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Postnatal ecdysis establishes the permeability barrier in snake skin: new insights into barrier lipid structures

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Summary

A competent barrier to transepidermal water loss (TEWL) is essential for terrestrial life. In various vertebrates, epidermal water barriers composed of lipids prevent excessive TEWL, which varies inversely with habitat aridity. Little is known, however, about the mechanisms and regulation of permeability relative to natal transition from the 'aqueous' environments of gestation to the 'aerial' environments of terrestrial neonates. We investigated newly hatched California king snakes *Lampropeltis getula* to test the hypothesis that the first ecdysis is important for establishing the barrier to TEWL. We found that skin resistance to TEWL increases twofold following the first postnatal ecdysis, corresponding with a roughly twofold increase in thickness and deposition of lamellar lipids in the mesos layer, the site of the skin permeability barrier in snakes. In addition, novel observations on lipid inclusions within the alpha layer of epidermis suggest that this layer has functional similarities with avian epidermis. It appears that emergence of the integument from embryonic fluids, and its subsequent pan-body replacement following contact with air, are essential for completion of barrier competence in the newborn. These conditions provide a potentially useful model for investigations on the mechanism of barrier formation. We also found that hatchling snakes are transiently endothermic, with skin temperatures elevated by approximately 0.6°C above ambient air temperature during the period of barrier formation. Behaviourally, hatchlings showed a higher tendency to seek humid microenvironments before the first ecdysis than after. The degree of water movement across the integument might explain the switch from reclusive to dispersive behaviours associated with postnatal ecdysis in snakes.

Key words: snake, *Lampropeltis getula*, ecdysis, skin, evaporative water loss, skin resistance, hatchling, epidermal differentiation, lipid, permeability barrier, mesos layer, alpha keratin, endothermy.

Introduction

The invasion of terrestrial habitats by vertebrates was an important evolutionary advance, made possible, in part, by development of a cleidoic egg and the acquisition of resistance to transepidermal water loss (TEWL). The barrier to water loss in most tetrapods is conferred by the outer layers of epidermis, consisting of dead, keratin-filled cells embedded within a lipid matrix that has been likened to 'bricks and mortar' (Elias, 1983; Elias and Menon, 1991). The barrier prevents desiccation, protects the body against infection and poisoning from the environment, and is essential for terrestrial life. As species invaded harsher and drier environments, the skin became an important target of natural selection, yielding qualitative and quantitative variation in the epidermal lipids. Thus, lipids provide the principal water barrier in terrestrial plants, arthropods and vertebrates (Hadley, 1989, 1991).

The water barriers of reptilian skin are of special interest, for several reasons. First, reptiles live successfully in a wide variety of conditions, ranging from aquatic to xeric terrestrial habitats. Second, lepidosaurian species exhibit periodic losses of 'epidermal generations' associated with synchronized patterns of pan-body cellular proliferation and differentiation; these losses are unique and quite distinct from the renewal of epidermis in other vertebrates (Baden and Maderson, 1970; Maderson et al., 1998). Third, numerous studies have demonstrated that reptilian skin is an important pathway for water loss and that rates of TEWL vary inversely with habitat aridity (Dmi'el, 1998; Gans et al., 1968; Lahav and Dmi'el, 1996; Mautz, 1982a,b; Roberts and Lillywhite, 1980). Barrier function in reptiles, as in mammals, appears to be genetically determined. However, the barrier can be rapidly restored following trauma (Maderson et al., 1978), and some species have been shown to exhibit plasticity for enhancing resistance to TEWL under conditions of water stress (Kattan and Lillywhite, 1989; Maderson, 1984). Very little is known,

however, about the properties of integument with respect to the important transition from the aqueous environment of the embryo to the terrestrial environment of the neonate and adult.

The skin of full-term human and rodent newborns possesses a competent permeability barrier at birth, and the timing of barrier formation is close to the disaggregation of periderm and direct epidermal contact with amniotic fluid (Hardman et al., 1999; Kalia et al., 1998; Williams et al., 1998). Comparable data are not available for reptiles, despite their various advantages as models for study (Dhouailly and Maderson, 1984). The purpose of the present investigation was, firstly, to determine whether the first postnatal ecdysis affects TEWL and skin resistance (R_s) in newborn snakes. Secondly, we investigated the hypothesis that changes in TEWL or R_s are related to changes in the mesos layer, which is the site of the permeability barrier in snake epidermis. Finally, we quantified the influence of postnatal ecdysis and associated changes in TEWL and R_s on reclusive behaviors of the newborn snakes.

Materials and methods

Animals

California king snakes *Lampropeltis getula californiae* Blainville, which are oviparous, were obtained immediately at hatching from a local breeder. This species inhabits a range of habitats throughout its natural distribution, including arid and semi-arid conditions. Snakes were kept in plastic shoeboxes containing a wood chip substrate and a small plastic bowl, wherein snakes could hide beneath moist sphagnum moss. Water was provided *ad libitum*. Newly hatched snakes did not eat for 2–3 weeks, presumably due to assimilation of yolk that visibly distended their guts. Thereafter, snakes were fed newborn mice at irregular intervals.

Measurements of evaporative water loss and skin resistance

We measured TEWL and skin temperature, and calculated skin resistance to TEWL in 20 snakes obtained from six different clutches. Measurements were repeated on four consecutive occasions, and each animal served as its own control. The four consecutive trials were: (1) within the first 3 days following hatching; (2) 2–4 days following the first ecdysis; (3) 14–16 days following the first ecdysis; and (4) 4–10 days following the second ecdysis.

For measurement of TEWL, each snake was lightly anesthetized by exposure to halothane vapor within a closed jar in order to induce immobility and apnea. The anesthetized snake was then fully extended and positioned loosely within a cylinder of 3 mm wire mesh. This tube, with snake extended, was then placed within a clear acrylic tube (41 cm × 2.5 cm i.d.) through which room air at ambient temperature was pumped at rates of 41.6–43.6 ml min^{-1} (Fig. 1). Airflow was maintained by an Applied Electrochemistry model R-1 flow control pump and calibrated with a volume meter. Excurrent air was directed through a Sable Instruments RH 100 RH/dewpoint meter before entering the flow pump. The RH/dewpoint meter was calibrated with dry nitrogen and water-saturated air prior to experimental measurements. Air temperatures and skin temperatures of snakes were measured with 30 g copper-constantan thermocouples.

During measurements, ambient air temperature and humidity varied within the range 24.5–25.6°C and 46.2–56.8%, respectively, in different trials, but remained virtually stable during individual trials. Ambient humidity was lower, however, during the fourth measurement (following second ecdysis) when it varied from 38.1–49.8% in different trials. All measurements used in calculations of TEWL were made after the flow-through system had equilibrated to constant excurrent humidity with a snake inside the chamber. Evaporative water loss was calculated from the relationship $TEWL=[\rho_e-\rho_i]\times\dot{V}a$, where ρ is water vapor density of incurrent or excurrent air and $\dot{V}a$ is rate of airflow. In separate experiments we examined mass changes of physical models to incorporate a correction factor for absorption of water vapor by the acrylic tube, using the equation: corrected TEWL=0.81×measured TEWL.

