



Original Investigation

Development of the integument of *Dasyopus hybridus* and *Chaetophractus vellerosus*, and asynchronous events with respect to the postcraniumCecilia M. Krmpotic^{a,b,c,*}, Fernando C. Galliari^{a,b}, Claudio G. Barbeito^{b,c}, Alfredo A. Carlini^{a,b}^a División Paleontología de Vertebrados, Museo de La Plata, La Plata, Buenos Aires, Argentina^b Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina^c Cátedra de Histología y Embriología, Facultad de Ciencias Veterinarias, UNLP, La Plata, Buenos Aires, Argentina

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ABSTRACT

The integument of extant armadillos (Xenarthra, Cingulata) is a unique organ in which complex glandular systems are associated with pilose follicles, dermal ossifications, and cornified scales. Up to date, papers have focused on neither comparative morphology of the skin (dorsal and ventral) nor chronology of the development of interspecific homolog structures. In order to clarify the way in which events occur during development of the integument structures, maturity of other tissues (e.g. skeletal tissues) should be considered. Therefore, we will be able to identify events that have been pre- or post-displaced during ontogenetic development. The aim of this paper is to describe in a developmental and comparative framework the integumentary system of neonates of *Dasyopus hybridus* and *Chaetophractus vellerosus*. In order to understand the morphology of the different integumentary structures serial histological sections were prepared. Staining techniques included H-E, Masson Trichrome, PAS, orcein and reticulin. To study ossification of postcranial elements, the specimens were cleared and double-stained with alcian blue and alizarin red. Determinations of ossification centers and their progress were recorded through the early uptake of alizarin. The dorsal dermis of neonates from *D. hybridus* is clearly differentiated into a superficial and deep layer, as in fetuses of *Dasyopus novemcinctus*. In *C. vellerosus*, however, these layers could not be identified. This suggests a less connective tissue differentiation in the latter species at this stage. Osteoderms in *D. hybridus* are well differentiated unlike *C. vellerosus* where no condensations of osteoprogenitory cells are observed. Conversely, pilose follicles and glandular tissues are less developed in *D. hybridus*. Regarding postcranial elements, ossification centers are less advanced in *C. vellerosus* than *D. hybridus*, this is particularly notorious for the vertebral column, sternal, and pelvic girdle elements. Asynchronies between neonates of both species observed on integumentary and postcranial skeletal tissues could match with specific adaptive strategies related to distribution in different environments, and/or different postnatal care.

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Introduction

Osteoderms (bone mineralizations in the dermis) are widely distributed among different groups of tetrapods (Sire et al. 2009; Vickaryous and Sire 2009). They are well represented among amphibians and reptiles (Moss 1969; Vickaryous and Sire 2009). In modern mammals, osteoderms are restricted to armadillos (Dasypodidae), being among the most conspicuous features of the group.

They form a protective dorsal cover, over which epidermal cornified scales lie. The scarce dorsal hairs emerge through osteoderm foramina and through the spaces between cornified scales

(Scillato-Yané 1982). Osteoderms cover the dorsum of the head (cephalic shield) and trunk (dorsal shield or carapace), and surround the tail (caudal shield), except for the naked-tailed armadillo *Cabassous*. The dorsal carapace is divided into a scapular buckler, a region of movable bands, and a pelvic buckler (Fig. 1). Furthermore, osteoderms may be also in the integument of the rostrum, ventral region of the trunk, and limbs. However, in these areas no continuous shields are formed (though incipient in some species of *Dasyopus*), and the relationship with cornified scales is variable (Ciancio and Carlini 2008; Carlini et al. 2009). Variability in osteoderm ornamentations has been important to establish phylogenetic relationships within the group (e.g. Ameghino 1889; Scillato-Yané 1982; Carlini and Scillato-Yané 1996; Ciancio and Carlini 2008; Carlini et al. 2009).

The fact of being the only living mammals with dermal ossifications makes the integument of armadillos a unique structure

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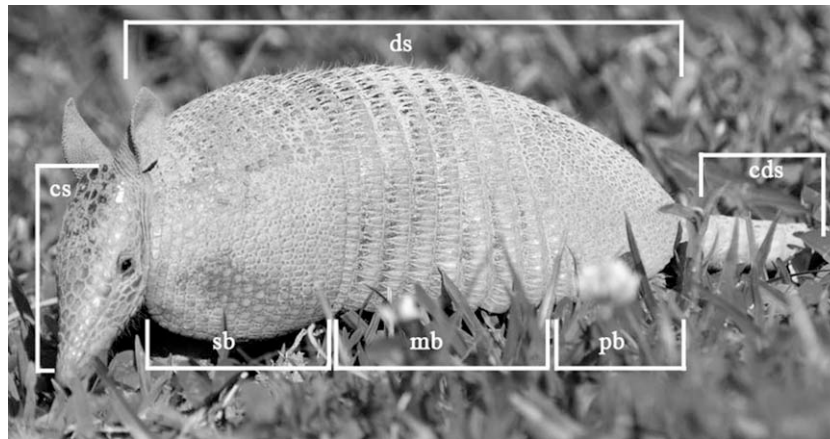


Fig. 1. Adult of *Dasypus hybridus*. Shows the head shield, portions of the dorsal shield and caudal shield. Abbreviations: cs, cephalic shield; cds, caudal shield; ds, dorsal shield; mb, movable bands; pb, pelvic buckler; sb, scapular buckler.

in which complex glandular systems are associated with pilose follicles, dermal ossifications, and cornified scales. Histologically, osteoderms are formed by compact bony tissue with primary and secondary osteons. Furthermore, they show concentric bony laminae around large cavities which host adipose tissue, pilose follicles, and sweat and sebaceous glands (Fernández 1931; Hill 2006; Krmpotic et al. 2009). Likewise, in such cavities, elements of red bony medulla were identified (Weiss and Wislocki 1956; Vickaryous and Hall 2006; Krmpotic et al. 2009). This histological pattern would be general for Dasypodidae; however, strong differences have been observed between *Dasypus* sp. (Dasypodinae) and *Chaetophractus villosus* (Euphractinae) (Ciancio et al. 2007; Krmpotic et al. 2009).

Some papers have dealt with certain aspects of the integument in young specimens, fetuses and embryos of *Dasypus novemcinctus* (Fernández 1922; Cooper 1930; Vickaryous and Hall 2006), and to a lesser extent, in *Chaetophractus villosus* (Fernández 1931). However, a comparative framework dealing with morphology and developmental chronology of the skin structures (dorsal and ventral) is yet to be studied. On the other hand, in order to clarify the way in which events occur during development of the compared structures, maturity of other tissues (e.g. skeletal, muscular, nervous systems) should be considered. In this way, we can identify events that have been pre- or post-displaced in the ontogenetic trajectory during evolution.

Armadillos show a peculiar combination of features in postcranial skeleton different from other mammals: a reduction in number of thoracolumbar vertebrae (Sánchez-Villagra et al. 2007; Galliani et al. 2010); supplementary or xenarthral intervertebral articulations on the posterior region of the dorsal vertebrae (Gaudin 1999);

synsacrum formed by the fusion of ilium and ischium to the sacral vertebrae and to a variable number of caudal vertebrae that become sacralized (Rose and Emry 1993; Szalay and Schrenk 1998); ossified sternal ribs and tibia-fibula represented by the fusion of the ends of these elements (Flower 1885).

Noteworthy, the axial skeleton, and even the pelvic girdle, shows different stages of structural relationships with the dorsal carapace; thus, additional information about the development of the postcranial skeleton is relevant to establish the overall maturity of the neonates.

The aim of this paper is to describe in a developmental and comparative framework the integumentary system of neonates of *Dasypus hybridus* and *Chaetophractus vellerosus*.

Material and methods

Three neonates 0–2 days old were used in this study, two of *Chaetophractus vellerosus* (PIMUZlab#2008.136; AAC-146) and one of *Dasypus hybridus* (JG-0209-D2), housed in the Department of Vertebrate Paleontology of the Museo de La Plata (Fig. 2). The specimens were obtained from farmers who hunt these species in their fields because they are considered harmful to agricultural practices, and keeps the viscera, neonates and juveniles for us to study (farmers record the capture and death dates). One specimen of *C. vellerosus* (PIMUZlab#2008.136) was partially disarticulated, and neither size measurements nor photographs could be taken. Neonates were fixed in formaldehyde 5% and preserved in ethanol 70%. Small portions (25–30 mm²) of the cephalic shield and different regions of the dorsal carapace of each specimen were taken. For decalcification purposes, some samples were immersed in Bouin

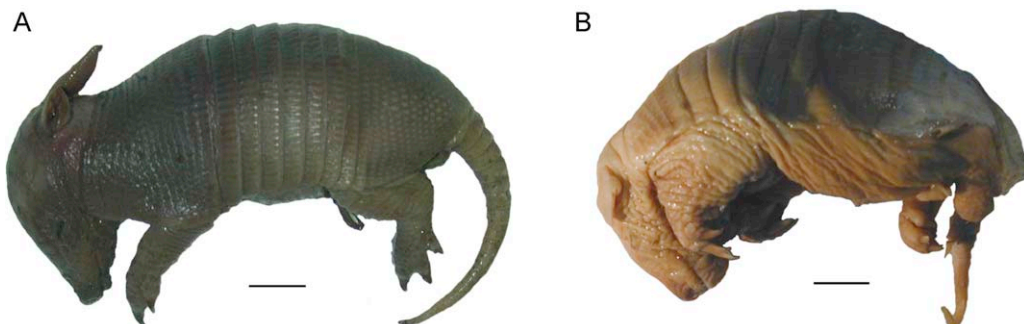


Fig. 2. Newborns used in this study. Left lateral view. (A) *Dasypus hybridus* JG-0209-D2 and (B) *Chaetophractus vellerosus* AAC-146.

solution for a week long period, and others with EDTA (2.8%) decalcifying solution for 5 days, solutions were daily renewed. Once decalcified, tissues were dehydrated with increasing concentrations of ethanol from 70% to 100%. Afterwards, materials were soaked in paraffin. More than 400 serial histological sections 3 μm thick were made, parallel to the sagittal plane. Serial sectioning allows a more reliable interpretation of the integumentary structures. In order to obtain as much as possible information from the different components of the skin, sections were stain with Haematoxylin & Eosin (H-E), Masson Trichrome, PAS, orcein and reticulin techniques. All the histological techniques were realized following the protocols described in Bancroft and Stevens (1990). To study postcranial elements, only two of the three specimens were used (Fig. 2). These specimens were processed through a clearing and double-staining technique, to show cartilage and bone. We followed a protocol modified from Dingerkus and Uhler (1977) described by Prochel (2006), which involves chemical treatment, including the use of trypsin for enzymatic clearing, staining with alcian blue to color cartilage, and alizarin red for bone. Determination of ossification centers and their progress was recorded through the early uptake of alizarin. Samples were observed with a Nikon SMZ645 stereomicroscope.

