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DEVELOPMENT OF SKIN AND DERMAL BONE OF THE SHELL IN *Pelodiscus maackii* (TESTUDINES: TRIONYCHIDAE)

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The article is devoted to the study of the ontogenetic development of the skin and the structure of the skin-bone shell of the trionychid turtle *Pelodiscus maackii* (Brandt, 1858). The process of formation of the epidermis and dermis, as well as their derivatives (epidermal scales and dermal ossifications), is described. The development of epidermal scales in *Pelodiscus maackii* has been shown to be similar to the development of asymmetric scales in other reptiles. Five stages of formation of the definitive structure of the dermal bones have been identified. Structural differences between the internal and external parts of the bony plates (rod-shaped elements and bony callosities) are due to the previous stratification of the dermis into histologically distinct strata: hypodermal, fibrous and papillary. In particular, the fibrous stratum, consisting of several layers of orthogonally oriented collagen bundles, is a precursor to the plywood-like structure of the trionychid bony plates. The growth of bone tissue around the vascular network in the papillary layer is the reason for the formation of surface ornamentation of the trionychid callosities. Some questions of the evolutionary significance of the unique features of the trionychid shell are discussed.

Keywords: *Pelodiscus maackii*; Trionychidae; skin development; epidermal scales; dermal bones; plywood-like structure.

INTRODUCTION

Turtles have a unique arrangement of the horny and bony shell (Zangerl, 1969). Recently, some progress has been made in studying the processes of their development in hard-shelled turtles, including main morphogenetic regularities in the development of the pattern of horny scutes (Moustakas-Verho et al., 2014; Moustakas-Verho and Cherepanov, 2015; Cherepanov et al., 2019). Significant results have been obtained in understanding the nature of bony plates (Cherepanov, 1997, 2005; Gilbert et al., 2001, 2007). Studies of the histological structure of the bony shell in living and extinct turtles have great importance for understanding of their phylogeny (see Scheyer, 2007).

Of particular interest are soft-shelled turtles (family Trionychidae), a phyletic group with an unusual shell design. Firstly, representatives of this family do not have large horny scutes, characteristic of most turtles, but only small, irregularly located tubercle-like scales (Webb, 1962; Moustakas-Verho and Cherepanov, 2015). Secondly, the plates of their bony shell have superficial callosities with a plywood-like arrangement of bone tissue, characteristic only of this taxon (Scheyer et al., 2007; Buffrenil et al., 2016). However, available studies are limited to descriptions of the definitive structures of the trionychid skin and its derivatives; in particular, bony plates (Alibardi and Toni, 2006; Scheyer et al., 2007), whereas data on the ontogenetic development of these structures are fragmentary and contradictory (Stoffert, 1889; Schmidt, 1921; Menger, 1931; Deraniyagala, 1939; Zangerl, 1939; Vallén, 1942; Cherepanov, 1992, 1995). The purpose of this work is to describe the process of formation of the histological structure of the epidermis and dermis, as well as their derivatives (epidermal scales and dermal ossifications) in soft-shelled turtle *Pelodiscus maackii* (Brandt, 1858).

MATERIAL AND METHODS

The work is based on a study of serially-sectioned embryos and post-embryos of *Pelodiscus maackii* that

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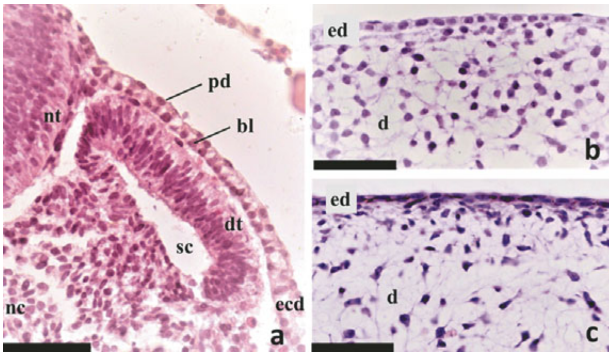

