

Body wiping behaviors associated with cutaneous lipids in hylid tree frogs of Florida

Tamatha R. Barbeau* and Harvey B. Lillywhite

Department of Zoology, University of Florida, Gainesville, FL 32611-8525, USA

*Author for correspondence (e-mail: tbarbeau@fmarion.edu)

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Summary

Body wiping behavior, integumentary secretions and rates of evaporative water loss (EWL) were examined in six species of Florida tree frogs (Anura: Hylidae). Additionally, morphology of the integument and dermal glands were compared among these and one other Florida tree frog (*Hyla andersonii*), an arid-adapted tree frog (*Phyllomedusa hypochondrialis*), and a highly aquatic frog (*Rana utricularia*). An extra-epidermal layer of lipid and mucus, presumably secreted from dermal granular glands, was detected on the skin of all Florida hylid frogs examined. Distinct body wiping behaviors were observed in the hylid frogs, but these were less complex than those described previously in phyllomedusine frogs, which occupy arid habitats, secrete lipids onto their skin, and are

regarded as relatively 'waterproof'. Florida hylids occupy seasonally arid habitats and appear to have reduced rates of EWL. The suite of traits we observed in these frogs have been previously documented in a rhacophorid tree frog from seasonally arid regions of India and likely represent an evolutionary convergent response to periodic dehydration stress. The presence of lipids that are spread by simple wiping behaviors to form an extra-epidermal water barrier may represent an early stage of the more advanced adaptations described in more waterproof arboreal frogs.

Key words: evaporative water loss, gland, Hylidae, integument, lipid, tree frog, wiping behavior.

Introduction

Amphibian exploitation of terrestrial environments depends on morphological, physiological and behavioral adaptations to reduce evaporative water loss (EWL). Although a limited fraction of extant amphibians has been well studied, it appears that a majority of anurans lack effective physiological control over EWL from the integument. Consequently, most species must live in or near water, and they utilize behavioral strategies such as nocturnal activity and fossoriality to avoid desiccation (Shoemaker, 1988; Toledo and Jared, 1993).

Some frogs, however, deviate from this pattern and exhibit specialized adaptations to reduce dehydration in arid environments (Table 1). Such adaptations include uricotelism, increased water uptake through the ventral skin patch, and skin resistance to EWL attributable to lipid secretions from cutaneous glands (Toledo and Jared, 1993). Arboreal species, in particular, have been shown to have comparatively low rates of EWL (Wygoda, 1984). Examples include South American (*Phyllomedusa sauvagei*) and Australian (*Litoria caerulea*) hylid tree frogs, which secrete lipids from specialized cutaneous glands and spread them over the body by complex self-wiping movements to form an effective barrier to EWL (Blaylock et al., 1976; Christian et al., 1988). Remarkably, the resulting EWL in *P. sauvagei* is similar to that of some desert-dwelling reptiles (Shoemaker et al., 1972).

The Indian tree frog *Polypedates maculatus* engages in

similar wiping movements involving lipid secretions from cutaneous mucous glands. These secretions, however, provide a lower reduction in EWL than do those of phyllomedusine or Australian tree frogs. Therefore, wiping behaviors might have evolved before such secretions provided a significant water barrier and are possibly more widespread among arboreal or xerophilic frogs than previously considered (Lillywhite et al., 1997; Lillywhite and Mittal, 1999).

The phylogenetic origin of wiping behaviors might have evolved from movements involved in removal of debris or shedding skin from the body (Blaylock et al., 1976). Wiping might also aid in the spread of mucous gland secretions that are necessary for respiration, thermoregulation and cutaneous water balance (Lillywhite, 1971). Comparative descriptions of anuran self-wiping behaviors provide important insights into their evolution and ecophysiological significance (Lillywhite et al., 1997).

Wiping behaviors associated with cutaneous lipids have been documented in relatively few species of amphibians. In this study we investigated and compared self-wiping behaviors in Florida tree frogs (family Hylidae). We also examined these frogs for the presence of an extra-epidermal layer of mucus and lipid secretions, and we describe the morphology of the epidermis, dermis and cutaneous glands. Lastly we determined rates of total EWL in unrestrained frogs during bouts of water

Table 1. Summary of the families of arboreal frogs exhibiting low rates of cutaneous evaporative water loss (CWL) related to cutaneous lipids and wiping behaviors, with comparisons of aquatic frogs having high rates of CWL

Habitat	Family	Species	Skin resistance to CWL (s cm ⁻¹)	Surface-area-specific CWL (mg cm ⁻² h ⁻¹)	Cutaneous lipids	Wiping behaviors
Arboreal	Hylidae	<i>Phyllomedusa sauvagei</i>	206 ^f	0.5 ^f	E, L ^a	+ ^a
		<i>P. hypochondrialis</i>	364 ^f	0.3 ^a	E, L ^a	+ ^a
		<i>P. iherengi</i>	277 ^f	0.3 ^f	E, L ^a	+ ^a
		<i>P. pailona</i>	336 ^f	0.3 ^f	E, L ^a	+ ^a
		<i>Litoria gracilentia</i>	118 ^e	0.7 ^e	S ^e	?
		<i>L. caerulea</i>	1.8 ^e	17 ^e	E, L ^b	+ ^b
		<i>Hyla arenicolor</i>	1.6 ^e	28 ^e	?	?
	Rhacophoridae	<i>Polypedates maculatus</i>	1.9 ^d	7.7 ^d	E, M ^d	+ ^d
	<i>Chiromantis petersi</i>	347 ^e	0.2 ^e	- ^c	- ^c	
	<i>C. rufescens</i>	404 ^e	0.2 ^e	S ^e	?	
Semi-aquatic	Ranidae	<i>Rana pipiens</i>	1.6 ^c	28 ^e	G ^{g,h}	?
		<i>R. utricularia</i>	?	26.9 ^f	?	?
Aquatic	Pipidae	<i>Xenopus laevis</i>	?	27.5 ^f	G ^h	?
		Free water surface	1.6 ^e	44 ^e		

Letters indicate the presence of lipids within the following: (E) extra-epidermal layer, (L) lipid glands, (M) mucous glands, (G) granular glands and (S) skin, where exact location of lipids is unknown.

+ presence, -, absence of lipids or behaviours; ? indicates values that have not been investigated.

^aBlaylock et al., 1976; ^bChristian et al., 1988; ^cDrewes et al., 1977; ^dLillywhite et al., 1997; ^eWithers et al., 1984; ^fWygoda et al., 1984; ^gDapson et al., 1973; ^hThomas et al., 1993.

