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Comparative Analysis of Cutaneous Evaporative Water Loss in Frogs Demonstrates Correlation with Ecological Habits

Jeanne E. Young[1,*]
Keith A. Christian[1]
Stephen Donnellan[2]
Christopher R. Tracy[1]
David Parry[1]
[1]School of Science, Charles Darwin University, Darwin, Northern Territory 0909, Australia; [2]Evolutionary Biology Unit and Centre for Evolutionary Biology and Biodiversity, South Australian Museum, North Terrace, Adelaide, South Australia 5000, Australia

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ABSTRACT

Most frog species show little resistance to evaporative water loss (EWL), but some arboreal species are known to have very high resistances. We measured EWL and cutaneous resistance to evaporation (R_c) in 25 species of frogs from northern Australia, including 17 species in the family Hylidae, six species in the Myobatrachidae, and one each in the Bufonidae and the Microhylidae. These species display a variety of ecological habits, including aquatic, terrestrial, and arboreal specialisations, with the complete range of habits displayed within just the one hylid genus, *Litoria*. The 25 species measured in this study have resistances that range from $R_c = 0$ to 63.1. These include low values indistinguishable from a free water surface to high values typical of "waterproof" anuran species. There was a strong correlation between ecological habit and R_c, even taking phylogenetic relationships into account; arboreal species had the highest resistance, aquatic species tended to have little or no resistance, and terrestrial species tended to have resistance between those of arboreal and aquatic frogs. For one species, *Litoria rubella*, we found no significant changes in EWL along a 1,500-km aridity gradient. This study represents the strongest evidence to date of a link between ecological habits and cutaneous resistance to water loss among species of frogs.

* Corresponding author. Present address: P.O. Box 237, Melrose Park, South Australia 5039, Australia; e-mail: jeanne.young@cdu.edu.au.

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Introduction

Historically, evaporative water loss (EWL) from the skin of anuran species was considered to be similar to the loss of water from a free water surface. Species that follow this pattern are considered "typical" amphibians with respect to water loss. In the last 30 yr, research has shown that not all species of frogs lose water from their skin in this manner. Some species have resistance to water loss from their skin that is equal to or higher than that of some desert-adapted reptiles (Loveridge 1970; Shoemaker et al. 1972; Withers et al. 1982*a*; Wygoda 1984; Buttemer 1990; Preest et al. 1992). Species with significant resistance to EWL are termed "atypical" or "waterproof." Frogs with cutaneous resistance to EWL represent a phylogenetically diverse range of anurans (Christian and Parry 1997; Lillywhite et al. 1997). Some studies have suggested that the rate of EWL from the skin may be related to the lifestyle (e.g., arboreal, terrestrial, aquatic) of a species (Wygoda 1984), while others have found no evidence of a relationship between EWL and activities of frogs in their natural environment (reviewed by Shoemaker et al. 1992). Wygoda (1984) demonstrated in a study of 17 species that an arboreal lifestyle was a common factor among species that were found to be "atypical" with regard to EWL from the skin.

Many Australian species of hylids are "atypical" in relation to water loss from the skin (Withers et al. 1984; Buttemer 1990; Amey and Grigg 1995; Buttemer et al. 1996; Christian and Parry 1997; Buttemer and Thomas 2003). Although variations in the methods and conditions of individual experiments make it difficult to make direct comparisons between studies, there is a general consensus that resistance of *Litoria* species is intermediate (10–40 s cm^{-1}; Withers 1995; Buttemer and Thomas 2003) between that of typical anuran amphibians (~1 s cm^{-1}) and the waterproof frogs from the genera *Chiromantis* and *Phyllomedusa* (>100 s cm^{-1}; Loveridge 1970; Shoemaker and McClanahan 1975).

In the wet-dry tropics of northern Australia, frogs must contend with the extremes of the dry season where water availability is limited for 4–5 mo of the year. Behavioural mechanisms such as burrowing, habitat selection, water-conserving posture (WCP), and limiting time exposed to ambient conditions are common mechanisms used by frogs to deal with such harsh, dry conditions (Shoemaker et al. 1992). In the Northern Territory (NT) of Australia, there is a high diversity of frogs belonging to the family Hylidae. Around Darwin, NT, 15 of these species exhibit a variety of lifestyles, including arboreal, terrestrial, burrowing, aquatic,

Table 1: Size range and reported lifestyle for all species evaluated in this study

Species	SVL[a] (mm)	Lifestyle
Hylidae:		
Cyclorana australis	71–102	Burrowing
Cyclorana longipes	36–55	Burrowing
Litoria bicolor	23–29	Arboreal
Litoria caerulea	60–110	Arboreal
Litoria coplandi	29–42	Terrestrial
Litoria dahlii	49–71	Aquatic
Litoria gilleni	70	Arboreal
Litoria inermis	24–34	Terrestrial
Litoria meiriana	16–21	Semiaquatic
Litoria microbelos	14–16	Terrestrial
Litoria nasuta	33–55	Terrestrial
Litoria pallida	27–37	Terrestrial
Litoria rothii	37–57	Arboreal
Litoria rubella	28–43	Arboreal
Litoria splendida	100	Arboreal
Litoria tornieri	28–34	Terrestrial
Litoria wotjulumensis	33–70	Terrestrial
Bufonidae:		
Bufo marinus	80–230	Terrestrial
Microhylidae:		
Austrochaperina adelphe	20	Terrestrial
Myobatrachidae:		
Limnodynastes convexiusculus	46–61	Burrowing
Limnodynastes ornatus	31–42	Burrowing
Notaden melanoscaphus	34–49	Burrowing
Crinia sp.	16–20	Terrestrial
Uperoleia sp. nov.	16–20[b]	Burrowing
Uperoleia lithomoda	17–29	Burrowing

[a] Snout-vent length (SVL) estimates as per Tyler and Davies (1986) and Cogger (2000).