Fig. 1. Transverse sections of trunk and dorsal skin in *Pelodiscus maackii* embryo at stages 10 (a, SPbU #2109), 13 (b, SPbU #2114), 17 (c, SPbU #2126). Labels: bl, basal layer; d, dermis; dt, dermatome; ecd, ectoderm; ed, epidermis; nc, notochord; nt, neural tube; pd, periderm; sc, somite cavity. Delafield's hematoxylin-eosin. Scale bar is 50 µm.

document the ontogenetic development of their shell integument. The embryonal stages were determined according to Yntema (1968). The material was preserved in 4% formaldehyde. Embryos were dehydrated in ethanol and embedded in paraffin. The transverse, frontal and sagittal histological sections of 10, 15, and 20 µm were stained by Delafield's hematoxylin with eosin and asan with asokarmin, according to Heidenhain. The number of specimens studied was 24 embryos and early postembryos, the number of histological preparations was 443 slides. All material is stored in the collection of the Department of Vertebrate Zoology of St. Petersburg State University (SPbU).

RESULTS

Stage 10. The body of the embryo is flattened laterally. The ectoderm has two layers: basal layer and periderm (Fig. 1a). The basal cells are cuboidal or slightly flattened. The cells of the periderm are more flattened and relatively rare. The somitic dermatomes are epithelialized and separated from the ectoderm by space filled with thin fibers.

Stage 13. The epidermis is bilayered with slightly flattened basal cells and relatively sparse peridermal cells (Fig. 1b). The dermatomes are destroyed and their cells form a thick layer of dermis. Dermal cells are stellate in shape.

Stage 17. The epidermis is still two-layered; its thickness is about 5 µm (Fig. 1c). On the dorsal side of the body, it forms local thickenings with a diameter of 120 – 150 µm. The dermis is composed of loosely arranged stellate mesenchymal cells; only three or four cell layers underlying the epidermis are more compacted.

The rudiments of the shell bones (nuchal and plastral plates) are present. The small accumulations of the mesenchymal cells surround a non-mineralized organic matrix — osteoid (Fig. 3a). They are located in the deep dermis.

Stage 19. The body of the embryo is flattened dorsoventrally. The soft carapace and plastron are formed. The dorsal thickenings of the epidermis have the shape of flat tubercles (Fig. 2a). In the tubercle's area between the basal layer and periderm, cells of the intermediate layer of the epidermis have appeared. A small condensation of the dermal cells is visible under the epidermal tubercles. The dermis is composed of elongated fibroblasts and contains thin collagen fibers, pigment cells and blood vessels. The dermis is poorly differentiated into thin fibrous and thick hypodermal strata. In the fibrous stratum, collagen fibers are oriented in the plane of the skin, while in the hypodermis they are elongated in different directions.

Bony elements are at an early stage of mineralization. They are primary osseous laminas, surrounded by osteoblasts and containing a small number of internal osteocytes.

Stage 21. The intermediate layer of the epidermis consists of two to three cellular layers. The thickness of the epidermis ranges from 10 – 25 µm, and it is maximum in the areas of the skin tubercles (tubercle-like scales) on the carapace. The scales are numerous and asymmetrical in shape (Fig. 2b). Their diameter is about 250 µm. A lowerdermal papilla supports each scale.

The dermis is differentiated into papillary (superficial), fibrous and hypodermal strata. The papillary stratum differs from the underlying fibrous stratum by the presence of a large concentration of the pigment cells. In addition, it is characterized by relatively less differentiation of dermal cells and the absence of large bundles of collagen fibers. In the fibrous stratum, collagen fiber bundles have a strict organization: they consist of several alternative layers, the fibers of which are oriented orthogonally (plywood-like arrangement). The bundles of fibers of the hypodermis are relatively thin; they are directed predominantly vertically and form a network. A thin layer of the cells of the subcutaneous fascia (subdermis) clearly separates the dermis from the deeper structures.

The dermal bony plates lie in the lower third of the hypodermis (Fig. 3b). The growth of the plates occurs due to the development of bone trabeculae on their apical surface. The hypodermis between the trabeculae is very diffuse and contains large blood vessels.

