



Ontogenetic variation in the stratum granulosum of the epidermis of *Chaetophractus vellerosus* (Xenarthra, Dasypodidae) in relation to the development of cornified scales

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ABSTRACT

The epidermis of mammals is characterized by having a stratum granulosum that produces an orthokeratotic stratum corneum, different from the typical reptilian parakeratotic stratum. Nonetheless, some mammals show distinct degrees of parakeratosis in epidermal regions with few or no pilose follicles (e.g., areas subjacent to cornified scales). With respect to the epidermis and the development of cornified scales in the Dasypodidae, previous studies have supported the presence of a continuous stratum granulosum without any variations during ontogeny. This condition, in which the cornified scales develop without a loss of the stratum granulosum, was interpreted as primitive for eutherians. The present contribution expands the knowledge on the epidermis of *Chaetophractus vellerosus* in distinct ontogenetic stages in order to determine whether the cornified scales show the same developmental pattern as in other eutherians. The presence of a stratum granulosum in *C. vellerosus* neonates and its reduction in more advanced ontogenetic stages, in direct relationship with cornified scale development, supports the hypothesis that the partial parakeratosis in the xenarthran integument is secondary, as in other eutherians, and can be interpreted as a derived character state.

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1. Introduction

The cornified epidermis unique to amniotes consists of a conspicuous external layer that is very rich in keratin and is of variable thickness. This layer of keratin corresponds to the stratum corneum, which may be formed by two different processes: parakeratosis and orthokeratosis. In the former, a subjacent stratum granulosum is not developed and corneocytes may retain pyknotic nuclei. By contrast, a stratum granulosum is well differentiated and pyknotic nuclei are not observed in the stratum corneum in an orthokeratotic epidermis (Alibardi, 2010 and references therein). In non-mammalian amniotes, only parakeratosis is observed. In mammals, depending on the type of the epidermal structure, two types

of stratified-cornified epithelia can be distinguished: soft-cornified epithelia (e.g., the epidermis) and hard-cornified epithelia (e.g., the plate of the human fingernail) (see Bragulla and Homberger, 2009); hard cornification involves parakeratosis (e.g., claws; see Alibardi, 2009).

In mammals, the epidermis is formed by distinct strata. From the deepest to the most superficial layer, these include: the stratum basale, the stratum spinosum, the stratum granulosum (characterized by the presence of granules of different composition, especially filaggrin and profilaggrin, traditionally named keratohyalin granules), the stratum lucidum (variably present), and the stratum corneum, which is formed by corneocytes (dead cells replete with keratin). According to previous studies, the flexibility of the mammalian epidermis is related to the development of the stratum granulosum, which produces an orthokeratotic stratum corneum, unlike the parakeratotic stratum typical of the reptilian epidermis (Spearman, 1964; Alibardi and Maderson, 2003).

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Nonetheless, the development of the stratum granulosum varies among mammalian species as well as regionally within the epidermis of an individual (e.g., there are more strata around hair follicles and openings of sweat glands; see Spearman, 1966). Zones that have little or no hair have small or unnoticeable keratohyalin granules associated with partial or total parakeratosis (Alibardi and Maderson, 2003). Also, parakeratosis in mammalian epidermis is often pathological, e.g., in psoriasis, eczema, ichthyosis, and sometimes in hyperkeratosis or lupus (Alibardi and Maderson, 2003 and literature cited therein). From a clinical point of view, the histological phenomenon of parakeratosis is defined as occurrence of cell nuclei within the stratum corneum (Fischer et al., 2004), in general with no stratum granulosum, although this can sometimes be observed (e.g., in granular parakeratosis; see Braun-Falco and Laaff, 2009). In the present paper we follow the description previously presented by Spearman (1964) and Alibardi and Maderson (2003), which is commonly used to define the normal cornification in amniotes.