[b] SVL estimates from J. E. Young and M. J. Tyler (unpublished data).

and semiaquatic habits. The aim of this study is to determine how EWL physiology relates to the lifestyle variation of a group of frogs, the hylids, which are cohabitants and share relatively close phylogenetic relationships. We use phylogenetic comparative methods (Felsenstein 1985; Garland et al. 1992; Blomberg et al. 2003) to examine the historical association between EWL physiology and lifestyle variation. Other cohabitant nonhylid species (Bufonidae, Microhylidae, and Myobatrachidae) were also studied for comparison across frog families. Seasonal comparisons were made for all species that were active in both the wet and dry seasons (J. E. Young, unpublished data). One species, *Litoria rubella*, had previously been shown to have different EWL rates in animals from Alice Springs, NT, and Wyndham, Western Australia (Warburg 1967), so we collected specimens along a north-south transect to determine whether *L. rubella* show variation in EWL physiology linked to inhabiting areas of increasing aridity to the south.

Methods

Animal Collection

Seventeen species of hylids, six species of myobatrachids, and one species of microhylid were collected across three sites (Knuckey Lagoons, Howard River Sand Sheet, Robin Falls Nature Area [approximately 100 km south of Darwin in NT, Australia]; Table 1) or were on loan from private collections (*Litoria splendida* and *Litoria gilleni*). One introduced species of bufonid, *Bufo marinus*, was collected near Katherine, NT, approximately 300 km south of Darwin. *Litoria coplandi* and *Litoria meiriana* were collected during the dry season when they were most abundant along George's Creek at the Robin Falls Nature Area. *Litoria bicolor*, *Litoria caerulea*, *Litoria dahlii*, *Litoria nasuta*, *Litoria pallida*, *Litoria rothii*, *Litoria rubella*, *Litoria tornieri*, and *Litoria wotjulumensis* were collected during both the wet and the dry season. *Litoria rubella* was also collected from Renner Springs (855 km from Darwin) and Alice Springs (1,525 km from Darwin).

Animals were captured during night surveys from September 2000 to February 2003 and returned to Charles Darwin University, where they were housed overnight in ventilated plastic boxes on moist or dry paper towels. Measurements of EWL were taken within 24 h of capture, and animals were returned to their respective collection site within 48 h. For the *L. rubella* captured at Renner Springs and Alice Springs, animals were housed and EWL was measured at nearby workstations.

Measurement of Evaporative Water Loss

An open-flow system was used for measurements of EWL (Christian and Parry 1997 and references therein). Dry air was pumped at a controlled rate into a chamber containing a frog, and the humidity and temperature of the incurrent and excurrent air were measured with a Vaisala HMP130Y capacitance humidity sensor (calibrated regularly against a Vaisala HM11 calibrator, which uses saturated salt solutions). For a given experiment, the same humidity sensor was used to measure both incurrent and excurrent air. Before placement of the frog into the chamber, humidity of the incurrent air was recorded after the humidity trace was steady for 10 min. The air was delivered from the building compressed air source or pumped by a Reciprotor electromagnetic pump through two columns of Drierite. The rate of flow was controlled using flow-controlled Sierra mass flow meters (regularly calibrated against a soap bubble burette [Long and Ireland 1985]). The chamber temperature during trials corresponded to the ambient air temperature in the laboratory. Trials were restricted to periods when the air conditions in the lab were stable, and the measured ambient air temperatures provided a steady trace.

Different-sized experimental perspex chambers were used for different-sized frogs in order to avoid large amounts of unoccupied space in the chambers. Tubes with internal diameters

of 32 and 57 mm were used for small (<25 mm snout-vent length [SVL]) and large animals (≥25 mm SVL), respectively. The flow rates were adjusted for chamber diameter so that all frogs were exposed to the same air speed, 0.25 cm s^{-1} (similar to that used by Shoemaker and McClanahan [1975] and Christian and Parry [1997]). The corresponding flow rates for these chambers were 120 and 380 mL min^{-1}, respectively.

Humidity, flow rate, and air temperature were recorded on PowerLab recording systems (Castle Hill, New South Wales, Australia), and the lowest humidity in the excurrent air that was stable for at least 20 min was used in the calculations. All water loss measurements were recorded from animals in the WCP (Heatwole et al. 1969).

