantify nonpolar lipids, we created standards by serial dilution with a mixture of cholesterol oleate, methyl esters, triglycerides, free fatty acids (FFAs), and cholesterol dissolved in chloroform : methanol (2 : 1) with BHT. We then developed the plates in 500 mL of hexane : ethyl ether : acetic acid (70 : 30 : 1) 5 cm from the top of the plate. To identify and quantify polar lipids, we prepared standards using a mixture of nonhydroxy fatty acid ceramides, galactocerebrosides, cholesterol, cholesterol sulfate, phospholipids, and sphingomyelin. We developed these plates in chloroform : methanol : water (40 : 10 : 1) to 10 cm from the bottom of the plate to separate the cerebrosides, then in chloroform : methanol : acetic acid (190 : 9 : 1) to 12 cm from the bottom of the plate to separate ceramides. Finally, we placed the plates in hexane : ethyl ether : acetic acid (70 : 30 : 1) run to the top to separate cholesterol and other nonpolar lipids. We also prepared phospholipid standards using phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, and sphingomyelin. To separate phospholipids, we developed plates in chloroform : methanol : acetone : acetic acid : water (17.5 : 12.5 : 5.5 : 2 : 1) to the top.

Table 1: Mean (± 1 SE) cutaneous water loss (CWL), mass, effective surface area, surface-specific (ss) CWL of *Cyclorana* species with and without cocoons, total resistance to water loss (R_t), the ratio of ssCWL of frogs within/without cocoons, and the dry masses of cocoons and epidermis (range in parentheses)

	<i>C. cultripes</i> (<i>n</i> = 10)	<i>C. longipes</i> (<i>n</i> = 3)	<i>C. maculosa</i> (<i>n</i> = 7)	<i>C. platycephala</i> (<i>n</i> = 9)	<i>C. australis</i> (<i>n</i> = 8)
Within cocoon:					
CWL (g d ⁻¹)	.5 \pm .13 ^a	.9 \pm .17 ^{a,b}	.4 \pm .11 ^a	.9 \pm .20 ^{a,b}	1.3 \pm .21 ^b
Mass (g)	5.7 \pm .67 ^a	6.9 \pm 1.05 ^a	7.7 \pm .52 ^a	31.0 \pm 4.59 ^b	39.9 \pm 2.93 ^b
Two-thirds surface area (cm ²)	17.4 \pm 1.13 ^a	19.5 \pm 1.63 ^a	20.8 \pm .76 ^a	44.4 \pm .86 ^b	52.1 \pm 5.76 ^c
ssCWL (mg H ₂ O h ⁻¹ cm ⁻²)	1.2 \pm .39 [*]	2.0 \pm .51	.8 \pm .23 [*]	.8 \pm .12 [*]	1.0 \pm .16 [*]
R_t (s cm ⁻¹)	95.6 \pm 16.43 ^a	39.9 \pm 11.42 ^a	115.3 \pm 16.04 ^a	118.9 \pm 19.65 ^b	72.5 \pm 7.43 ^b
Cocoon mass (mg)	50.9 \pm 11.0	79.9 \pm 26.0	63.2 \pm 15.9	75.1 \pm 24.0	276.2 \pm 34.2
Range	(6.8–107.3)	(41.8–129.5)	(11.3–121.6)	(14.6–224.6)	(176.1–440.2)
Epidermis mass (mg)	5.7 \pm .8	10.6 \pm 3.1	9.9 \pm .8	34.1 \pm 9.7	49.6 \pm 7.8
Range	(1.8–12.1)	(7.1–16.8)	(6.2–12.4)	(10.2–88.6)	(13.3–80.9)
Without cocoon:					
CWL (g d ⁻¹)	4.3 \pm .31 ^a	5.5 \pm .52 ^a	5.1 \pm .26 ^a	6.9 \pm .47 ^b	7.9 \pm .37 ^b
Mass (g)	7.3 \pm .58 ^a	9.9 \pm 1.45 ^a	10.1 \pm .95 ^a	28.5 \pm 4.51 ^b	40.3 \pm 2.30 ^c
Two-thirds surface area (cm ²)	20.3 \pm .94 ^a	24.3 \pm 1.66 ^a	24.5 \pm .45 ^a	42.5 \pm 3.86 ^b	52.7 \pm 1.67 ^c
ssCWL (mg H ₂ O h ⁻¹ cm ⁻²)	8.9 \pm .63 ^{a*}	9.3 \pm .27 ^a	8.7 \pm .44 ^{a*}	6.9 \pm .26 ^{b*}	6.3 \pm .13 ^{b*}
R_t (s cm ⁻¹)	4.4 \pm .44	3.2 \pm .52	3.7 \pm .25	2.9 \pm .33	2.3 \pm .35
Ratio ssCWL within/without cocoon	.14 \pm .048	.17 \pm .045	.09 \pm .025	.11 \pm .019	.17 \pm .028