In eutherian mammals with cornified scales such as those present in the tail of the house mouse (*Mus musculus*), no stratum granulosum is present under such scales in adults (Spearman and Hardy, 1977; Alibardi, 2004); the same is true for the epidermis subjacent to the cornified scales of pangolins (*Manis tetradactyla* and *Manis pentadactyla*), although there are always keratohyalin granules in the epidermis between scales (Spearman, 1967). Recently, Meyer et al. (2010) found a stratum granulosum underlying the cornified scales of *Manis javanica* that was less developed than in the ventral skin, which lacks scales. The occurrence of a stratum granulosum among xenarthrans is extremely varied. Among members of the Pilosa, Machida et al. (1966) described a continuous stratum granulosum three layers thick in *Tamandua tetradactyla*, but Wislocki (1928) and Sokolov (1982) did not observe a stratum granulosum in *Bradypus* or *Choloepus* by optical microscopy (OM). Based on previous works, Alibardi (2010) noted that the stratum granulosum is discontinuous in xenarthrans.

However, among the Cingulata (xenarthrans with cornified scales), studies have described a continuous stratum granulosum in embryos and fetuses (Wilson, 1914; Cooper, 1930; Fernández, 1931) as well as adults (Fernández, 1931) of dasypodids. Based on the results of Cooper (1930), Spearman (1966) hypothesized that the presence of a stratum granulosum in Dasypodidae represents an early evolutionary stage prior to the acquisition of the type of cornified scale formation more typical of mammals. It is worth mentioning that Schaffer (1940) pointed out the absence of a stratum granulosum under the cornified scales of adults of *Euphractus sexcinctus*. More recently, Vickaryous and Hall (2006) supported the presence of a continuous stratum granulosum in fetuses of *Dasypus novemcinctus*, whereas Krmpotic et al. (2012) observed the same in neonates of *Dasypus hybridus* and *Chaetophractus vellerosus*. In those neonates, the stratum granulosum was only observed in epidermic areas that lacked cornified scales and was completely absent where these structures were starting to develop.

The systematic position of Xenarthra among eutherians and the group's relationships with the other major mammalian clades is still a controversial matter. Morphological characters that had initially been considered plesiomorphies of Xenarthra were treated as derived in later studies (e.g., Wible et al., 1990). In this context, the primary objective of the present paper is to analyze the occurrence of a stratum granulosum in distinct ontogenetic stages of *C. vellerosus* and its relation to the development of cornified scales, with the aim of determining whether the epidermal stratum has the same pattern as in other scale-bearing eutherians or whether it has a more primitive pattern, with a stratum granulosum still present, as has been suggested by other authors.

2. Materials and methods

For the present study, integument samples for optical microscopy analysis were taken from homologous regions of two neonates, a 17-day-old juvenile, and two adults of *C. vellerosus* (small hairy armadillo). The neonate and juvenile specimens were obtained from the collection of Vertebrate Paleontology of the Museum of La Plata, Buenos Aires; the material was fixed in 5% formaldehyde and preserved in 70% ethanol. Adult samples were extracted from live animals by punch biopsy under anesthesia.

Small portions (25–30 mm²) of the cephalic shield, distinct regions of the dorsal carapace and the ventral integument were taken from each specimen. In addition, biopsies of the ventral skin of an adult specimen were also taken in zones subjacent to the scales, and between successive scales. Bouin's solution was used to decalcify any ossifications. Subsequently, the tissues were dehydrated using a graded ethanol series (from 70% to 100%) and embedded in paraffin. Serial histological sections of 3–5 µm were taken parallel to the sagittal plane and stained with hematoxylin and eosin (H&E stain).

Samples were also taken for transmission electron microscopy (TEM) in order to evaluate the presence of small-sized keratohyalin granules not discernible under OM. The material was fixed in glutaraldehyde, dehydrated in a graded ethanol series, immersed in propylene oxide, and then embedded in Spurr's resin. The samples were cut into semi-thin sections that were stained with toluidine blue and into ultra-thin sections for TEM that were contrasted with 2% uranyl acetate and 0.5% lead citrate. Calibrated images were taken using Leica software LAS EZ (Leica Microsystems, Wetzlar, Germany). The epithelial height (including the stratum basale, stratum spinosum and stratum granulosum) was measured using ImageJ 1.48c (Rasband, 1997). Five replicate measurements were taken for each sample and then averaged, following Jones and Pfeiffer (1994).

To interpret the results in a broader context within the Dasypodidae, a variety of other specimens were also analyzed, including neonates of *C. villosus* (large hairy armadillo), a neonate and juveniles of *D. hybridus* (lesser long-nosed armadillo), and adults of *C. villosus*, *D. novemcinctus* (long-nosed armadillo) and *E. sexcinctus* (yellow armadillo). All of these specimens except *E. sexcinctus* were obtained from the collection of Vertebrate Paleontology of the Museo de La Plata, Buenos Aires. The osteoderms of *E. sexcinctus* were obtained from a road-killed animal. The samples were processed for optical microscopy and morphometric analysis using the protocol previously described for *C. vellerosus*.