Stage 23. The thickness of the epidermis ranges from 20 to 40 µm. The embryonic stratum corneum, consisting

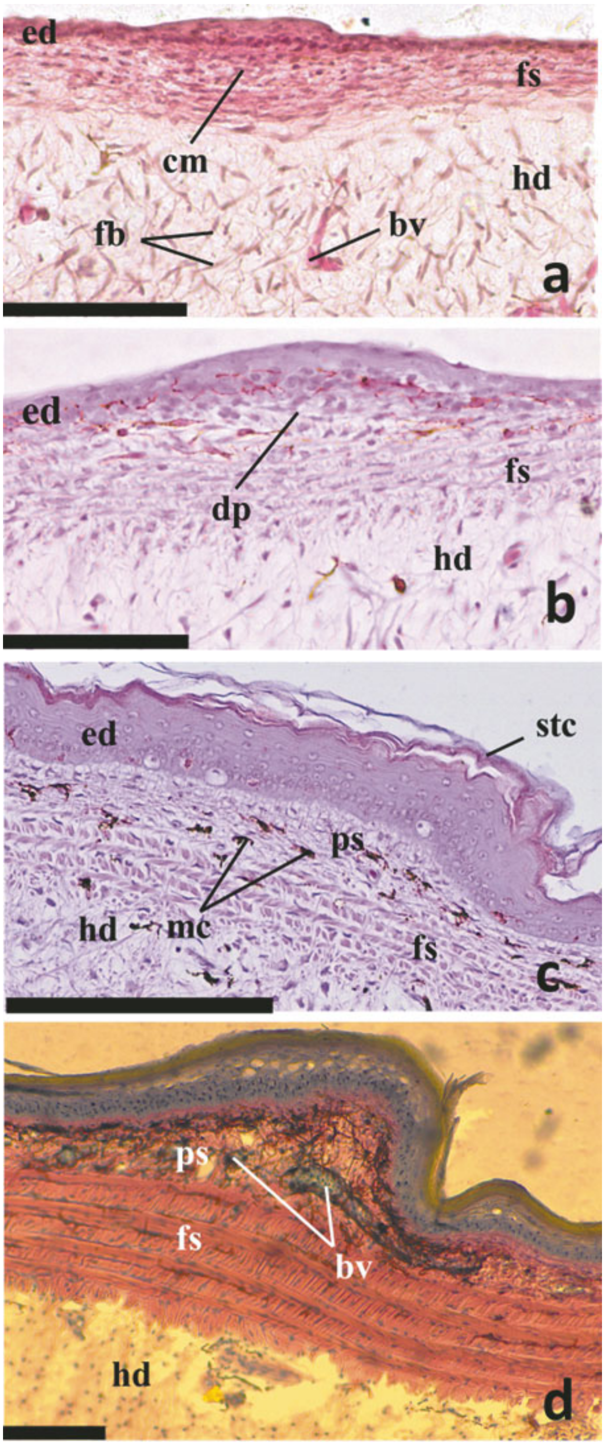

Fig. 2. Longitudinal sections of dorsal skin in *Pelodiscus maackii* embryo at stages 19 (a; SPbU #2129); 21 (b; SPbU #2131); 26 (c; SPbU #2133); and 1-year-old specimen (d; SPbU #0043). Labels: bv, blood vessel; cm, condensation of mesenchyme; dp, dermal papilla; ed, epidermis; fb, fibroblasts; fs, fibrous stratum; hd, hypodermis; mc, melanocytes; ps, papillary stratum; stc, stratum corneum. Delafield's hematoxylin-eosin (a – c) and Azan; azo-carmine (d). Scale bars: a, b, 100 μm; c, d, 250 μm.