The skin resistance to evaporative water flux was calculated as $R_s=R_t-R_b$, where R_t is the total resistance and R_b is the boundary layer resistance. The total resistance was calculated from the relationship $R_t=[\rho_s-(RH\times\rho_a)]\times TEWL^{-1}$, where ρ_s is the saturated water vapor density at skin temperature, ρ_a is the saturated water vapor density at ambient chamber temperature, and RH is the relative humidity of ambient air (Spotila and Berman, 1976). The boundary layer resistance was determined as above, utilizing measurements of TEWL from 'wet' snakes that evaporated as a free water surface. This separate set of experiments included seven different measurements from either agar models of snakes used in the study, or anesthetized snakes that were wrapped with a single layer of fine, water-saturated tissue paper. Values of R_b were less than 5% of R_t.

To measure skin surface areas of snakes, we carefully wrapped each individual with a layer of thin Parafilm® fitted carefully to head and body contours, while the snake was

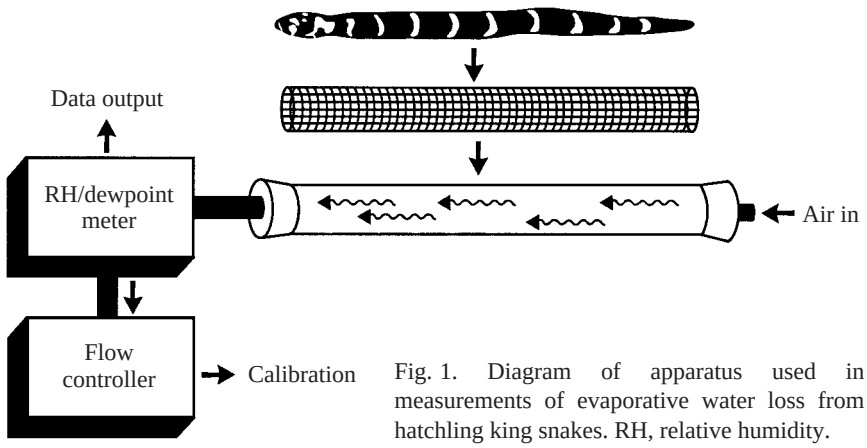

Fig. 1. Diagram of apparatus used in measurements of evaporative water loss from hatchling king snakes. RH, relative humidity.

still anesthetized at the conclusion of TEWL measurement (Lillywhite and SanMartino, 1993). This Parafilm was then removed from the snake and laid out on a piece of paper. This paper was cut to match the area of Parafilm and weighed on a balance to convert mass to area, using mass:area calibrations derived from the same paper.

Ultrastructure and histochemistry

Pre- and post-shed (first ecdysis) skin samples were frozen on dry ice and stored at –70°C until sectioning. These samples were embedded in OCT compound, and 10–12 mm sections were cut on a cryostat maintained at –20°C. Sections were transferred onto slides and stained with Fat Red–7B for neutral lipids, washed in 70% alcohol followed by water, mounted in glycerine jelly, observed and photographed. For routine ultrastructure, freshly obtained skin samples were fixed in Karnovski's fixative for 24 h, washed in sodium cacodylate buffer, osmicated in 1% osmium tetroxide, dehydrated through a graded series of alcohol, and routinely embedded in Epon 12. To demonstrate the barrier lipid structures, skin samples were post-fixed with 0.5% ruthenium tetroxide (RuO_4) instead of osmium tetroxide (OsO_4) for 1 h and then processed as above. With respect to routine histology, semi-thick sections (0.5–1 mm) of OsO_4-fixed samples were stained with Toluidine Blue for light microscopy, while silver gray sections were double-stained with uranyl acetate and lead citrate, then visualized using a Zeiss EM 12 microscope. Silver gray sections from RuO_4-fixed samples were observed with and without double staining to evaluate the lipid structural organization.

Behaviour

We observed the behaviours of snakes used for measurements of TEWL and noted a tendency for individuals to be less reclusive in damp moss following the initial ecdysis. Therefore, we devised an experiment to test whether snakes altered humidity selection following ecdysis. We used a total of 26 newborn snakes in this experiment, different from the ones used for measurement of TEWL.

Each snake was kept individually in a plastic shoebox, as above, provided with wet and dry microenvironments. To create a dry microhabitat, we placed about 4 g of dry sphagnum moss in a plastic cup 11 cm in diameter by 4.5 cm in height. The wet microhabitat was prepared the same way except that 10 ml of water was added to the moss inside a second cup. We allowed ample time for the moisture to distribute and wet the moss evenly, resulting in moss that was moist to the touch. Each of the two cups was positioned at opposite ends of the box, and their relative position was determined randomly in each trial. The humidity and temperature within the box and both cups were checked regularly throughout the experiment. The humidity inside the box was 69–88% (mean=78.6%), and the temperature was 24.4–26.1°C (mean=25.3°C). The humidity (66–85%; mean=76.1%) and temperature (24.3–26.0°C; mean=25.3°C) of dry cups were similar to those within the box, while the wet cups had constantly higher humidity (95.0–98.0%; mean=98%) and a slight tendency to lower temperature (24.0–25.5°C; mean=24.8°C) than either the dry cups or the greater box environment.

At the beginning of the experiment, each snake was placed in the center of the box. The location of each snake was recorded once during the late afternoon, at night, and the following morning. Then, before repeating the test, each box was wiped clean, and two new cups were placed in the box. We tested each hatchling snake for seven days before shedding. After shedding we waited for 1–2 days and then retested the same individuals for another week.

Because the location of each snake in the afternoon, evening and morning was generally the same, we used only the location of each morning observation in the analysis of behaviour. Each snake had seven trials before and after shedding. The probability of a snake staying in the wet or dry cup was computed for each individual.

Stastistics

Data are reported as means ± S.E.M. To evaluate changes in measured variables related to TEWL, we performed a repeated-measures analysis of variance (ANOVA), followed by *post hoc* tests to examine differences between specified trials. In other circumstances, we employed paired *t*-tests, as described elsewhere in the text. Behavioural data for the percentage occurrence of snakes in wet *versus* dry containers were analyzed first using non-parametric Wilcoxon signed-rank tests. All analyses were performed using SAS StatView© 5.0.1 for Windows.

Results

Ecdysis, growth and behaviour

Newborn snakes exhibited dull-appearing skin and tended to be reclusive, usually hiding within the small dish of damp moss provided inside the plastic cages. The first ecdysis occurred 9–13 days following hatching (mean=10.6±0.31 days), with second ecdysis 25–78 days later in different individuals (mean=65.9±3.17 days). During the period from birth to second ecdysis, snakes maintained a stable body mass (initial mass=13.9±0.51 g; final mass=13.4±0.58 g) while increasing in length from 32.6±0.40 mm to 35.9±0.58 mm. The pattern of length changes is illustrated in Fig. 2. Repeated-measures ANOVA followed by Bonferroni *post hoc* tests indicated that body length increased significantly from birth to second ecdysis ($P<0.0001$), while changes in length between the first and second ecdysis (trials 2 and 3) were not significant ($P=0.6715$).

Snakes showed a greater tendency to hide in wet moss before rather than after the first ecdysis. In behavioural tests, the occurrence of snakes in wet moss decreased from 74% to 59%, and the occurrence of snakes in dry moss increased from 4% to 19%, following postnatal ecdysis (Table 1). The occurrence of snakes outside the moss containers remained the same at approximately 22%.