Note. Epidermis mass shown in milligrams. Superscript letters denote statistical differences in each category (CWL, mean, effective surface area, ssCWL), among species with statistical difference accepted when $P \leq 0.05$; Student-Newman-Keuls post hoc test. No differences were found among species in ssCWL or R_t for frogs within a cocoon or for those without a cocoon. Asterisk denotes statistical significance between surface-specific CWL of frogs within and without a cocoon for each species.

To visualize lipids, we sprayed plates with a solution of 3% cupric acetate in 8% phosphoric acid and heated them in an oven at 180°C for 35 min to char lipids. We quantified concentrations of lipid classes by scanning plates onto a computer using a Hewlett-Packard scanner and measured the density of lipid bands using IMAL TN-Image 3.5.10c (T. J. Nelson, 2008: shared software available at <http://www.randombio.com/imal.html>).

Statistics

All statistical tests were performed using SPSS 17.0 with the null hypothesis rejected at $P \leq 0.05$. Means are reported ± 1 SE. We tested for normality and homogeneity of variance with Shapiro-Wilk and Levene's test of homogeneity, respectively, before analysis. We lost one sample of *C. maculosa*, which reduced our sample size by one.

We performed a repeated-measures two-way ANOVA to determine differences in lipid composition between cocoons and epidermis. We used an ANCOVA to determine whether effective surface area and species were significant covariates for frogs within cocoons and without cocoons. We used a *t*-test with a modified Bonferroni correction to determine whether there was a difference between CWL of frogs within cocoons and frogs not within cocoons and also to determine whether there was a difference between cocoon disks with and without lipids.

To determine interdependency of lipid classes in the cocoons and epidermis, and also of CWL, body mass, and lipids, we

used a general linear regression model to identify correlations. To test for differences in proportions of polar, nonpolar, and phospholipids, we first transformed percentages using a logit function, ($\ln [Y/(1 - Y)]$; Zar 1996) and then used a repeated-measures two-way ANOVA.

Results

Body Mass and Surface Area

For frogs within a cocoon, body plus cocoon mass ranged from 5.7 g in *Cyclorana cultripes* to 39.9 g in *Cyclorana australis* (table 1). Frogs allowed to rehydrate after cocoons were removed weighed 7.3 g in *C. cultripes* to 40.3 g in *C. australis* (table 1). The effective surface area of the frogs in WCP ranged from 17.4 cm² in *C. cultripes* to 52.1 cm² in *C. australis* for frogs within a cocoon and from 20.3 cm² in *C. cultripes* to 52.7 cm² in *C. australis* for frogs not within a cocoon (table 1). Cocoon dry mass ranged from 50.9 \pm 11.0 mg in *C. cultripes* to 276.2 \pm 34.2 mg in *C. australis* (table 1). Epidermis mass ranged from 5.7 \pm 0.8 mg in *C. cultripes* to 49.6 \pm 7.8 mg in *C. australis* (table 1).