3. Results

In the epidermis of neonates of *C. vellerosus* there is a stratum basale consisting of a single cell layer, a stratum spinosum consisting of four or five cell layers, a stratum granulosum of one or two cell layers that is continuous along most of the epidermis, and a well-developed stratum corneum that stained strongly eosinophilic (Fig. 1A). In the neck of the hair follicles there can be three or more cell layers and larger granules (Fig. 1B).

In the 17-day specimens, the dorsal epidermis is composed of a stratum basale consisting of a single cell layer and a stratum spinosum that is less developed than in neonates, consisting of approximately three cell layers. The stratum granulosum is absent in these specimens, and the stratum corneum has developed into scales (Fig. 1C). In the sections stained with H&E the cornified scales that have already developed can be distinguished from the stratum corneum by their yellowish and pyknotic nuclei (Fig. 1C).

In adults, the epidermis is thinner than in juveniles and much thinner than in neonates. It is composed of a stratum basale and two

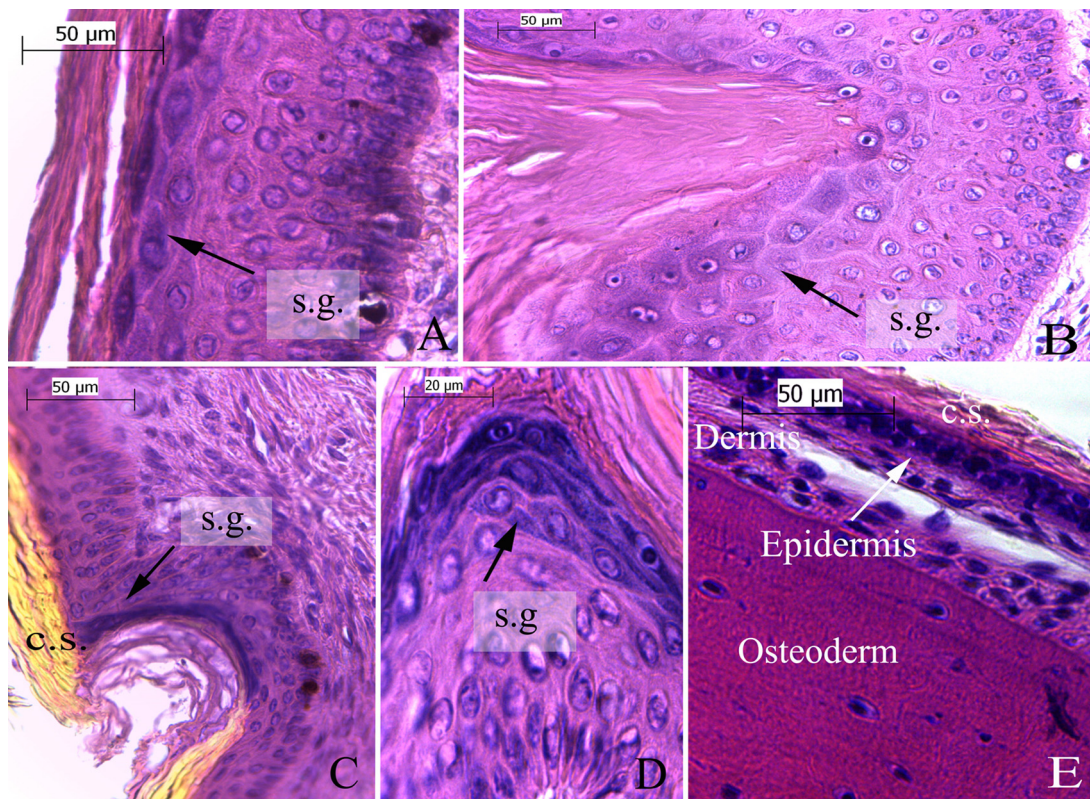


Fig. 1. Histological details of the dorsal epidermis of *Chaetophractus vellerosus* specimens showing different strata. (A) and (B) Neonate epidermis, H&E stain. (B) Details of the neck of a hair follicle with the stratum granulosum more developed. (C) and (D) Juvenile epidermis (17 days old), H&E stain. (C) Two cornified scales and the area between them. A conspicuous stratum granulosum is present in the area between the cornified scales. (D) Stratum granulosum in the epidermis without cornified scales. (E) Adult epidermis with cornified scales, without granulose stratum, H&E stain. Abbreviations: c.s., cornified scales; s.g., stratum granulosum.

