

Cocoon formation by the treefrog *Litoria alboguttata* (Amphibia: Hylidae): A 'waterproof' taxonomic tool?

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Abstract

The hylid frog *Litoria alboguttata* (formerly *Cyclorana alboguttatus*) forms a cocoon during aestivation. After 21 days of water deprivation a thin, transparent cocoon is formed; the cocoon covers and closely adheres to the entire body surface, except the external nares. The cocoon consisted after 21 days of about 24 layers of squamous epithelial cells, about 14-18 μ thick. It reduced markedly the rate of evaporative water loss (measured at 22 °C) from 39.3 mg g⁻¹ h⁻¹ (non-cocooned frogs) to 2.1 mg g⁻¹ h⁻¹ (cocooned frogs). The formation of a cocoon by *Litoria alboguttata* raises the question of the possible phylogenetic significance of cocoon formation amongst the Australopapuan frogs, because no other *Litoria* has yet been reported to form a cocoon but cocoon formation is common amongst both *Cyclorana* and *Neobatrachus*. Cocoon formation may have independently arisen at least three times amongst Australopapuan frogs (*Litoria*, *Cyclorana*, *Neobatrachus*). Alternatively, *Litoria alboguttata* may be more closely allied with *Cyclorana* than *Litoria*, or cocoon formation was a primitive capability of the frogs ancestral to both *Litoria* and *Cyclorana*, and so cocoon formation independently evolved only twice in Australopapuan frogs.

Introduction

This study was prompted by the report of Lee & Mercer (1967) that *Litoria alboguttata* (called *Cyclorana alboguttatus* by them) form a cocoon; they also reported that *Cyclorana platycephala*, *C. australis*, *Neobatrachus pictus* and *Limnodynastes spenceri* form a cocoon. The cocoon of these frogs, and of a number of other non-Australian frogs (*Pyxicephalus*, *Leptopelis*, *Lepidobatrachus*, *Ceratophrys*, *Pternohyla*, *Smiliscus*) is a multi-layered covering of sloughed skin formed during aestivation; it markedly reduces evaporative water loss (Loveridge & Crayé 1979; McClanahan *et al.* 1976; Ruibal & Hillman 1981; McDiarmid & Foster 1987). Since the pioneering study of Lee & Mercer (1967), cocoon formation has been described for a variety of species of *Cyclorana* (van Beurden 1982; Withers 1995; Richards, unpublished observations) and *Neobatrachus* (Withers 1995), but cocoon formation has not been observed for *Limnodynastes spenceri* (Withers, unpublished observations) and there have been no further studies of cocoon formation by *Litoria alboguttata*.

Cocoon formation by *Litoria alboguttata* is of particular interest because the taxonomic position of *Litoria alboguttata* within the monophyletic *Cyclorana* or *Litoria aurea* species group is problematical (Tyler & Davies 1993). The Australopapuan hylid frogs (Hylidae, Pelodyadinae: *Cyclorana*, *Litoria* and *Nyctimystes*) appear to be a monophyletic group (Tyler 1979; Hutchinson & Maxson 1987; Tyler & Davies 1978, 1993), but only relatively few of the species have been considered in phylogenetic analyses within the taxon. *Litoria alboguttata* was described as *Chiroleptes alboguttatus* Gunther (1867); it was moved to *Mitrolysis* by Cope

(1889) and then to *Cyclorana* by Parker (1940). Tyler (1973) considered it to be a species of *Litoria* and a member of the *Litoria aurea* complex, as *L. alboguttata*.

The objectives of this study were to confirm that *Litoria alboguttata* formed a cocoon during aestivation, to examine the structure of the cocoon, and to measure the rate of evaporative water loss of normal and cocooned frogs.

Methods

Twelve specimens of *Litoria alboguttata* were collected at Townsville, Queensland, and transported to Perth for study (two frogs are deposited in the WA Museum as voucher specimens R119533, R119534). Initially, the frogs were maintained individually, in the dark, with access to free water, in plastic containers that had a small hole in the lid for gas exchange. After initial measurements were made for hydrated frogs, no free water was provided and the frogs were slowly dehydrated to induce cocoon formation. Frogs were maintained, and all experiments conducted, at room temperature (approx. 22 °C).

