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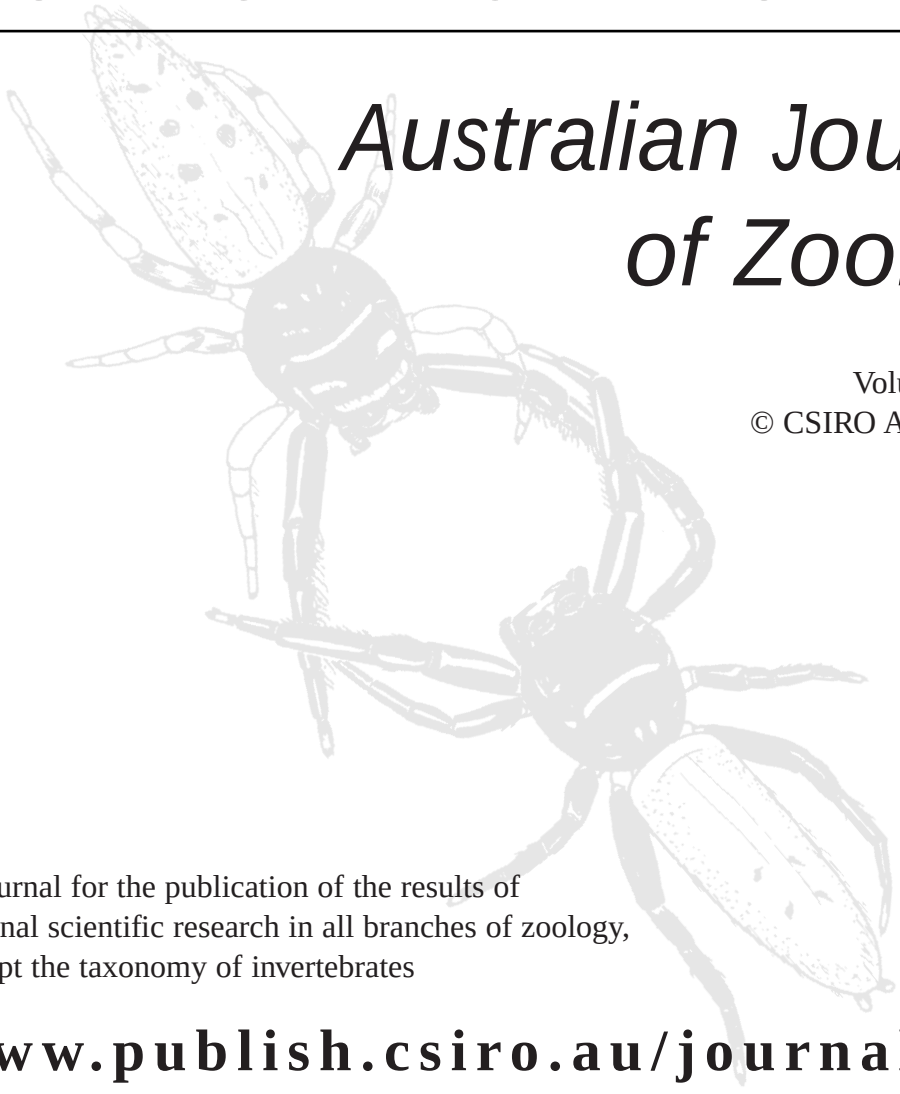

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Reduced Rates of Water Loss and Chemical Properties of Skin Secretions of the Frogs *Litoria caerulea* and *Cyclorana australis*

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Abstract

We measured the rates of water loss in two Australian hylid frogs: the arboreal *Litoria caerulea* and the terrestrial burrowing frog *Cyclorana australis*. We measured the latter species with and without cocoons. Both species showed reduced rates of water loss compared with 'typical' amphibians that lose water as if from a free surface. Cocooned *C. australis* had very low rates of water loss. We examined the chemical composition of skin secretions rinsed (using only high-pure water) from both species and the cocoon material from *C. australis*. The chemical composition of the material from these three sources was generally similar and consisted of 5–10% neutral lipids and 78–85% proteinaceous material. The fact that the terrestrial species has a high resistance to water loss is unusual given that almost all other known species of non-cocooned frogs with reduced rates of water loss are arboreal. The chemical similarity of the skin secretions and cocoons from this species suggest that the reduced rate of water loss in this species is linked to its ability to form a cocoon. Amino acid composition of the material indicated that a sclerotisation process may occur upon oxidation of the secretions. This would result in a physical barrier to water loss in the cocoons and possibly a thin physical proteinaceous barrier on the skin of both species in the absence of cocoons. We suggest that the high proportion of proteins in the skin secretions cannot be ignored, and that it may, in conjunction with the lipids, produce an effective waterproofing barrier in both species. We suggest that chemical components other than lipids also may be important in frogs from other continents, and complete compositional analyses of frog 'mucus' are required before we can fully understand the nature of the mechanisms involved in reduced rates of water loss in amphibians with and without cocoons.