Cutaneous Water Loss of Frogs and of Frogs within Cocoons

CWL of frogs in a cocoon ranged from 0.5 g H₂O day⁻¹ in *Cyclorana maculosa* to 1.3 g H₂O day⁻¹ in *C. australis* (table 1). CWL of frogs without cocoons ranged from 4.3 g H₂O d⁻¹ in *C. cultripes* to 7.9 g H₂O d⁻¹ in *C. australis* (table 1). Effective

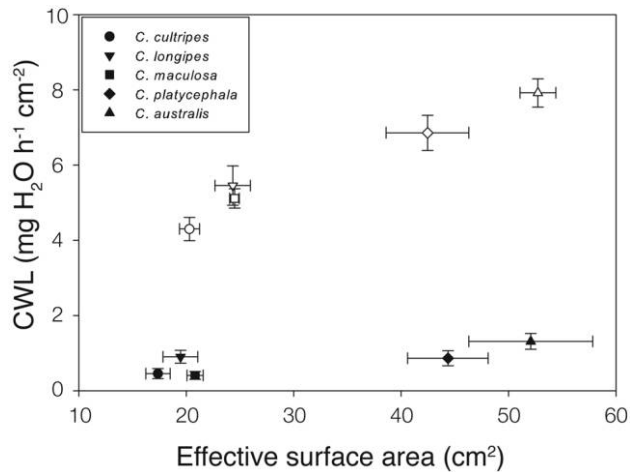


Figure 1. Mean (± 1 SE) cutaneous water loss (CWL; $\text{g H}_2\text{O d}^{-1}$) versus mean (± 1 SE) effective surface area of frog (cm^2) by species. Filled symbols denote frogs within a cocoon, and unfilled symbols denote frogs without a cocoon.

surface area was a significant covariate of CWL for frogs within cocoons and frogs without cocoons ($F_{1,31} = 8.02$, $P = 0.008$; $F_{1,31} = 43.2$, $P \leq 0.0001$, respectively; fig. 1), but species was not a significant covariate for frogs within cocoons or frogs without cocoons ($F_{4,28} = 1.46$, $P = 0.24$; $F_{4,28} = 0.74$, $P = 0.57$, respectively).

When frogs were in cocoons, surface-specific CWL (ssCWL) varied from $0.8 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *C. maculosa* and *Cyclorana platycephala* to $2.0 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *Cyclorana longipes* (table 1). Surface-specific CWL of frogs without cocoons ranged from $6.3 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *C. australis* to $9.3 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *C. longipes* (table 1).

Surface-specific CWL in frogs without cocoons was significantly higher than frogs within cocoons in four of the five species (fig. 2A): *C. cultripes* ($n = 9$, $t = 10.1$, $P < 0.001$); *Cyclorana maculosa* ($n = 7$, $t = 17.2$, $P < 0.001$), *C. platycephala* ($n = 9$, $t = 21.3$, $P < 0.001$), *C. australis* ($n = 7$, $t = 25.60$, $P < 0.001$). The sample size for *C. longipes* ($n = 2$) was too small for meaningful statistics, but there was a trend toward their also having higher ssCWL without cocoons than with them.

Mean total resistance to water loss (R_t) of frogs with cocoons ranged from 39.9 s cm^{-1} in *C. longipes* to 118.9 s cm^{-1} in *C. platycephala* (table 1). There were no significant differences among species ($F_{4,31} = 2.65$; $P = 0.052$). Frogs without cocoons had much lower R_t , ranging from 2.3 s cm^{-1} in *C. australis* to 4.4 s cm^{-1} in *C. cultripes* (table 1), and there were significant differences among species ($F_{4,29} = 4.81$; $P = 0.004$), with *C. cultripes* having significantly higher resistance than *C. platycephala* and *C. australis*.

Cutaneous Water Loss through Cocoons In Vitro

CWL through disks of cocoons ranged from $79 \text{ mg H}_2\text{O d}^{-1}$ in *C. cultripes* to $135 \text{ mg H}_2\text{O d}^{-1}$ in *C. platycephala* (table 2).