Note: in some references (a,d-f), measurements of CWL were made using different methods.

deprivation and wiping. The species of tree frogs included *Hyla andersonii*, *H. avivoca*, *H. chrysoscelis*, *H. cinerea*, *H. femoralis*, *Hyla gratiosa* and *H. squirella*.

Materials and methods

Animals

Adult *Hyla chrysoscelis* ($N=6$; mean \pm S.D. snout-vent length 4.10 ± 0.10 cm, mass 4.32 ± 1.37 g), *H. cinerea* ($N=5$; 3.70 ± 0.45 cm, 3.97 ± 1.09 g), *H. femoralis* ($N=3$; 2.82 ± 0.28 cm, 1.88 ± 0.37 g), *H. gratiosa* ($N=7$; 4.70 ± 0.42 cm, 8.33 ± 1.63 g), and *H. squirella* ($N=12$; 3.20 ± 0.12 cm, 2.14 ± 0.45 g) were collected in Gainesville, Alachua County, Florida from March through May 1997. *Hyla avivoca* ($N=5$; 3.78 ± 0.08 cm, 3.30 ± 0.44 g), *Phyllomedusa hypochondrialis* ($N=4$; 2.58 ± 0.10 cm, mass 2.70 ± 0.23 g), and *Rana utricularia* ($N=5$; 7.40 ± 0.42 cm, mass 26.62 ± 6.76 g) were purchased from a herpetological supplier. *Hyla andersonii* ($N=4$; 3.87 ± 0.31 cm, 4.00 ± 0.20 g) were collected from Santa Rosa County, Florida, maintained under state permit #WX97203, and were examined for the presence of extra-epidermal mucosubstances and lipids, skin layer thickness, and diameter and density of cutaneous glands. We did not measure EWL in this species. Frogs were acclimated indoors and were kept for 2 weeks prior to experimentation under a 12:12 h light:dark cycle at 23°C and 56% relative humidity in plastic terraria (25 cm \times 20 cm \times 13 cm). Frogs were provided with *ad libitum* water, leaves and stems for perching or hiding, and domestic crickets at 3-day intervals.

Observations of skin secretions

To evoke cutaneous glands to secrete their contents onto the dorsal skin surfaces of frogs, we stroked the dorsal skin surfaces of frogs using a blunt metal probe or, in separate tests, injected epinephrine into the dorsal lymph sac ($0.15 \mu\text{g g}^{-1}$ body mass). These methods have been used previously to study glandular secretions in tree frogs (Lillywhite et al., 1997).

Secretions were collected by gently pressing a glass slide against the dorsal skin surface. Slides were air-dried, stained with Hematoxylin and Eosin (H&E), and examined microscopically to confirm that secretions were not contaminated with epidermal cells. Periodic Acid Schiff (PAS) was used to identify mucopolysaccharides, neutral mucosubstances, hyaluronic acid and sialomucins. Alcian Blue at a pH 1.0 (AB-1) was used to identify sulfated glycoproteins, and at a pH of 2.5 (AB-2.5) to identify nonsulfated and sulfated glycoproteins. Sudan Black B (SBB) and Oil Red O (ORO) were used to identify lipids (Presnell and Schreiber, 1997). Duplicate slides were treated with chloroform:methanol (2:1 v/v) to extract lipids prior to staining with SBB. The duplicate slides did not stain with SBB, indicating that the stain effectively detected lipids in experimental slides.

Wiping behavior and evaporative water loss

To evoke possible grooming behaviors, we dropped plant debris and water droplets onto the dorsal body surfaces of resting and active frogs, and we observed their responses for 15 min. Grooming movements that dislodged debris were

considered distinct from wiping behaviors that spread glandular secretions. We then conducted systematic observations of wiping behavior. First, we observed wiping behaviors of frogs during 10 h trial periods when they had *ad libitum* access to water. We monitored the mass of each animal by weighing at the beginning (0 h), middle (5 h) and end (10 h) of each trial. Wiping behaviors of individual frogs were observed for 15 min immediately after each weighing for a total of 45 min of observation of each frog. A total of 10 trials were repeated for each individual for a total of 450 min of observation of wiping behavior per individual per species. Each trial was conducted approximately 12–14 h apart, and frogs were provided free access to water between trials.

A second set of observations was conducted on individuals subjected to repetitive trials of moderate dehydration (<35% of standard body mass loss) to examine whether dehydration stress influenced the frequency or pattern of wiping behavior. With the exception of water deprivation, each dehydration trial was identical in procedure, length of time and repetition to the free water trials. At the start of each dehydration trial, bladder water was expressed from each frog, and access to water was withheld for a total of 10 h. Calculations of EWL ($\text{mg cm}^{-2} \text{h}^{-1}$) were based on losses of body mass relative to standard mass during each 10 h trial.

Partly to account for size differences among species, we converted rates of EWL measured during dehydration trials to surface area-specific rates of EWL for each frog. Surface areas of frogs were estimated from the general equation in Talbot and Feder (1992), assuming an average exposure of two-thirds of the total body surface area to EWL (Withers et al., 1984). We recognize that a small component of these measurements reflects pulmonary water losses, and we use such data solely to assess whether dehydration stress and wiping reduced EWL in frogs, and to contrast the temporal patterns of integrated (total) EWL among the species studied.

Histology

Skin biopsies were sampled from five individuals each of *H. andersonii*, *H. avivoca*, *H. chrysoscelis*, *H. cinerea*, *H. femoralis*, *H. gratioiosa*, *H. squirella*, *P. hypochondrialis* and *R. utricularia*. Frogs were euthanized with chloroform hydrochloride gas. Pieces of skin (5 mm×5 mm) were excised from the dorsal midline between the shoulders (three samples) and from the abdomen (two samples). One skin sample from each region was rinsed in Ringer's solution and fixed in 10% neutral buffered formalin, while a second skin sample was fixed in Opti-freeze solution and flash-frozen in isopentane (2-methylbutane) immersed in liquid nitrogen. Formalin-fixed skin samples were dehydrated in ethanol, embedded in paraffin, serially sectioned at 6 μm , mounted onto glass slides, and stained with H&E, PAS, AB-1 and AB-2.5. Frozen skin sections were serially sectioned at 10 μm , mounted on glass slides, air-dried and stained with ORO or SBB. Lipids were extracted from control skin sections prior to staining for lipids using chloroform:methanol (2:1 v/v) for 15 min. These