Introduction

'Typical' amphibians have been defined as having a rate of evaporative water loss (EWL) equivalent to that of a free water surface having the same shape and size and exposed to the same environment (Wygoda 1984). In recent years several species have been shown to have reduced rates of water loss (reviewed by Toledo and Jared 1993). In phyllomedusine frogs of South America an extra-epidermal lipid layer results in extremely reduced rates of EWL (Shoemaker *et al*. 1972, 1975; Blaylock *et al*. 1976; McClanahan *et al*. 1978; Stinner and Shoemaker 1987). Several species of arboreal frogs of the African genera *Chiromantis* and *Hyperolius* also exhibit extremely low rates of EWL, but the mechanism is different and reportedly involves a physical barrier of dermal iridophores and possibly mucus secretions (Loveridge 1976; Drews *et al*. 1977; Withers *et al*. 1982, 1984; Geise and Linsenmair 1986; Kobelt and Linsenmair 1986; Schmuck *et al*. 1988; Kaul and Shoemaker 1989). Some North American treefrogs have rates of EWL that are significantly lower than those of typical amphibians although less pronounced than those of the phyllomedusine frogs and the African frogs mentioned above (Bentley and Yorio 1979; Wygoda 1984, 1988, 1989).

Early investigations of the water economy of Australian frogs indicated variation in the rate of EWL, with treefrogs of the genus *Litoria* having the lowest rates (Warburg 1965, 1967, 1972). This was more recently confirmed for *L. gracilenta* (Withers *et al*. 1984), for *L. fallax* and *L. peroni* (Amey and Grigg 1995), and for *L. chloris* and *L. caerulea*, which were found to have rates of EWL much lower than those of similarly sized arboreal frogs from North America

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(Buttemer 1990). *L. caerulea* can be found in hot and desiccating conditions (Shine *et al*. 1989; Buttemer 1990), and it can tolerate extreme levels of dehydration (Main and Bentley 1964).

The mechanism resulting in reduced rates of EWL in most of these Australian tree frogs has not been determined, although a cutaneous lipid layer was found in two (*L. fallax* and *L. peroni*) of five species examined (Amey and Grigg 1995). These two arboreal species had the lowest rates of water loss of the species examined, suggesting that the lipid layer provided the waterproofing mechanism (Amey and Grigg 1995). Dermal glands that contain lipid secretions have also been found in *L. caerulea* (J. R. Roberts, M. R. Warburg and H. Heatwole, unpublished data). Seymour and Lee (1974) suggested that because Australian arid regions are characterised by frequent unpredictable drought, it is likely that unique strategies may have evolved among Australian frogs to meet the demands of the physical environment. However, no mechanisms that are unique to Australian frogs have been reported, although the differences in the location of the lipid layer in *L. fallax* and *L. peroni* (Amey and Grigg 1995) suggest an interesting variability in the way lipids are used.

The 'mucus' of frogs is a complex mixture of compounds that serve functions of the prevention of integumentary desiccation (Lillywhite and Licht 1975), thermoregulation (Lillywhite 1971; Kaul and Shoemaker 1989; Buttemer 1990) and antipredator toxins (Duellman and Trueb 1986; Tyler 1987). Wygoda (1988) suggested that a layer of dried mucus increases the resistance to EWL in moderately waterproof treefrogs from North America. The chemical nature of the mucus that may decrease water loss in moderately waterproof frogs has not been investigated.