EWL Trials

At the beginning of each trial, each frog was patted dry with a paper towel, weighed to the nearest 0.01 g, and then placed into the end of the chamber nearest the sensor. Each chamber was then covered with a cotton cloth to minimise disturbance. During each trial, behaviour (active or settled), posture (water conserving or otherwise), position in the chamber (vertical: bottom, side, or top; end of chamber: flow, middle, or sensor; body position relative to airflow: facing flow, facing away, or perpendicular), and evidence of excretion or defecation (if observed, the trial was aborted) were recorded every 15 min. A trial was considered over when the humidity and temperature trace was stable for a 20-min period or when the animal appeared to be distressed (identified by a calm period followed by a sudden impulse to attempt escape). Skin temperature was taken from inactive animals within 10–15 s of opening the chamber using a Raytek noncontact infrared thermometer, and mass was recorded to the nearest 0.01 g for each animal for which a stable trace was obtained. Skin temperatures were taken rather than cloacal temperatures because previous work (Wygoda 1984) found that skin temperature and cloacal temperature were not significantly different for frogs in a moving airstream. Laboratory measurements confirmed this relationship for three species of *Litoria* (K. A. Christian, unpublished data). Measurements of SVL, maximum head width, maximum body width, and body depth were taken to the nearest 0.01 mm to enable the carving of an agar model for each individual. For each species, enough animals were measured to have a minimum of eight successful traces. A successful trace was defined as one where the animal maintained a WCP long enough for stable humidity and temperature traces to be recorded. EWL measurements were attempted once for each individual frog (i.e., eight traces represented eight individuals).

Calculations

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). Total EWL is a combination of cutaneous and pulmonary EWL. In anuran species, pulmonary EWL contributes insignificantly to total EWL relative to cutaneous water losses (Spotila and Berman 1976; Bentley and Yorio 1979; Wygoda 1984). In this study, EWL is used to indicate that pulmonary water loss has not been taken from the calculated values. The effective frog surface area was estimated using the empirical equation based on mass derived by McClanahan and Baldwin (1969) and the assumption that the WCP exposes only two-thirds of the surface area to the air (Withers et al. 1984). The empirical equation was verified as an accurate estimate of surface area for *L. caerulea* and *Litoria chloris* (Buttemer 1990). The total resistance to water loss (reported s cm^{-1}) can be 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). The vapour density of the skin of the frog is taken as the saturation vapour density at the skin temperature. Total resistance to water loss is the sum of the cutaneous resistance and the boundary layer resistance (Spotila and Berman 1976).

Boundary layer resistance can be determined by using 3% agar frog models (Buttemer 1990; Christian and Parry 1997), which are assumed to lose water at the same rate as a free water surface. The use of agar models has become a standard technique that provides a measure of boundary layer resistance for a given frog size and shape in the experimental apparatus. Agar models were carved to represent an animal in WCP, and the shape of the model was species specific on the basis of observations of the live animals in the chamber. The agar models were placed inside the chambers in the positions recorded for the live animals for which they were representative. Cutaneous resistance was calculated as the difference between total resistance (calculated from measurements of the real frog) and the boundary layer resistance (calculated from measurements of the agar model).

Molecular Phylogeny

Details of the hylid specimens sequenced are available from S. Donnellan. Several phyllomedusine taxa were used as outgroups on the basis of the phylogenetic analysis of Darst and Cannatella (2004), in which the Australian hylids and phyllomedusines were found to be sister lineages. DNA was extracted from tissues with a standard phenol chloroform method. An approximately 800-bp portion of the *12S rRNA* gene was PCR amplified in two overlapping segments, with one segment amplified with H1478 and L1091 (Kocher et al. 1989) and the other amplified with either L669 (Donnellan et al. 1999) or L675 (5′-TTG GTC CTR RCC TTG AAA TC-3′) with H1160 (Donnellan et al. 1999). A portion of the *16S rRNA* gene was amplified and sequenced with the primers 16sar and 16sbr (Cunningham et

Table 2: Summary of mass, surface area–specific evaporative water loss (EWL), and total resistance (R_t) of hylid and nonhylid species and R_t for similar-sized agar models