Terminology

Regarding pilose follicles, the terms primary and secondary pilose follicles are widely used in the literature on mammals. This terminology is quite confusing, and commonly refers only to differences in size. Furthermore, according to Cooper (1930), these terms imply not only differences in size, but also, to a certain extent, a sequential order. Consequently, and due to the arrangement of the pilose follicles in the osteoderms of Dasypodidae, we will use the terms marginal pilose follicles (MF), to designate those large follicles set on the posterior and lateral margins of the osteoderms, and surface pilose follicles (SF), for those small ones, emerging from the foramina of the external surface of the osteoderms. In the case of follicles of the ventral skin, in which osteoderms are lacking, the larger follicles were considered MF, and the smaller ones, SF (approximately one third of the first).

Results

Dasypus hybridus

Carapace

On the dorsal epidermis of this species, different strata can be distinguished from basal to external: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum (Fig. 3A and B). The stratum basale shows cubic or columnar keratinocytes with circular or oval nuclei, some of them with melanin granules (Fig. 3B). In addition, cells of pale cytoplasm, probably melanocytes, are identified. The overlying stratum spinosum shows polyhedral cells with rounded nuclei, and is formed by approximately three to four cellular layers that become compressed toward the surface (Fig. 3A and B). The stratum granulosum (Fig. 3B) is not continuous; cells with keratohyalin granules are differentiated mainly near the pilose follicles. This stratum is completely absent in the epidermis below the developing cornified scales (Fig. 3A). The stratum lucidum is only observed underlying the developing cornified scales. The cells have eosinophilic and refringent cytoplasm with very condensed nuclei (Fig. 3A). The stratum corneum has a variable thickness, scarcely stains with H-E, and has a yellowish color in the regions where the cornified scales develop. However, in areas where developing cornified scales are absent, it is quite eosinophilic (Fig. 3B). The basal

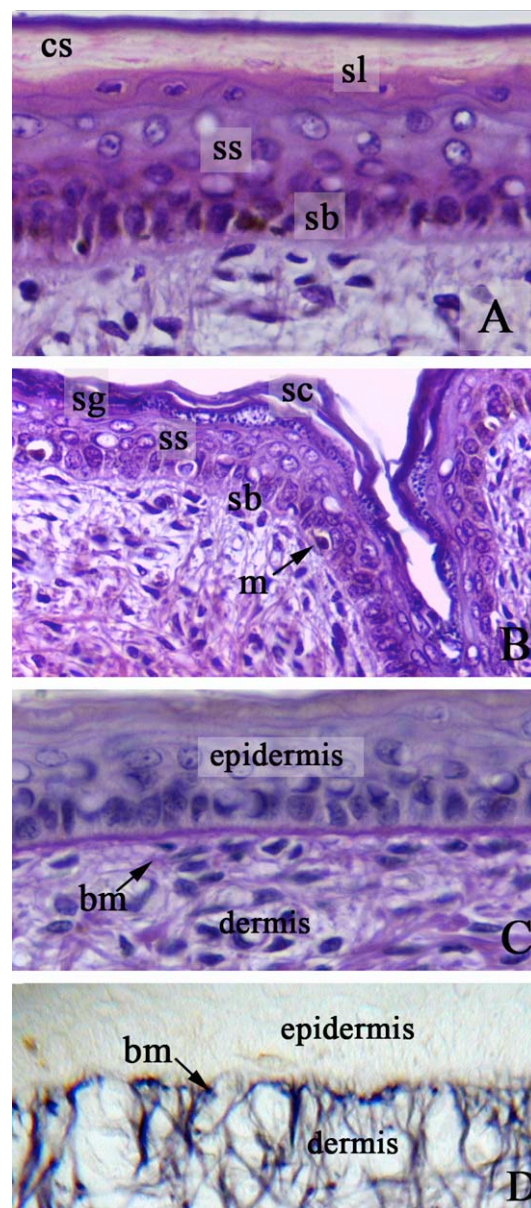


Fig. 3. *Dasypus hybridus*. Histological details of dorsal epidermis showing different strata. (A and B) H-E 40 \times . (C) PAS 40 \times . (D) Reticulin 40 \times . Abbreviations: bm, basal membrane; cs, corneal scale; m, melanocyte; sb, stratum basale; sc, stratum corneum; sg, stratum granulosum; sl, stratum lucidum; ss, stratum spinosum.

membrane becomes strongly evident with PAS and reticulin (Fig. 3C and D), which implies the presence of reticular fibers from the reticular lamina of the basal membrane; glycoproteins from the basal lamina (Fig. 3C).

The dermis is formed by two clearly different layers: the superficial layer, in contact with the basal membrane of the epidermis, and the deep underlying layer (Fig. 4A). The first is very rich in cells, shows less collagen bundles untidily arranged, though most of them perpendicular to the epidermis (Fig. 4B). In the deep layer, thick collagen bundles are seen parallel to the epidermis (Fig. 4C), and oriented craniocaudal and mediolaterally. In longitudinal sections of the mobile bands region, large neurovascular ingressions are seen in the dorsal part of the deep layer of the dermis, which continue in the superficial dermis among the primordia of the future osteoderms (Fig. 4A). PAS and reticulin techniques showed a large amount of reticular fibers in both layers, although they are richer in the superficial layer (Fig. 4D and E). Sections stained with

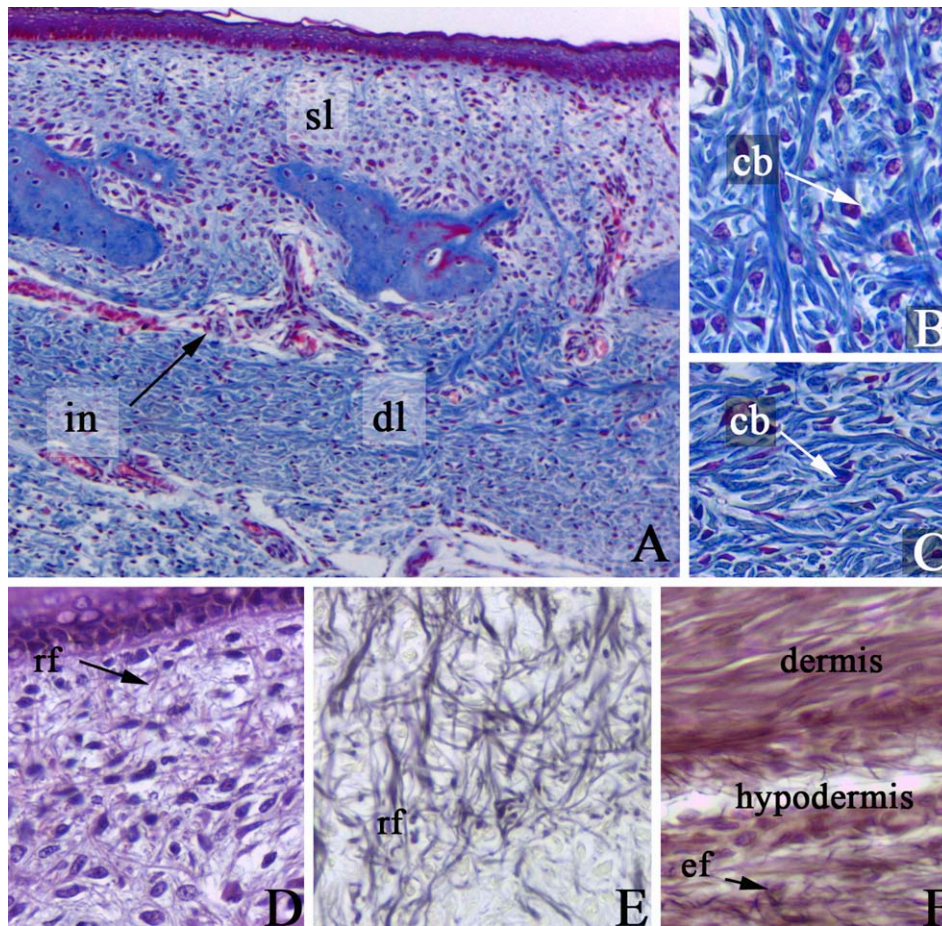


Fig. 4. *Dasypus hybridus*. Histological details of dorsal dermis. (A) Trichromic 10 \times . (B and C) Trichromic 40 \times . (D) PAS 40 \times . (E) Reticulin 40 \times . (F) Orcein 40 \times . Abbreviations: cb, collagen bundles; dl, deep layer; ef, elastic fibers; in, neurovascular ingressions; rf, reticular fibers; sl, superficial layer.

orcein showed scarce elastic fibers, which are more abundant in the hypodermis (Fig. 4F).