layers of stratum spinosum only (Fig. 1E). A stratum granulosum was not observed in the epidermis subjacent to the cornified scales nor in any of the studied regions of the dorsal integument. A stratum granulosum was only found to be present in the areas between cornified scales (Fig. 1C). The ventral epidermis of juveniles and adults has a conspicuous stratum granulosum three layers thick (Fig. 1D).

TEM of the epidermis of adult specimens confirmed the complete absence of a stratum granulosum under the cornified scales of the dorsal integument; not even smaller keratohyalin granules were observed, although these are conspicuous in epidermal regions lacking cornified scales (Fig. 2A–D). These results indicate that the epidermis of *C. vellerosus* displays partial parakeratosis directly related to the development of the cornified scales in the dorsal integument.

With respect to the other analyzed species, a conspicuous and continuous stratum granulosum was observed in neonates of *D. hybridus* and *C. villosus*, which lacked developed cornified scales in the development stages studied (Fig. 3A and B). This matches the condition in *C. vellerosus*. In adult specimens of *E. sexinctus*, *C. villosus*, *D. novemcinctus* (Fig. 4A–C), and in neonates and juveniles (with developed cornified scales) of *D. hybridus* (Fig. 5A and B), not even a reduced stratum granulosum was observed below developed or developing cornified scales.

There was a clear difference in epithelial thickness (without the stratum corneum) between different ontogenetic stages of *C. vellerosus* (Fig. 6), with neonates having the greatest epithelial thickness, adults the lowest values, and juveniles in between. The decreased epithelial thickness under developing or developed scales is due to the absence of a stratum granulosum and the reduction of the stratum spinosum. This pattern of reduction of the epithelial thickness with ontogenetic age was consistent with

observations in other species (e.g., *C. villosus*), although the ontogenetic sequence was not as complete as in *C. vellerosus* (Fig. 6). This pattern was not observed in *D. hybridus* where the juveniles exhibited greater epithelial thickness than neonates. However, a more complete sequence with a greater number of individuals would be necessary to clarify this pattern. It is likely that the differences are due to an already developing cornified scale in neonates.

4. Discussion

The occurrence of a stratum granulosum and its differentiation are variable among mammals. In more primitive mammals (monotremes, metatherians) the alternation between parakeratotic and orthokeratotic zones, as well as the variation in the diameter of the keratohyalin granules are interpreted as plesiomorphic, whereas the existence of a conspicuous stratum granulosum, like that present in eutherians, is considered derived (Alibardi and Maderson, 2003). Juvenile and adult specimens of *C. vellerosus* have a conspicuous stratum granulosum in areas of epidermis that lack cornified scales, but it is always absent in areas subjacent to cornified scales. This agrees with the condition observed in other eutherians that have scales in parts of their integument, such as *M. musculus* (Spearman and Hardy, 1977; Alibardi, 2004), *M. tetradactyla*, and *M. pentadactyla* (Spearman, 1967).

It is important to point out that in the tail of *M. musculus* a stratum granulosum is present in neonates but has not been observed under scales in adults (Wrench, 1977). Alibardi (2004) observed that the stratum granulosum underlying the cornified scales of *M. musculus* is well developed during intrauterine development, as it is present and thick at 4 days after birth. At later stages, the stratum granulosum is completely absent. In the Dasypodidae, the stratum granulosum is continuous and well