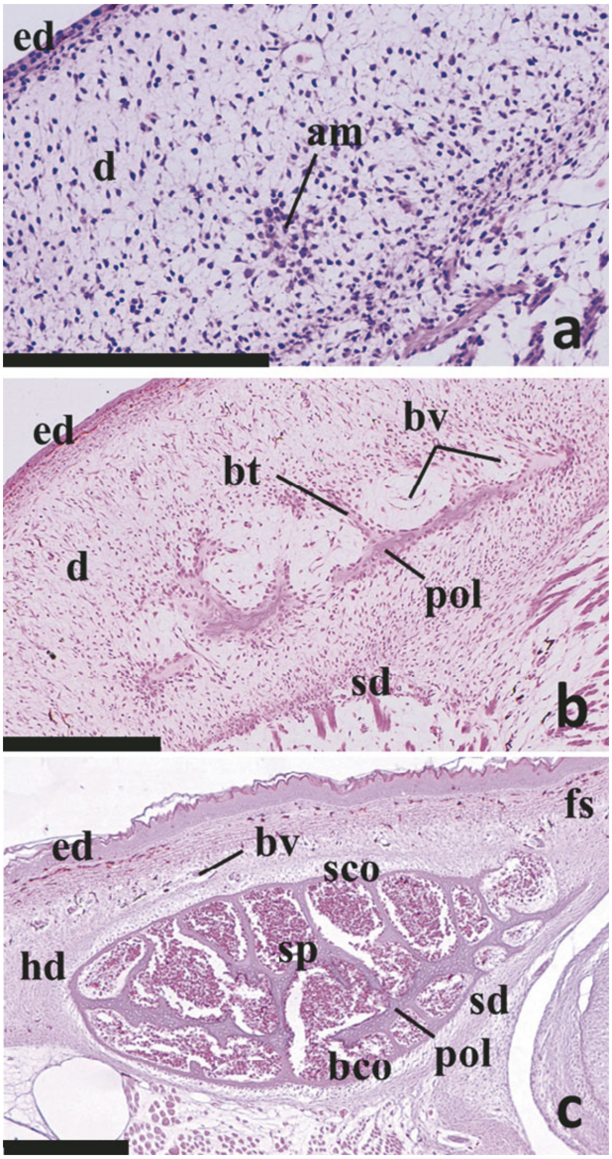

Fig. 3. Longitudinal sections of dorsal skin and nuchal plate in *Pelodiscus maackii* embryo at stages 17 (a; SPbU #2126); 21 (b; SPbU #2131) and 26 (c; SPbU #2133). Labels: am, accumulation of mesenchyme; bco, basal cortex; bt, bone trabecula; bv, blood vessel; d, dermis; ed, epidermis; fs, fibrous stratum; hd, hypodermis; pol, primary osseus lamina; sco, superficial cortex; sd, subdermis; sp, spongiosa.

of flattened scale-like cells, is formed. The epidermal scales reach up to 500 μm in diameter. Their dermal papillae are well defined. The fibrous stratum of the dermis has close contact with the epidermis between the scales. Its fibers are collected in thick bundles and form a compact layered plywood structure in the dermis. The collagen fibers of the hypodermis are thin and not strictly organized.

The bony plates of the shell lie in the lower part of the hypodermis. Their growth occurs along the periphery because of the formation of large bone outgrowths elongated in the plane of the skin. Bone thickness is increased mainly due to the growth of the apical trabeculae. These trabeculae form a spongy layer of bone, the cavities of which are filled with the hematopoietic tissue.

Stage 26. The epidermis is multilayered, keratinized, and its surface is highly folded (Fig. 2c). The thickness of the epidermis varies from 50 to 100 μm. The cells of the basal layer are cuboidal or columnar. The intermediate layer is formed by four to eight rows of slightly flattened cells with clearly visible nuclei. The outer epidermal layer is stained basophilic and form the stratum corneum (alpha-keratin layer). Scales are present on the carapace only, and they form relatively regular longitudinal rows along its lateral sides. The scales have an asymmetrical shape.

The dermis is clearly stratified. The papillary stratum is thin. Its fibroblasts are located more densely than in other strata of the dermis, and the pigment cells are also concentrated here (there are no melanocytes in the abdominal region). Thin collagen fibers are partly anchored in the basement membrane and make the lower border of the epidermis uneven. The fibrous stratum of the dermis is distinguished by a strict orthogonal organization of the collagen matrix. The hypodermis is the thickest layer of the skin; its collagen fibers are arranged irregularly and form a dense network.