Regarding pilose follicles, both the MF and the SF are externally surrounded by a sheath of connective tissue, which is always laxer than the adjacent connective tissue (Fig. 5A and C). The MF are still immature and present associated sweat glands (Fig. 5A). In few sections an associated immature sebaceous gland was identified (Fig. 5A). The MF are composed by the external root sheath, the internal root sheath, and the hair shaft (Fig. 5A and B).

The external root sheath has two layers of large cells, which are scarcely colored with H–E (Fig. 5A). Between these cells and the external sheath of connective tissue lies the basal membrane, which is called glassy membrane. Internally, the internal root sheath (Fig. 5A and B) is formed by: the Henle layer, a single row of scaly cells with flattened nuclei, the Huxley layer, formed by polyhedral cells with flattened nuclei, and the cuticle. The hair shaft is formed by partially cornified cells (Fig. 5A). At the distal end of the MF the canal of the hair is closed while at the proximal end the pilose bulb surrounds the dermal papilla. Melanocytes are absent among the germinative cells of the bulb. The MF do not reach the hypodermis, and the pilose bulb is placed in the deep layer of the dermis. The apocrine sweat gland associated with the MF is well developed; its deep portion exceeds even the follicle length. Its distal (superficial) two thirds are straight, but the proximal end is rolled up. The secretory glandular unit is in the deep layer of the dermis and is formed by one internal layer of cubic cells surrounded by a basal membrane (the complex structure described here of this gland can be seen in serial sections).

Several SF are observed, all of them in equivalent developmental stages, associated with sweat and sebaceous glands (Fig. 5C and D). The external root sheath is similar to that of the MF, and is separated from the external sheath of connective tissue by the glassy membrane (Fig. 5D). The layers of the internal root sheath could not be differentiated. The hair shaft is formed by cornified cells (Fig. 5C). At the distal end the canal of the hair is closed. At its proximal end, germinative cells of the bulb are identified, and melanocytes are absent. The pilose bulb reaches the deep layer of the dermis. Associated to the SF there is a well developed sweat gland, which penetrates in the dermis deeper than the pilose follicle (Fig. 5D). The distal portion of the sweat gland, the duct, is straight, and the proximal portion, the secretory portion, is rolled up. Lateral evaginations are seen at both sides of the follicle, which correspond to developing sebaceous glands (Fig. 5C).

Osteoderm primordia shows bony tissue, and cartilaginous condensations were not observed in any case. They develop in the superficial layer of the dermis (Fig. 5E). A large amount of osteoblasts surround them (Fig. 5F). Osteoblasts that form the closest layers to the osteoderms are the largest. The ventral surface of the primordia is in contact with the deep layer (Fig. 5E). The long axis of the primordia is parallel to the epidermis, and is formed by trabeculae of regular bony tissue with numerous osteocytes. In sections stained with Masson's trichrome thick bundles of collagen fibers penetrate from the dermis into the osteoderm (Fig. 5F). Osteoderm primordia are PAS positive, whereas the periosteum is recognized with reticulin staining, because of the presence of numerous reticular fibers (Fig. 5G).

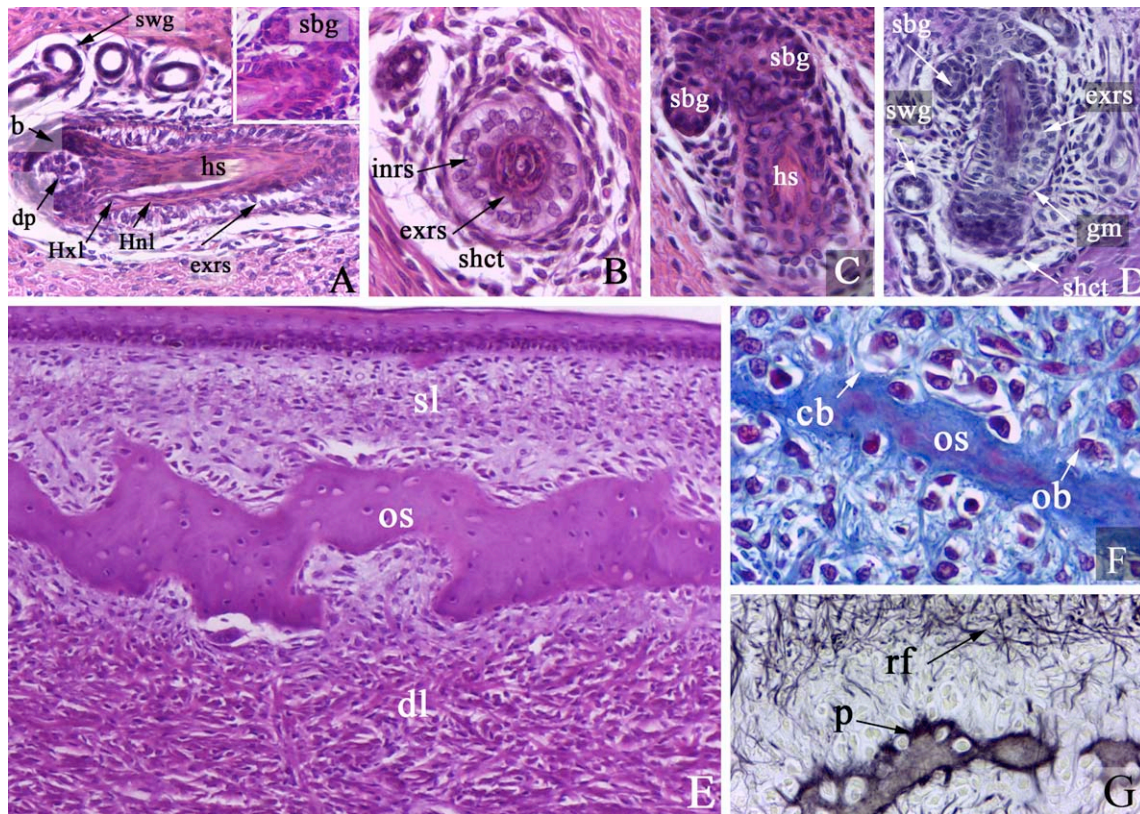


Fig. 5. *Dasypus hybridus*. (A and B) Histological details of marginal pilose follicles. (C and D) Histological details of superficial pilose follicles. (E–G) Histological details of the dermis with osteoderm primordia. (A–C) H–E 40 \times . (D) PAS 40 \times . (E) H–E 10 \times . (F) Trichrome 40 \times . (G) Reticulin 40 \times . **Abbreviations:** b, pilose bulb; dp, dermal papilla; exrs, external root sheath; gm, glassy membrane; Hnl, Henle layer; hs, hair shaft; Hxl, Huxley layer; inrs, internal root sheath; ob, osteoblasts; os, osteoderm primordium; p, periosteum; sbg, sebaceous gland; shct, sheath of connective tissue; swg, apocrine sweat gland; see also preceding figures.

Ventral skin

The ventral epidermis shows the same strata than the dorsal one (Fig. 6A and B). The stratum basale is formed by a layer of keratinocytes with rounded nuclei, some of which have melanin granules in the cytoplasm. Furthermore, cells with pale cytoplasm, probably melanocytes, are identified. The stratum spinosum is formed by four layers of cells. The stratum granulosum has a single layer of cells (Fig. 6B), and is only interrupted in the epidermis underlying the cornified scales. The stratum corneum is yellowish where cornified scales develop, and eosinophilic in between them.

The dermis cannot be clearly divided in layers, instead the dermis grades from superficial into deep (Fig. 6C). In the deep layer, collagen fibers are seen parallel to the epidermis. Orcein technique shows elastic fibers (Fig. 6D). The MF differs from SF in size, whereas MF is larger than SF (Fig. 7A). In the largest follicles the external sheath of connective tissue is well developed. The external root sheath (Fig. 7A) is a pluristratified layer of cells with oval euchromatic nuclei and pale cytoplasm, which extend in the proximal two thirds of the follicle. The internal root sheath with the Henle and Huxley layers may be observed. The hair shaft is formed by cornified cells (Fig. 7B). A large amount of melanocytes are seen in the pilose bulb (Fig. 7A). The sebaceous gland associated to this follicle has vacuolized cells (Fig. 7C). The sweat glands bear secretory units with a large lumen (Fig. 7D). Cross-sections of the pilose follicles show the external sheath of connective tissue, the glassy membrane, the external root sheath, formed by several layers, the internal root sheath, the hair shaft, and the medulla (Fig. 7E). In some cases the hair canal is open and the hair shaft emerges to the surface (Fig. 7B).

The smallest follicles are generally associated with a single sebaceous gland (in some cases two), and with very well developed sweat glands; in the latter their secretory units are located deeper, near the dermal papilla (Fig. 7F).

Chaetophractus vellerosus

The major differences between *Dasypus hybridus* and *Chaetophractus vellerosus* neonates are summarized in Tables 1 and 2.