		Live Animal			Agar Model
Species	*N*	Mass (g)	EWL (mg cm^{-1} h^{-1})	R_t (s cm^{-1})	R_t (s cm^{-1})
Hylidae:					
Cyclorana australis	14	30.5 ± 6.04	5.2 ± .4	7.1 ± 1.5	1.5 ± .28****
Cyclorana longipes	12	7.41 ± .79	6.3 ± .7	7.1 ± 1.4	3.6 ± .43****
Litoria bicolor	31	.62 ± .16	1.6 ± .76	66 ± 9.3	2.7 ± .11****
Litoria caerulea	28	28.2 ± 17.4	3.4 ± .7	16 ± 4.7	1.6 ± .67****
Litoria coplandi	15	3.79 ± 1.23	3.8 ± .5	14 ± 2.9	4.8 ± .74****
Litoria dahlii	24	15.3 ± 8.95	6.4 ± 1.1	4.9 ± 1.9	2.8 ± 1.1***
Litoria gilleni	2	28.0 ± 2.98	2.4 ± .17	12.8 ± 3.2	1.3 ± .11**
Litoria inermis	10	2.11 ± .42	7.7 ± 1.5	4.2 ± 1.2	3.1 ± .78**
Litoria meiriana	9	.87 ± .13	9.7 ± .5	3.2 ± .46	2.7 ± .49
Litoria microbelos	9	.23 ± .02	14.8 ± 2.8	3.0 ± .79	3.1 ± .10
Litoria nasuta	18	4.45 ± 1.51	6.1 ± .8	8.8 ± 1.3	4.1 ± .46****
Litoria pallida	17	3.02 ± .94	4.8 ± .67	8.2 ± 2.2	2.1 ± .82****
Litoria rothii	47	4.36 ± 1.39	3.3 ± .61	20 ± 4.5	4.2 ± .63****
Litoria rubella	45	2.94 ± 1.30	3.6 ± .7	17 ± 3.5	3.9 ± .46****
Litoria splendida	6	41.6 ± 11.2	3.0 ± .18	12 ± 2.6	1.3 ± .21****
Litoria tornieri	19	2.66 ± .67	6.7 ± 1.6	5.4 ± 2.3	1.9 ± .84****
Litoria wotjulumensis	23	8.99 ± 2.55	4.4 ± .52	12 ± 2.2	2.8 ± .59****
Bufonidae:					
Bufo marinus	7	40.3 ± 9.10	5.6 ± .4	3.4 ± .33	1.8 ± .59***
Microhylidae:					
Austrochaperina adelphe	12	.39 ± .10	14.9 ± 4.0	3.1 ± 1.3	3.0 ± .73
Myobatrachidae:					
Limnodynastes convexiusculus	10	11.7 ± 3.53	6.6 ± .6	5.6 ± .81	2.8 ± .48***
Limnodynastes ornatus	12	6.49 ± 1.03	6.6 ± .7	7.2 ± 1.3	4.0 ± .47****
Notaden melanoscaphus	6	16.5 ± 1.65	6.5 ± .6	4.9 ± .70	2.8 ± .092***
Crinia bilingua	12	.30 ± .04	13.1 ± 1.9	2.9 ± .67	2.9 ± .43
Uperoleia sp. nov.	12	1.01 ± .28	8.9 ± 1.4	4.1 ± 1.1	4.1 ± .54
Uperoleia lithomoda	10	1.27 ± .25	9.2 ± 1.5	2.9 ± 1.2	3.2 ± .95

Note. Values are expressed as mean ± SD. *N* represents the number of individuals (1 individual = 1 trace) used to calculate the mean values. Asterisks signify the level of significance of differences between the total resistance for the live animal versus the agar model.

** $P < 0.01$.

*** $P < 0.001$.

**** $P < 0.0001$.

al. 1992). PCR conditions were as follows: one cycle 94°C 3 min, 55°C 45 s, 72°C 1 min; 29 cycles 94°C 45 s, 55°C 45 s, 72°C 1 min. PCR products were purified for sequencing using a Bresa-Clean DNA Purification Kit (Bresatec). Each sample had both strands cycle sequenced directly from the PCR product with the original PCR primers using the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems). Sequence product was electrophoresed and viewed on an Applied Biosystems Model 373A sequencing system.

GenBank accession numbers for all sequences included in this study are AY326037/39, AY326043-7, AF136316, DQ116830–DQ116853, and DQ116854–DQ116876. Sequence alignments were made by eye using the conserved motif (Hickson et al. 1996) and secondary structure (Kjer 1995, 1997) approaches to align stems and loops according to the latest secondary structure models for RNA secondary structure. Regions of doubtful homology were discarded.

Phylogenetic analyses of the combined data were performed with Bayesian inference maximum likelihood using MrBayes version 3.0 (Huelsenbeck and Ronquist 2001). Modeltest version 3.0 (Posada and Crandall 1998) was used to assess the most suitable model of nucleotide substitution for the data by the AIC. The model parameters were not specified a priori and were treated as unknown variables with uniform priors. Bayesian analyses were run for 5,000,000 generations, saving trees from every 100 generations. Four simultaneous Markov chain Monte Carlo chains were run with the temperature of the heated chains set at the default of 0.2. The likelihoods of trees were inspected to determine whether the Markov chains had reached stationarity, that is, relatively stable likelihood scores

Table 3: Wet and dry season mass and cutaneous resistance (R_c) for hylid species commonly found during both periods

	Wet			Dry		
Species	N	Mass (g)	R_c (s cm^{-1})	N	Mass (g)	R_c (s cm^{-1})
Litoria bicolor	16	.71 ± .12	47.6 ± 22.4	15	.52 ± .12	79.7 ± 67.6^{NS}
Litoria caerulea	12	40.09 ± 15.85	13.2 ± 3.4	16	19.24 ± 12.65	15.2 ± 5.3^{NS}
Litoria dahlii	11	19.57 ± 8.79	2.3 ± 2.1	13	11.69 ± 7.64	2.4 ± 1.9^{NS}
Litoria nasuta	10	5.28 ± .89	4.4 ± 1.4	8	3.41 ± 1.53	4.9 ± 1.6^{NS}
Litoria pallida	7	2.95 ± .37	5.4 ± 1.4	10	3.07 ± 1.28	6.55 ± 3.0^{NS}
Litoria rothii	21	5.58 ± .75	15.8 ± 4.1	26	3.38 ± .92	16.4 ± 5.1^{NS}
Litoria rubella	13	1.94 ± .68	10.5 ± 2.8	29	3.38 ± 1.27	14.2 ± 3.2^{NS}
Litoria tornieri[a]	17	2.62 ± .70	3.5 ± 2.3	2	2.97 ± .10	3.9 ± 4.4
Litoria wotjulumensis	13	8.10 ± 4.21	7.9 ± 3.4	20	9.09 ± 2.17	8.6 ± 2.5^{NS}

Note. Values are expressed as mean ± SD. N indicates the number of individual traces used to calculate the mean mass and R_c for each season. NS = not significant.