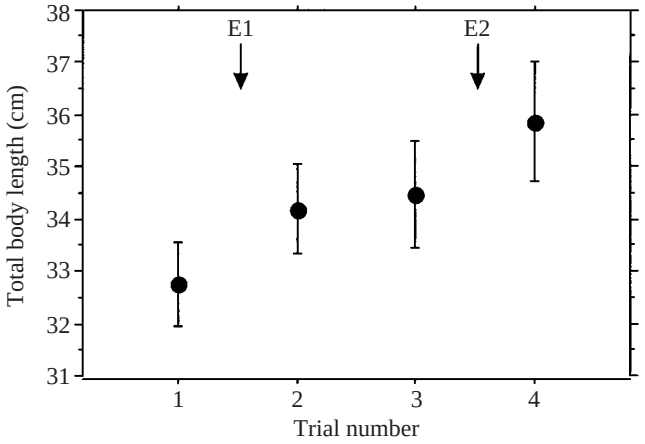

Fig. 2. Changes in mean total body length (±2 S.E.M.) measured in 20 hatchling California king snakes (*Lampropeltis getula*) during the course of two postnatal shedding cycles. Each measurement was made at the time of TEWL measurements (see Fig. 3), indicated as trial number on the abscissa. Arrows indicate relative timing of the first (E1) and second (E2) ecdysis with respect to trial number. Repeated-measures ANOVA followed by Bonferroni *post hoc* tests indicate that body length increased significantly from birth to second ecdysis (P<0.0001), while changes in length between the first and second ecdysis (trials 2 and 3) were not significant (P=0.6715).

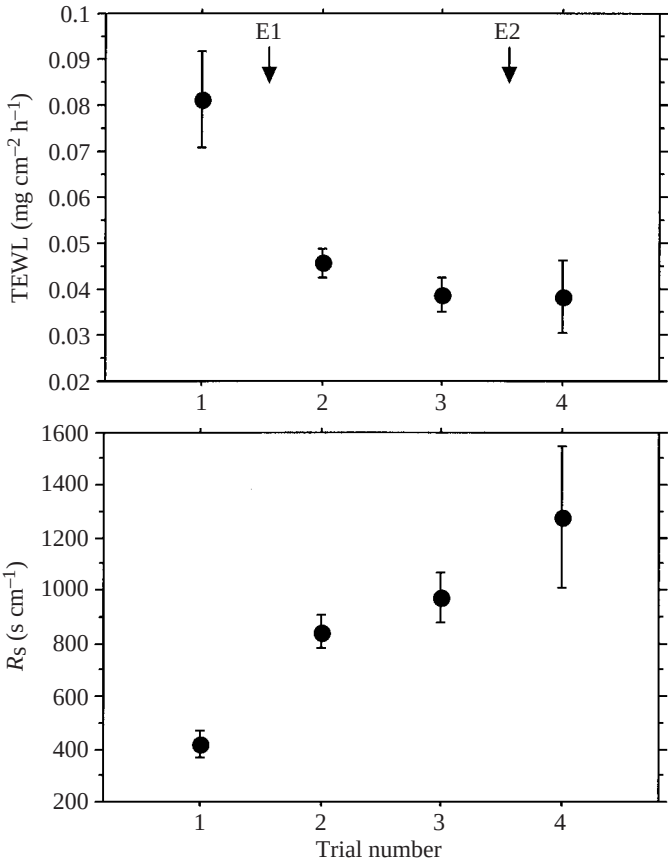

Fig. 3. Rates of transepidermal water loss (TEWL) and skin resistance (R_S) measured in the same 20 hatchling king snakes (*Lampropeltis getula*) as in Fig. 2, during four consecutive trials. Values are means ± 2 S.E.M., and arrows indicate the relative timing of first (E1) and second (E2) ecdysis with respect to trial number. ANOVA followed by *post hoc* tests indicate that mean TEWL measured in trial 1 is significantly greater than the values obtained in subsequent trials (P<0.0001). Similarly, there was a significant increase in R_S following the first trial (P<0.0001) and a second increase following trial 3 (P=0.0022), whereas measurements of R_S during trials 2 and 3 were not statistically different (P=0.1655). The pattern of changes suggests that the mechanism producing changes in R_S is related to ecdysis.

Evaporative water loss, skin resistance and skin temperature

TEWL of newborn snakes was nearly twice as great at hatching as in the same individuals following the first ecdysis (81.2±5.2 *versus* 45.7±1.5 μg cm^{-2} h^{-1}, respectively), reflecting a doubling of the skin resistance (R_S) after skin shedding (441.7±24.9 *versus* 865.7±30.7 s cm^{-1}) (Fig. 3). Subsequent measurements showed a downward trend of TEWL, but the changes were not statistically significant, partly because of a decrease in the humidity of room air that increased the tendency for TEWL during the fourth measurement period. However, R_S, which is independent of ambient conditions, decreased significantly following the second ecdysis (Fig. 3). The variance of measurements within and between clutches was similar (Fig. 4), and clutch effects for TEWL and R_S before and after postnatal ecdysis were not significant (ANOVA, all P>0.05). Inspection of Fig. 5 illustrates how subsequent measurements of R_S of the same individuals before and after ecdysis tend to covary. This pattern suggests that much of the variation in TEWL and R_S among individuals represents true biological variation rather than errors in experimental measurements.

The guts of hatchling snakes were visibly distended with yolk that gradually diminished during the first 2 weeks following hatching. Skin temperatures were elevated 0.60±0.06°C above ambient air temperature during the initial measurements of TEWL, then converged toward ambient in subsequent measurement trials as visible evidence of yolk disappeared (Fig. 6).

Table 1. *Average occurrence of hatchling king snakes in wet or dry containers*

Position of snake	Pre-shed	Post-shed	P
Wet	0.742±0.051	0.588±0.049	0.015
Dry	0.044±0.015	0.192±0.040	0.002
Outside either container	0.214±0.049	0.220±0.048	0.968

N=26 individuals.

Each snake was observed for a period of 7 days before (Pre-shed) and 7 days following (Post-shed) the first postnatal ecdysis.

P values indicate the outcome of Wilcoxon signed-rank tests of the null hypothesis that the probabilities of snakes using the wet or dry container tend to remain the same following ecdysis.

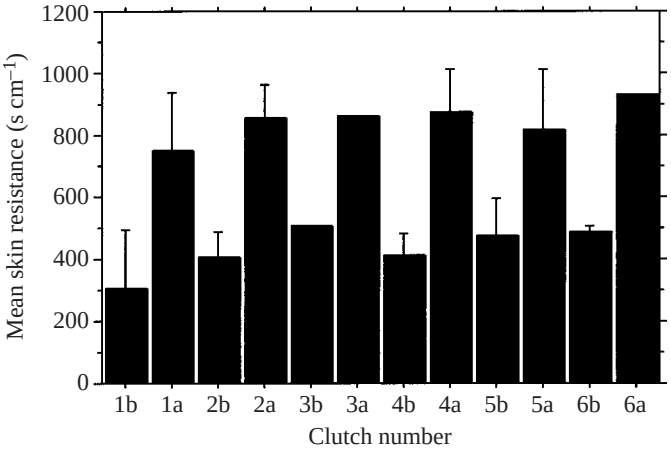

Fig. 4. Mean values of skin resistance (± 2 S.E.M.) measured in each clutch of hatchling king snakes before (b) and after (a) the first postnatal ecdysis, identified by numbers and letters on the abscissa. ANOVA followed by *post hoc* tests indicate there are no differences among clutch means, either before or after ecdysis (all P>0.07).