CWL from cocoon disks with lipids extracted ranged from $96.8 \text{ mg H}_2\text{O d}^{-1}$ in *C. cultripes* to $219 \text{ mg H}_2\text{O d}^{-1}$ in *C. longipes* (table 2). For each species, CWL was not statistically different between disks of intact and cocoons with lipids extracted (fig. 2B), but when all species were pooled together and a paired t -test was performed, cocoon disks with lipids extracted had significantly higher CWL than cocoon disks with lipids intact ($t_{35} = -2.22$, $P = 0.03$).

Surface-specific CWL of disks from intact cocoons ranged from $6.2 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *C. cultripes* to $10.5 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *C. platycephala*, whereas after lipids were extracted, the values for cocoon disks ranged from $7.7 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *C. cultripes* to $17.2 \text{ mg H}_2\text{O h}^{-1} \text{ cm}^{-2}$ for *C. longipes* (table 2). Surface-specific CWL of disks taken from intact cocoons was positively correlated with ssCWL of frogs with cocoons (Pearson's correlation; $P = 0.02$).

Lipids in the Cocoons and Epidermis

The amount of lipids in cocoons ranged from $49.6 \text{ mg lipid g}^{-1}$ dry cocoon in *C. longipes* to $109.8 \text{ mg lipid g}^{-1}$ dry cocoon

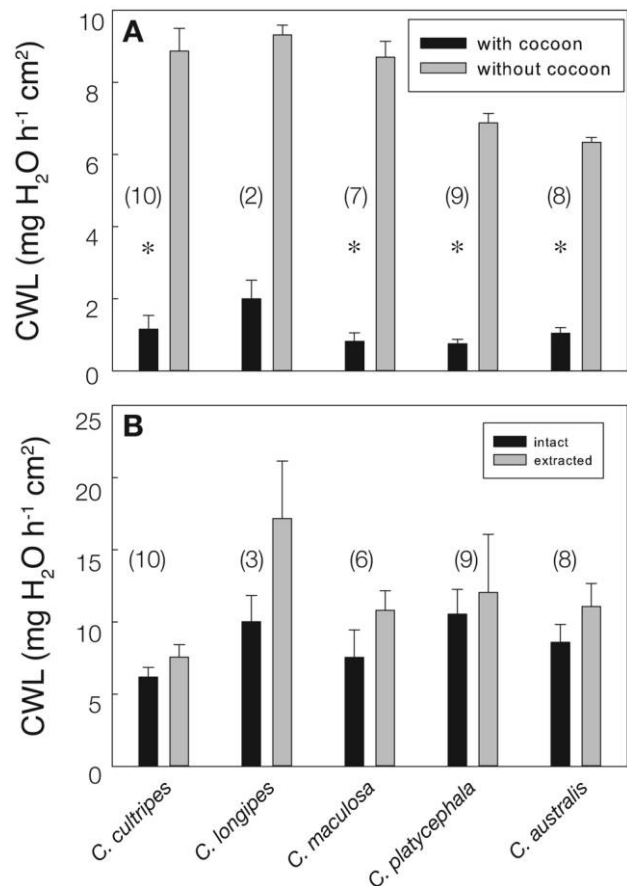


Figure 2. Mean (± 1 SE) effective surface-specific cutaneous water loss (CWL) for frogs when within a cocoon and after emerging from the cocoon (A) and effective surface-specific CWL of disks of in vitro intact cocoon disks and in vitro cocoon disks with lipids extracted (B). Sample sizes are in parentheses.

Table 2: Cutaneous water loss (CWL) of intact cocoon disks and cocoon disks after lipid extraction