The bones of the shell are rod-shaped. They are characterized by a three-layer structure with a thin superficial and basal cortex and a wide layer of spongiosa located between them (Fig. 3c). The bones occupy almost the entire thickness of the hypodermis and are separated from the fibrous stratum and subcutaneous fascia only by narrow zone of the hypodermis.

Juvenile stage. Carapace length is 10 cm. The papillary stratum of the dermis is stratified. Beneath the epidermis, it has four or five orthogonally organized layers of thin bundles of the collagen fibers. In the lower part of the stratum, the position of the fibers is not ordered, and a large number of blood vessels are concentrated here (Fig. 2d). The fibrous stratum consists of dense connective tissue and comprises 8 – 12 layers of orthogonally organized collagen. Its bundles are exceptionally thick: their diameter reaches 35 μm. The non-ossifying hypodermis consists of loose connective tissue, the thin fibers of which are mainly oriented perpendicular to the surface of the skin. Blood vessels are numerous in the outer layer of the hypodermis; most of them extend horizontally, underlying the fibrous stratum of the dermis (Fig. 4a).

The plates of the shell are composed of compact bone tissue. The entoplastron and epiplastra lie in the hypodermis and maintain a three-layer structure: the lamellar bone forms the basal and superficial cortex, the dense spongiosa is located between them. Other plates of the shell form superficial callosities; a system of horizontally oriented vascular canals separated the callosities from the underlying lamellar bone. The bone tissue of callosities is stratified into two zones. The inner zone is formed on the basis of the fibrous stratum of the dermis and retains the plywood-like pattern of the collagen matrix (Fig. 4b). The outer zone is formed in the papillary stratum and is characterized by the absence of an orthogonal fiber structure and the presence of numerous vascular canals opening onto the bone surface (Fig. 4c). The outer surface of the callosities is ornamented with tubercles and ridges that surround bone cavities.

DISCUSSION

Development of the Epidermis and Its Derivatives

The most striking feature of the skin of soft-shelled turtles (Trionychidae) is the absence of large horny scutes characteristic of most turtles. The presence of scutes (at least vertebral ones) was noted in newborns of *Carettochelys insculpta* (Carettochelyidae), closely related to soft-shelled turtles (Zangerl, 1969). However, trionychids are characterized by complete reduction of the scutes; the study of their embryogenesis did not even reveal the primary anlages of scutes — epidermal placodes (see Cherepanov, 2005).

The differentiation of the epidermis in early embryogenesis of *Pelodiscus maackii* is indistinguishable from that in other turtles (Cherepanov, 1985, 2005; Alibardi and Thompson, 1999a; Alibardi and Dipietrangelo, 2005). Significant differences appear only at the hatching stages, when the definitive structure of the epidermis is formed. In particular, the epidermis on the shell of *P. maackii* does not form the corneous beta-layer that occurs in hard-shelled turtles (Landmann, 1986). Unlike the skin of hard-shelled turtles, the definitive integument of *P. maackii* retains the structure of a thin and easily peelable horny layer (Fig. 2d), which other turtles have only at the embryonic stages of shell development. It is the result of the alpha-cornification (Alibardi and Toni, 2006) and the shell integument of the *P. maackii* is histologically similar to the definitive alpha-structure of the soft skin on the movable parts of the body (neck, limbs and tail) in any turtles (Spearman, 1969; Baden and Maderson, 1970; Landmann, 1986; Alibardi, 1999, 2002). It should be noted that, like trionychids, only the leatherback turtle *Dermochelys coriacea* (Dermochelyidae) has alpha-cornified shell integument and also lacks horny scutes