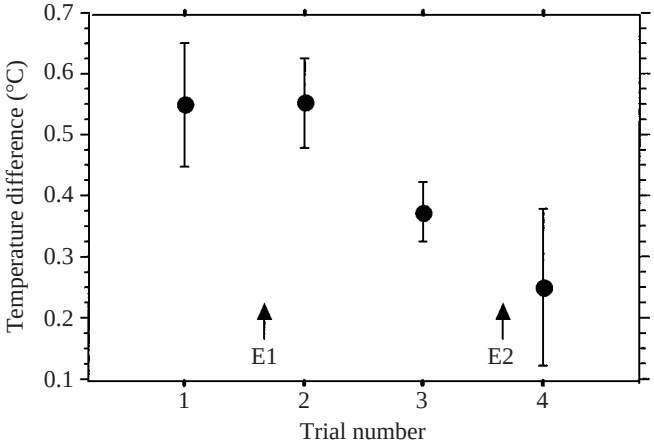

Fig. 6. Temperature differences between skin surfaces of snakes and the chamber (flow-through) air at the time of TEWL measurements (see Fig. 2) in 20 hatchling king snakes. Each value is the mean ± 2 S.E.M.; arrows denote the relative timing of the first (E1) and second (E2) ecdysis with respect to trial number. The measured temperature differential decreases significantly following the second measurement trial (ANOVA followed by *post hoc* tests, P=0.0076) and correlates with the assimilation of residual yolk (see text).

Ultrastructure and histochemistry of the skin permeability barrier

Histology of epidermis

Histologically, the pre-shed epidermis showed about five layers of nucleated cells and compact α and β layers. The mesos layer was not discernible at a histological level (Fig. 7, insets). The epidermis showed a perceptible staining for neutral lipids when stained with Fat Red-7B (Fig. 8B, inset). Post-shed epidermis showed fewer nucleated layers, more pronounced α and β layers, and perceptible lipid staining with Fat Red-7B (Fig. 9B, inset).

Ultrastructure of neonatal, pre-shed epidermis

The epidermal organization in snake skin is complex, and further complicated by the stage of the skin-shedding cycle at the time of biopsy. In this study we restricted our observations to the general morphology of the layers, and focussed especially on the organization of the barrier lipid structures, as revealed by the RuO_4 post-fixation. Due to its highly reactive nature, RuO_4 is destructive to the cytosolic elements, especially keratin, and hence evaluation of RuO_4-stained tissues has to be complemented with routine OsO_4-fixed samples (Menon and Ghadially, 1997).

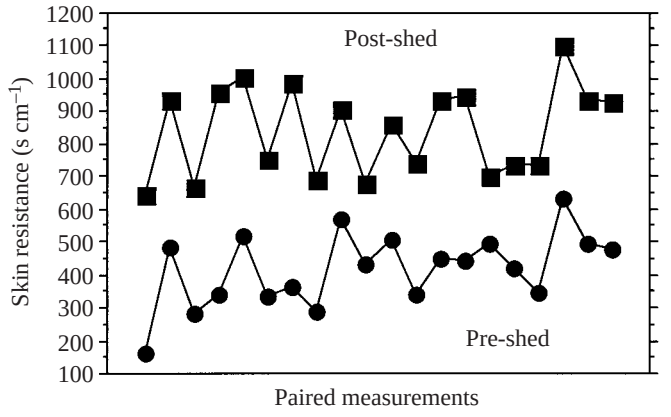

Fig. 5. Skin resistance in hatchling king snakes measured before (circles) and after (squares) the first postnatal ecdysis. The post-shed measurement is shown directly above the pre-shed measurement for each individual snake. Note that the pattern of variation among individuals is generally similar before and after ecdysis.

In low-magnification, survey electron micrographs (Fig. 7A), the outer β layer appears to be artificially separated from the underlying mesos layer. Within the β layer, individual cell boundaries are apparent. The mesos layer is composed of about three layers of extremely flattened, thin cells, which periodically show a slight 'ballooning' of electron-lucent cores. Desmosomal connections between overlapping mesos cells are apparently absent or very rare.

Below the mesos layer is the α layer. As seen in Fig. 7A, there are about 2–3 cell layers of mature α cells, with characteristically dense, keratinized cytosol, subjacent to which are 1 or 2 cell-thick immature α cells. Below the immature α cells lie two layers of nucleated cells. As the basal cell is rather large and not flattened, this may be indicative of a very early renewal phase.

As mentioned earlier, and noted elsewhere in the literature, RuO_4 staining causes considerable disruption to cytosolic structures and keratin, while superbly staining the extracellular barrier lipid structures (mortar) and cellular lipid inclusions, including lipid-enriched organelles. In the mesos layer of pre-shed skin, the extracellular lipids stained by RuO_4 showed some lamellar organization, but lacked the tight bilayer organization that characterizes barrier efficacy.