<i>Cyclorana</i> species	Intact cocoon disk		Cocoon disk with lipids extracted	
	CWL (mg H ₂ O d ⁻¹)	ssCWL (mg H ₂ O h ⁻¹ cm ⁻²)	CWL (mg H ₂ O d ⁻¹)	ssCWL (mg H ₂ O h ⁻¹ cm ⁻²)
<i>C. cultripes</i> (n = 10)	79 ± 8.5	6.2 ± 0.67	96.8 ± 11	7.6 ± 0.86
<i>C. longipes</i> (n = 3)	128 ± 23	10.0 ± 1.80	219 ± 51	17.2 ± 3.99
<i>C. maculosa</i> (n = 7)	96.4 ± 24.3	7.5 ± 1.90	138 ± 17.1	10.8 ± 1.34
<i>C. platycephala</i> (n = 9)	135 ± 22	10.5 ± 1.72	157 ± 51.3	12.3 ± 4.01
<i>C. australis</i> (n = 8)	110 ± 15.7	8.6 ± 1.23	141 ± 20.5	11.1 ± 1.60

in *C. platycephala*, but based on one-way ANOVA, differences among species were not statistically significant ($F_{4,36} = 0.96$, $P = 0.44$). Epidermal values ranged from 147 mg lipid g⁻¹ dry epidermis in *C. australis* to 263.2 mg lipid g⁻¹ dry epidermis in *C. longipes* but differences among species were not statistically significant ($F_{4,32} = 1.5$, $P = 0.23$).

Lipid Composition of Cocoons and Epidermis

The epidermis contained a large amount of cholesterol but also had high amounts of FFAs and triglycerides (table 3). We detected no ceramides in the epidermis of any individual and cerebroside only in the epidermis of one individual *C. platycephala*. The lipids in cocoons were composed primarily of cholesterol, methyl esters, cerebroside, and phospholipids (table 4). We found cerebroside in the cocoons of every species, and ceramides in cocoons of three of the five species.

FFAs were significantly lower in cocoons than in epidermis (ANOVA, $F_{9,50} = 6.3$, $P \leq 0.001$). Based on an SNK post hoc test, epidermis of *C. cultripes* and *C. longipes* had significantly more FFA than did the cocoons (fig. 3).

To determine relative contribution of each lipid class to the total amount of lipids, we divided the amount in each class by the total quantity of extracted lipids. We found that, on average, polar lipids were the largest constituent of cocoons, 15% of dry mass ($F_{2,15} = 76.2$, $P \leq 0.001$), but nonpolar lipids were the largest constituent of the epidermis, 14% of dry mass ($F_{2,18} = 9.97$, $P = 0.001$).

Table 3: Epidermal lipid composition

<i>Cyclorana</i> species	Cholesterol	Methyl	Triglycerides	Free fatty	Cholesterol	Ceramide	Cerebrosides ^a	Phospholipids
	esters	esters		acids				
<i>C. cultripes</i> (n = 10)	4.6 ± 1.25	13.6 ± 3.05	26.0 ± 18.84	13.5 ± 2.81	7.5 ± 1.16	10.1 ± 5.0
<i>C. longipes</i> (n = 3)	1.3 ± 0	12.3 ± 1.67	73.2 ± 68.58	15.8 ± 2.13	16.4 ± 4.13	4.1 ± 0
<i>C. maculosa</i> (n = 7)	2.5 ± .45	6.2 ± 1.06	2.5 ± .89	4.0 ± .73	10.2 ± 2.51	15.4 ± 5.95
<i>C. platycephala</i> (n = 9)	3.8 ± 2.58	9.7 ± 1.92	14.1 ± 11.56	6.4 ± 2.90	8.8 ± 1.17	...	1.4 ± 0	7.4 ± 2.35
<i>C. australis</i> (n = 8)	2.5 ± 1.15	10.8 ± 2.27	2.0 ± .33	7.8 ± 1.54	12.0 ± 3.39	6.5 ± 1.97

Note. Data shown as mean (±SE) of each lipid class in milligrams lipid/grams dry epidermis. There are no statistical differences among species for any class of lipid.

^aA mixture of cerebroside was used for this analysis.

Relationship between Epidermal Lipids and Body Mass among Species

The quantity of methyl esters, cholesterol, and cerebroside in cocoons decreased as body mass of frog increased among the five species of *Cyclorana* ($P = 0.02$, $P \leq 0.001$, $P \leq 0.001$, respectively). We found no relationships between mass and any class of epidermal lipids for frogs without cocoons.