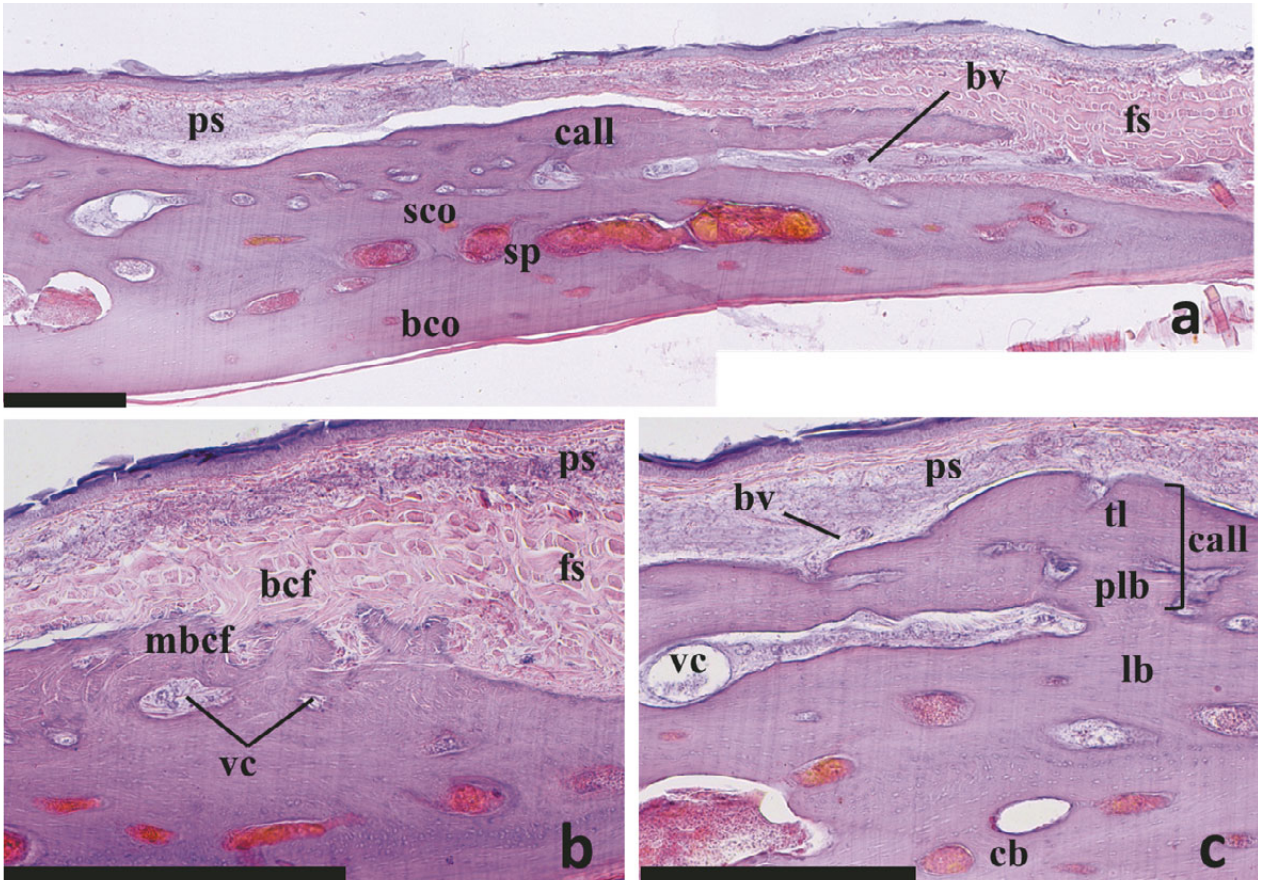

Fig. 4. Longitudinal sections of xiphiplastron in 1-year-old specimen of *Pelodiscus maackii*; SPbU #2105). Labels: bcf, bundles of collagen fibers; bco, basal cortex; bv, blood vessel; call, callosity; cb, compact bone; fs, fibrous stratum; mbcf, mineralized bundles of collagen fibers; lb, lamellar bone; plb, plywood-like bone; ps, papillary stratum; sco, superficial cortex; sp, spongiosa; tl, tuberculated layer; vc, vascular canal. Delafield's hematoxylin-eosin. Scale bar is 500 μm.

(Baden and Maderson, 1970; Maderson, 1985). This indicates a direct connection between beta-cornification and the presence of horny scutes on the turtle shell. The appearance and disappearance of these structures in evolution are, apparently, strictly correlated processes, which explains the similarity in the integumental structure in such phylogenetically distant taxa as Trionychidae and Dermochelyidae.