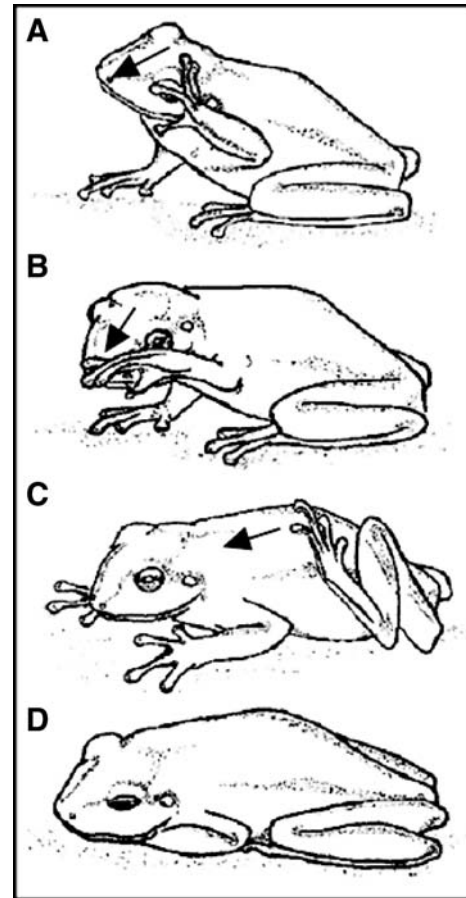


Fig. 1. Common wiping movements in Florida tree frogs. (A) Beginning of a head-wipe, (B) end of a head-wipe and (C) dorsal-wipe. Arrows indicate direction of wiping movement. (D) After wiping, most frogs displayed a water conserving posture. Illustration by T. Barbeau.

negative control sections showed little or no reaction to ORO or SBB.

Quantification of skin glands

The densities of mucous and granular glands per mm length of dorsal skin were determined using light microscopy. Measurements were made of the widest diameter of glands and of the thickness of the epidermis, stratum spongiosum, stratum compactum and total skin. Ten randomly selected skin sections were measured from each of five frogs per species, and the results are reported as means \pm S.D.

Statistical analyses

The total number of wiping movements exhibited by all species under the conditions of normal hydration and dehydration were compared with a paired *t*-test. For each type of wiping movement, analysis of variance (ANOVA) was performed to compare the number of wiping movements among species. Differences in surface area-specific rates of EWL among trials for each species were determined with one-way repeated measures ANOVA, following which time effects

were tested by Helmert contrasts. The total number of wiping movements and rates of EWL were compared among species using ANOVA, and significant ANOVAs were followed by Scheffe's pairwise contrasts. Thickness of the epithelium, stratum corneum, stratum spongiosum, and total skin and diameter of glands, were compared among species with ANOVA followed by Student–Newman–Keuls *post hoc* tests. Gland density was compared among species with Kruskal–Wallace non-parametric analyses followed by Mann–Whitney *U*-pairwise contrasts. All data were analyzed using SPSS 7.5 software and *P* set *a priori* at 0.05, unless otherwise specified.

Results

Observations of skin secretions

Skin surfaces of all frogs examined appeared slightly wet after self-wiping movements or after gentle tactile manipulation with the probe. Tactile stimulation of skin elicited secretions from multiple but localized glands. For example, stimulation of skin on the dorsal head of all species examined elicited secretions from glands in that region and around the neck, but not in the back or limbs. Repeated stimulation of an area resulted in the accumulation of localized, discrete secretions that coalesced to cover the entire area.

In all species examined, slides with air-dried secretions stained positively with PAS, AB-2.5 and AB-1. Dried secretions stained dark blue to black for lipids with SBB, whereas duplicate slides with secretions subjected to lipid extraction prior to staining demonstrated a weak or negative reaction to SBB or to ORO.

Description of body wiping movements

Variable wiping behaviors were observed in Florida tree frogs. The most common behavior involved wiping of the dorsal head by the front limbs (head wipe). The wipe progressed, in a caudal to cranial direction, over the eye orbit (the eyes momentarily closed), the snout and the nostril before the hand returned to a resting position on the substrate (Fig. 1A,B). It was not unusual for animals to wipe one side of the head several times in succession and then immediately wipe the opposite side of the head with the other forelimb, once or several times, in the same manner.

Another behavior observed was the wiping of the ventral surface of the chin with the forelimbs (ventral-lateral chin wipe). As with the dorsal head wipe, this wipe was often immediately repeated with the same or opposite forelimb several times.

Wiping behaviors involving the hind limbs were also observed in all species. One of these behaviors was the wiping of the dorsal back surface with the hind foot (dorsal wipe). The wipe progressed over the back in a caudal to cranial direction, before the hind foot extended laterally off the body at the thoracic region and returned to a resting position (Fig. 1C). This wipe was typically repeated several times with singular, alternating movements of the left and right hind limbs. The

hind limbs were also involved in wiping of the lateral body surfaces (lateral wipe) in all species. Similarly to the dorsal back wipe, this wipe was often repeated several times with singular, alternating movements of the hind limbs.

Another wiping behavior observed was a brief wiping of the eyes with one of the front limbs (eye flick). This movement typically was not repeated with the same or opposite limb; however it often preceded a sequence of seating movements associated with a water-conserving posture (WCP; Fig. 1D).

When debris was dropped on the heads of frogs, they would quickly 'flick' off the debris with a forelimb, but this behavior appeared distinct from head-wiping and was seen only occasionally. After such flicking movements, individuals promptly assumed a WCP.

Each species appeared to engage in a different repertoire of wiping movements during normal hydration and dehydration trials. *Hyla cinerea* exhibited the highest number of head-wiping movements compared to the other species ($P=0.0001$), whereas *H. gratiiosa* and *H. squirella* exhibited an intermediate number of wiping movements compared to *H. femoralis* and *H. chrysoscelis* ($P=0.008$ and $P=0.001$, respectively). The number of head wipes in *H. avivoca* was similar to all species except *H. cinerea*. Although the number of dorsal wiping movements was similar among species, *H. squirella* exhibited the highest number of lateral wiping movements ($P=0.0001$). The number of ventral-lateral chin-wiping movements was similar among *H. avivoca*, *H. gratiiosa* and *H. squirella*; however, *H. cinerea*, *H. chrysoscelis* and *H. femoralis* did not exhibit this wiping movement. *Hyla gratiiosa* engaged in more eye-flicking movements ($P=0.024$) than *H. avivoca*, but *H. femoralis* exhibited a similar number of wiping movements to the aforementioned two (Table 2).

During normal hydration trials, frogs typically were active and displayed wiping behaviors within the first 5–10 min of each observation period, after which they became quiescent in a WCP for the remaining time. During dehydration trials, frogs typically displayed wiping behaviors within several minutes after weighing, following which they displayed a WCP for the remaining time. However, several individuals of *H. cinerea* displayed exploratory or escape behaviors during the final minutes of several observation periods, suggesting that these individuals were stressed by the trial. The mean number of wiping movements exhibited by all species during dehydration trials (52.8) was significantly higher ($P<0.025$) than those under normal hydration (34.8). This difference partly reflects the relatively higher frequency of wiping movements observed during the first 5 min of the dehydration trials.