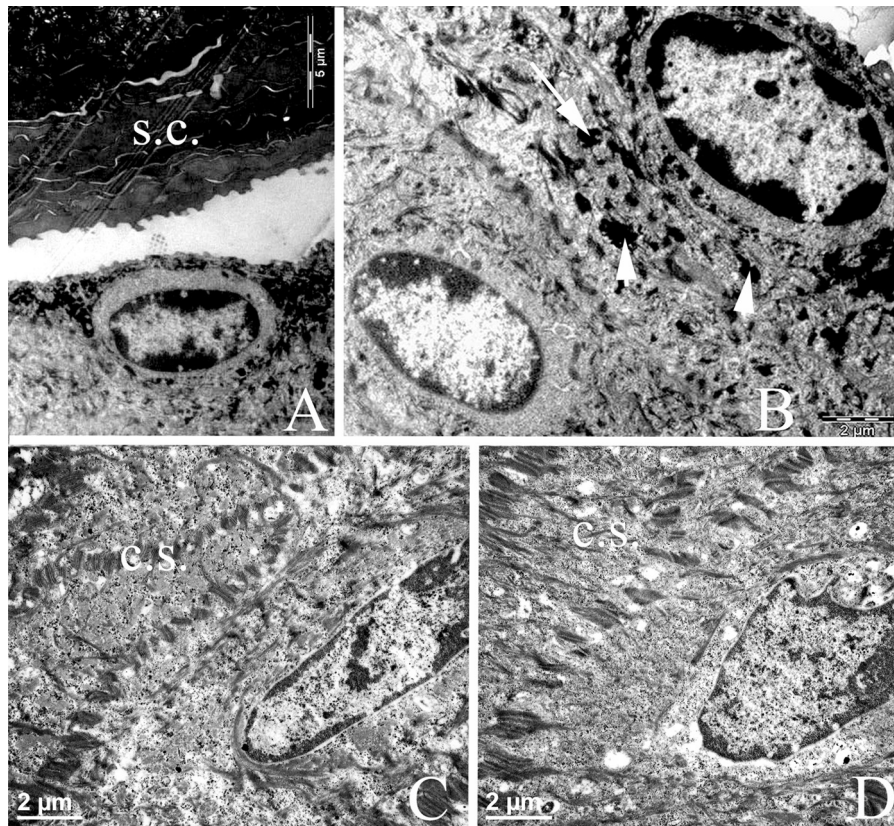


Fig. 2. TEM micrographs of the adult epidermis of *C. vellerosus*. (A) and (B) Ultrastructure of granulated cells of epidermis without cornified scales. The arrows point to keratohyalin granules. (C) and (D) Ultrastructure of epidermis underlying cornified scales; the absence of granulated cells is evident. Abbreviations: c.s., cornified scales; s.c., stratum corneum.

developed in early ontogenetic stages (neonates) of *C. vellerosus* and other species (Cooper, 1930; Fernández, 1931; Vickaryous and Hall, 2006; Krmpotic et al., 2012). Therefore, the disappearance of the granular layer from fetus to adult has to be derived from a change of the epidermal differentiation program, from an orthokeratotic fetal epidermis to a scaled adult epidermis. This supports the hypothesis that the partial parakeratosis in the integument of the Dasypodidae is secondary, as in other eutherians, and can be interpreted as a derived rather than a plesiomorphic character

state. Additionally, the pattern of development of cornified scales that is associated with the disappearance of the stratum granulosum is the same as that present in other scaled eutherians. Therefore, this pattern does not correspond to a stage prior to the alteration of the epidermal keratinization to the typical cornified scale formation in mammals, as argued by Spearman (1966).

Parakeratotic cornification in Eutheria is not restricted to regions covered by scales or other hard cornifications, since the skin of cetaceans lacks a stratum granulosum and has a stratum