[a] Statistical analysis not completed for *L. tornieri* because of low dry season sample size.

over time. Sample points generated before stationarity was reached were discarded as "burn-in" samples and were not considered in calculation of a posteriori node probabilities or parameter estimates. To ensure that Bayesian analyses were not trapped in local optima, analyses were performed four times, with each analysis starting from a random tree. Apparent stationarity levels were compared for convergence, which was considered to have occurred when likelihood values from independent Bayesian analyses had similar mean values. In addition, the posterior probabilities of nodes from independent analyses were compared for convergence. After verifying convergence and discarding burn-in samples, the remaining samples were pooled for summary analysis. The percentage of samples recovering a particular clade, determined from 50% majority rule consensus trees, represents the posterior probability of the clade. Because the posterior probabilities represent true P values, clades with P values $\geq$ 95% were considered significantly supported.

Statistics

Comparisons of log-transformed data of water loss rates and cutaneous resistance within and among species were made using ANCOVA on whole-animal data with mass as a covariate and one-way ANOVA. Post hoc multiple comparisons (Fisher's protected LSD) were made between all pairs. These tests were performed with SuperANOVA for Macintosh computers.

To correct for phylogeny, we analyzed data from 24 species of hylids obtained in this study and from the literature (Buttemer 1990; Amey and Grigg 1995; Withers and Richards 1995; Buttemer et al. 1996; Buttemer and Thomas 2003). The relationship between size (SVL) and resistance in hylids was analyzed with independent contrasts (Felsenstein 1985; Garland et al. 1992) using the PDTREE module in PDAP (Garland et al. 1993; Garland and Ives 2000). Branch lengths obtained from the Bayesian phylogenetic inference were used to standardise parameters (Felsenstein 1985). To determine whether there was a phylogenetic signal in values of cutaneous resistance of hylids, we calculated the K statistic for log SVL and log R_c using PHYSIG.M (Blomberg et al. 2003). For species with no resistance, we substituted a value of $R_c = 0.1$ into the analysis in order to allow logarithmic transformation.

Results

Agar Model Comparisons

Total resistance (R_t) to EWL of live frogs was not significantly different from that of similarly sized and shaped agar models for *Crinia bilingua*, the *Uperoleia* species, *Litoria microbelos*, *Litoria meiriana*, and *Austrochaperina adelphe*. R_t was significantly different between live animals and agar models for all other species ($P < 0.01$–0.0001; Table 2).

EWL Comparisons

Wet versus Dry Season. Table 3 compares EWL between individuals collected in the wet and dry seasons. There was no significant difference in EWL between seasons in *Litoria caerulea*, *Litoria dahlii*, *Litoria nasuta*, *Litoria pallida*, *Litoria rothii*, *Litoria rubella*, and *Litoria wotjulumensis*, and mass was not a significant covariate. For *Litoria bicolor*, there were no significant seasonal differences; however, mass was different between seasons and was a significant covariate (Table 3). A regression analysis of resistance versus mass for *L. bicolor* showed a significant relationship, with smaller animals having a higher resistance to water loss ($r^2 = 0.258$, $P < 0.001$).

Across Species. Mass was not a significant covariate when cu-

Table 4: Assignment of species to resistance categories based on statistical comparisons of cutaneous resistance values

Species	R_c (s cm^{-1})	T_{skin} (°C)[a]	Groups	Statistical Groupings[b]
Litoria bicolor	63.1 ± 51.5	24.2 ± 1.27	High	A
Litoria rothii	16.2 ± 4.7	22.6 ± .80	Moderate	B
Litoria caerulea	14.3 ± 4.6	22.3 ± 1.68	Moderate	B
Litoria rubella	13.1 ± 3.5	21.6 ± 1.21	Moderate	BC
Litoria gilleni	11.5 ± 3.2	21.0 ± .64	Moderate	BC
Litoria splendida	10.2 ± 2.6	22.0 ± .27	Moderate	C
Litoria coplandi	9.6 ± 2.6	19.8 ± 1.32	Moderate	C
Litoria wotjulumensis	8.3 ± 2.9	21.3 ± 1.35	Moderate	C
Litoria pallida	6.1 ± 2.5	20.8 ± .88	Low	D
Litoria tornieri	5.5 ± 1.3	22.3 ± 1.11	Low	D
Cyclorana australis	5.4 ± 1.6	23.0 ± 1.27	Low	D
Litoria nasuta	4.7 ± 1.5	21.3 ± .63	Low	DE
Cyclorana longipes	3.5 ± 1.4	20.3 ± 1.09	Low	EF
Limnodynastes ornatus	3.1 ± 1.3	21.0 ± .79	Low	FG
Limnodynastes convexiusculus	2.7 ± 1.0	20.2 ± 1.36	Low	FG
Litoria dahlii	2.3 ± 2.0	20.7 ± .82	Low	G
Notaden melanoscaphus	2.0 ± .7	20.8 ± .45	Low	GH
Bufo marinus	1.7 ± .7	20.9 ± .41	Low	HI
Litoria inermis	1.4 ± 1.1	22.1 ± 1.78	Low	HI
Litoria meiriana	.6 ± .7	22.1 ± 2.16	Typical[c]	IJ
Austrochaperina adelphe	.1 ± .7	20.6 ± 1.71	Typical[c]	J
Crinia bilingua	.1 ± .5	19.6 ± .98	Typical[c]	JK
Litoria microbelos	−.1 ± .8	20.9 ± 1.06	Typical[c]	JK
Uperoleia sp. nov.	−.1 ± .9	20.8 ± 1.00	Typical[c]	K
Uperoleia lithomoda	−.4 ± .7	19.5 ± .88	Typical[c]	K