The alpha-cornification is the primary and basic process of development of horny integument in vertebrates and occurs in all tetrapods, including amphibians (see Vandebergh and Bossuyt, 2012; Alibardi, 2016, 2022). In addition, alpha-cornified layer appear in ontogenesis much earlier than beta-layer (Carver and Sawyer, 1987; Alibardi and Dipietrangelo, 2005; Alibardi, 2009; Greenwold et al., 2014). Consequently, the occurrence of beta-cornification is secondary and more specialized; in turtles it is associated with the formation of such mechanically strong structures as horny scutes. The origin of trionychids from turtles that had horny scutes is beyond doubt (Meylan, 1987; Gaffney and Meylan, 1988). Thus, we can conclude that soft-shelled turtles demonstrate a return to a more primitive (plesiomorphic) state of the shell integument compared to hard-shelled turtles.

Most of recent Trionychidae are characterized by the presence of small scales on the dorsal side of the shell (Webb, 1962). In *Pelodiscus maackii*, their formation begins with the appearance of local epidermal thickenings with simultaneous condensation of dermal cells underlying them (Fig. 2a). At the early stages of morphogenesis, the rudiments of scales are similar to the scale-like tubercles that appear on the embryonic scutes of the shell in *Testudo graeca* and *Emys orbicularis* (Cherepanov, 1985). The morphological similarity of these structures probably reflects their phyletic unity. However, the formation of the described elements of the horny shell begins at later stages of embryogenesis in *T. graeca* and *E. orbicularis* (stages 19 – 20) than in *P. maackii* (stage

17), and reaches only the stage of "symmetrical scales" (Cherepanov, 1985). The development of the scale-like tubercles in *T. graeca* and *E. orbicularis* stops at the hatching stage and this is probably due to the appearance of a thick stratum corneum that prevents the growth of the dermal papillae of the scales. In adult hard-shelled turtles, the dermal papillae are completely absent, and the scale-like tubercles on the surface of the scutes are only local thickenings of the horn. During the development of the trionychid integument, the scales acquire an advanced asymmetrical shape (Fig. 2d), and their growth continues in the postnatal period. The development of scales on soft (alpha-keratinized) skin areas occurs in a similar way in other turtles (see Alibardi, 1999).

Development of the Dermis and Dermal Ossifications

Many authors associate the appearance of the soft-skinned state in the evolution of trionychids with their aquatic lifestyle and the functioning of their skin as an additional respiratory organ (Khozatsky, 1967; Davenport and Wong, 1992; Scheyer et al., 2007). On the contrary, the function of mechanical protection, especially if we take into account the partial reduction of the bony armor, is largely transferred to the dermis (Alibardi and Toni, 2006). The dermis of trionychids is structured much more complex in comparison with other turtles (Goette, 1899; Schmidt, 1921). This is due to the development of a unique fibrous stratum, well demarcated from adjacent layers of the dermis. In juvenile *P. maackii* it consists of 11 – 12 orthogonally organized layers of collagen fibers (Fig. 4a, b). In hard-shelled turtles (*T. graeca*, *E. orbicularis*, *Emydura macquarii*), such a fibrous stratum is not formed, and the dermis retains a relatively uniform structure (Cherepanov, 1985, 2005; Alibardi and Thompson, 1999b). At the hatching stage, it consists of randomly arranged bundles of collagen fibers and is structurally similar to the hypodermis of *P. maackii*.

Based on the study of the ontogeny of *P. maackii*, it was possible to describe the development of the histological structure of the dermis and identify five successive stages of dermal ossification.

Stage I (embryonic stages 16 – 17). The rudiments of bony plates (nuchal and plastral) are small accumulations of dermal cells. They lie in the middle level of the dermis. The dermis is not stratified and is composed of stellate cells (Fig. 3a).

Stage II (embryonic stages 17 – 18). The primary zone of ossification (bone lamina) is formed inside the mesenchymal rudiment of the shell plates.

Stage III (embryonic stages 19 – 24). Development of bone trabeculae on the outer surface of the lamina, which form the spongiosa (Fig. 3b). The dermis is differentiated into hypodermis, fibrous and papillary strata (see Fig. 2c). The rudiments of bony plates are located in the hypodermis.