Carapace

Four strata are differentiated in the dorsal epidermis: stratum basale, spinosum, granulosum, and corneum. The stratum basale shows cubic keratinocytes with circular nuclei and cytoplasmic melanin granules, and melanocytes (Fig. 8A). Above the stratum spinosum shows polyhedral cells with rounded nuclei (three to four layers). The stratum granulosum is formed by a single (Fig. 8A) layer of flattened cells with granules of keratohyalin (discontinuous in small areas), and is always more conspicuous (up to two layers) near the pilose follicles. The stratum lucidum is absent. The stratum corneum is well developed and eosinophilic (Fig. 8A). No developing cornified scales are observed. The basal membrane is evidenced by reticulin and PAS techniques (Fig. 8B and C).

The dermis shows no clear-cut differentiation in superficial and deep layers (Fig. 8D). PAS and reticulin techniques show a large amount of reticular fibers, especially on the dermis immediately below the epidermis (Fig. 8B and C), and elastic fibers are also present (Fig. 8E).

Several MF are observed in advanced developmental stages, with associated sebaceous glands, surrounded by a sheath of

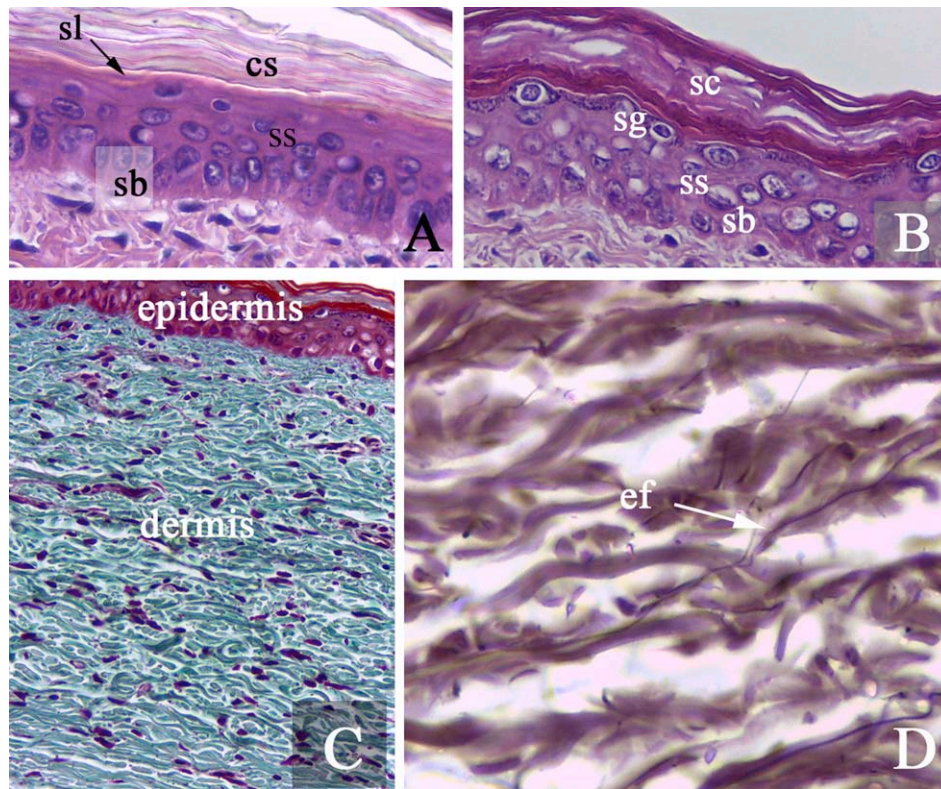


Fig. 6. *Dasypus hybridus*. Histological details of ventral epidermis and dermis. (A and B) H–E 40×. (C) Trichromic 10×. (D) Orcein 40×. *Abbreviations:* see preceding figures.

Table 1
Main differences in the epidermis and dermis of the dorsal integument between *D. hybridus* and *C. vellerosus*.

Dorsal integument					
Epidermis and dermis					
	Stratum corneum	Stratum lucidum	Stratum granulosum	Dermis	Osteoderm primordia
<i>Dasypus hybridus</i>	Eosinophilic in between developing corneal scales. Yellowish zones corresponding to developing corneal scales.	Present, underlying developing corneal scales.	Single layer of cells, except in zones surrounding pilose follicles (two layers). Absent in epidermis underlying developing corneal scales.	Mature, superficial and deep strata well differentiated. Large amount of collagen fibers, reticular fibers, and scarce elastic fibers.	Present
<i>Chaetophractus vellerosus</i>	Eosinophilic. No evidence of developing corneal scales.	Absent	Single continuous layer, except in zones surrounding pilose follicles (two layers)	Immature. Large amount of collagen, reticular, and elastic fibers.	Absent

Table 2
Main differences in the follicles and associated glands of the dorsal integument between *D. hybridus* and *C. vellerosus*.

Dorsal integument		
Follicles and associated glands		
	Marginal pilose follicles	Surface pilose follicles
<i>Dasypus hybridus</i>	The hair shaft consists of cornified cells with visible nuclei, absent melanocytes, and closed hair canal. The internal radicular sheath is composed by large cells, and well differentiated layers. In association with sweat glands that show a straight distal (superficial) segment (two thirds of the length), and a rolled up proximal segment.	Immature; hair shaft partially cornified, without melanocytes. External radicular sheath composed by large cells. Internal radicular sheath undifferentiated. In association with immature sebaceous glands. Sweat glands rolled up only in the proximal segment.
<i>Chaetophractus vellerosus</i>	Cornified hair shaft, large amount of melanocytes, and open hair canal. Internal radicular sheath with small cells, and well differentiated layers. In association with immature sebaceous glands only.	Immature; hair shaft cornified, large amount of melanocytes. External radicular sheath with small cells. Internal radicular sheath with well differentiated layers. In association with mature sebaceous glands with vacuolized cells. Sweat glands are rolled up in the medial and proximal segments.

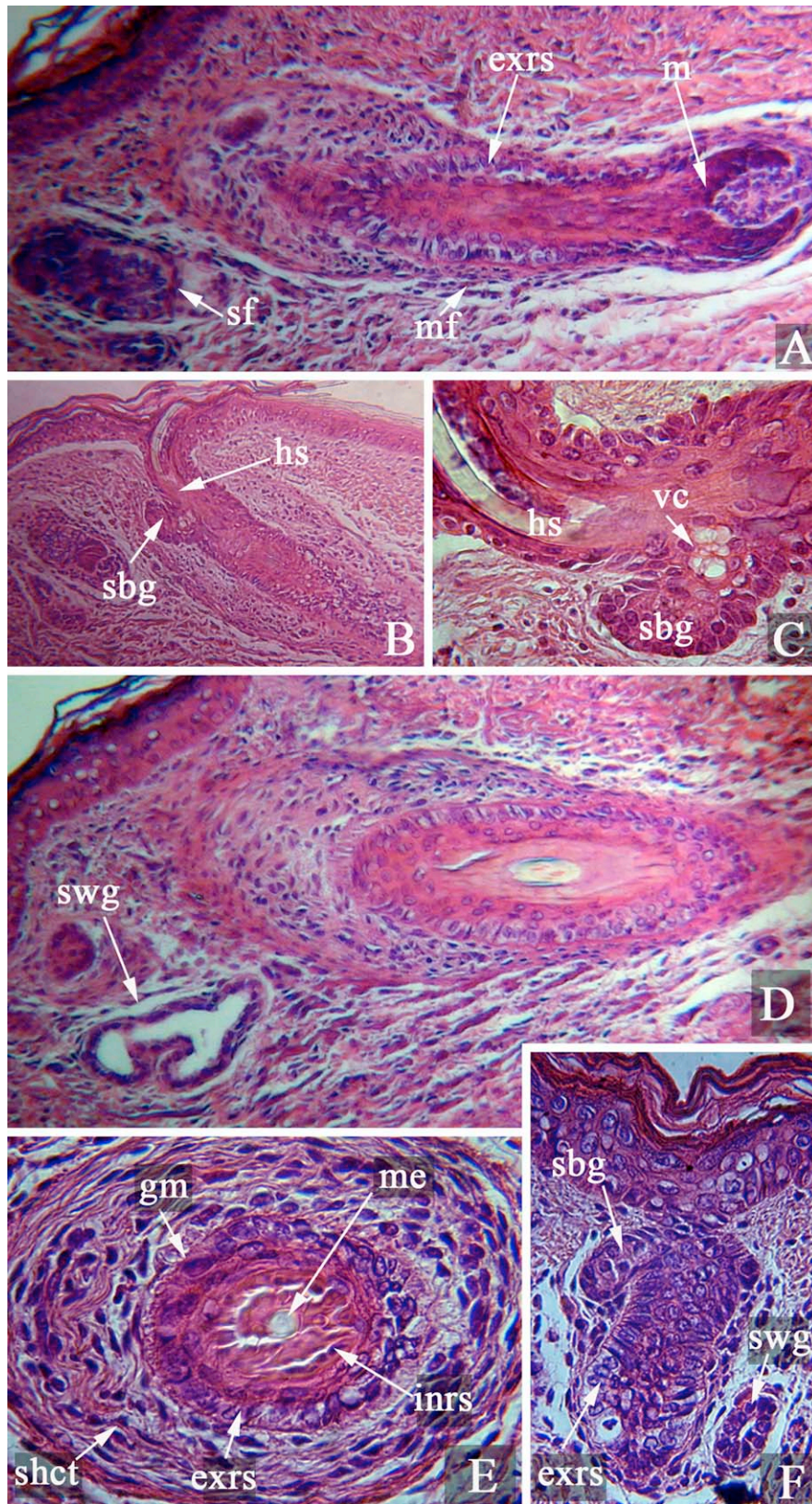


Fig. 7. *Dasypus hybridus*. Histological details of marginal and superficial pilose follicles of ventral integument. (A and B) H–E 10 \times . (C) H–E 40 \times . (D) H–E 10 \times . (E) H–E 40 \times . (F) H–E 40 \times . Abbreviations: me, medulla; mf, marginal follicle; sf, superficial follicle; vc, vacuolated cells; see also preceding figures.