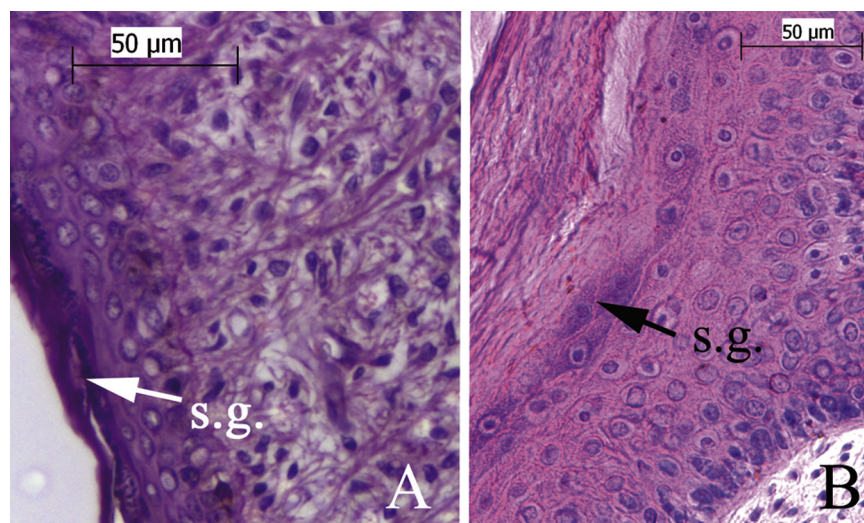


Fig. 3. Histological details of the epidermis of neonates without developed cornified scales with stratum granulosum present. (A) *Dasybus hybridus*, PAS stain. (B) *Chaetophractus villosus*, H&E stain. Abbreviation: s.g., stratum granulosum.

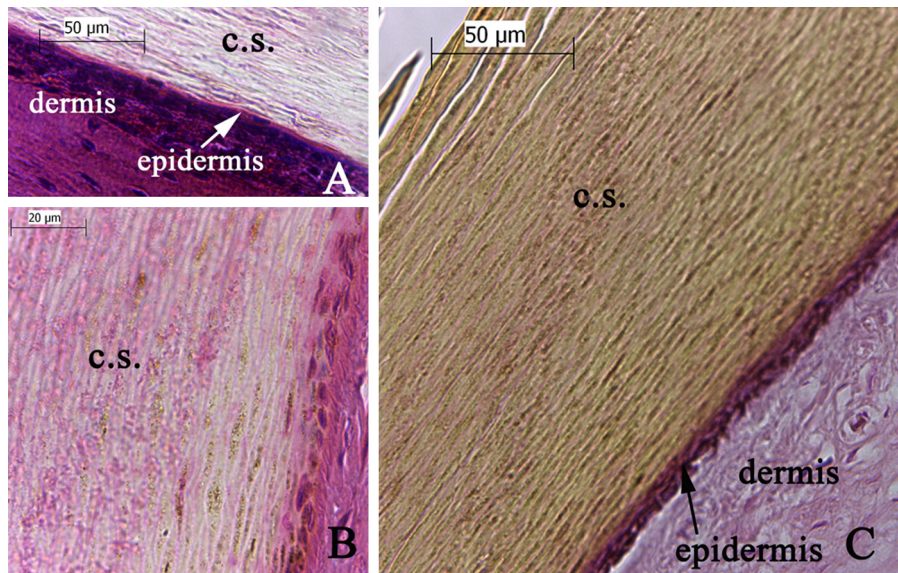


Fig. 4. Histological details of the epidermis of adult specimens with developed cornified scales, where no stratum granulosum is present, H&E stain. (A) *Euphractus sexcinctus*. (B) *Chaetophractus villosus*. (C) *Dasypus novemcinctus*. Abbreviation: c.s., cornified scales.

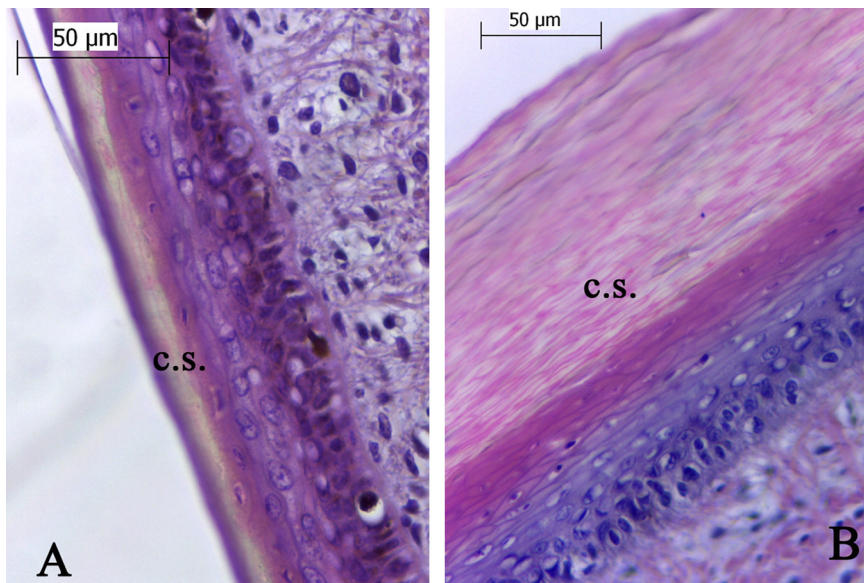


Fig. 5. Histological details of the epidermis of *Dasypus hybridus* with developed cornified scales, where no stratum granulosum is present, H&E stain. (A) Neonate, (B) juvenile. Abbreviation: c.s., cornified scales.