The formation of a cocoon during aestivation is a mechanism of some burrowing frogs that greatly reduces water loss (McClanahan *et al*. 1976, 1983; Loveridge and Withers 1981; Shoemaker *et al*. 1992). Some species of Australian frogs from the genera *Cyclorana*, *Neobatrachus* and *Litoria* (Lee and Mercer 1967; Withers 1995; Withers and Richards 1995) form cocoons during aestivation. The cocoons are composed of multiple layers of single-cell-thick epidermal cells (Withers 1995). Mucus layers are found between the cellular layers in the cocoons of *Pternohyla fodiens* (Ruibal and Hillman 1981) and *Lepidobatrachus llanensis* (McClanahan *et al*. 1983), and intercellular material is also found between layers of epidermal cells in the cocoons of Australian frogs (see fig. 5 in Withers 1995). The role of this mucus, as distinct from the epidermal layers, has not been determined with respect to its relationship to the skin secretions of non-cocooned frogs or to the waterproofing characteristics of the cocoons of the cocoon-forming Australian frogs.

In order to investigate the mechanisms of waterproofing in frogs we measured the rates of EWL and analysed the chemical composition of skin secretions from two Australian hylid frogs: the arboreal *Litoria caerulea* and the terrestrial burrowing frog *Cyclorana australis*. The rates of EWL of cocooned and non-cocooned *Cyclorana australis* were measured and the chemical characteristics of the cocoons were compared with the skin secretions of frogs without cocoons.

Materials and Methods

Frogs were collected from the Myilly Point Campus of the Northern Territory University, Darwin. Prior to the experiments, frogs were housed in tilted plastic containers with water at the low end only.

The measurements of EWL were made with an open flow system in which dry air was pumped at a controlled rate into a chamber containing the frog, and the humidity and temperature of the incurrent and excurrent air were measured with Vaisala HMP113Y capacitance humidity sensors. The air was pumped by a Reciprotor electromagnetic pump, and the rate of flow was controlled with a rheostat and measured with a Haliu rotameter calibrated against a soap bubble burette (Long and Ireland 1985). Flow rates of 1 L min^{-1} (corresponding to a wind speed of 0·24 cm s^{-1}) were used in all experiments. Flow was converted to STPD conditions for calculations of water loss. A perspex cylinder with a diameter of 9·5 cm formed the experimental chamber, and the humidity sensor was calibrated daily against a Vaisala HM11 calibrator which uses saturated-salt solutions. Humidity and air temperature were recorded on a strip-chart recorder, and the lowest humidities in the excurrent air that were stable over 30 min were used in the calculations. All measurements of water loss reported here are from animals in the water-conserving posture (Heatwole *et al*. 1969).

At the beginning of each trial, each frog was weighed to the nearest 0·1 g after a catheter had been used to void the bladder water. Total frog surface area was estimated from the empirical equation based on mass derived by McClanahan and Baldwin (1969). This equation was recently confirmed to be a good estimate of surface area for *Litoria caerulea* and *L. chloris* (Buttemer 1990). For calculations of area-specific water loss it was assumed that the water-conserving posture resulted in only two-thirds of the surface area being exposed to the air (Withers *et al*. 1982). At the end of each trial the body temperature of the frog was measured within 20 s of removing the frog from the chamber by inserting a calibrated thermocouple thermometer into the cloaca. Rates of EWL were calculated from the equations of Bernstein *et al*. (1977) for an open flow system, in conjunction with standard tables (List 1971) of saturation vapour density (needed to calculate the mass of water from the measurements of relative humidity) and saturation vapour pressure (needed to correct flow rates to STPD). The total resistance to water loss was calculated by dividing the vapour density difference between the skin of the frog and the air in the chamber by the area-specific rate of water loss (Spotila and Berman 1976; Wygoda 1984). The vapour density of the skin of the frog was taken as the saturation vapour density at the skin temperature. Skin temperature is not significantly different from cloacal temperature for both typical and atypical frogs in a moving air stream (Wygoda 1984). Total resistance to water loss is the sum of the cutaneous resistance and the boundary layer resistance (Spotila and Berman 1976).

Variations in air velocities and other experimental parameters make it difficult to directly compare the results of different experiments in the literature (Buttemer 1990). In order to determine whether or not the rates of EWL of the species under study were significantly lower than those of typical frogs, the frog measurements were compared with measurements made under the same experimental conditions by 3% agar frog models (Buttemer 1990) which are assumed to lose water as a free surface (cutaneous resistance = 0; Spotila and Berman 1976). The agar models were carved into the shape of a frog in the water-conserving posture, and measurements were made from seven models ranging in mass from 20 to 45 g. The frogs used in measurements of EWL were also of the same size range.