Rates of surface area-specific EWL, body water deficit and rehydration

Body water deficits incurred among all species during dehydration cycles ranged between 4.0 and 26.9% of standard body mass, with *H. gratiiosa* averaging one-half to one-third the deficit displayed by the other species. Following access to water, rehydration levels among species ranged from 103.5 to 124.7% of standard body mass (Table 3).

Table 2. Mean number of wiping movements observed in Florida tree frogs during pooled conditions of dehydration and normal hydration

Species (total wipes)	Head wipe	Dorsal wipe	Lateral wipe	Ventral-lateral chin wipe	Eye flick
<i>Hyla cinerea</i> (14)	22.2±3.6 ^a (3; N=5)	1.2±1.1 ^a (2; N=4)	0.8±0.8 ^a (5; N=3)	None (N=5)	None (N=5)
<i>Hyla gratiosa</i> (101)	9.0±5.1 ^b (73; N=7)	1.0±0.8 ^a (7; N=5)	0.7±0.8 ^a (5; N=4)	0.9±0.7 ^a (6; N=5)	1.43±0.5 ^b (10; N=7)
<i>Hyla squirella</i> (234)	11.6±3.1 ^b (139; N=12)	2.2±1.4 ^a (26; N=11)	5.3±2.1 ^b (64; N=12)	0.4±0.5 ^a (5; N=5)	None (N=7)
<i>Hyla avivoca</i> (34)	3.0±2.2 ^{b,c} (15; N=4)	0.6±0.9 ^a (3; N=2)	1.6±1.1 ^a (8; N=4)	1.2±0.5 ^a (6; N=5)	0.4±0.6 ^a (2; N=2)
<i>Hyla chrysoscelis</i> (18)	1.2±0.8 ^c (7; N=5)	0.7±0.8 ^a (4; N=3)	0.5±0.6 ^a (3; N=3)	None (N=6)	None (N=6)
<i>Hyla femoralis</i> (14)	1.0±0.0 ^c (3; N=3)	0.7±0.6 ^a (2; N=2)	1.7±0.6 ^a (5; N=3)	None (N=3)	1.3±0.6 ^{a,b} (4; N=3)

For each species, the total number of all wiping movements observed is indicated. The number of each wiping movement observed followed by the sample size *N* of individuals observed is reported within parentheses.

Values are means ± s.d. for multiple observations.

Superscript letters indicate significant differences in wiping movements among species as determined by one-way ANOVA followed by Scheffe *post-hoc* comparisons ($P=0.05$).

Species that did not display certain wiping movements are indicated by 'none' followed by sample size.

Table 3. Surface area-specific rates of evaporative water loss (EWL), body water deficit and levels of body water during rehydration in Florida tree frogs subjected to repetitive dehydration trials

Species (<i>N</i>)	EWL (mg cm ⁻² h ⁻¹)	Incurred deficit (% standard mass)	Rehydration level (% standard mass)
<i>Hyla gratiosa</i> (7)	10.0±1.5 ^a (7.3–11.2)	5.5±1.5 (4.0–7.0)	106.2±2.5 (103.5–109.2)
<i>Hyla squirella</i> (12)	13.1±3.4 ^{a,b} (9.8–22.4)	12.3±2.9 (8.5–18.8)	110.1±4.2 (105.4–117.8)
<i>Hyla avivoca</i> (5)	14.9±2.2 ^b (12.8–18.2)	11.9±3.1 (9.7–15.6)	108.9±2.4 (106.3–112.7)
<i>Hyla femoralis</i> (3)	14.9±2.7 ^b (12.7–17.8)	14.5±3.5 (12.7–13.1)	115.0±4.3 (110.4–118.9)
<i>Hyla chrysoscelis</i> (6)	15.5±1.5 ^b (13.3–17.3)	11.3±2.9 (9.4–13.4)	112.5±3.6 (107.6–116.3)
<i>Hyla cinerea</i> (5)	25.29±5.9 ^c (18.9–35.1)	19.1±2.9 (14.5–26.9)	114.0±6.2 (110.2–124.7)
<i>P</i> -value	$P<0.001$		

For each species (*N*), a total of 10 cycles were conducted for a duration of 100 h per individual.

Values are means ± s.d. followed by ranges in parentheses for multiple measurements.

Superscript letters indicate significant differences as determined by one-way ANOVA followed by Scheffe *post-hoc* comparisons ($P=0.05$).

Frogs were subjected to repetitive trials of dehydration to determine if acclimation to dehydration stress affected rates of EWL over time. Rates of EWL were similar among trials for *H. cinerea* and *H. femoralis*, but different among trials for *H. avivoca* (Fig. 2A, $P<0.001$), *H. gratiosa* (Fig. 2B, $P<0.001$), *H. squirella* (Fig. 2C, $P<0.001$) and *H. chrysoscelis* (Fig. 2D, $P<0.001$). Overall, the rates of EWL fluctuated considerably over time for all species examined, and no discernable trend in EWL was evident.

Rates of EWL ranged from 7.3–35.1 mg cm⁻² h⁻¹ and were significantly different among species ($P<0.001$) as demonstrated by a one-way ANOVA. Scheffe pair-wise comparisons demonstrated that rates of EWL were lowest in *H. gratiosa* compared to all species except *H. squirella*, while rates were similar among *H. avivoca*, *H. chrysoscelis*, *H. femoralis* and *H. squirella*. The highest rate of EWL was observed in *H. cinerea* ($P<0.001$; Table 3).

Skin morphology

For all species examined, the epidermis was organized into several layers and exhibited an outermost stratum corneum (Sc) of flattened, keratinized epithelial cell layers (Fig. 3A).

Directly above the Sc of most species was a thin coat of sulfated and nonsulfated glycoproteins, indicated by a turquoise color when stained with AB-1 and AB-2.5, respectively. This extra-epidermal mucous coat was confirmed by the staining of extra-epidermal skin secretions. For most species, neutral glycoproteins, hyaluronic acid or sialomucins were detected by PAS. Beneath the Sc was the stratum intermedium layer consisting of eosinophilic epithelial cell layers. Lower epithelial cells of this layer were cuboidal in shape with cells appearing more flattened near the Sc. This layer was eosinophilic with H&E, and stained positively for lipids with SBB in *P. hypochondrialis* and *H. andersonii*. The basal layers of the epidermis were composed of a stratum germinativum of a single layer of columnar epithelial cells bordered below by an innermost basement membrane (BM) of collagenous fibers (Fox, 1994).

