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Morphology, development and heterochrony of the carapace of Giant Amazon River Turtle *Podocnemis expansa* (Testudines, Podocnemidae)[1]

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ABSTRACT.- Vieira L.G., Santos A.L.Q., Moura L.R., Orpinelli S.R.T., Pereira K.F. & Lima F.C. 2016. **Morphology, development and heterochrony of the carapace of Giant Amazon River Turtle, *Podocnemis expansa* (Testudines, Podocnemidae).** *Pesquisa Veterinária Brasileira 36(5):436-446.* Laboratório de Ensino e Pesquisa em Animais Silvestres, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia, Av. Amazonas 2245, Jardim Umuarama, Uberlândia, MG 38405-302, Brazil. E-mail: luceliabio@yahoo.com.br

With aim to report the ontogeny of the osseous elements of the carapace in Peurodiras, 62 embryos and 43 nestlings of *Podocnemis expansa* were collected and submitted to the clearing and staining technique of bones and cartilages and study of serial histological slices. The carapace has mixed osseous structure of endo and exoskeleton, formed by 8 pairs of costal bones associated with ribs, 7 neural bones associated with neural arches, 11 pairs of peripheral bones, 1 nuchal, 1 pygal and 1 suprapygal. This structure begins its formation in the beginning of stage 16 with the ossification of the periosteal collar of the ribs. With exception of the peripheral bones, the other ones begin their ossification during the embrionary period. In histologic investigation it was found that the costal bones and neural bones have a close relation to the endoskeleton components, originating themselves as intramembranous expansions of the periosteal collar of the ribs and neural arches, respectively. The condensation of the mesenchyme adjacent to the periosteal collar induces the formation of spikes that grow in trabeculae permeated by fibroblasts below the dermis. The nuchal bone also ossifies in an intramembranous way, but does not show direct relation to the endoskeleton. Such information confirms those related to the other Pleurodira, mainly with *Podocnemis unifilis*, sometimes with conspicuous variations in the chronology of the ossification events. The formation of dermal plates in the carapace of Pleurodira and Criptodira follow the same pattern.

INDEX TERMS: Carapace, Giant Amazon River Turtle, *Podocnemis expansa,* Testudines, Podocnemidae, shell, ontogeny, pleurodira, ossification, turtle.

RESUMO.- [**Morfologia, desenvolvimento e heterocronia da carapaça da Tartagura da Amazônia, *Podocnemis expansa* (Testudines, Podocnemidae).**] Com objetivo de relatar a ontogenia dos elementos ósseos da carapaça em Pleurodiras, coletaram-se 62 embriões e 43 filhotes de *Podocnemis expansa* que foram submetidos à técnica de clareamento e coloração dos ossos e cartilagens e pelo estudo cortes histológicos seriados. A carapaça possui estrutura óssea mista de endo e exoesqueleto, sendo formada por 8 pares de ossos costais associados às costelas, 7 ossos neurais associados aos arcos vertebrais, 11 pares de ossos periféricos, 1 nucal, 1 pigal e 1 supra-pigal. Esta estrutura

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começa sua formação no início do estágio 16 com a ossificação do colar periostal das costelas. Com exceção dos ossos periféricos, os demais iniciam sua ossificação durante o período embrionário. A investigação histológica explicitou que os ossos costais e neurais possuem uma estreita relação com componentes do endoesqueleto, originando-se como expansões intramembranosas do colar periostal das costelas e dos arcos neurais, respectivamente. A condensação do mesenquima adjacente ao colar periostal induz a formação de espiculas que crescem em trabéculas permeadas por fibroblastos abaixo da derme. O osso nucal, também se ossifica de maneira intramembranosa mas não apresenta relação direta com o endoesqueleto. Tais informações corroboram àquelas relatadas para os demais Pleurodiras, principalmente com *Podocnemis unifilis*, outrora com variações conspícuas na cronologia dos eventos de ossificação. A formação das placas dérmicas da carapaça em Pleurodira e Criptodira seguem um mesmo padrão.

TERMOS DE INDEXAÇÃO: Carapaça, Tartagura da Amazônia, *Podocnemis expansa,* Testudines, Podocnemidae, casco, ontogenia, pleurodira, ossificação, tartaruga*.*

INTRODUCTION

Testudines possess a particular morphological structure formed by the shell that composes a ventral portion, the plastron, and other dorsal portion, the carapace. It is an autapomorphy, which becomes this impracticable composition for anatomical comparisons among other vertebrates (Scheyer & Sander 2007, Scheyer et al. 2008, Lima et al. 2011). The shell is considered a classic model of morphological novelty, being a synapomorphic feature which defines the order (Romer 1956, Gilbert et al. 2001) and makes this clade a restrict group (Gilbert et al. 2001, Lee 2006, Li et al. 2008). Maybe because of this feature the understanding of its phylogenetic position among the amniotes remains still unknown (Lyson et al. 2010, 2012, Shen et al. 2011, Chiari et al. 2012, Crawford et al. 2012).

Carapace integrates elements of the endoskeleton (neural arches and ribs) of endochondral formation with dermal structures of intramembranous development (Vickaryous & Sire 2009) that, in different species, along with the plastron, offers physical support for protection and shelter, nutritional and, mostly, calcium and ionic stock (Cebra-Thomas et al. 2005). The shell is the most evident autapomorphy of the Testudines (Joyce 2007) and one of the most conspicuous intertegumentar structures of the vertebrates (Vickaryous & Sire 2009), thus, all the knowledge about the morphology and ontogeny of the skeletal apparatus of this group can potentially be a subside for new investigations aiming to explain the evolution of these animals.