Cyclorana australis were induced to form cocoons by placing a frog in a plastic container with small pin holes in the lid and moist towelling on the bottom and then placing the container in a dark cabinet at room temperature. The frogs entered the water-conserving posture and began to form a cocoon within a matter of days. Experiments were done on three frogs after three weeks in the containers, and the EWL of another group of five frogs was measured after three months. After EWL experiments, the frogs were induced to break out of their cocoons by gently handling them and exposing them to light. The cocoons were collected and frozen until analysed.

During preliminary experiments we observed secretions welling-up from the skin of *Litoria caerulea* under desiccating conditions. On several occasions (but not in every experiment) we observed these frogs wiping the secretions over their body in a way similar to that described for *Phyllomedusa sauvagei* (Blaylock *et al*. 1976). The welling-up of the secretions allowed us to gently collect them for analysis by rinsing the frogs with a fine stream of distilled water. This method avoids contaminating the sample with epidermal lipids or proteins which would probably occur if whole-skin extractions were used or if solvents were applied directly to the skin. Examination of the wash water revealed no visible suspended particles such as pieces of sloughed skin. The wash water was frozen initially, then later lyophilised prior to analysis.

Combined samples from up to 11 individuals of each species and cocoons from *C. australis* were lyophilised prior to analysis. Lipids were extracted from the lyophilised secretions and cocoons with chloroform : methanol (2 : 1) and determined gravimetrically (Bligh and Dyer 1959). Lipid extracts were separated into neutral and polar fractions on Merck Kieselgel 60 silica thin layer chromatography plates with petroleum ether : diethyl ether : acetic acid (90 : 10 : 1) and chloroform : methanol : water (64 : 31 : 5). Fatty acid methyl esters were prepared by saponification and methylation of the lipid extracts with 14% boron trifluoride in methanol, and analysed with a Varian 6000 gas chromatograph and a SGE BP 225 column (25 m × 0·22 mm) (Renaud *et al*. 1994).

Total protein of lyophilised secretions and cocoons was determined from Kjeldahl nitrogen as 6·2 × percent nitrogen (Helrich 1990). Lyophilised secretions and cocoons were hydrolysed in 6N constant boiling hydrochloric acid at 108°C for 24 h under vacuum. The hydrolysate was derivatised with phenylisothiocyanate and the derivatised amino acids were quantitatively determined with a Varian high performance liquid chromatograph and a Waters amino acid hydrolysate column.

Results

The mean masses of the different groups of frogs were not significantly different (ANOVA, $F_{2,24} = 0{\cdot}22$, $P = 0{\cdot}80$) (Table 1). The agar frogs, *Litoria caerulea* and *Cyclorana australis*

without cocoons were compared by ANOVA for the three indices for EWL [water loss per unit mass (mg g^{-1} h^{-1}), water loss per unit area (mg cm^{-2} h^{-1}) and total resistance to water loss (s cm^{-1})], and Student–Newman–Keuls (*SNK*) tests were performed to determine which means were significantly different at $P < 0{\cdot}05$. The agar frogs had the highest rates of EWL (and lowest resistance to water loss), *Litoria caerulea* had the lowest rates of EWL (and the highest resistance), and *Cyclorana australis* were intermediate with respect to all three indices (Table 1). The *SNK* tests revealed that all three groups of frogs were significantly different from one another for all the indices.