For all species examined, the dermis was similarly organized into two principle layers, the upper stratum spongiosum (SS) and the lower stratum compactum (SC). The SS contained numerous granular and mucous glands, and thickness of the layer was different among species. Among Florida tree frogs, the SS was thickest for *H. chrysoscelis* and thinnest for *H.*

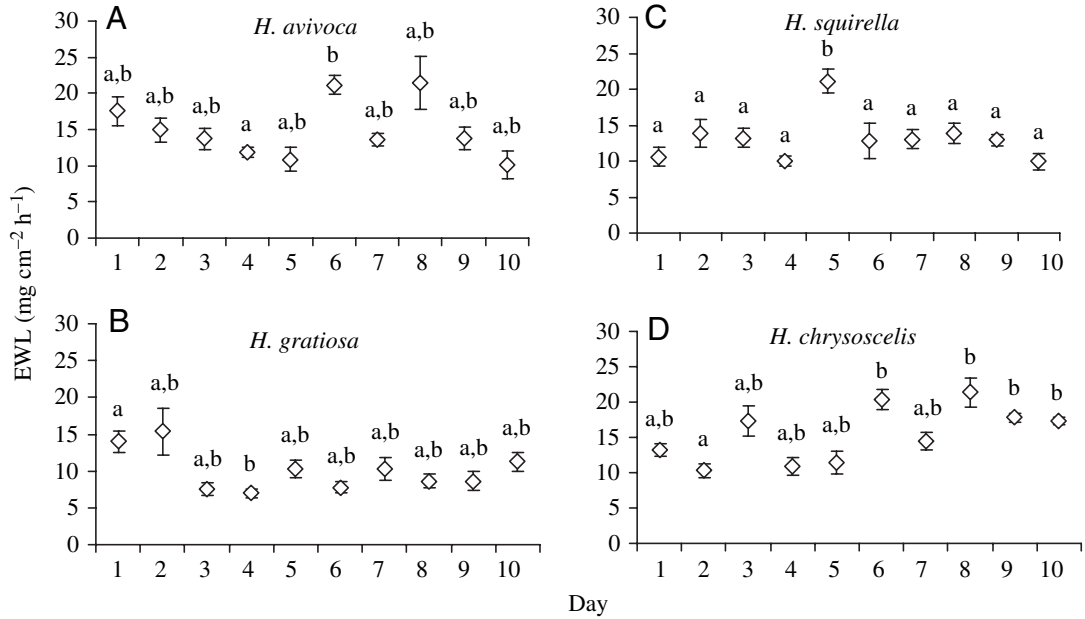


Fig. 2. Mean surface-area specific evaporative water loss (EWL) in (A) *Hyla avivoca*, (B) *H. gratiosa*, (C) *H. squirella* and (D) *H. chrysoscelis* over a 10 day trial. Bars indicate mean \pm S.D. Different letters above bars indicate significant differences among the means ($P < 0.001$).

femoralis and *H. avivoca* ($P = 0.01$). Among all species compared, the SS was thinnest for *H. andersonii* (Fig. 4, Table 4, $P = 0.009$). The SC was composed of dense

collagenous connective tissue and the thickness of this layer was different among species. Among Florida tree frogs, the SC was thinnest for *H. squirella*, *H. femoralis* and *H. avivoca*, and thicker for *H. gratiosa*, *H. cinerea* and *H. chrysoscelis* (Fig. 4, Table 4, $P < 0.001$). Among all species the SC was thickest for *R. utricularia* and thinnest for *P. hypochondrialis* (Table 4, $P < 0.001$). The SS layer contained two basic types of alveolar glands: mucous and granular. The inclusion of *R. utricularia* and *P. hypochondrialis* in statistical analyses suggests that statistical variation in skin layer thickness among all species represented considerably different skin morphology in these two species. For all species examined, the granular glands were larger and fewer in number than mucous glands, and contained luminal secretory granules (Fig. 3A).

The basal portion of granular glands often extended into the SC layer, whereas the mucous glands were found in the upper SS beneath the basement membrane. The inner wall of mucous glands was lined with cuboidal or columnar epithelial cells having eosinophilic nuclei. The gland collar and duct typically were filled with cuboidal myoepithelial cells. The lumen of these glands was often empty and contained few if any granules. Granular glands were round or elliptical in shape, sometimes with an

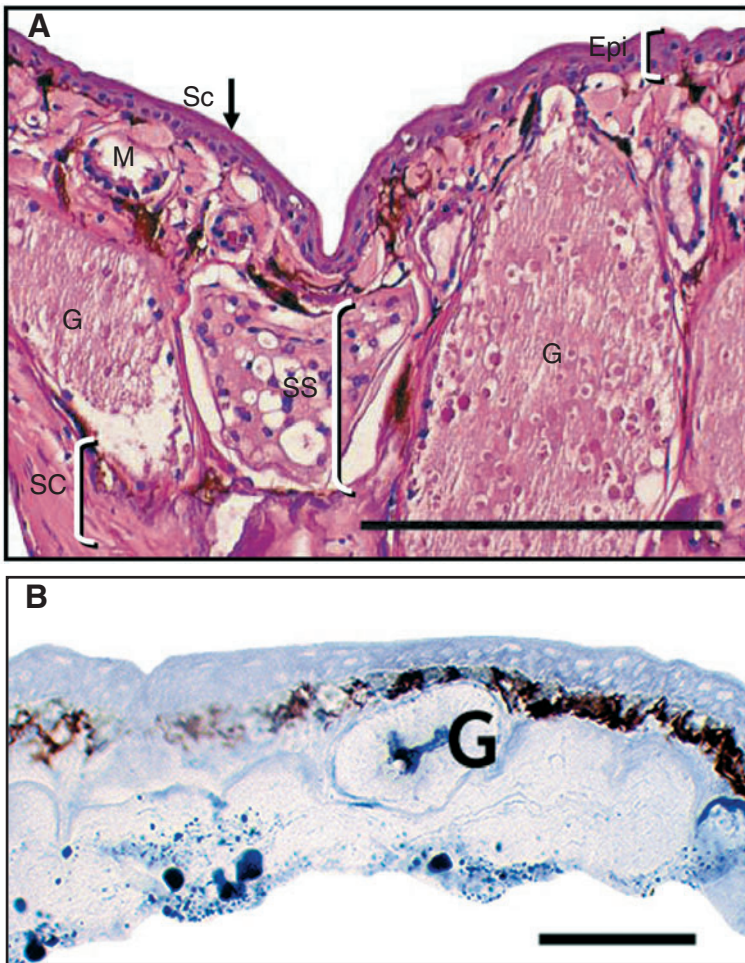
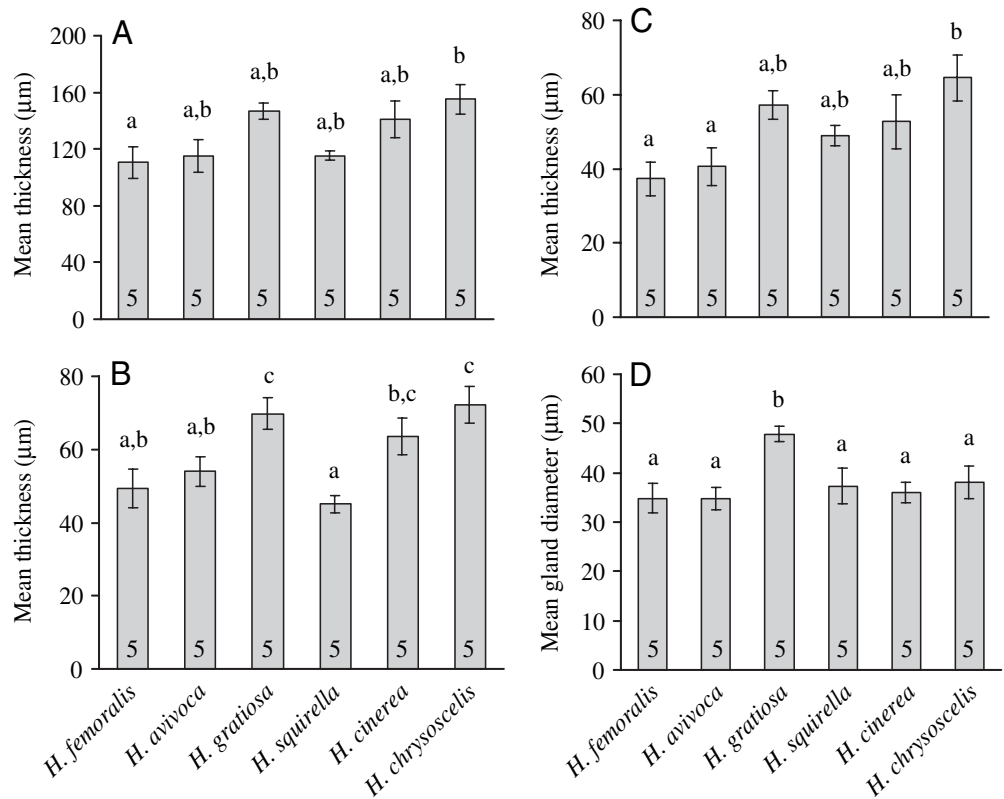