Note. Values represent mean ± SD.

[a] Mean chamber air temperature was 24.1° ± 1.11°C.

[b] $P < 0.05$; letters indicate groups are not significantly different on the basis of ANOVA of log R_c followed by a Fisher's protected LSD.

[c] The "typical" group includes those species whose total resistance was not statistically different from that of the representative agar model.

taneous resistance was compared across all the species, but after mass was removed from the model, there were significant differences across species ($F_{24,386} = 74.8659$, $P < 0.0001$). Post hoc comparisons (Fisher's protected LSD) and a consideration of statistical differences between the total resistance of the live animals and the agar models (Table 2) revealed four groupings, with one group (high) comprising a single species, *L. bicolor*, having a significantly higher cutaneous resistance than the other three groups (moderate, low, typical; Table 4).

Litoria rubella *along the North-South Transect.* The range of ambient air temperature of the air-conditioned workstations where observations were taken was 19.0°–26.1°C. The measurements for animals from Renner Springs were all taken at the low end of this range (19.0°–22.1°C). The incurrent air temperature was not found to be a significant cofactor by ANCOVA where the incurrent air temperatures was the covariate ($F_{1,23} = 0.195$, $P = 0.6626$). Cutaneous resistance to water loss in *L. rubella* from three different sites along the north-south transect was not significantly different (Alice Springs mean EWL = 13.8 ± 4.24 s cm^{-1}; Renner Springs mean EWL = 14.9 ± 1.67 s cm^{-1}; Darwin mean EWL = 14.04 ± 3.08 s cm^{-1}; $F_{2,26} = 0.293$, $P = 0.75$).

Chamber Position Analysis. An a posteriori statistical analysis was completed in order to investigate whether there was an influence of end of chamber position (EC; incurrent end vs. excurrent end) and body position (BP) relative to airflow on the results for cutaneous resistance. For *L. bicolor*, *L. rothii*, and *L. rubella*, there were enough individual traces available for this analysis. A two-way ANOVA comparing cutaneous resistance for EC position (flow, middle, sensor) and BP relative to airflow (facing, facing away, or perpendicular) was not significant for either EC or BP (Table 5).

Chamber Behaviour and Posture. The level of activity in the chamber varied across the species. Anecdotally, the trend was for species with high and intermediate resistances (Table 4) and

Table 5: Two-way ANOVA of the effects of the frog body position (EC) and orientation (BP) in the chamber on R_c

	EC			BP			Interaction EC × BP		
Species	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
Litoria bicolor	.441	2, 37	.6466	.783	5, 37	.5862	2.274	3, 37	.0961
Litoria rothii	.275	2, 39	.07614	.1363	3, 39	.2683	2.810	2, 39	.0724
Litoria rubella	.980	2, 33	.3861	.635	2, 33	.5363	1.381	4, 33	.2618

Note. Neither the main effects nor their interaction was significant at $P \leq 0.05$.

species with an SVL > 40 mm to settle into a quiescent posture within minutes of being placed in the chamber. The smaller species (SVL < 40 mm) with low or no (typical) resistance (Table 4) remained very active in the chamber for 10–30 min before settling into a posture. Four postures were identified; WCP (Heatwole et al. 1969), modified, chin bulge, and low flat. WCP has been described previously as the ventral surfaces of the body concealed and forming a seal to the perching substrate, the limbs folded underneath, and the chin flattened to the resting surface. All arboreal species of hylids and *Litoria coplandi*, *Cyclorana australis*, and *Limnodynastes convexiusculus* were consistently observed in the WCP during EWL trials. A modified WCP was observed in the terrestrial hylids, in which the position of the limbs differed. The length and robustness of the hind limbs seemed to inhibit the animal's ability to fold the ventral surface of the hind limbs tightly underneath. The forelimb position also varies, with the "elbow" joint resting on the anterior of the "knee" bend of the hind limb rather than being folded underneath. Two species, *Limnodynastes ornatus* and *Notaden melanoscaphus*, were observed to use a chin bulge posture, where the ventral surfaces of the body and limbs are folded under but, because of the rotundness of the animals, the ventral surface of the chin is not pressed to the resting surface. All other species would be in a low flat posture, where they would press their ventral surfaces down onto the bottom of the chamber, but limbs were not folded underneath as described for WCP.