Relationship between Cutaneous Water Loss and Lipids

Phosphatidyl ethanolamine in the epidermis was positively correlated with evaporative water loss in frogs without cocoons ($P = 0.04$). Total amount of epidermal lipids was negatively correlated with CWL ($P = 0.006$), so as the quantity of lipids in the epidermis increased, CWL decreased. There were no relationships between CWL of frogs within cocoons and any lipid classes of cocoons.

Discussion

We investigated CWL of five *Cyclorana* species that burrow underground and form cocoons during the dry season in Northern Territory, Australia (Lee and Mercer 1967; Tracy et al. 2007). We found major differences in the classes of lipids found in cocoons relative to the epidermis for all species. When frogs were in cocoons, we found, as expected, reduced CWL relative to when frogs were not within cocoons.

We quantified lipid classes within cocoons and epidermis of each individual, because lipids provide an effective barrier to

Table 4: Cocoon lipid composition

<i>Cyclorana</i> species	Cocoon				Epidermis			
	Cholesterol esters	Methyl esters	Triglycerides	Free fatty acids	Cholesterol	Ceramide	Cerebrosides ^a	Phospholipids
<i>C. cultripes</i> (n = 10)	.8 ± .19	3.7 ± .48	1.1 ± .23	1.2 ± .18	14.8 ± 4.06	...	1.8 ± .41	1.0 ± .15
<i>C. longipes</i> (n = 3)	.2 ± .092	2.0 ± .48	.3 ± .12	.6 ± .10	7.0 ± 1.50	...	1.0 ± .34	1.3 ± .45
<i>C. maculosa</i> (n = 7)	.8 ± .23	2.8 ± .70	.3 ± .12	.9 ± .52	17.9 ± 6.08	.4 ± .13	1.8 ± .32	1.5 ± .58
<i>C. platycephala</i> (n = 9)	.9 ± .36	3.7 ± 1.16	1.0 ± .71	2.4 ± 1.60	18.0 ± 4.99	.6 ± .34	2.0 ± .36	2.6 ± 1.23
<i>C. australis</i> (n = 8)	.5 ± .15	.9 ± .11	.3 ± .03	.5 ± .12	4.9 ± .87	.2 ± .03	.5 ± .08	.5 ± .07

Note. Data shown as mean (\pm SE) of each lipid class in milligrams lipid/grams dry cocoon. There are no statistical differences among species for any class of lipid.

^aA mixture of cerebrosides was used for this analysis.

water loss in some species (Lillywhite and Maderson 1988; Lillywhite 2006). There are clear differences in lipid composition between cocoons and epidermis, which suggests that the secretions formed between shed skin layers in the cocoons are composed of different types and quantities of lipids than are found in the epidermis. It is possible that these secretions include polar lipids that contribute to the lipid barrier to CWL. One vital difference is that ceramides and cerebrosides, two lipid classes implicated in reducing water permeation in the skin of mammals and birds, were largely found only in cocoons. The presence of both of these lipid classes in cocoons, and minimally in epidermis, suggests the hypothesis that frogs secrete these lipid classes to form a barrier to water vapor diffusion. Cocoons of frogs also contained cholesterol and its esters along with large quantities of cerebrosides. The composition of the epidermis was markedly different, with cholesterol and methyl esters still constituting a large percentage of total lipids, but with FFAs comprising more of the total than is seen in cocoons (fig. 3). Nonpolar classes, such as FFAs, were a larger proportion of total lipids in the epidermis when compared with cocoons. FFAs likely influence the fluidity of the lipid layer, so as FFA content increases, so would CWL (Bouws-tra et al. 2003; Haugen et al. 2003).