Fig. 3. (A) Light microscopy of a dorsal skin section from *Hyla chrysoscelis*, illustrating staining by Periodic-Acid Schiff. Positive magenta staining in dorsal mucous gland (M), granules of granular gland (G), and surface of stratum corneum (Sc). Skin layers are indicated as epithelium (Epi), stratum spongiosum (SS) and stratum compactum (SC). Bar, 100 μ m; magnification, 200 \times . (B) Light microscopy of the dorsal granular gland (G) from *Hyla squirella* stained with Sudan Black. Bar, 100 μ m; magnification, 100 \times .

Fig. 4. Mean thickness of the dorsal (A) total epidermis + dermis, (B) stratum compactum and (C) stratum spongiosum, and (D) the mean diameter of the dorsal mucous glands in Florida tree frogs. Values are means \pm S.E.M. Numbers within bars indicate sample size, and the different letters above bars indicate significant differences among the means ($P < 0.001$).



enlarged basal region. The inner wall of these glands was lined with flattened myoepithelial cells with eosinophilic nuclei. The gland collar and duct were filled with cuboidal epithelial cells, similar to that in mucous glands. The lumen of granular glands was relatively large and usually filled with heterogenous granular secretory material. Often, the basal portion of the gland contained more secretory material than apical regions (Fig. 3A).

Histochemical differences were observed between granular and mucous glands. Secretory mucus was located within the lumen or apical region of the mucous gland. In the granular glands of all species except *H. cinerea* and *H. gratiosa*, mucous and lipid secretions were associated with the secretory granules and often concentrated at the basal region of the gland. Both types of glands contained nonsulfated glycoproteins; however, sulfated glycoproteins were found predominantly in mucous glands whereas neutral glycoproteins, hyaluronic acid or sialomucins were found predominantly in granular glands. In *P. hypochondrialis*, sulfated and nonsulfated glycoproteins were found only in granular glands while neutral mucosubstances were found in mucous glands. In *R. utricularia* these mucosubstances were detected only in the mucous glands, with the exception of sulfated and nonsulfated mucins in the ventral granular glands of *R. utricularia*. In all species, the secretory material in mucous glands stained positively for neutral glycoproteins and for non-sulfated glycoproteins, but did not demonstrate lipids with either ORO or SBB staining.

The lumen and periphery of dorsal granular glands stained positively for lipids with SBB in *H. andersonii*, *H. femoralis*, *H. chrysosecelis* and *H. squirella* (Fig. 3B). Dorsal granular glands in *H. andersonii* and *H. avivoca* stained positively for lipids with ORO. In *P. hypochondrialis*, lipid glands unique to this genus stained positively for lipids with SBB and with ORO, but granular glands did not show similar lipids. Granular glands in *H. cinerea*, *H. gratiosa* and *R. utricularia* did not stain positively for lipids with SBB or ORO. Control slides of

skin sections rinsed in chloroform:methanol showed little or no reaction to lipid staining.

Glands differed in diameter and density among species. Diameter of the granular glands did not differ among Florida tree frogs, but among all species granular glands were larger in *R. utricularia*, smaller in *P. hypochondrialis* and *H. squirella*, and of intermediate size in the other hylids (Table 4, $P = 0.04$). For Florida tree frogs, mucous glands were larger in *H. gratiosa* and smaller in the other tree frogs ($P = 0.02$). For all species combined, mucous glands were larger in *P. hypochondrialis* and smaller in *H. femoralis* and *H. avivoca* (Table 4, $P < 0.001$). Density of granular glands was similar among species but the density of mucous glands was greater in *H. femoralis* than in *H. cinerea* and *P. hypochondrialis* (Table 5, $P = 0.002$).