connective tissue (Fig. 8F). Each MF shows an external root sheath, with two layers of small, quadrangular cells, and an internal root sheath (Fig. 8G). In the latter, the Henle layer (formed by a single layer of flattened cells), the Huxley layer (formed by

two layers of larger cells with circular nuclei), and the cuticle, are present (Fig. 8G). The hair shaft is completely cornified (Fig. 8F). At the distal end, the canal of the hair is open and the hair shaft emerges. At the proximal end the pilose bulb shows

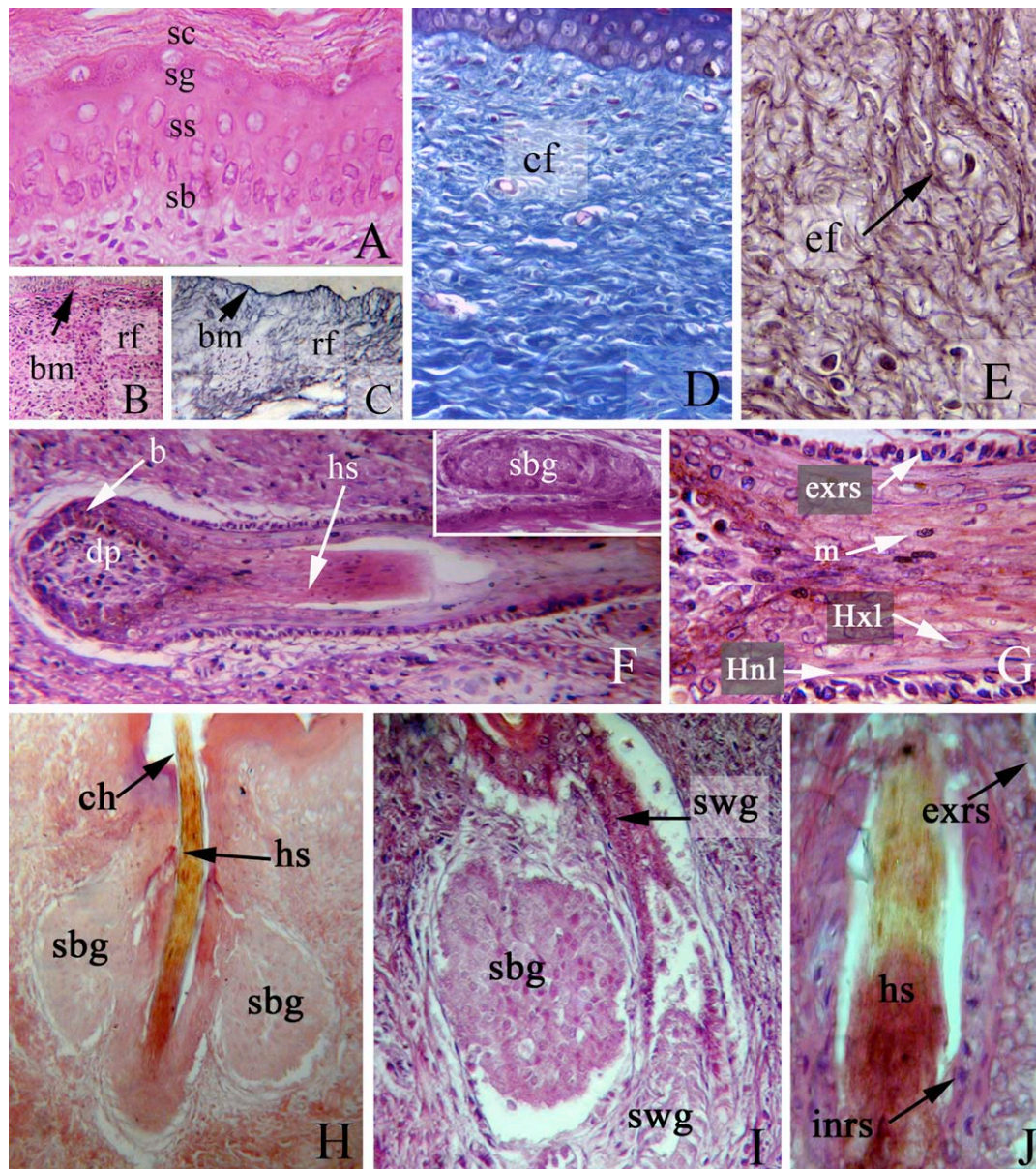


Fig. 8. *Chaetophractus vellerosus*. (A–E) Histological details of epidermis and dermis. (F and G) Histological details of marginal pilose follicles. (H–J) Histological details of superficial pilose follicles. (A) H–E 40 \times . (B) PAS 10 \times . (C) Reticulin 10 \times . (D) Trichomic 40 \times . (E) Orcein 10 \times . (F) H–E 10 \times . (G) H–E 40 \times . (H) Orcein 10 \times . (I and J) H–E 40 \times . *Abbreviations:* ch, canal of the hair; see also preceding figures.

germinative cells and a large amount of melanocytes (Fig. 8G), and the pre-medullar and pre-cortical epithelia are identified. Between the sheath of connective tissue and the external root sheath lies the glassy membrane. The dermal papilla is surrounded by the pilose bulb (Fig. 8F), which reaches the deep part of the dermis. A small sebaceous gland is observed at the distal end of these MF (Fig. 8F).

The SF are associated with one sweat gland and two sebaceous glands (Fig. 8H and I). Externally, the glassy membrane develops. In the SF the external and internal root sheaths are identified. The hair shaft is completely cornified (Fig. 8H and J). The canal of the hair is open with an emerging hair shaft (Fig. 8H). The sweat gland is extremely developed, surpassing the length of the SF (Fig. 8I). It has a straight distal portion but its middle and proximal portion immediately rolls (Fig. 8I). The well developed sebaceous glands rest at both sides of the pilose follicle, and have many vacuolized cells (Fig. 8H and I). The connection with the SF is ventral to the opening of the sweat

gland. The sebaceous glands extend up to the level of the pilose bulb.

Neither osteoderm primordia nor condensation of osteoprogenitor cells are identified at neonatal stage.

Ventral skin

Like the dorsal skin, the ventral epidermis is formed by four strata (Fig. 9A). The stratum basale shows keratinocytes with rounded nuclei. The stratum spinosum is four layers thick with cubic cells that become flattened at the superficial layers. The stratum granulosum is continuous, well differentiated, and formed by one to three layers of cells. The underlying basal membrane is very rich in reticular fibers as shown in Fig. 9B. The most superficial dermis is somewhat more cellular than the deepest one, and a large amount of collagen and reticular fibers are identified (Fig. 9C). Pilose follicles are organized in sets of approximately six units (Fig. 9D). Three of them are MF, distinguished by their large size; the rest would be SF. They show the external and internal root sheaths well

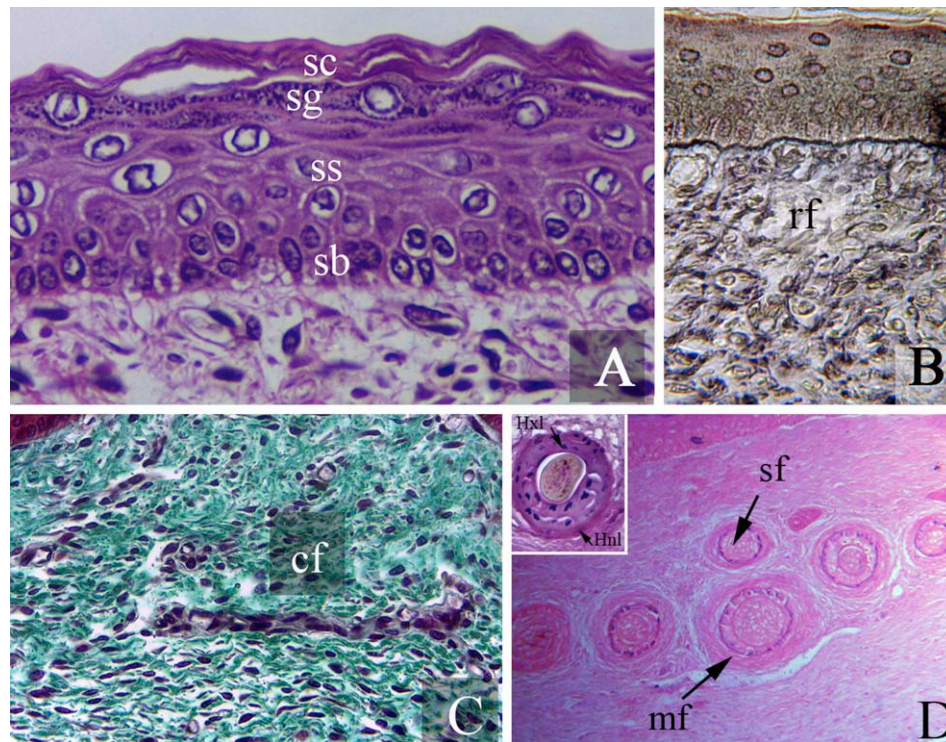


Fig. 9. *Chaetophractus vellerosus*. (A–C) Histological details of ventral epidermis and dermis. (D) Histological details of ventral dermis with marginal and superficial pilose follicles. (A) H–E 40 \times . (B) Reticulin 10 \times . (C) Trichromic 10 \times . (D) H–E 10 \times . *Abbreviations:* see preceding figures.

differentiated (Fig. 9D). Each follicle is associated with a sebaceous gland, but no sweat glands could be identified.