There were more total lipids in cocoons (in grams); however, when corrected for the dry mass of the cocoon and epidermis, the epidermis had more milligrams of lipids per gram of dry mass. Lipid mass accounted for approximately 7% of total cocoon dry mass, and approximately 20% of epidermis dry mass. The total dry lipid mass of cocoons is consistent with what was found in *C. australis* (Christian and Parry 1997), in which cocoons were composed of approximately $8.3\% \pm 3.1\%$ lipids. In this species, the amount of lipids in milligrams per gram of dry epidermis was also higher than the amount of lipid per gram of dry cocoon. This finding is counterintuitive if it is exclusively total amount of lipids that reduces water loss through the skin (Lillywhite and Maderson 1988; Amey and Grigg 1995), given that frogs with cocoons had lower CWL than those without a cocoon. Research on lipids of the SC of birds suggests that the proportions of certain lipid classes, rather than the total amount of lipids, is vital to reducing cutaneous water loss (Muñoz-García et al. 2008a, 2008b). It also seems that in frogs it is the combination of lipids in the cocoon, rather than the quantity, that reduces CWL.

The secreting glands of amphibians are found in the dermis (Amey and Grigg 1995; Barbeau and Lillywhite 2005; Lillywhite 2006), and thus, contents of these glands are not likely to be detected in the epidermis by our methods. The secretions are released onto the top of the skin. Phyllomedusine frogs, for example, secrete wax esters to prevent cutaneous water loss during dry periods (Shoemaker et al. 1972; Blaylock et al. 1976; Gomez et al. 2006). Similar secretions have been found in some *Hyla* spp. (Wygoda 1988), and a cutaneous lipid layer was found in some *Litoria* spp. (Amey and Grigg 1995). Our data support the suggestion that secretions produced by *Cyclorana* spp. that form cocoons contribute to the barrier that effectively reduces water lost to the surrounding environment when the frogs are within cocoons during the dry season. However, we can not rule out that protein-lipid interactions, rather than lipids exclusively, provide an effective barrier to water lost during estivation (Christian and Parry 1997). Christian and Parry (1997) found that secretions produced by *C. australis* are composed of about $9.8\% \pm 2.3\%$ lipids, primarily fatty acid compounds (although they did not test for cerebrosides or ceramides), and

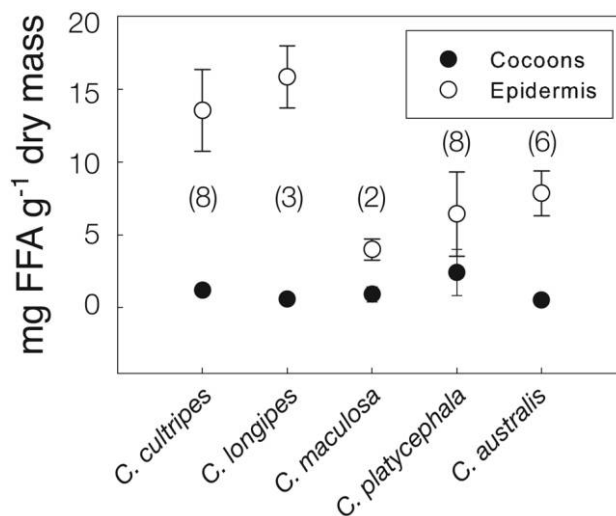


Figure 3. Mean (\pm SE) milligrams of free fatty acids (FFA) per gram of dry mass in cocoons (filled symbols) and epidermis (unfilled symbols). Sample sizes are in parentheses.

Table 5: Number of days it would take a frog in a burrow to lose 30% of its body mass by evaporation

<i>Cyclorana</i> species	Days to desiccation	
	With cocoon	Without cocoon
<i>C. cultripes</i>	1,387	101
<i>C. longipes</i>	688	96
<i>C. maculosa</i>	1,920	105
<i>C. platycephala</i>	3,137	159
<i>C. australis</i>	2,266	173

77.9% \pm 6.2% proteins, and the protein component was mostly made of amino acid residues arranged in an opaque sheetlike form. It is possible that the secretions developed in order to bind the layers of shed skin together, but the evidence that they also provide lipids that influence water permeation cannot be ignored.