Discussion

Wiping behaviors

Florida tree frogs engage in wiping behaviors that are distinct from grooming, and the dropping of debris onto the dorsal surfaces of frogs does not elicit any of the distinct self-wiping behaviors described here. Movements of the limbs that draw shed skin from the body to the mouth followed by ingestion of the skin are not considered wiping movements. Wiping movements associated with skin secretions are identical in sequence and duration of motion among species, and are executed by both front and hind limbs with no preferential use of left or right limbs. Although wiping behaviors in Florida tree frogs are not as elaborate or frequent

Table 4. Morphological measurements of dorsal skin layers and glands of each species examined

Species	Thickness (μm)				Diameter (μm)	
	Total skin	Epidermis*	Stratum spongiosum	Stratum compactum	Granular glands	Mucous glands
<i>R. utricularia</i>	222.3 (59.2) ^a	33.8 (14.8)	54.8 (18.6) ^{a,b}	113.6 (34.5) ^a	60.8 (22.4) ^c	41.6 (4.7) ^{b,c}
<i>P. hypochondrialis</i>	78.7 (23.6) ^c	17.0 (6.0)	45.8 (13.9) ^{a,b}	16.0 (7.7) ^c	104.6 (5.9) ^a	52.2 (6.8) ^a
<i>H. andersonii</i>	100.3 (28.8) ^c	20.1 (7.9)	28.3 (10.9) ^a	51.8 (11.9) ^b	94.6 (21.5) ^{a,b}	44.4 (4.7) ^{b,c}
<i>H. femoralis</i>	110.6 (25.3) ^{b,c}	23.9 (7.2)	37.3 (10.0) ^{a,b}	49.3 (12.0) ^b	70.6 (8.9) ^b	34.8 (6.6) ^c
<i>H. avivoca</i>	115.0 (25.2) ^{b,c}	20.5 (5.3)	40.6 (11.5) ^{a,b}	53.9 (9.0) ^b	71.7 (11.3) ^b	34.8 (5.1) ^c
<i>H. gratioiosa</i>	146.8 (12.4) ^{b,c}	19.8 (3.2)	57.2 (8.5) ^b	69.8 (9.6) ^b	92.5 (29.0) ^{a,b}	47.9 (3.3) ^{a,b}
<i>H. squirella</i>	115.3 (7.2) ^{b,c}	21.4 (3.4)	48.9 (6.3) ^{a,b}	45.0 (5.1) ^b	100.2 (14.9) ^a	37.3 (8.0) ^{b,c}
<i>H. cinerea</i>	141.0 (28.4) ^{b,c}	24.7 (7.5)	52.7 (16.3) ^{a,b}	63.6 (11.2) ^b	100.2 (4.9) ^{a,b}	36.0 (4.6) ^{b,c}
<i>H. chrysoscelis</i>	155.2 (23.4) ^{b,c}	18.5 (7.8)	64.5 (13.8) ^b	72.2 (11.3) ^b	94.3 (35.6) ^{a,b}	38.1 (7.4) ^{b,c}
<i>P</i> values	<i>P</i> <0.001	<i>P</i> =0.08	<i>P</i> =0.009	<i>P</i> <0.001	<i>P</i> =0.04	<i>P</i> <0.001

Values are reported as mean \pm s.d. (within parentheses).

Different superscript letters indicate significant differences among means.

*No significant difference for epidermis values.

Table 5. Density of dorsal glands among species

Species	Relative gland density/mm skin	
	Granular*	Mucous
<i>R. utricularia</i>	4.09 (1.9)	2.62 (1.0) ^{a,b,c}
<i>P. hypochondrialis</i>	2.67 (1.4)	4.40 (0.6) ^a
<i>H. andersonii</i>	2.58 (1.9)	5.06 (0.6) ^{a,b,c}
<i>H. femoralis</i>	1.15 (0.3)	7.27 (4.5) ^c
<i>H. avivoca</i>	1.45 (1.2)	5.21 (1.4) ^{a,b,c}
<i>H. gratioiosa</i>	1.36 (0.6)	4.43 (1.1) ^{a,b,c}
<i>H. squirella</i>	1.87 (0.9)	5.30 (0.8) ^{a,b,c}
<i>H. cinerea</i>	1.79 (1.5)	3.10 (0.5) ^{a,b}
<i>H. chrysoscelis</i>	1.55 (0.6)	6.66 (1.5) ^{b,c}
<i>P</i> value	<i>P</i> =0.25	<i>P</i> =0.02

Density of dorsal glands is per 1 mm skin length.

Values are means \pm s.d.

*No significant difference for granular values.

as those documented in *Phyllomedusa* sp. (Blaylock et al., 1976) or *Polypedates maculatus* (Lillywhite et al., 1997), there are subtle variations in the pattern and frequency of wiping among species. These different patterns appear to distribute secretions over specific skin surface regions.

Most species of the Florida hylids we studied engage in frequent wiping of the head, back and lateral body surfaces compared to the ventral surfaces and eyes. The surfaces wiped more frequently are those that are more exposed to EWL when individuals are positioned in a WCP. With the exception of three species that displayed a low frequency (<10% of total wipes observed) of ventral-lateral chin wiping, wiping of other ventral surfaces is absent. In natural environments, several of these species occupy plant or tree cavity microhabitats wherein the frogs insert the posterior body into a protective depth of a cavity and conceal the ventral body surface with a WCP, while the anterior surfaces of head and back remain exposed (Neil, 1951; Goins, 1958; Boughton, 1997). To the extent that skin

secretions containing lipids reduce EWL, wiping behaviors that spread these secretions over skin regions subjected to evaporative exposure should provide a further selective advantage.

No visible skin secretions are expelled from glands prior to tactile stimulation, whereupon the skin becomes visibly wet at the region of contact. This pattern of secretion is similar to that shown in *P. maculatus* (Lillywhite et al., 1997) but distinct from that of phyllomedusine frogs, which appear to expel secretions prior to the wiping event (Blaylock et al., 1976). Thus, one function of wiping might be to stimulate secretion from cutaneous glands (Lillywhite et al., 1997).

After completing a sequence of wiping movements, most frogs become quiescent in a WCP. This behavior is probably necessary to prevent the physical disruption of the dried extra-epidermal layer of secretions that cover the skin (Lillywhite and Mittal, 1999).

Frogs that exhibit self-wiping behaviors accompanied by lipid secretions are associated with arboreal habitats and are subject to dehydrating conditions (Blaylock et al., 1976; Christian et al., 1988; Lillywhite et al., 1997). Although Florida tree frogs occupy mesic habitats, they are active in microenvironments that are subject to seasonal or periodic aridity. Moreover, *Hyla cinerea*, *H. femoralis* and *H. squirella* have been observed basking on exposed vegetation during the day (Einem and Ober, 1956; Lee, 1969; McComb and Noble, 1981; Ritke and Babb, 1991). *Hyla femoralis* are often heard calling from high in the canopy of upland forested areas and are infrequently found on the ground except during periods of rainfall or reproduction (T.R.B., personal observation). *Hyla gratioiosa* inhabit comparatively drier upland regions away from water except during the reproductive season (Farrell and MacMahon, 1969; Layne et al., 1989). Compared to the other Florida hylids, *H. cinerea* is typically found on vegetation closely associated with permanent or temporary water bodies (Wright and Wright, 1949; Neil, 1951; Goins, 1958; Farrell and MacMahon, 1969). The relatively higher EWL measured in *H.*

cinerea reflects the association with aquatic vegetation, whereas the lower EWL measured in *H. gratiosa* corresponds with drier arboreal environments that are used by this species.