Dasypus hybridus and *Chaetophractus vellerosus*

Postcranial skeleton

Vertebral column. In both species the ossifications of the vertebrae have already started along the whole column. Nevertheless, ossification is more advanced in *D. hybridus* than in *C. vellerosus* (Figs. 10 and 11). This is shown in the cervical region (Figs. 10A and 11A), where in *D. hybridus* the ossifications of the neural arches at both sides of the vertebrae are closer than in *C. vellerosus*. A similar pattern is observed along the thoracic, lumbar and sacral regions. In fact, the last vertebrae from the thoracic region of *D. hybridus* have their neural arches almost complete. It is noteworthy that in the last 3–4 vertebrae of the presumptive synsacrum, and first caudals, an extra ossification center appears at both sides of each element (Figs. 10A and 11A). At this ontogenetic stage no fusion between vertebral elements and pelvic girdle is observed. In the caudal region of both species all the vertebral bodies are ossified at birth.

Ribs and sternum. Vertebral ribs are entirely ossified in neonates of both species. Nevertheless, sternal ribs show no ossifications (Figs. 10B and 11B). Ventrally, the sternum of *D. hybridus* is partially ossified. The omosternum has one advanced ossification center; posteriorly, there are two ossification centers on the midline of the two first mesosternal elements. The xiphisternum has one central ossification (Fig. 10B). In *C. vellerosus*, the omosternum has one central ossification, less advanced than in *D. hybridus*. The first mesosternal element has two ossification centers clearly separated on the midline, whereas the second has a single center at one side, not central. Finally, the xiphisternum has a single central ossification (Fig. 11B). The rest of the sternum is not ossified.

Girdles and limbs. Both in *D. hybridus* and *C. vellerosus* ossification has started in the body of the scapula and clavicle. Likewise, ossification centers have been recorded in the diaphyses of the

elements corresponding to stylo- and zeugopodium. All the carpal elements of the autopodium of *D. hybridus* are cartilaginous. Ossification has started in all metacarpals and phalanges, except for those of the fifth finger. In *C. vellerosus*, all metacarpals and phalanges have started ossification; however, carpal elements remain cartilaginous. In the pelvic girdle of both species the main elements began ossification, although, as shown in Figs. 10B and 11B, the ossifications are more advanced in *D. hybridus* than in *C. vellerosus*. Ilium and ischium are almost in contact in *D. hybridus*, whereas in *C. vellerosus* they are widely separated. The elements of the stylo- and zeugopodium of the hindlimb began ossification in both species. In the autopodium both the astragalus and calcaneum are ossified, whereas the remaining tarsals are still cartilaginous. Metatarsals and phalanges of all fingers are ossified, except for the fifth proximal phalanx of *D. hybridus*.

Discussion

In this paper we focused on a comparative morphological description between different structures of the dorsal and ventral skin of neonates from *Dasypus hybridus* and *Chaetophractus vellerosus*, which have never been described in this ontogenetic stage. Likewise, this is the first comparative study of the integumentary system (dorsal and ventral skin) in armadillos, which identifies interspecific differences in the maturity of tissues at birth. Histological results are compared to those reported in previous papers for other species and ontogenetic stages. It is important to highlight that only three specimens have been used in this study and conclusions should be interpreted cautiously.

Different papers have dealt partially with the ontogeny of the integumentary system of *Dasypus novemcinctus* (e.g. Fernández 1922; Cooper 1930; Vickaryous and Hall 2006). Vickaryous and Hall (2006) described the changes observed in the epidermis and dermis of the dorsal integument, in three embryonic stages, but they only treated superficially other integumentary associated structures. They focused, as well as Fernández (1922), on osteoderm

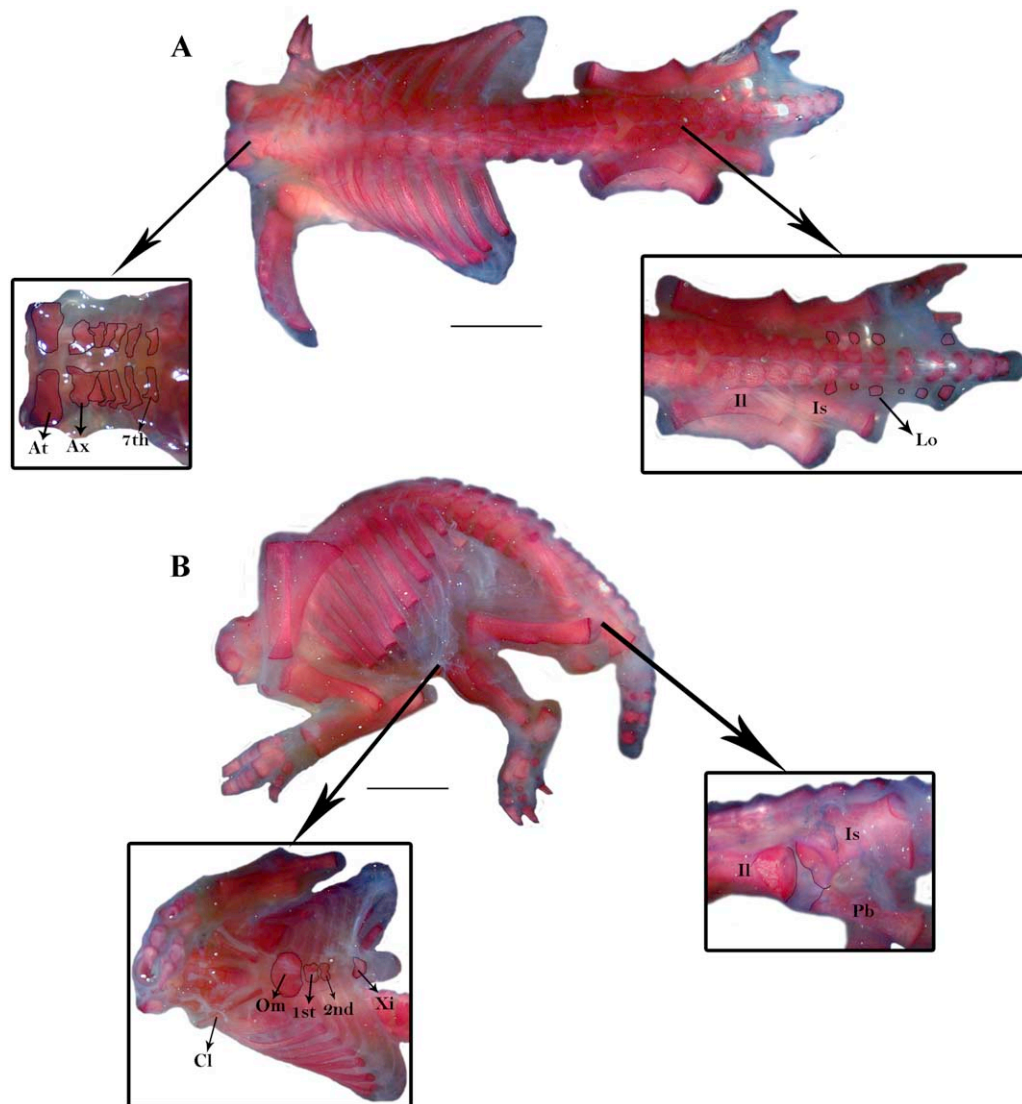


Fig. 10. Postcranial ossifications in *Dasypus hybridus* neonate. (A) Dorsal view; left arrow: magnification of the cervical region, right arrow: magnification of the sacroischial and more proximal caudal region. (B) Left lateral view; left arrow: magnified ventral view of the thorax, right arrow: magnified pelvic girdle corresponding to the right side; the latter image was flipped horizontally to obtain the specular homolog for better exposition purposes. *Abbreviations:* 1st, first mesosternal element; 2nd, second mesosternal element; 7th, seventh cervical vertebrae; At, atlas; Ax, axis; Cl, clavicle; Il, ilium; Is, ischium; Lo, lateral ossification; Om, omosternum; Pb, pubis; Xi, xiphisternum.

development. The ontogeny of the pilose follicles and the glandular structures of the dorsal and ventral skin have been described mainly by Cooper (1930) and specifically in *Chaetophractus villosus* by Fernández (1931). Regarding juvenile specimens, only Cooper (1930) studied the histology of the integument of a neonate from *D. novemcinctus* and of a 1-week-old specimen. Adults from this species are partially described by Hill (2006) and Vickaryous and Hall (2006). In *C. villosus*, adult specimens have been described by Fernández (1931) and Krmpotic et al. (2009).

With respect to the epidermis of the neonates studied here, the stratum granulosum of the ventral skin is much more developed than the dorsal. Likewise, it is absent in the epidermis underlying the developing cornified scales of *Dasypus hybridus*. It is noteworthy that in the dorsal skin of the neonate of *C. vellerosus* (without developing cornified scales) the stratum granulosum is almost continuous. The stratum lucidum was observed under the developing scales, which are only present in *D. hybridus* neonate.

A large amount of reticular fibers were seen in the dermis of *D. hybridus* and *C. vellerosus*. Elastic fibers are scarce in the dorsal integument and proportionally more abundant in the ventral

skin of both species. This result agrees with those found in fetuses of *Dasypus novemcinctus* (Vickaryous and Hall 2006), pangolins (Meyer et al. 2010), and reptiles with osteoderms (Vickaryous and Hall 2008), which demonstrates that the absence of these fibers is related to dermal rigidity.