Table 1. Mass, evaporative water loss (EWL) and total resistance of agar replicates, *Litoria caerulea* and non-cocooned *Cyclorana australis*
Values are means ± standard error, with the sample sizes in parentheses

	Agar 'frogs'	*Cyclorana australis*	*Litoria caerulea*
Mass (g)	32·0 ± 3·7 (7)	35·0 ± 1·8 (11)	33·2 ± 4·1 (9)
EWL (mg g^{-1} h^{-1})	12·9 ± 0·9 (7)	7·8 ± 0·3 (11)	5·8 ± 0·6 (9)
EWL (mg cm^{-2} h^{-1})	8·7 ± 0·3 (7)	5·6 ± 0·2 (11)	4·0 ± 0·3 (9)
Total resistance (s cm^{-1})	5·4 ± 0·2 (7)	7·8 ± 0·3 (11)	11·2 ± 1·6 (7)

Eight *Cyclorana australis* were induced to form cocoons. EWL was measured in three frogs on a single occasion after three weeks of aestivation, and the other five were measured after three months of aestivation. The cocooned *Cyclorana australis* had extremely low rates of EWL and extremely high total resistance to water loss (Table 2). After three months in aestivation the cocooned frogs lost significantly less water ($F_{1,6} = 10{\cdot}8$, $P = 0{\cdot}017$ for mass-specific EWL; $F_{1,6} = 11{\cdot}1$, $P = 0{\cdot}016$ for area-specific EWL) and had significantly higher total resistance values ($F_{1,6} = 11{\cdot}9$, $P = 0{\cdot}014$) than frogs in cocoons only three weeks.

Table 2. Mass, evaporative water loss (EWL) and total resistance of cocooned *Cyclorana australis*
Values are means ± standard error, with the sample sizes in parentheses

	After 3 weeks	After 3 months
Mass (g)	31·8 ± 2·4 (3)	31·5 ± 3·5 (5)
EWL (mg g^{-1} h^{-1})	1·5 ± 0·4 (3)	0·5 ± 0·1 (5)
EWL (mg cm^{-2} h^{-1})	1·1 ± 0·3 (3)	0·4 ± 0·1 (5)
Total resistance (s cm^{-1})	65·6 ± 16·1 (3)	219·3 ± 32·2 (5)

Whole cocoons from *C. australis* varied in mass up to a maximum of 136 mg (dry). The lyophilised secretions from *L. caerulea* and *C. australis* contained both opaque sheet-like material and a white powdery material.

The total lipid from *L. caerulea* secretions was variable, ranging from 2·4 to 10% of the total dry mass with a mean of 5·1 ± 3·2% (Table 3). The lipid fraction was composed entirely of neutral lipids (polar lipids were not detected). The fatty acid composition of the lipid fraction showed it was primarily palmitic acid (16:0; with the standard notation L:B, where L is the carbon chain length and B is the number of double bonds), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2).

Table 3. Total lipid and protein composition
Expressed as a percentage of dry mass (± standard error, with the sample sizes in parentheses) of skin secretions from *Cyclorana australis* and *Litoria caerulea* and cocoons from *C. australis*

	Cyclorana australis		*Litoria caerulea*
	Cocoons	Secretions	Secretions
Total protein (%)	79·1 ± 4·8 (5)	77·9 ± 6·2 (4)	84·6 ± 4·7 (4)
Total lipid (%)	8·3 ± 3·1 (5)	9·8 ± 2·3 (4)	5·1 ± 3·2 (4)

Skin secretions from *C. australis* had a total lipid content of 9·8 ± 2·3%, and the cocoons had 8·3 ± 3·1% (Table 3). The lipid fractions from secretions and cocoons were composed entirely of neutral lipids. The fatty acids were primarily palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2) in the lipid fraction from secretions and cocoons.

The total protein composition of the skin secretions and cocoons of *C. australis* and skin secretions of *L. caerulea* are shown in Table 3. The amino acid composition of the opaque sheet-like material and white powdery material from lyophilised secretions of *L. caerulea* were different. The sheet-like material had no lysyl residues and had lower concentrations of aspartic and glutamic acid (which includes asparagine and glutamine) and proline, with higher concentrations of alanine and phenylalanine.

Hydrolysis of cocoons and lyophilised secretions from *C. australis* was unsuccessful. The solid material initially turned purple-red in colour, and after derivatisation of the hydrolysate solution, amino acid analysis showed virtually no hydrolysed amino acids. The purple coloration was also observed during hydrolysis of *L. caerulea* secretions, both the sheet-like material and the white powder, and a significant amount of solid material remained after hydrolysis.