As previous work has done, we have shown that cocoons, which are composed of layers of shed skin held together by layers of secretions from mucous glands within the skin, act as a barrier to water vapor loss through the skin (Lee and Mercer 1967; Withers 1998; Christian and Parry 1997). Rates of water loss in this study were consistent with previous measurements on *Cyclorana* spp. from Australia. *Cyclorana australis* lost approximately 1.1 mg H₂O h⁻¹ cm⁻² for frogs that estivated for 3 wk, and 0.4 mg H₂O h⁻¹ cm⁻² for those that estivated for 3 mo (Christian and Parry 1997). Similar results were found in another study, where *C. australis* lost 0.59 mg H₂O h⁻¹ cm⁻² after 46 to 50 d of estivation, and *Cyclorana cultripes* lost 0.39 mg H₂O h⁻¹ cm⁻² (Withers and Thompson 2000). *Cyclorana platycephala* had lower CWL rates, averaging 0.8 mg H₂O h⁻¹ cm⁻², than was previously found, 6.2 mg H₂O h⁻¹ cm⁻² (Withers 1998). However, measurements among studies are difficult to compare directly because *Cyclorana* cocoons increase in thickness over the dormancy period, with evaporative water loss decreasing proportionally (Christian and Parry 1997; Tracy et al. 2007), and the rate that skin layers are added to the cocoon varies with temperature (McMaster 2007). Cocoon formation may even vary among species, although this has not been tested thoroughly. This study reports the first measurement on CWL in *Cyclorana longipes* within a cocoon.

Using values of R_t with and without a cocoon from this study, we calculated the length of time it would take each of the study species to desiccate to 70% of their standard mass while dormant in a burrow, which we assumed would be a lethal level of desiccation. Even without a cocoon, we estimated that frogs could survive more than 3 mo in burrows, and with a cocoon, frogs could survive up to several years (table 5). Many burrowing frogs use bladder fluid as a water store during dormancy, and *Cyclorana* species are known to have large bladder capacities, even more than 100% of standard mass in some species (Tracy et al. 2007). However, our calculations assumed that the frogs began with an empty bladder, so the actual desiccation times would be substantially longer. Reynolds et al.

(2010) demonstrated that the cocoon of *C. australis* functions as a barrier preventing liquid water exchange with the soil. That barrier allows frogs to avoid having their body water drawn out as soils dry and soil water potential drops. Our calculations suggest that lengthening desiccation time by reducing evaporative water loss may not be necessary for most *Cyclorana* species in burrows, enforcing the hypothesis that the primary function of the cocoon is to act as a barrier to liquid exchange with the soil, rather than being a way to reduce evaporative water loss.

Although we did not find statistical differences in CWL between disks of intact cocoons and cocoons from which lipids were extracted, there was a trend for higher CWL rates for disks of cocoons with lipids extracted. Previous research in which CWL was measured on cocoons in vitro found that CWL was significantly higher when lipids were extracted (McMaster 2007). The difference between studies may be attributed to the length of each trial, as McMaster conducted trials that lasted up to 9 mo, which may be more ecologically relevant. Also, the strain of mounting these cocoon disks on vials may influence water loss through the cocoon disks. An issue with both methods is that because the lipids are no longer present, the cocoon will likely not rehydrate in a similar manner as when the cocoon contains lipids. Thus, there are technical difficulties associated with experiments associated with extracting lipids from tissues.

Lipids play an important role in forming effective barriers to water loss in tetrapods (Lillywhite 2006), but with respect to the role of lipids in frog cocoons, perhaps the most interesting question relates to the source of the lipids. There were more nonpolar lipids in the epidermis, and cerebrosides and ceramides were almost exclusive to cocoons. This suggests that the lipid classes that reduce water loss are in the mucous secretions found in between the layers of shed skin in the cocoon rather than in the shed layers of epidermis. Thus, in addition to holding the layers of shed skin together, the secretions between the skin layers contain specific lipids that enhance the effectiveness of the cocoon as a barrier to water loss. If the mechanism behind evaporative water loss in amphibians can be better elucidated, we will then better understand the gaps in our knowledge of how different taxa reduce CWL, and also provide insight into how terrestrial animals facing extended droughts (Sandeman 2008) may cope with global climate change.

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