The dorsal dermis of neonates from *D. hybridus* is clearly divided into superficial and deep layers, as in fetuses from *Dasypus novemcinctus* (Vickaryous and Hall, 2006). Alternatively, in *C. vellerosus* these layers could not be identified, which suggests a less connective tissue differentiation at this stage.

Osteoderms are differentiated in *D. hybridus*. In *C. vellerosus*, however, there are no traces of osteoprogenitory cell condensations whatsoever that could suggest the beginning of osteoderm formation. Vickaryous and Hall (2006) stated that before birth, osteoderms of *D. novemcinctus* have already began formation in each shield. In agreement with our observations in *C. vellerosus*, Fernández (1931) was not able to identify dermal ossifications in neonates of *C. villosus*. Concerning the mechanism of osteogenesis, Hill (2006) proposed, on the basis of previous works and his own observations in adults of *D. novemcinctus* and several fossil

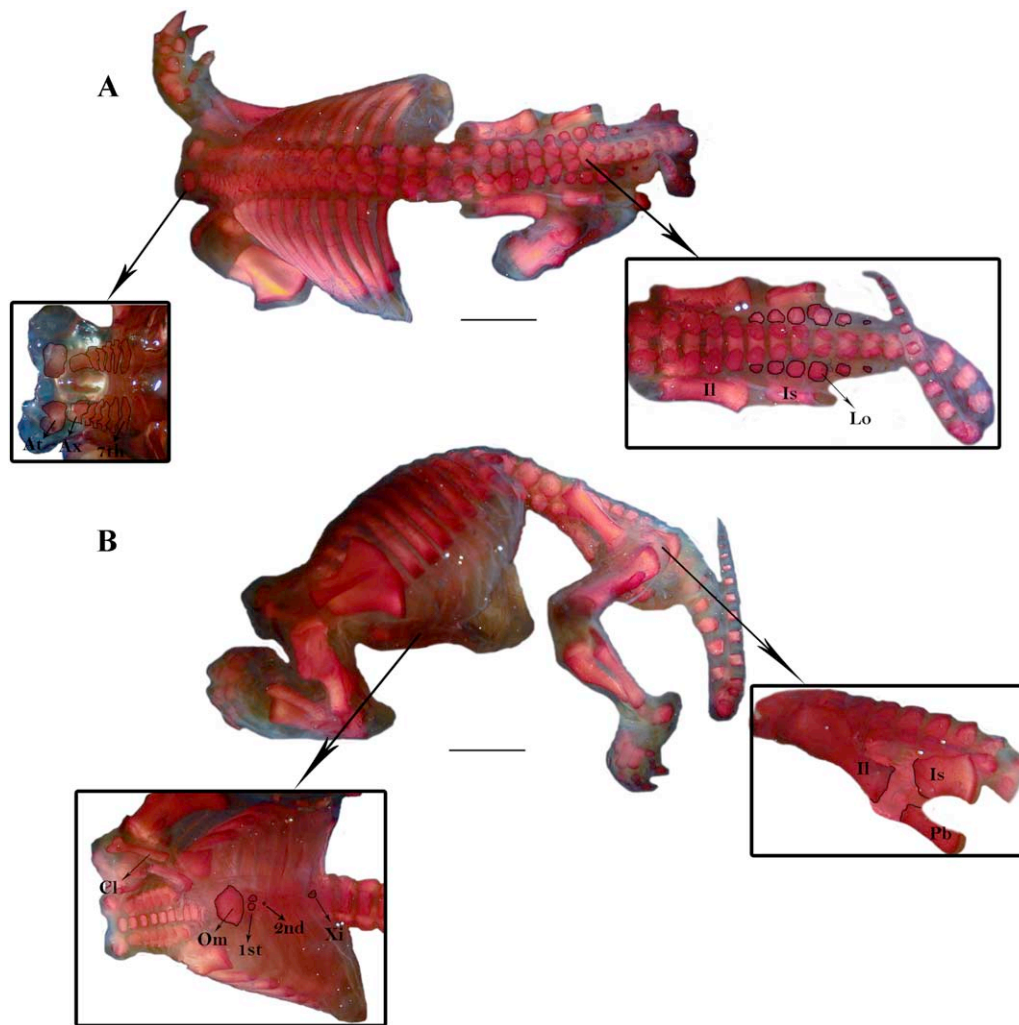


Fig. 11. Postcranial ossifications in *Chaetophractus vellerosus* neonate. (A) Dorsal view; left arrow: magnification of the cervical region, right arrow: magnification of the sacroischial and more proximal caudal region. (B) Left lateral view; left arrow: magnified ventral view of the thorax, right arrow: magnified pelvic girdle corresponding to the right side; the latter image was flipped horizontally to obtain the specular homolog for better exposition purposes. *Abbreviations:* see Fig. 10.

species, that osteoderms develop through metaplastic ossification. In this mechanism, bony tissue is formed without the activity of a periosteum in a strict sense. Dense fibers of connective tissue transform directly into bony tissue without previous differentiation into osteoblasts (Moss 1969; Vickaryous and Sire 2009). Alternatively, other contributions (Vickaryous and Hall 2006; Vickaryous and Sire 2009) claim that ossification of osteoderms is intramembranous. This implies that osteogenesis begins inside the superficial stratum of the dermis, as a cell aggregation that differentiates into osteoblasts, which start secreting osteoid. With osteoblasts growth, the bundles of collagen are trapped or incorporated (Vickaryous and Sire 2009) into the osteoderm. Although in *Dasypos* osteoderms are already formed, a large amount of osteoblasts are seen, which are not present in metaplastic ossifications (Moss 1969; Vickaryous and Sire 2009). Hence, we believe ossification is intramembranous. In *Chaetophractus vellerosus*, however, osteoderm primordia are absent therefore it is impossible to identify the ossification mechanism in the studied specimens.

Regarding the pilose follicles, MF and SF are distinguished. In *D. hybridus* sebaceous glands are sometimes associated with MF while sweat glands are always present. Alternatively, sebaceous and sweat glands are associated with SF. In *C. vellerosus*, also both glandular types are associated with the SF, but contrarily, only sebaceous glands are associated with MF. As mentioned above, MF and

SF are much more developed in *C. vellerosus*. Vickaryous and Hall (2006) made no distinction among different types of pilose follicles, and observed only sebaceous glands in association. On the contrary, Cooper (1930) stated that sweat glands develop before sebaceous glands and are associated both with primary follicles (MF in this paper) and secondary follicles (SF). This author described pilose follicles and associated glands of a neonate from *D. novemcinctus*, which show a similar developmental pattern to that observed in this paper for *D. hybridus*.

Little attention has been focused on the development of postcranial skeleton for *D. hybridus*. Fernández (1915) while referring to early embryos and fetuses, enumerates the appearance of *anlagen* of some skeletal elements such as vertebral bodies, ribs, sternum, girdles, stylopodium and zeugopodium. He also referred to cartilaginous stages of some of these elements. In *C. vellerosus*, no previous references for this subject are reported. In a recent paper, Hautier et al. (2011) deal with postcranial skeletal ossifications in armadillos, *Dasypos novemcinctus* specifically, but they only analyzed prenatal data.

Both specimens show ossification of all the vertebral ribs and partially ossified sternum; however, sternal ribs are not yet ossified. This is relevant because adult armadillos have a thoracic cavity formed by completely ossified ribs and sternum (Flower 1885; Rose and Emry 1993). On the pelvic girdle, no fusion between elements

of the vertebral column with the ilium and ischium are observed, having in mind that these armadillos have a synsacrum when adults (Flower 1885; Rose and Emry 1993). However, lateral ossifications are observed at the level of postsacral vertebrae, which in turn will become fused to the girdle. These ossifications are likely to be included within the synsacrum, and hence, this would be formed not only by vertebrae and girdle elements, but also by independent ossifications lateral to the vertebral series. Regarding the elements forming the forelimbs, there are some differences between both genera. In *Dasypus* the distal carpals 1 and 2 are separated, whereas in *Chaetophractus* they are fused. In adult *Dasypus novemcinctus* the fifth finger is reduced to a small vestigial element (Schulthess 1920; Costa and Vizcaíno 2010). In the neonate of *D. hybridus* no ossifications are recorded here, but there are two cartilaginous elements. In *Chaetophractus vellerosus* the five fingers are well developed. If we bear in mind that the reduced structures begin their formation in late stages of development (Alberch et al. 1979), the delayed ossification of the fifth finger in *D. hybridus* with respect to the remaining fingers, would be related to its reduction.

Conclusions

The stratum granulosum is never related to developing cornified scales in the epidermis of neonates of *Dasypus hybridus*. In *C. vellerosus* the cornified scales have not yet started their development, and hence it cannot be determined whether this stratum will be lost. A future contribution should focus on the study of the stratum granulosum in different ontogenetic stages of both species in relation to developing cornified scales.

In *D. hybridus* osteoderms are partially developed at birth, whereas in *C. vellerosus* no osteoderms traces were found, not even osteoblast condensations. Previous authors (Vickaryous and Hall 2006; Vickaryous and Sire 2009) claim that osteoderms of Dasypodidae are formed through an intramembranous ossification. According to this mechanism, dermal ossifications need an “extrinsic support” to begin their development. From this standpoint, the delay in the appearance of osteoderms in neonates of *C. vellerosus* respect to those of *D. hybridus* would be related to the different degree of maturation of the dermis. On the contrary, pilose follicles and glandular tissues are less developed in *D. hybridus*.