Phylogenetic Relationships and Comparative Analysis of Hylids. Phylogenetic analysis of the mitochondrial ribosomal RNA nucleotide sequences of the 24 hylid taxa revealed two main clades: one (clade A) comprising the arboreal green tree frogs of the *L. caerulea* and *Litoria chloris* species groups and the second (clade B) comprising the remaining *Litoria* and *Cyclorana* (Fig. 1). Within clade A, monophyly of the *L. caerulea* and *L. chloris* species groups is well supported. Within clade B, there are two well-supported clades, one comprising *Cyclorana*, *Litoria alboguttata*, and members of the *Litoria aurea* species group and the second comprising a collection of *Litoria* with a wide range of ecological habits. Within the first of these clades, there is strong support for the paraphyly of *Cyclorana* with the *L. aurea* species group. Members of the clade are either terrestrial burrowers (*Cyclorana*, *L. alboguttata*) or aquatic (*L. aurea* species group). Within the second clade, the two small terrestrial species *L. meiriana* and *L. microbelos* are monophyletic and the sister lineage to the remaining taxa. The terrestrial ground hylids form a single well-supported clade with two major well-supported subclades: *L. coplandi–L. wotjulumensis* and *Litoria tornieri–L. nasuta–Litoria inermis–L. pallida*. The arboreal *L. bicolor* species group is monophyletic with strong support and is sister to the ground hylids. There is strong support for monophyly of the arboreal *Litoria peroni* species group, which forms a clade with the widespread arboreal species, *L. rubella*, which are together the sister to the ground hylid–*L. bicolor* species group clade. On the basis of this tree topology, ecological habit is polyphyletic within the Australian hylids.

K statistic analysis for phylogenetic signal across the group was significant for both body size (log SVL: $K = 1.315$, $P < 0.001$, $N = 24$) and cutaneous resistance to water across (log R_c: $K = 1.096$, $P < 0.001$, $N = 24$). However, body size and resistance to water loss were not significantly correlated in hylids, using phylogenetically independent contrasts (Pearson product moment correlation $= -0.17$, $P > 0.05$).

Discussion

Of the 25 species for which EWL and resistance were measured in this study, only six species would be considered "typical" on the basis of comparisons of total resistance between live animals and agar models. Two of these six species are the smallest of the hylids: *Litoria meiriana* and *Litoria microbelos*. The remaining species should be considered "atypical, " with mean cutaneous resistance ranging from 1.5 to 63 s cm^{-1}. Within the atypical species, three groupings were observed: low, moderate, and high. Size within (with the exception of *Litoria bicolor*) and among species was not a significant covariate to the ability to resist EWL. This confirms the suggestion of Buttemer and Thomas (2003) that in the *Litoria*, there is no consistent relationship between body size and R_c. Resistance to EWL was indicative of the ecological habit of a species, with arboreal species having higher resistances than nonarboreal species, which is consistent with previous work (Tables 1, 4; Wygoda 1984). Interestingly, the majority of nonarboreal species showed some resistance to cutaneous EWL. This contrasts with prior