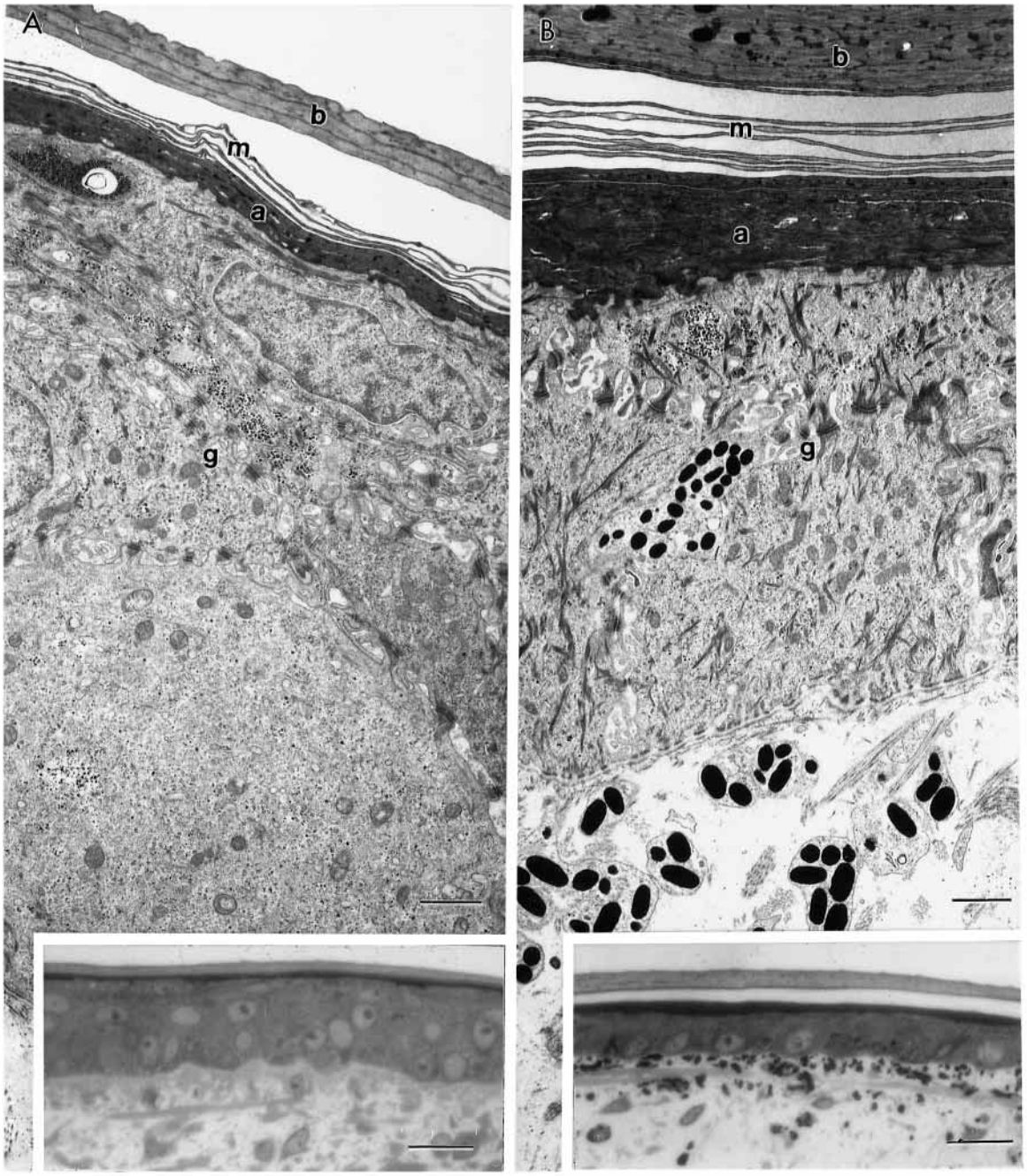

Fig. 7. (A) Ultrastructure of hatchling skin, sampled on the day of hatching and before the first ecdysis, showing mesos (m), α (a) and β (b) layers above the germinative layer (g, granular layer). Inset shows semi-thick plastic section (0.5–1 μm) of the same. (B) Post-shed skin sampled 2 days after the first ecdysis shows increased thickness in all three layers at the same magnification as in A. Note the near doubling of cells in the mesos layer. The germinative layer appears more compact compared to A. Inset shows light microscopic features of the post-shed skin in semi-thick plastic section (0.5–1 μm). The gaps in mesos layers seen in the micrographs are artifacts in tissue preparation (OsO_4 post-fixation). Scale bars, 1.0 μm; in insets, 0.1 μm.

The somewhat chaotic organization seen here (Fig. 8A) is reminiscent of what has been reported in fetal mammalian skin before the attainment of barrier competence (Azsterbaum et al., 1992). Within the α layers, the outer, mature α cells showed large inclusions of lamellar lipid structures intermixed with electron-lucent lipid material. Unlike the mesos layer, the α layer showed prominent desmosomal connections between adjacent and subjacent cells. In immature α cells, the cytosol contained many vesicular and membrane-bound structures, most notably large lipid inclusions of varying morphologies. Multi-lamellar bodies of these snake α cells (mlb; Fig. 10) closely resemble avian multi-lamellar bodies (Fig. 10, inset). There were also large elongated lipid structures with a lamellar

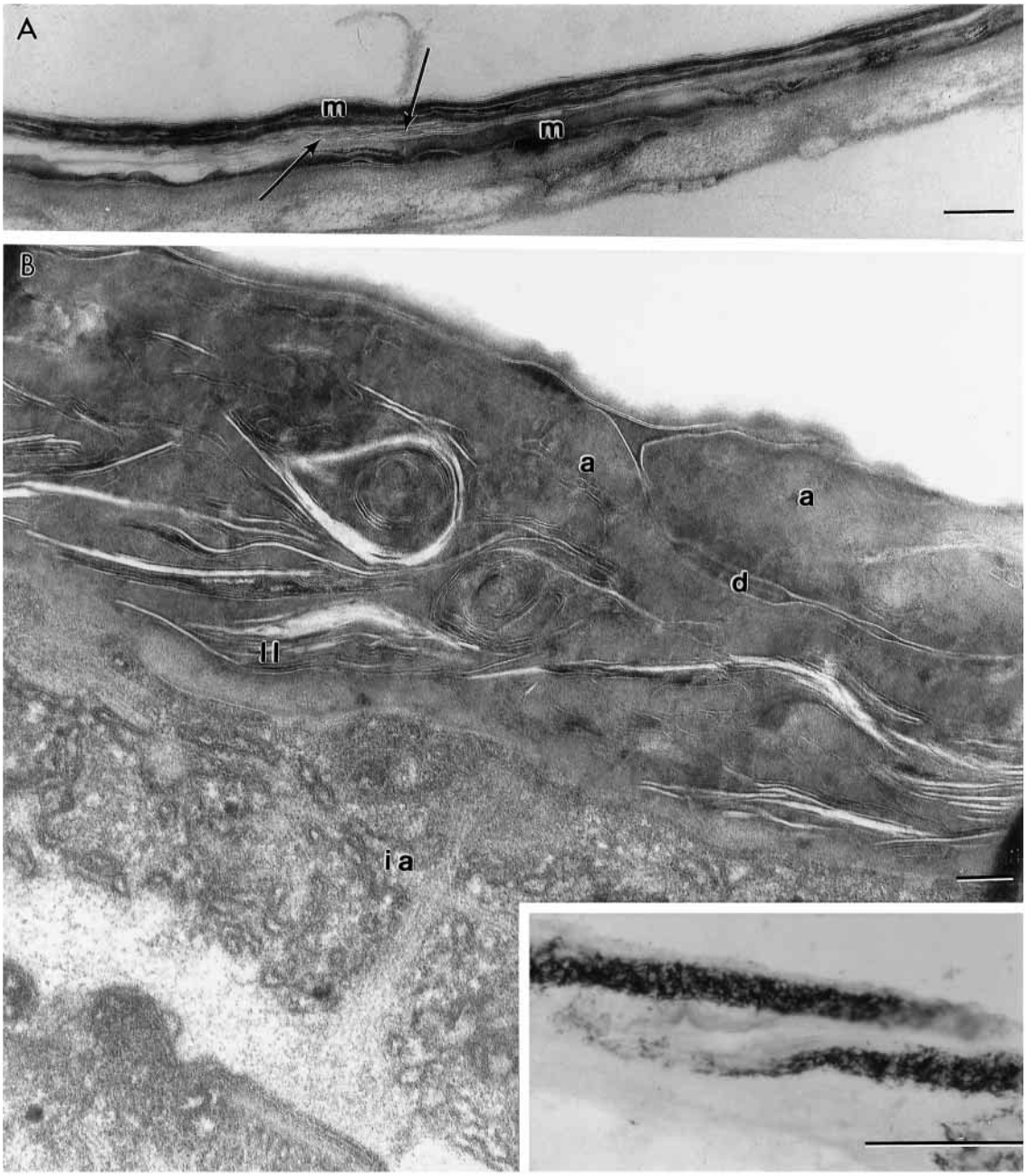

Fig. 8. (A) Mesos layer (m) showing somewhat disorganized bilayer structures in the intercellular spaces (arrows) in pre-shed skin sampled on the day of hatching. (B) High magnification ultrastructure of the α layer in pre-shed skin of same hatchling showing a desmosome (d) and lamellar lipid inclusions (ll) in the outer α (a) layer. Such an abundance of lipid is not seen in the section of inner α cells (ia), shown here, which exhibits an abundance of membrane structures resembling *trans*-Golgi (RuO_4 post-fixation). Scale bars, 0.1 μm. Inset: a frozen section (10–12 μm) of skin stained with Fat Red-7B showing presence of neutral lipids, but not well demarcated compared to that in Fig. 9 inset of post-shed skin. Scale bar for inset, 10 μm.

substructure (ll) and electron-lucent cores (l), and large electron-lucent lipid inclusions with lamellar lipids at their periphery (arrow).