Asynchronies between neonates of both species observed in the integumentary and postcranial skeletal tissues could match with adaptive strategies related to distribution in different environments, or to different postnatal care. Neonates of *Dasypus novemcinctus*, dasypodinae congeneric with *D. hybridus* “. . . are fully formed at birth, with eyes open and with a complete though not very hard armor. They are able to walk in a more or less uncertain fashion within a few hours after birth.” (sic Newman 1913). Instead, young *Chaetophractus villosus*, euphractine congeneric of *C. vellerosus*, open their eyes between 16 and 30 days after birth, and they scarcely drag themselves looking for suckle at birth (Olocco-Diz and Duggan 2004). The maturity degree of the neonates observed at osteoderms and postcranial ossifications level seems to be closely related to its locomotion capabilities and hardness of carapaces (Newman 1913; McBee and Baker 1982; Layne 2003; Olocco-Diz and Duggan 2004). In this way, considering these characters and their relationships with the definition of the altricial–precocial spectrum of previous works (Derrickson 1992), *D. hybridus* may be considered as a precocial species and *C. vellerosus* as essentially altricial. However, the maturity of the pilose follicles in *C. vellerosus* is not considered a character that reflects altriciality (Derrickson 1992). In this sense, differences in the development of the pilosity of neonates for these species may be related to environmental conditions of their habitats. Geographical distribution of both species is different: *Dasypus hybridus* inhabits more temperate climates,

whereas *Chaetophractus vellerosus* is recorded in arid to semi-arid ones, with broader thermal ranges (Abba and Cassini 2008).

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References

- Abba, A.M., Cassini, M.H., 2008. Ecology and conservation of three species of armadillos in the Pampean region, Argentina. In: Vizcaíno, S.F., Loughry, W.J. (Eds.), *The Biology of the Xenarthra*. University of Florida Press, Florida, pp. 300–305.
- Alberch, P., Gould, S.J., Oster, G.F., Wake, D.B., 1979. Size and shape in ontogeny and phylogeny. *Paleobiology* 5, 296–317.
- Ameghino, F., 1889. Contribución al conocimiento de los mamíferos fósiles de la Republica Argentina. In: *Actas de la Academia Nacional de Ciencias de Córdoba* 6: 1027 y Atlas: 98 lams.
- Bancroft, J.D., Stevens, A., 1990. *Theory and Practice of Histological Techniques*, 3rd ed. Churchill Livingstone, Edinburgh/London/Melbourne/New York.
- Carlini, A.A., Scillato-Yané, 1996. *Chorobates recens* (Xenarthra, Dasypodidae) y un análisis de la filogenia de los Euphractini. *Rev. Museo La Plata (NS) Paleontol.* 9, 225–238.
- Carlini, A.A., Ciancio, M.R., Flynn, J.J., Scillato-Yané, G.J., Wyss, A.R., 2009. The phylogenetic and biostratigraphic significance of new armadillos (Mammalia, Xenarthra, Dasypodidae, Euphractinae) from the Tinguirirican (Early Oligocene) of Chile. *J. Syst. Palaeontol.* 7, 489–503.
- Ciancio, M.R., Krmpotic, C.M., Carlini, A.A. and Barbeito, C., 2007. Morfología interna de los osteodermos de Dasypodinae y Euphractinae (Xenarthra, Dasypodidae). *Actas III Congreso de Mastozoología de Bolivia. Resúmenes*: 50.
- Ciancio, M.R., Carlini, A.A., 2008. Identificación de ejemplares tipo de Dasypodidae (Mammalia, Xenarthra) del Paleógeno de Argentina. *Rev. Mus. Arg. Cienc. Nat.* 10, 221–237.
- Cooper, Z.K., 1930. A histological study of the integument of the armadillo, *Tatusia novemcincta*. *Am. J. Anat.* 45, 1–37.
- Costa, F.R., Vizcaíno, S.F., 2010. A diagnostic character revisited: is there a fifth toe in the forefoot of *Dasypus novemcinctus* (Xenarthra: Dasypodidae)? *Zootaxa* 2671, 61–64.
- Derrickson, E.M., 1992. Comparative reproductive strategies of altricial and precocial eutherian mammals. *Funct. Ecol.* 6, 57–65.
- Dingerkus, G., Uhler, L.D., 1977. Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. *Stain Technol.* 52, 229–232.
- Fernández, M., 1915. Die Entwicklung der Mulita. La embriología de la Mulita (*Tatusia hybrida* Desm.). *Rev. del Museo de La Plata XXI*, 1–519.
- Fernández, M., 1922. Sobre la histogénesis y filogenia de la caparaza ósea de desdentados. In: *Publicaciones de la Junta para el homenaje a Cajal* (Ed.), Libro en honor de D. S. Ramón y Cajal. Tomo II, Madrid, pp. 385–406.
- Fernández, M., 1931. Sobre la anatomía microscópica y embriología de la coraza de *Dasypus villosus*. *Actas de la Academia Nacional de Ciencias de la Rep. Argentina X*, 61–121.
- Flower, W.H., 1885. *An Introduction to the Osteology of the Mammalia*. Macmillan and Co., London.
- Galliari, F.C., Carlini, A.A., Sánchez-Villagra, M.R., 2010. Evolution of the axial skeleton in armadillos (Mammalia, Dasypodidae). *Mamm. Biol.* 75, 326–333.
- Gaudin, T.J., 1999. The morphology of xenarthrous vertebrae (Mammalia: Xenarthra). *Fieldiana: Geology New Series* 41, 1–38.
- Hautier, L.J., Weisbecker, V., Goswami, A., Knight, F., Kardjilov, N., Asher, R.J., 2011. Skeletal ossification and sequence heterochrony in xenarthran evolution. *Evol. Dev.* 13 (5), 460–476.
- Hill, R.V., 2006. Comparative anatomy and histology of xenarthran osteoderms. *J. Morphol.* 267, 1441–1460.
- Krmpotic, C.M., Ciancio, M.R., Barbeito, C., Mario, R.C., Carlini, A.A., 2009. Osteoderm morphology in recent and fossil euphractinae xenarthrans. *Acta Zool. (Stockholm)* 90, 339–351.
- Layne, J.M., 2003. Armadillo: *Dasypus novemcinctus*. In: Feldhamer, G.A., Thompson, B.C., Chapman, J.A. (Eds.), *Wild Mammals of North America. Biology, Management, and Conservation*. 2nd ed. The Johns Hopkins University Press, Baltimore/London, pp. 75–97.
- McBee, K., Baker, R.J., 1982. *Dasypus novemcinctus* Mammalian Species 162, *Dasypus novemcinctus*, pp. 1–9.
- Meyer, W., Liumsiricharoen, M., Hornickel, I., Suprasert, A., Schnapper, A., Fleischer, L.G., 2010. Demonstration of substances of innate immunity in the integument of the Malayan pangolin (*Manis javanica*). *Eur. J. Wildl. Res.* 56, 287–296.
- Moss, M.L., 1969. Comparative histology of dermal sclerifications. in reptiles. *Acta Anat.* 73, 510–533.

- Newman, H.H., 1913. The natural history of the ninebanded armadillo of Texas. *Am. Nat.* 47 (561), 513–539.
- Olocco-Diz, M.J., Duggan, A., 2004. The first hand-rearing of larger hairy armadillos (*Chaetophractus villosus*) at the Temaikèn Foundation. *Edentata* 6, 27–30.
- Prochel, J., 2006. Early skeletal development in the mole *Talpa europaea*. *Zool. Sci.* 23, 427–434.
- Rose, K.D., Emry, R.J., 1993. Relationships of Xenarthra, Pholidota, and fossil “Edentates”: the morphological evidence. In: Szalay, F.S., Novacek, M.J., McKenna, M.C. (Eds.), *Mammal Phylogeny: Placentals*. Springer-Verlag, New York, pp. 81–102.
- Sánchez-Villagra, M.R., Narita, Y., Kuratani, S., 2007. Thoracolumbar vertebral number: the first skeletal synapomorphy for afrotherian mammals. *Syst. Biodivers.* 5, 1–7.
- Scillato-Yané, G.J., 1982. Los Dasypodidae (Mammalia, Edentata) del Plioceno y Pleistoceno de la Argentina. Tesis Doctoral, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, 159 pp.
- Schulthess, B., 1920. Beiträge zur Kenntnis der Xenarthra auf Grund der “Santiago Roth’schen Sammlung” des Zoologischen Museums der Universität Zürich. In: *Mémoires de la Société Paléontologique Suisse*, Vol. XLIV.
- Sire, J.Y., Donoghue, P.C.J., Vickaryous, M.K., 2009. Origin and evolution of the integumentary skeleton in non-tetrapod vertebrates. *J. Anat.* 214, 409–440.
- Szalay, F.S., Schrenk, F., 1998. The middle Eocene *Eurotamandua* and a darwinian phylogenetic analysis of “Edentates”. *Kaupia* 7, 97–186.
- Vickaryous, M.K., Hall, B.K., 2006. Osteoderm morphology and development in the nine-banded armadillo, *Dasypus novemcinctus* (Mammalia, Xenarthra, Cingulata). *J. Morphol.* 267, 1273–1283.
- Vickaryous, M.K., Hall, B.K., 2008. Development of the dermal skeleton in *Alligator mississippiensis* (Archosauria, Crocodylia) with comments on the homology of osteoderms. *J. Morphol.* 269, 398–422.
- Vickaryous, M.K., Sire, J.Y., 2009. The integumentary skeleton of tetrapods: origin, evolution, and development. *J. Anat.* 214, 441–464.
- Weiss, L.P., Wislocki, G.B., 1956. Seasonal variation in hematopoiesis in the dermal bones of the nine-banded armadillo. *Anat. Rec.* 126, 143–163.