The rate of evaporative water loss (EWL) was determined by flow-through hygrometry. EWL was first measured at the start of the study for control, hydrated frogs, and then at the end of 21 days of dehydration for cocooned, aestivating frogs. Each frog was weighed to \pm 0.001 g and then placed in a vertically-oriented glass tube (5 cm diameter) on a plastic mesh platform. Compressed dry air (dewpoint = -10 °C) was passed at a flow rate of 2000 ml min⁻¹ through the tube and then a General Eastern model 1100A dewpoint hygrometer. The analog voltage output of the hygrometer was monitored by a Thurlby 1905a digital multimeter, and its RS232 output was interfaced to a PC. The excurrent

dewpoint was monitored at 30 sec intervals, and converted to relative and absolute humidity using the equations of Parrish & Putnam (1977). The absolute evaporative water loss rate (EWL; mg min^{-1}) and mass-specific evaporative water loss (MSEWL; $\text{mg g}^{-1} \text{h}^{-1}$) were calculated from the air flow rate, incurrent and excurrent absolute humidity, and body mass. The surface-area-specific evaporative water loss (SAEWL; $\text{mg cm}^{-2} \text{h}^{-1}$) was calculated assuming a surface area (cm^2) of 9.9 $\text{grams}^{0.567}$ (McClanahan & Baldwin 1969). Total resistance to evaporative water loss ($R = \Delta C / \text{SAEWL}$; sec cm^{-1}) was calculated from SAEWL (converted to $\mu\text{g cm}^{-2} \text{sec}^{-1}$), assuming the difference in water vapor concentration (ΔC ; $\mu\text{g cm}^{-3}$) driving evaporation was the difference between absolute humidity for saturated air at 22 °C and the incurrent absolute humidity.

Samples of cocoon were removed from aestivating frogs, and examined by scanning and transmission electron microscopy. For scanning electron microscopy, air-dried samples and glutaraldehyde-fixed samples of skin were mounted on an aluminium stub using double-sided adhesive tape, and sputter-coated with gold-palladium. The thickness of the cocoon was determined using the air-dried specimens, and the number of layers counted using the glutaraldehyde-fixed specimens. Specimens were examined using a Phillips 505 scanning electron microscope.

Samples of air-dry cocoon were prepared for transmission electron microscopy by fume fixation with osmium tetroxide and direct embedding in araldite. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Specimens were examined using a JOEL FX2000 transmission electron microscope.

All experiments were conducted with the approval of the Animal Ethics Committee, University of Western Australia.

Results

All frogs were hydrated and healthy at the start of the study, when EWL was determined for all individuals, and then were water-deprived. Most specimens of

Litoria alboguttata became quiescent, adopted the water-conserving posture, and commenced cocoon formation (Fig 1) within 7 days of the start of water deprivation. A well-developed, transparent cocoon was apparent after 21 days, when EWL was redetermined and the cocoon was removed for microscopical examination. At this time the cocoon was a thin, transparent sheet that covered and closely adhered to the entire body surface, including the closed eyes, mouth and cloaca. Only the external nares were free of the cocoon, to allow pulmonary ventilation. The cocoon was easily peeled from the skin; the freshly-exposed skin appeared and felt moist.

The piece of cocoon examined by scanning electron microscopy consisted of about 24 discrete layers (Fig 2A) forming a compact sheet (Fig 2B) approximately 14-18 μ thick; each layer is consequently calculated to be about 0.6-0.7 μ thick. A transmission electron micrograph of the cocoon (Fig 3) more clearly shows the individual electron-dense cell layers, about 0.7 to 1.0 μ thick, separated by 0.1 to 0.2 μ thick inter-cellular spaces. The outer surface of the cells is more crenulated than the inner surface. Inter-cellular junctions are evident in some of the layers.

The mean body mass of *L. alboguttata* was $20.6 \pm \text{se}$ 0.7 grams ($n=9$). The rate of evaporative water loss declined markedly from 13.4 mg min^{-1} for non-cocooned *L. alboguttata* ($39.3 \text{ mg g}^{-1} \text{h}^{-1}$; $4.07 \text{ mg cm}^{-2} \text{h}^{-1}$) to 0.74 mg min^{-1} for cocooned frogs ($2.1 \text{ mg g}^{-1} \text{h}^{-1}$, $0.22 \text{ mg cm}^{-1} \text{h}^{-1}$; Table 1). The resistance was considerably higher for cocooned frogs (89.4 sec cm^{-1}) than non-cocooned frogs (3.1 sec cm^{-1} ; Table 1).

Table 1. Evaporative water loss for hydrated and cocooned *Litoria alboguttata*. Values are mean \pm standard error; n is the sample size. All values for cocooned frogs are significantly different from the values for control frogs, by t-test ($P < 0.05$).