Ultrastructure of post-shed epidermis

In low-magnification, survey electron micrographs (Fig. 7B), the outer β layer showed a clearer syncytial organization, as compared to the pre-shed stage. The artifactual separation (resulting from tissue processing) between the β and mesos layers was also seen in the post-shed samples. However, the outermost mesos cell layer remained partly attached to the β layer (Fig. 7B). The mesos layer is approximately 6–7 cells thick, which reflects a doubling of cell numbers following the first ecdysis. Individual cells of the mesos layer were quite similar to those of their counterparts in the pre-shed stage in all features, including the paucity of desmosomal connections.

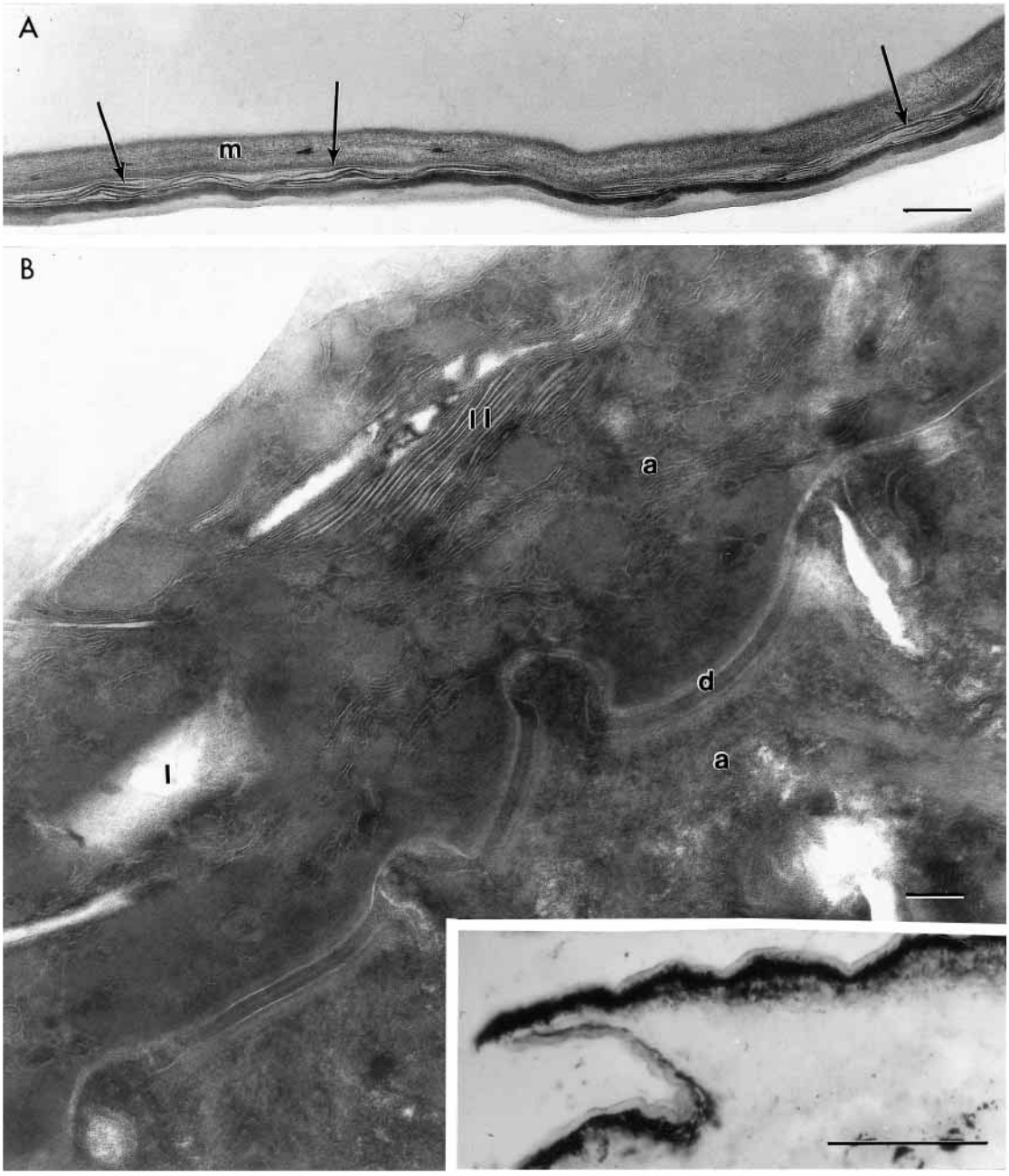

Fig. 9. (A) Mesos layer (m) in post-shed skin (2 days) showing well-organized and continuous bilayers in intercellular domains (arrows). (B) High magnification ultrastructure of the α layer in the same post-shed skin (RuO_4 post-fixation). Note decreased lamellar lipid inclusions (ll) as well as electron-lucent lipid inclusions (l) in the outer α cell compared to the pre-shed skin in Fig. 8. Desmosomes (d) are clearly seen in this field. Scale bars, 0.1 μm. The inset shows light microscopic histochemistry of frozen section (10–12 μm) stained for neutral lipids. Note the improved staining in the stratum corneum compared with Fig. 8, inset. Scale bar for inset, 10 μm.

The α layer was also thicker than in the pre-shed stage, and consisted of about 4–5 mature α cell layers. Below this were two cell layers of viable, nucleated cells, including the basal layer that rests on the basement lamina.

Intercellular domains of the mesos layer showed lipid structures with well-defined, continuous bilayer organization (Fig. 9A, arrows) as opposed to the pre-shed samples. The bilayers were not anchored to any desmosomal structures (in contrast to lipid bilayers in mammalian stratum corneum).

Within the mature α cells, lamellar inclusions were retained (Fig. 10), similar to what is seen in pre-shed epidermis. However, as immature α cells were not seen in the samples we examined, mlbs of the kind seen in the pre-shed stages were not observed.