Stage IV (embryonic stages 24 – 26). The skeletal elements are rod-shaped and have a three-layer bone structure. Trabeculae spongiosa close the bone cavities, which are filled with the hematopoietic tissue. On the surface of the skeletal element, they form a thin layer of compact bone — the basal and superficial cortex (Fig. 3c).

Stage V (juvenile specimens). Bony rod-shaped elements (except for entoplastron and epiplastra) on their outer surface have callosities, formed as a result of the ingrowth of the upper compact layer of bone into the fibrous and papillary strata of the dermis (Fig. 4). The bone of callosities is stratified into two zones: the inner zone, formed because of the fibrous stratum, retains the plywood-like structure of the collagen matrix; the outer zone, developing in the papillary stratum of the dermis, is distinguished by the irregular organization of collagen fibers and an abundance of vascular canals. Callosities bear a superficial ornamentation of tubercles and ridges surrounding cavities with numerous openings of blood vessels.

The morphogenetic data (see also Cherepanov, 1995; Sánchez-Villagra et al., 2009) do not confirmed the widespread hypothesis of a complex origin of the trionychid bony plates (Zangerl, 1939, 1969; Williams, McDowell, 1952; Romer, 1956). No additional isolated centers of ossification were found above the primary rudiments of the shell plates at any stage of *P. maackii* development. Bony callosities, which appear late in ontogenesis, are only the result of the growth of rod-shaped elements into the superficial layers of the dermis.

The three-layer bone structure with the inner spongy and outer compact layers is typical for shell ossifications of most turtles (Suzuki, 1963; De Ricqles, 1976; Scheyer, 2007). This "simple" bone structure can be observed in some trionychids, such as the entoplastron and epiplastron of adult *P. maackii*, which are located in a relatively uniform hypodermis (plesiomorphic state). The complication of the histological structure of dermal ossifications caused by the appearance of the callosities is secondary in Trionychidae. The reason for this phenomenon is a change in the conditions for the dermal bone development in soft-shelled turtles, caused by the stratification of the dermis into structurally different strata: papillary, fibrous, and hypodermal (Cherepanov, 1992). The dermis of hard-shelled turtles is relatively homogeneous, so no complication of the bone structure is observed (see Vallén, 1942; Cherepanov, 1985; Alibardi and Thompson, 1999b). Since the origin of Trionychidae from turtles with the three-layer bone structure of shell plates is beyond doubt, we can agree with the opinion of Meylan (1987) that callosities are a phylogenetic innovation.

Rich vascularization is one of the significant features of the integument of soft-shelled turtles. Skin vessels play an important role in gas exchange processes (Khozatsky, 1978; Davenport and Wong, 1992). In addition, the dermal blood vessels have important morphogenetic significance as an osteogenic factor. It is known that blood vessels are necessary for the initiation of ossification, and their distribution in the dermis forms the primary system of bone canals and cavities (Francillon-Vieillot et al., 1990). Most likely, the vascular network determines the main directions of growth of the bone tissue that encloses it. In relation to trionychids, this is clearly visible in the early stages of the formation of the spongy layer, when bone trabeculae overgrow the blood vessels and hematopoietic cavity of the dermis (Fig. 3b, c). Apparently, such uneven bone growth around the vessels of the papillary stratum induces the formation of ornamentation on the surface of trionychid callosities. It is assumed that the sculptured ridges and tubercles on the dermal bones of tetrapods act as a protection for these vessels against mechanical damage (Witzmann et al., 2010).

In conclusion, it can be stated that the unique structure of the bones of the trionychid shell is due to the unique structure of their soft skin, its unusually clear stratification and rich vascularization. I can agree with Scheyer et al. (2007) that the evolutionary success of soft-shelled turtles is tied to the plywood-like structure of the integument and bone. It may have been the key adaptation that allowed trionychids to greatly reduce and flatten their bony shell, while still retaining biomechanical strength and improving locomotor characteristics. In addition, vascularization of the dermis and reduction of epidermal scutes allowed for more efficient cutaneous respiration in trionychid turtles.

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