	Hydrated (n=9)	Cocooned (n=6)
Absolute EWL (mg min^{-1})	13.4 ± 0.6	0.74 ± 0.12
Mass-specific EWL ($\text{mg g}^{-1} \text{h}^{-1}$)	39.3 ± 2.0	2.1 ± 0.3
Surface-area-specific EWL ($\text{mg g}^{-1} \text{h}^{-1}$)	4.1 ± 0.2	0.22 ± 0.03
Resistance (sec cm^{-1})	3.1 ± 0.3	89.4 ± 14.9

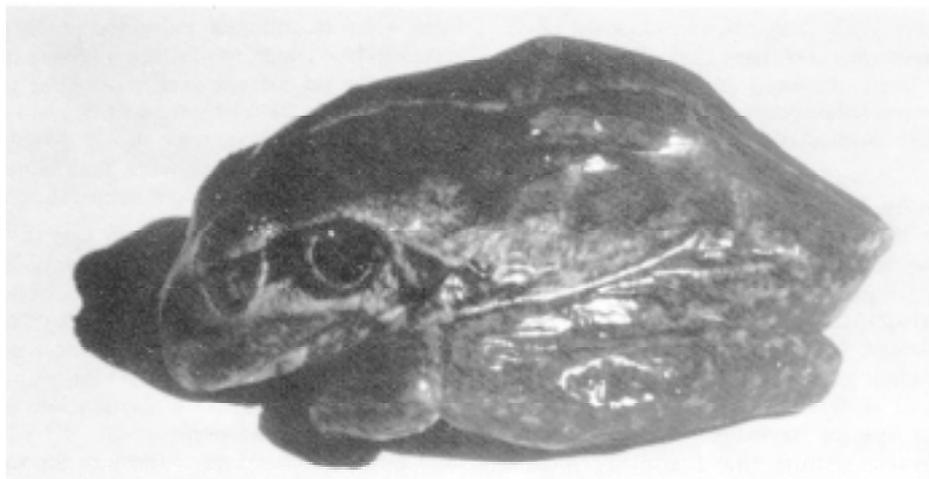


Figure 1. An aestivating *Litoria alboguttata* in the water-conserving posture and covered with a transparent cocoon that covers the entire outer body surface (including eyes, mouth and cloaca) except for the openings of the nares.

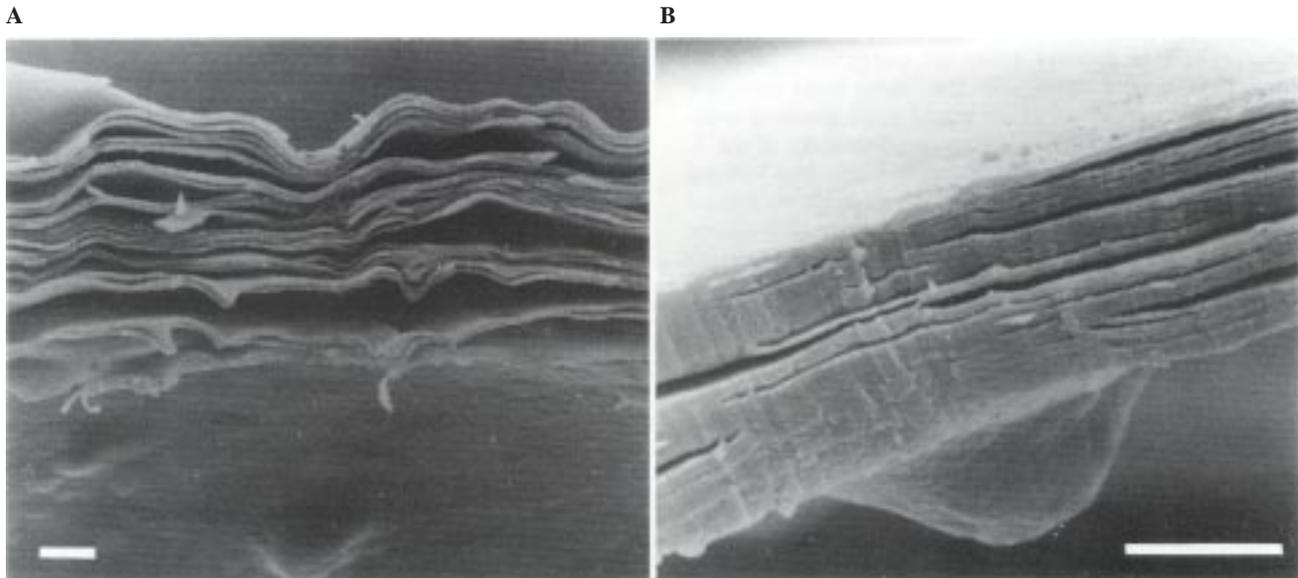


Figure 2. Scanning electronmicrographs of the cocoon of *Litoria alboguttata*. **A.** glutaraldehyde-fixed specimen showing individual layers **B.** an air-dried specimen showing the compact *in vivo* structure of the cocoon. Scale bars are 10 μ .