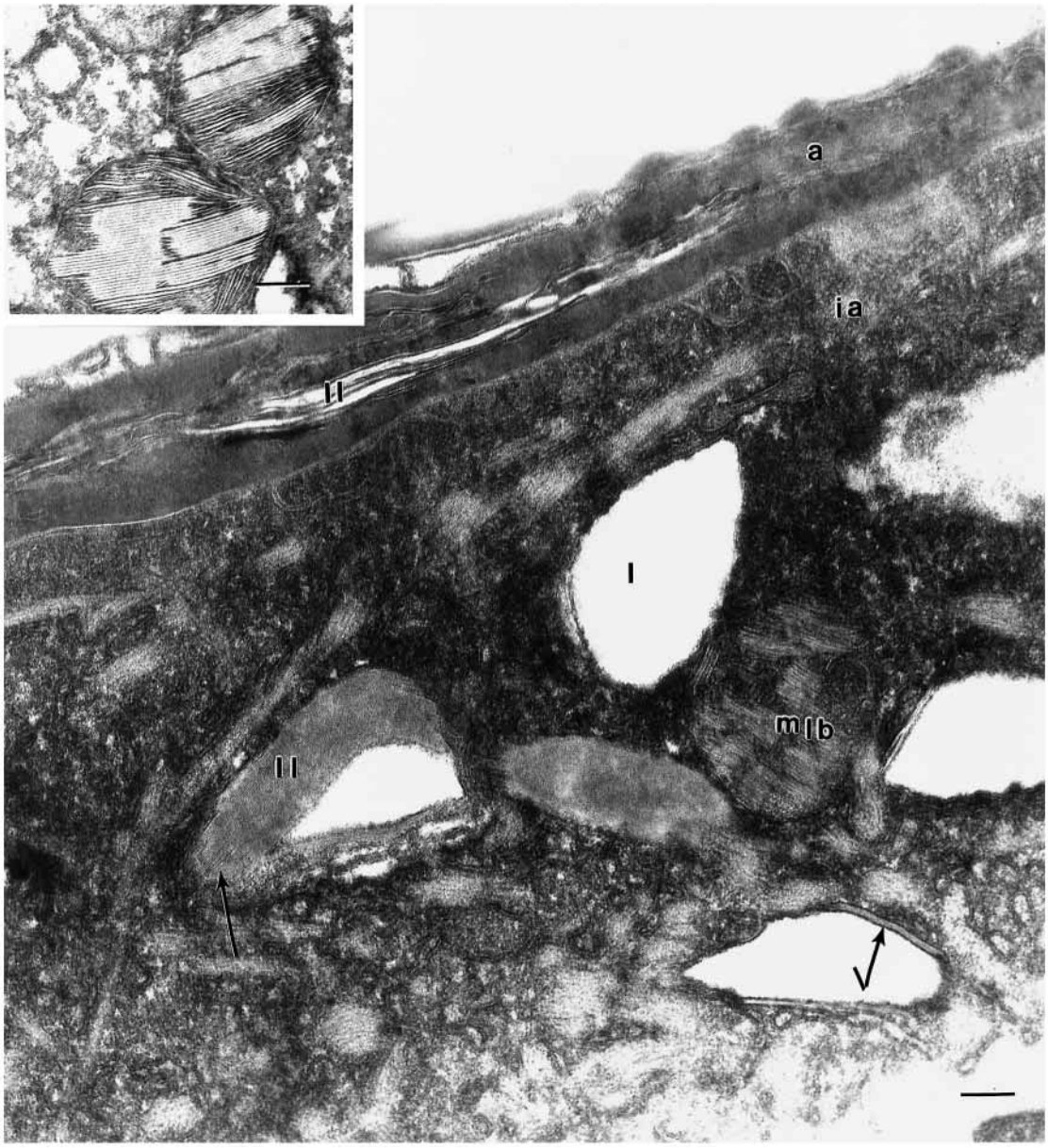

Fig. 10. Higher magnification electron micrograph of a portion of the inner α (a) cell in pre-shed skin showing lipid inclusions including a multigranular lamellar body (mlb), large lamellar (ll) and electron-lucent lipid (l) inclusions (RuO_4 stain) in close association with elements of the tubulo-reticular membrane system (arrow). This skin was sampled on the day of hatching, as in Figs 7 and 8. Inset: avian multilamellar bodies at comparable magnification, to highlight the structural similarity to the snake organelle. Scale bars, 0.1 μm.

Discussion

Hatchling integument and water relations

The skin of reptiles is an important route for osmotic and evaporative water exchange, as in many other vertebrates (Lillywhite and Maderson, 1982; Mautz, 1982b; Minnich, 1979). The permeability of skin to water varies considerably with environmental demands, and variation in skin resistance can be physiologically labile as a consequence of acclimation (Kattan and Lillywhite, 1989; Kobayashi et al., 1983). Thus, skin resistance might vary intraspecifically as well as interspecifically with season and habitat, at least in some species (Dunson and Freda, 1985). Snakes are useful animals for studies of skin permeability, largely because of their pan-body synchronized ecdysis – characteristic of squamates generally – and the variability exhibited among species that occupy a broad range of habitats. The variation of skin resistance among snake species correlates closely with the evaporative stress that is associated with different environments (Dmi'el, 1998; Lahav and Dmi'el, 1996; Lillywhite and SanMartino, 1993; Prange and Schmidt-Nielsen, 1969; Roberts and Lillywhite, 1983). Thus, heterogeneity of skin properties is related, in part, to hydric properties of the environment.

Perhaps the most profound change in hydric environment

during the ontogeny of any squamate reptile is the natal transition from the 'aqueous' environment of the embryo to the aerial (terrestrial) environment of the neonate or hatchling. It is well known (anecdotally) that newborn snakes generally shed their skin within 24–36 h after birth or hatching, although in some species the timing is longer (Ernst and Zug, 1996). There are two factors that are likely to stimulate early postnatal shedding. First, neonatal snakes continue to assimilate yolk while growth processes contribute to body elongation, even before feeding (Fig. 2). Ecdysis is presumably necessary to accommodate these increases in body length. Second, it seems likely that the natal transition from embryonic fluids to air stimulates ecdysis as an important means of adjusting the permeability barrier (Maderson, 1984). In king snakes we have demonstrated a twofold increase in R_s as a result of the first postnatal ecdysis, correlated with a structural doubling of the permeability barrier (Figs 3, 7).

Previous studies of TEWL in snakes have reported measurements for adult animals, but there is little information about neonates or juveniles. Dmi'el (1985) reported measurements of TEWL and R_s for a range of body mass that included hatchlings of the desert snake *Spalerosophis diadema*, and he found that R_s was independent of body mass. However, shedding histories of the snakes were not mentioned, so it is not known whether the hatchlings in Dmi'el's study were measured before or after postnatal ecdysis. While the R_s of newborn king snakes is less than half that of older snakes (Fig. 3), it is nonetheless several-fold greater than R_s that has been measured in an aquatic species of snake (Lillywhite and SanMartino, 1993). It appears the periderm and embryonic epidermis slough within the egg (Alibardi, 2002), and a permeability barrier (beta and mesos layers) of partial competence is formed within the epidermis prior to hatching (Maderson, 1985), similar to barrier formation in mammals (Williams et al., 1998). The further increase of R_s at the second postnatal ecdysis (Fig. 3) demonstrates a continued capacity for improvement of barrier function, as previously shown for lizards (Kattan and Lillywhite, 1989; Kobayashi et al., 1983) and for birds (Menon et al., 1996). Our measurements demonstrate there is a threefold improvement of barrier effectiveness (R_s) over the two shedding cycles examined in the present investigation (Fig. 3). However, the maximum effectiveness of the barrier in this species, and its facultative mechanism, remain to be determined.

The postnatal changes in barrier effectiveness that we describe here for king snakes differ strikingly from those of altricial species of birds endemic to xeric environments. Nestlings of zebra finches (*Taenyopygia guttata*) have a remarkably tight water barrier that progressively decreases in efficacy as they fledge, allowing evaporative cooling for thermoregulation (Menon and Menon, 2000). However, under conditions of water deficit, adult zebra finches are capable of rapid facultative waterproofing. We do not yet know whether adult snakes are capable of facultative changes in permeability barrier effectiveness as shown for some lizards and birds.