Discussion

Given that the total resistance to water loss is the sum of the skin resistance and the boundary layer resistance, and that the agar frogs provide a free water surface such that skin resistance = 0 (Spotila and Berman 1976), then the resistance measured for the agar frogs represents a boundary layer resistance for the conditions of the experiment (including the chamber, flow rate, and size and posture of the frogs). Given that *L. caerulea* and *C. australis* have total resistance that is significantly greater than this boundary layer resistance, it follows that these species have significant skin resistances, and are therefore 'atypical' amphibians as defined by Wygoda (1984). This conclusion is further supported by the reduced rates of EWL compared with those of the agar frogs, and by previous results for *L. caerulea* (Buttemer 1990). The values presented here for *L. caerulea* are similar to those reported by Buttemer (1990), with the differences being consistent with the different flow rates. Although the resistance to water loss was not as great in *C. australis* as in the arboreal *L. caerulea*, the terrestrial species is nevertheless atypical with respect to its EWL. It is very unusual for a terrestrial species to have this level of resistance to water loss (Wygoda 1984; Toledo and Jared 1993; Amey and Grigg 1995). Given the chemical similarity between the skin secretions and cocoon material in *C. australis*, it is possible that the waterproofing quality of this terrestrial species is related to the fact that it is a cocoon-forming burrowing species.

What, then, is the physical and chemical nature of this barrier to water loss in these two species? The skin secretions from *L. caerulea* and *C. australis* and the cocoons from *C. australis* had similar gross chemical composition (Table 3). In all cases the lipid fraction was composed of neutral lipids. The fatty acid compositions were also similar with palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids accounting for more than 60% of the total fatty

acids. The lipid fraction of the cocoons and skin secretions were chemically similar to the extra-epidermal lipids that have been found to act as a waterproofing barrier in phyllomedusine frogs (McClanahan *et al*. 1978). However, McClanahan *et al*. (1978) did not report what proportion the lipid extract was of the total skin secretions of *Phyllomedusa sauvagei* or whether proteins were present in the secretions.

The main component of the skin secretions and cocoons in the present study was proteinaceous material (Table 3). The skin secretions were carefully washed from the frogs which resulted in a milky solution that was free of solid material. The lyophilisation of the secretions from both species resulted in dried material that contained a mixture of small pieces (up to 12 mm^2) of opaque sheet-like material and white powdery material. The formation of the opaque sheet-like material would suggest that a partial polymerisation of the skin secretions had occurred during lyophilisation which may have been triggered by air oxidation. While the amino acid analysis was not conclusive, because of only partial hydrolysis, there were significant differences between the sheet-like material and the powdery material. The lack of any lysyl residues and the lower concentrations of aspartic and glutamic acids (which include asparagine and glutamine) and proline provide preliminary evidence of a sclerotisation process occurring.

The 'mucus' of skin secretions (and as a component of cocoons) almost certainly plays a role in the reduction of EWL (Wygoda 1988), but the chemical nature of the secretion and the mechanisms involved in reducing water loss are unclear. While it may be possible to attribute the low rates of water loss solely to the presence of an extra-epidermal lipid secretion, the high protein content of the secretions cannot be ignored. The presence of an inert protein, together with the lipid, may be responsible for the barrier against water loss in these and other frogs. We suggest that the sheet-like proteinaceous material between the layers of epidermal sheets of cocoons (Withers 1995) may be an integral component of the physical barrier that inhibits water loss from cocooned frogs. The fact that sheet material similar in gross chemical composition to cocoon material is produced when skin secretions of both species are oxidised *in vitro* suggests that a very thin proteinaceous barrier may also account for the reduced water loss of frogs without cocoons. The chemical relationship between the proteins and lipids is not known, but these results suggest that the nature of the waterproofing mechanism of these species is more complex than a simple lipid layer. We further suggest that, in the absence of complete compositional analysis of the secretions and cocoons of frogs from other continents, it may be premature to attribute the waterproofing mechanisms of frogs with lipid glands solely to a lipid barrier. Some frogs have cutaneous lipids but do not have reduced rates of EWL (Withers *et al*. 1984), indicating that the presence of lipids alone may not be sufficient. Similarly, a physical barrier composed of sclerotised proteins may contribute to the waterproofing mechanisms of those atypical species that lack lipid glands. There is much to be learned about the chemical nature of the secretions of frogs skin collectively referred to as 'mucus'. Complete compositional analyses are required to determine the full range of mechanisms involved in elevated resistances to water loss in both cocooned and non-cocooned amphibians.

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