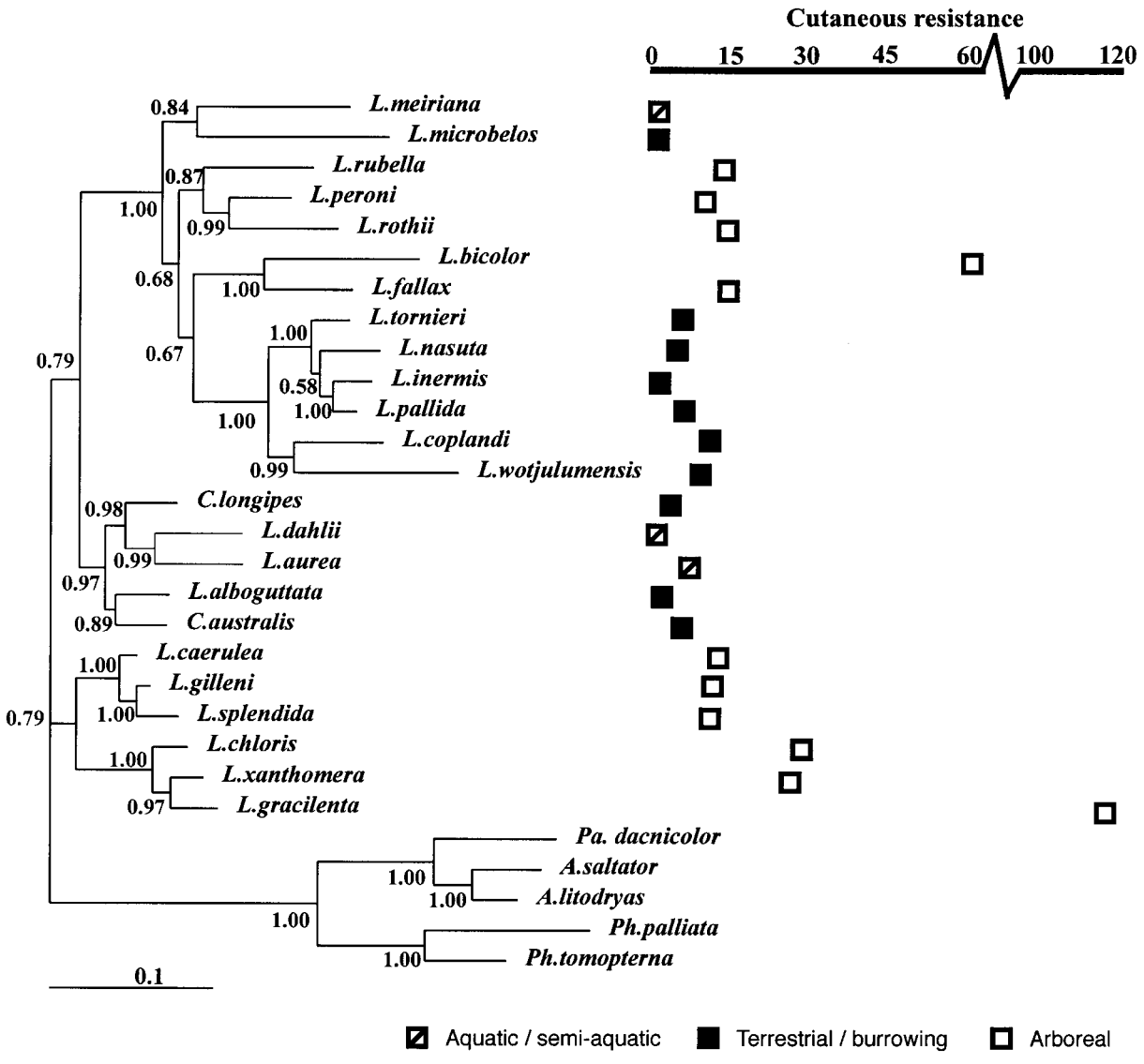

Figure 1. Phylogenetic relationships and mean values of cutaneous resistance (R_c; Table 4) for 24 species of Australian hylids used in comparative analyses. Branch lengths are proportional to substitutions per site (scale bar at bottom of figure) estimated with the GTR model of nucleotide substitution with corrections for proportion of invariant sites and among site rate heterogeneity. Branch support is indicated by posterior probabilities (see text). The outgroups comprise the phyllomedusines *Agalychnis litodryas*, *Agalychnis saltator*, *Pachymedusa dacnicolor*, *Phyllomedusa palliata*, and *Phyllomedusa tomopterna*.

studies of nonarboreal anurans that found no cutaneous resistance to water loss in ranid frogs and bufonids (Spotila and Berman 1976; Heatwole 1984; Wygoda 1984, 1988).

The range of resistance values found across the hylid species from this investigation (0.3–63.1 s cm^{-1}) is consistent with other studies of Australian hylids (Withers et al. 1984; Buttemer 1990; Amey and Grigg 1995; Withers 1995; Buttemer et al. 1996; Withers and Richards 1996). In particular, values for EWL and cutaneous resistance for *Litoria caerulea* and *Cyclorana australis* from this study were similar to those values found by Christian and Parry (1997), who used the same wind speed but larger chambers. Other studies have indicated that seasonal variation in cutaneous resistance may occur. In this study, while mean resistance values were consistently higher during the dry season for all species, they were not significantly different from wet season values.

Behavioural mechanisms are important to the measure of EWL, since those species utilising a WCP tended to have the higher resistance to cutaneous water loss. The interaction between posture and cutaneous resistance is important for all the "atypical" species in this study. If animals shifted in the chamber or did not settle into a WCP, the relative humidity values re-

corded in the chambers were observed to be twice as high as for animals in a WCP.

Cocoon formation is known to significantly reduce cutaneous EWL in *C. australis* as well as in other species of *Cyclorana* and in species of *Neobatrachus* (Christian and Parry 1997; Withers 1998; Withers and Thompson 2000). One individual of *Litoria dahlii*, which had been held in a metabolic chamber for 2 mo, was found to have a mucus covering over it and was measured for EWL with the mucus covering intact to investigate whether this layer provided a similar benefit. The measured value ($R_c = 2.0$ s cm^{-1}) was within the range of all other *L. dahlii* measured; therefore, it was concluded that the mucus covering provided no extra resistance to EWL.

Groups of hylid species showed similarities in resistance to EWL and in size, and this was evident by significant *K* statistics for both. For example, the green tree frog group of *Litoria caerulea*, *Litoria gilleni*, and *Litoria splendida* all showed moderate resistance (Fig. 1). However, body mass and resistance were not significantly correlated either with standard statistics or with phylogenetically independent contrasts. Resistance was also clearly polyphyletic, showing high levels in several clades (Fig. 1). However, resistance did show a good correspondence with ecological habit across the tree topology. This link is apparent when both R_c and ecological habit (arboreal vs. nonarboreal) are plotted on the phylogenetic tree (Fig. 1). Thus, despite strong evidence that phylogenetic history is important for the distribution of resistance on the topology, it is likely that the evolution of resistance is coupled with ecology of the species rather than simply being a historical artefact carried across ecological habits.

This study represents the first comprehensive examination of cutaneous resistance to water loss in a group of species from the same region and using the same techniques with the benefit of a comparative analysis framework. It provides the strongest evidence yet of a link between ecological habits and water loss rates. Furthermore, information on the range of resistances and the relationships with habit of these species will allow future studies to explore the underlying physiological or chemical mechanisms resulting in cutaneous resistance as well as the effects of cutaneous resistance on other ecological variables such as seasonal and daily activity patterns.

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