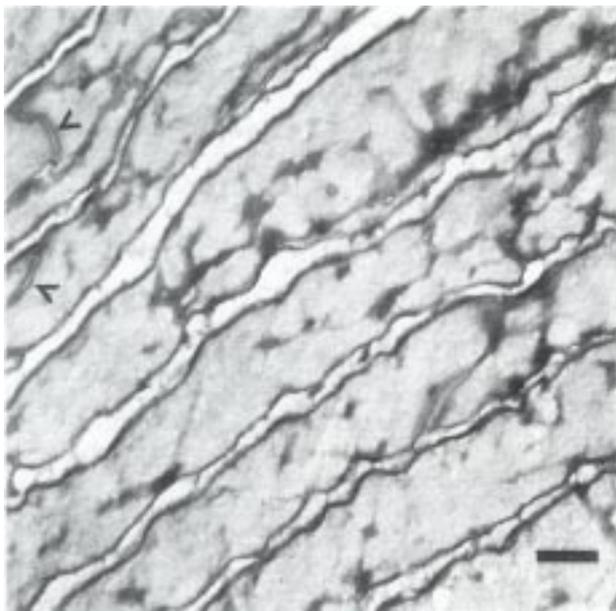


Figure 3. Transmission electronmicrograph of an air-dried specimen of *Litoria alboguttata* cocoon, showing detail of squamous cells and intercellular space. Imbricate intercellular junctions are indicated by arrow heads. Scale bar is 500 μ m.

Discussion

It is clear from Lee & Mercer (1967) and this study that *Litoria alboguttata* forms a cocoon during aestivation; however, cocoon formation has not yet been described for any other species of *Litoria*. The general appearance and structure of the cocoon of *L. alboguttata* is similar to that of other Australian cocoon-forming frogs (*Neobatrachus* and *Cyclorana*; Withers 1995), and also *Pyxicephalus adspersus* (Parry & Cavill 1978; Loveridge & Crayé 1979), *Lepidobatrachus llanensis* (McClanahan *et al.* 1976), *Pternohyla fodiens* (Ruibal & Hillman 1981) and

Smilisca baudinii (McDiarmid & Foster 1987). The thickness of individual layers of the cocoon of *L. alboguttata*, 0.6-0.7 μ , is more similar to that of *Neobatrachus* spp (0.57-0.62 μ) than *Cyclorana maini* (0.39 μ ; Withers 1995) but the rapid rate of cocoon formation for *L. alboguttata* (at least 1.1 layers d^{-1}) is more similar to that of *C. maini* (0.57 d^{-1}) than *Neobatrachus* spp (0.22-0.35 d^{-1} ; Withers 1995).

The cocoon of *Litoria alboguttata* significantly reduces its rate of evaporative water loss, as has been observed for other cocoon-forming *Cyclorana* spp, *Neobatrachus* spp, and other genera. The resistance to water loss (3.1 $sec\ cm^{-1}$) of non-cocooned *L. alboguttata* is slightly higher than that for typical 'non-waterproof' frogs and a free water surface (about 1 $sec\ cm^{-1}$). This might reflect an underestimation of evaporative surface area, a substantial resistance of the cutaneous boundary layer, or initial stages of cocoon-formation in some of the "non-cocooned" individuals (resistance ranged for non-cocooned individuals from 2.4 to 4.9 $sec\ cm^{-1}$). In contrast, the resistance to water loss of cocooned frogs was much higher, at about 90 $sec\ cm^{-1}$; such resistance values are typical for other species of cocooned frogs (Loveridge & Withers 1981; Withers, unpublished observations for *Cyclorana* and *Neobatrachus* spp).

Cocoon formation has presumably evolved independently at least three times in the Australopapuan frogs *i.e.* in both hylid genera *Cyclorana* and *Litoria* and the myobatrachid genus *Neobatrachus*. If *Litoria alboguttata* is ascribed to the *Cyclorana australis* species group as suggested by Maxson *et al.* (1982, 1985), then cocoon formation need only have evolved independently twice in Australopapuan frogs, once in *Cyclorana* (there is at least one cocoon-forming frog in each of the three *Cyclorana* species groups listed by Tyler & Davies 1993), and once in *Neobatrachus*. Or, if cocoon formation was a primitive capability of the common ancestor of both *Litoria* and *Cyclorana*, but has been subsequently lost in most *Litoria* spp, then cocoon formation need only have

evolved twice in Australopapuan frogs. However, the analyses of Maxson *et al.* (1982, 1985) suggest that *C. platycephala* is remote from its congeners (and *L. alboguttata*) and is allied with the *Litoria aurea* complex; if so, then cocoon-formation might have evolved independently in *C. platycephala* (or other members of the *L. aurea* complex also form cocoons but this has not yet been recorded, or they have lost the capacity to form a cocoon). The ability to form a cocoon, although it cannot be considered to be a taxonomic tool *per se*, provides suggestive evidence of a close phylogenetic relationship between *Cyclorana* and *Litoria alboguttata*, and it will be of interest to see if such a relationship holds when the patterns of cocoon formation and phylogenetic relationships are clearer for Australopapuan frogs.

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