corneum that retains pyknotic nuclei (Haldiman et al., 1985). This condition can also be considered a derived character state and is probably related to life in an aquatic environment, as it is associated with other features such as the absence of hairs and glands and

an abundance of lipids in the epidermis. But in contrast to what has been observed in parakeratotic regions of Dasypodidae, the stratum granulosum in cetaceans seems not to form during ontogenesis (Meyer et al., 1995). The presence of a parakeratotic epidermis in

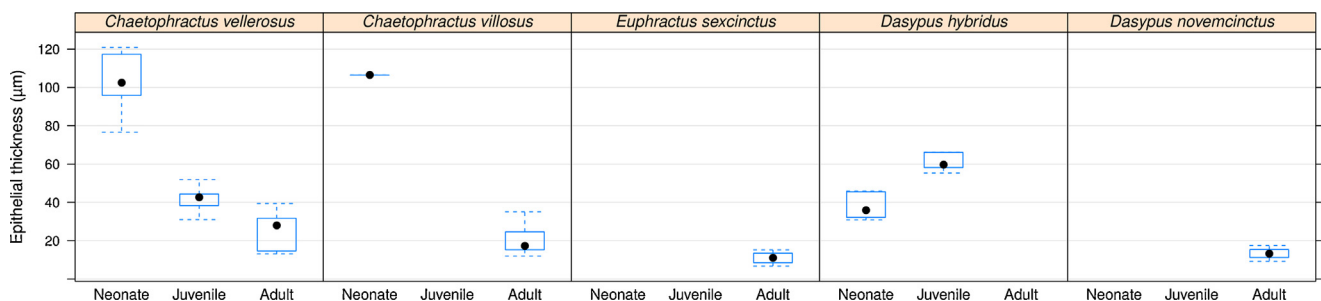


Fig. 6. Box plots of the epithelial thickness in the examined age stages of each species.

Dasypodidae seems to be an adaptation of zones of integument that require strengthening. As pointed out above, the suprabasal cell layers of hard-cornified stratified epithelia do not include a stratum granulosum (e.g. cortex of hair, plate of the human fingernail, cornified sheath of a cat claw, cornified sheath of a bird beak, wall of the horse hoof) (see Bragulla and Homberger, 2009). Some ontogenetic studies of these structures (e.g., human nail – see Zaias, 1963; claw – see Alibardi, 2009) indicate the presence of a stratum granulosum in the early ontogenetic stages with subsequent loss later, as observed in the ontogeny of horny scales. In contrast, hard cornification, including horny scales found in other non-mammalian amniotes, cannot be considered homologous, because these structures are composed of different kinds of keratins. Hard cornification in sauropsids is related to beta-keratins, while in mammals it is linked to alpha-keratins.

These results become especially relevant when the systematic position of Xenarthra in the different phylogenies of high-rank mammals is directly linked to the interpretation of the supposed plesiomorphies of the group. One phylogenetic hypothesis proposes that the Xenarthra diverged prior to the other three clades of placental mammals (Euarchontoglires, Laurasiatheria, and Afrotheria) which comprise the group Epitheria. This hypothesis is generally supported by morphologists (McKenna, 1975; Novacek and Wyss, 1986; Novacek, 1992; Shoshani and McKenna, 1998) and was accepted in the last general classification of mammals based on morphologic data (McKenna and Bell, 1997). Recently, it was also supported by the molecular phylogeny of Kriegs et al. (2006), which used retroposons as phylogenetic markers. Among morphologists, McKenna (1975) advocated the basal position of Xenarthra using character states (anatomical and physiological) that are assumed to be plesiomorphic for mammals. However, some of those character states have been re-evaluated by subsequent authors (Wible et al., 1990). Based on our study, epidermal parakeratosis is yet another derived feature of xenarthrans that was previously considered to be plesiomorphic. Combined with other such features, this reinterpretation raises further doubts about the supposed basal-most position of Xenarthra among Eutheria.

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