There are subtle distinctions in wiping behaviors associated with lipid secretions among arboreal frogs. Phyllomedusine species tend to exhibit more elaborate patterns of wiping compared to the Florida hylids. *Polypedates maculatus* engages in complex wiping behavior but, similar to Florida tree frogs, exhibits a higher EWL than phyllomedusine tree frogs. These wiping behaviors might have evolved in the absence of lipid secretions and possibly served other functions, such as grooming, shedding skin or spreading secretions that maintain a moist integument for respiration and thermoregulation (Lillywhite, 1971; Lillywhite and Licht, 1974). The comparatively simple body wiping behaviors shown in Florida tree frogs, associated with lipid secretions, may represent an early stage of the more advanced wiping behaviors seen in waterproof species.

Cutaneous lipids

An important discovery in this study was that Florida tree frogs, like some other arboreal anurans, have lipid skin secretions that form an extra-epidermal layer and appear to be spread by stereotypical wiping behaviors (Blaylock et al., 1976; McClanahan et al., 1978). The release of secretions and initiation of wiping behaviors can be elicited by tactile stimuli such as brief handling, massaging or gentle probing of skin surfaces. Tactile stimuli are possibly perceived by the frogs as predatory, and the resulting secretions and wiping behaviors could serve a defensive function. However, we did not examine the secretions for bioactive amines, peptides or alkaloids associated with defensive responses (Esparmer, 1994). Similarly to *P. maculatus* (Lillywhite et al., 1997; Lillywhite and Mittal, 1999), the skin secretions in Florida tree frogs contain both mucosubstances and lipids that reduce EWL (Wygoda, 1984, 1988; Toledo and Jared, 1993), whereas the rates of EWL we measured are comparatively higher than those of so-called waterproof species (Blaylock et al., 1976; Lillywhite et al., 1997; Withers et al., 1984; Wygoda, 1984).

There is considerable variation, and no distinct trend, in rates of EWL over time in the Florida tree frogs subjected to moderate dehydration. These results indicate an absence of acclimation to prolonged dehydration. Such acclimation most likely would be demonstrated by a gradual but consistent decrease in rates of EWL over the 10 dehydration trials. The variation observed in rates of EWL over time might be attributable to several factors, including the influences of activity and posture as well as possible changes in the cutaneous water barrier. Generally, the rates of EWL observed in the Florida hylids are within the range of measurements reported in studies of non-waterproof tree frogs (Wygoda, 1984; Lillywhite et al., 1997). Whereas these data cannot provide estimates of skin resistance, they provide an index of the moderate waterproofing related to wiping and skin secretions, and they indicate an absence of acclimation during periods of water deprivation.

The presence of extra-epidermal lipids and wiping behaviors represent a combination of traits that appear to be convergent among several distantly related genera of arboreal frogs. Clearly there is a wide range of reduced EWL associated with lipid secretions in arboreal frogs. Therefore, an extra-epidermal lipid layer may aid in reducing EWL as in *P. maculatus* and evidently Florida hylids, but not necessarily lead to tight waterproofing as in the phyllomedusine species.

In *P. hypochondrialis*, lipids are detected with SBB and ORO staining in specialized lipid glands. In *H. andersonii*, *H. chrysoscelis*, *H. femoralis* and *H. squirella*, SBB indicates that lipids are present in the dorsal granular glands, but these are extracted from tissue on control slides that are treated with chloroform-methanol prior to staining. This result suggests the presence of phospholipids because polar lipids dissolve readily in chloroform-methanol (Withers et al., 1984). Lipids are also detectable with ORO in the dorsal granular glands of *H. andersonii* and *H. avivoca*. Unbound lipid secretory material would likely be extracted from tissue during staining with ORO because it is an alcohol-based stain. Therefore, the positive reaction to ORO in the granular glands of these species indicates the presence of bound lipids, or lipids that are bound to structural elements within the gland. These might be neutral lipids, such as fatty acids, esters, cholesterol or triglycerides.

The size and density of dorsal granular glands are similar among Florida tree frog species, which suggests the relative quantities of mucus and lipids (when present) are also similar among species. According to Withers et al. (1984), the polar and neutral cutaneous lipids found in the waterproof arboreal frogs *Litoria gracilentata* and *Phyllomedusa hypochondrialis* are also present in some non-waterproof species. Like *Litoria* and *Phyllomedusa*, Florida tree frogs secrete these lipids onto the skin, and likely spread them with wiping behaviors to form the extra-epidermal layer. Despite these similarities, skin resistance to EWL in Florida tree frogs appears significantly lower than that of *L. gracilentata* and *P. hypochondrialis* (Wygoda, 1984; Christian et al., 1988; Buttemer, 1990). What could account for the clear disparity in the protective capacity of these lipids associated with wiping behaviors? Variable rates of EWL may be attributable to quantitative or qualitative differences of the lipids that are present in the extra-epidermal skin secretions of arboreal frogs. Further, it is likely that the relatively simple body-wiping behaviors observed in Florida tree frogs do not spread lipids adequately over the entire body to form a complete barrier to EWL.

The quantity of lipids secreted onto the skin of Florida tree frogs might also be influenced by the dilution of these lipids with mucus or other proteinaceous secretory products. Lipids are combined with mucus within mucous and granular glands, and therefore might be diluted when secreted onto the skin surfaces. Conceivably, a thinner and less concentrated extra-epidermal lipid layer might confer the comparatively moderate skin resistance to EWL shown in these species. The dilution of secretory lipids is considered a factor contributing to the relatively modest skin resistances demonstrated in other non-waterproof arboreal frogs (Lillywhite et al., 1997). A

specialized gland is dedicated to lipid production in the phyllomedusines, and therefore a larger quantity and concentration of lipids are likely to result in a higher resistance to CWL in these species. Further investigation is required to determine how lipid chemistry and quantity of secreted products might influence EWL rates in arboreal frogs.

Strikingly similar traits are shared among arboreal frogs inhabiting arid or seasonally arid environments. The present study demonstrates that glandular secretion of lipids spread over the body by wiping behaviors is part of a suite of characters that are more widespread among arboreal frogs than previously known. The occurrence of these characters among different genera of arboreal frogs on several continents likely represents evolutionary convergence of function in response to dehydration stress (Shoemaker et al., 1987; Christian et al., 1988; Lillywhite et al., 1997).

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