Newborn humans and rodents possess a competent permeability barrier at birth, with rates of TEWL at least as low as in adults (Williams et al., 1998). Barrier formation begins during late gestation and involves a progressive increase in the thickness of skin layers, formation of a multilayered stratum corneum, secretion of lipid lamellar bodies in the interstices of stratum corneum, and transformation of short lamellar disks into compact, continuous, lamellar unit structures (Aszterbaum et al., 1992). The keratinization and barrier formation in skin coincide with changes in the composition of amniotic fluid and are thought to be essential for protection from amniotic fluid during late gestation (Hardman et al., 1999; Parmley and Seeds, 1970). Also, contact of rat fetal skin with air accelerates barrier formation (Williams et al., 1998). Little is known about the processes underlying permeability barrier ontogenesis in reptiles. However, present data for king snakes suggest that emergence of the integument from embryonic fluids and its subsequent contact with air are essential for completion of barrier competence in the newly hatched animals, which might render them potentially useful models for mechanistic investigations of barrier development.

Ultrastructure and histochemistry of hatchling integument

From the morphological data, there is a clear correlation between the reduced cutaneous water loss in post-shed snakes and qualitative and quantitative changes in the mesos layer, which is the acknowledged site of permeability barrier in snakes (Lillywhite and Maderson, 1982). The increased number of cell layers in the mesos layer in post-shed skin, together with the continuous, organized bilayer structures of the barrier lipids, would contribute to a tighter permeability barrier, in contrast to the less organized lipids of the mesos layer in pre-shed skin. A similar structure–function relationship in barrier competency is seen when xerically stressed birds upregulate their permeability barrier (Menon et al., 1996) as well as during the fetal mammalian barrier maturation in late gestation (Azsterbaum et al., 1992; Hardman et al., 1999). The chaotic organization of bilayers in pre-shed ophidian skin is reminiscent of the similarly disorganized lipids of mammalian fetal skin before attainment of functional competency.

An interesting feature of the snake mesos layer is the apparent paucity of desmosomal connections within this layer (Fig. 7). In mammalian permeability barrier formation, desmosomes play important roles in (i) providing initial anchoring for the secreted lamellar body contents that subsequently undergo enzyme-mediated processing into mature lamellar bilayer structures, and (ii) providing cohesion to the corneocyte 'scaffolding' (bricks) structure that supports the organization of lipids (mortar) providing the permeability barrier. Again, it is the gradual dissolution of the desmosomes in upper stratum corneum, mediated by lamellar body-derived proteases, that allows controlled desquamation in mammals. Such a pattern of desquamation does not occur in snakes, due to the syncytial nature of the outer beta layer and the unique pan-body shedding cycles that characterize ophidian skin.

However, it is quite possible that a sequence of desmosomal degradation similar to that in mammalian stratum corneum might occur within the mesos layers of snake epidermis during its early formation in the pre-shed condition, when the second generation is formed beneath the one that is destined to be shed. As the mesos layer is physically protected, by virtue of being sandwiched between the β and α layers, no desquamation could result from the desmosomal degradation within the mesos layer. It is interesting to speculate on the functional benefit of a barrier layer that is free of desmosomes and yet protected from desquamation and loss. From a structural point of view, the stress propagation through desmosomes could conceivably weaken the delicate mesos layer, while in its absence, this vital barrier layer could be protected from the physical stresses of locomotion, preventing shearing or other damage to the 'waterproofing' lipid bilayers.

Another interesting feature concerns the lipid inclusions within cells of the α layer. Within the mature α layers, these inclusions show lamellar as well as electron-lucent morphologies, bearing close resemblance to what has been described for avian stratum corneum (Menon and Menon, 2000). In the immature α cells of pre-shed skin, multilamellar bodies (Fig. 10) and different stages of 'dissolution' of lamellar inclusions into electron-lucent lipids are dominant features, again very similar to what is seen in avian transitional cell layers (Menon et al., 1996). These observations point to an intriguing possibility that α cells themselves might be involved in the barrier homeostasis, which has not previously been suggested for ophidian epidermis. The facultative waterproofing ability of avian epidermis (Menon et al., 1996) resides in its capacity to modulate the type of lipids secreted, i.e. non-bilayer, electron-lucent lipids under basal conditions, but lamellar lipid structures under xeric stress, leading to significantly decreased evaporative water loss. The retained bilayer lipids in the avian stratum corneum under basal conditions, as well as that seen in the ophidian α layer (previously named cholesterol clefts by Jackson and Sharawy, 1978), might represent a reserve barrier mechanism. Whether snakes can modulate TEWL by secreting lamellar lipids from the α layer has not yet been evaluated. We speculate that this might be possible, and such a mechanism could perhaps underlie the large variation of TEWL that is observed among neonate snakes. Experimental tape stripping of scales results in α layer hyperplasia, and in this type of barrier repair, no mesos layers are formed until the next skin shedding cycle (Maderson et al., 1978). Careful ultrastructural investigations on the α layers during the repair response that follows tape stripping might reveal whether newly formed α cells are secreting lamellar lipids to reseal the barrier-defective areas, without necessitating a pan-body epidermal renewal needed to form the mesos layer.

Endothermy and behaviour of hatchlings

The transient elevation of skin temperatures, averaging 0.6°C above ambient air temperature, are sufficient to designate hatchling king snakes as endothermic (Fig. 6). The guts of the hatchlings we studied were visibly distended with yolk, and skin temperatures gradually converged toward ambient temperature, as visible evidence of yolk disappeared and the snakes increased in length (Figs 1, 6). These results strongly suggest that the endothermic conditions of snakes reflect an elevated metabolic rate related to the digestion and assimilation of yolk (Bakker and Andrews, 1984; Beaupre and Zaidan III, 2001). The condition appears to be analogous to postprandial calorigenesis ('specific dynamic action'), which is capable of producing remarkable elevations in metabolism in snakes (Secor and Diamond, 2000). The presence of internal yolk in newborns has been reported for a number of squamate reptiles and appears to be an important energy supplement used for synthesis in growing neonates (Beaupre and Zaidan III, 2001; Stewart and Castillo, 1984; Troyer, 1983). Newborn water snakes (*Nerodia rhombifera*) contain both fat bodies and yolk remnant, which account for 71% of the total lipid present at birth and 43% of the original yolk lipid (Stewart and Castillo, 1984).

The skin permeability barrier is considered important for water balance and potentially influences the behaviours of newborns and hatchlings. Our observations of newborn king snakes demonstrate that they are reclusive and seek humid microenvironments prior to their first ecdysis. While the majority of hatchlings continued to seek shelter in humid environments following ecdysis, a significant fraction abandoned humid containers in favor of drier ones (Table 1). Thus, it appears quite probable that the degree of water movement across the integument has an important influence on the dispersal of newborn snakes away from birth sites. In this context, it is of interest that neonatal pit vipers of numerous species remain with their mother until the first ecdysis, after which maternal care is abandoned and the young disperse (Greene et al., 2002). The degree of water movement across the integument has perhaps been important in influencing the evolution of parental care in viviparous species of squamates. The low resistance of neonatal skin to water loss, coupled to a transiently high metabolic rate, would promote high rates of evaporative loss in the newborn. Insofar as yolk assimilation precludes the necessity for immediate prey capture, aggregation of snakes in protected places would reduce surface area/volume ratios and thereby mitigate a tendency to dehydration.

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