Some aspects of the homology of the shell of the Testudines were explained. It is common knowledge that the plastron is a dermic structure with contributions of the clavicle, interclavicle, and presumably, homologous to the gastralia (Zangerl 1939, 1969, Cherepanov 1984, Gaffney & Meylan 1988, Cherepanov 1997, Vieira et al. 2009), and that the peripheral, the nuchal and the pygal bones are also of dermic origin (Scheyer & Sander 2007). On the other hand, the costal and neural elements were the target of much controversy. Traditionally were interpreted as independent elements of secondary origin, merged to the ribs and neural arches, respectively (Goette 1899, Vallén 1942), or derived from these elements of the endoskeleton, as reported in some investigations about its ontogeny (Hoffmann 1878, Suzuki 1963, Zangerl 1969, Cherepanov 1997, Scheyer et al. 2008, Vickaryous & Sire 2009, Lima et al. 2011).

On the last decades, several methods are being used aiming to reveal particularities about the phylogeny (Gaffney & Meylan 1988, Li et al. 2008, Wilson & Sánchez-Villagra 2011), embryology (Yntema 1968, Werneburg et al. 2009), morphology and ontogeny (Scheyer et al. 2008, Vieira et al. 2009, Lima et al. 2011) of the Testudines among others (Nagashima et al. 2005, Colbert & Rowe 2008, Harrison & Larsson 2008). Recently, discussions about this theme took also molecular directions (Nagashima et al. 2005, 2007, Ohya et al. 2006). These same authors use embrionary and ontogenetic data as support to their discussions, even though some of these features are still not completely explained.

Living Testudines are subdivided in two monophyletic groups, Cryptodira and Pleurodira (Gaffney & Meylan 1988, Werneburg et al. 2009, Lima et al. 2011), which can easily be distinguished by the head retraction mechanism. Ontogeny and anatomy of the Pleurodira group is still little known, with some investigations being needed to provide contributions to understanding of the variations of this taxon (Kordikova 2002, Sánchez-Villagra et al. 2007). The ontogeny of the Pleurodira shell, like big part of the other anatomic systems of this group, are based mostly in the anatomy of the Cryptodira (Gaffney 1979), even though some of the particularities of this group were described in *Podocnemis unifilis* (Fabrezi et al. 2009, Lima et al. 2011), *Podocnemis expansa* (Vieira & Santos 2007, Vieira et al. 2009), *Emydura subglobosa, Pelusios sinuatus, Pelomedusa subrufa* (Scheyer et al. 2008) and *Phrynops hilarii* (Bona & Alcalde 2009).

Investigation about the embryology and ontogeny of the Testudines are being boosted by the big concern in the evolution, origin and development of the shell (Gilbert et al. 2001, 2007, Nagashima et al. 2005, 2007, 2009, Li et al. 2008, Scheyer et al. 2008), because of the necessity of undoubtful explaining of its evolutive relation with the other amniotes, mainly the reptiles (Rieppel 1995, 2004, Rieppel & De Braga 1996, Hedges & Poling 1999, Meyer & Zardoya 2003, Müller 2003, Hill 2005, Werneburg & Sánchez-Villagra 2009).

P. expansa, also called giant Amazon river turtle, is a fluvial Testudine of the Podocnemididae family found in the Amazon River and most of its tributaries. It is a large species, with the largest specimens reaching more than 90 cm in length (Molina 1992, Zardoya & Meyer 2001, Malvásio et al. 2002a, 2002b, Gaspar et al. 2005).

According to Valenzuela (2001), its geographic distribution covers north and west-central of Brazil, in the basins of the Amazon, Araguaia and Tocantins rivers, and other South American countries. It is an omnivorous species with diurnal habits, living in communities with many animals. Reproduce from September to March. They dig holes and build nests in the sand, where they lay 60 to 100 eggs per season.

Its meat and eggs are highly appreciated, constituting the basis for various dishes of Amazonian cuisine. The shell is used as adornment or bowl (Pritchard & Trebbau 1984). *P. expansa* can be commercially raising because it is an important component of the regional economy and it has high potential for zootechnical performance (Luz et al. 2003).

Here we provide a detailed description of the morphology, formation sequence and development of the carapace bones of *P. expansa*, aiming to describe also possible intra-specific alterations that occur during all of the embrionary period, since the importance of ontogenic data in the interpretation of the new fossils which document the evolution of the lineage for the turtles, as well as the understanding of the anatomy of the current living groups.

MATERIALS AND METHODS

Embryos and nestlings of *Podocnemis expansa* (Schweigger, 1812) were acquired in the reproduction field in the River Araguaia – GO (13º 20' 38,7" S and 50º 38' 05,7" W) in the spawning periods occurred in 2005 and 2006, under license number 117/2005-IBAMA/RAN. The investigation was authorized by the Ethics Committee in Research Animals of the Federal University of Uberlândia as issued by protocol CEUA-UFU 032/2009.

An embryo was collected daily, of a nest randomly chosen, until the hatching, in a total of 62 samples. All embryos were removed from their eggs by cutting the shells with surgical scissors, isolating them from the yolk sac and their wrappings. The development steps of the embryos were named according to the external morphologic criteria (Danni et al. 1990). For the helping of description of the development processes, the skeleton of three *P. expansa* belonging to the collection of the Wild Animal Teaching and Researching Laboratory of the Federal University of Uberlândia, Brazil were observed.

After hatching, 43 nestlings were kept in tanks with average temperature of 27ºC and fed with commercial food for fishes. These were collected in regular intervals, which allowed the keeping of the ontogenetic sequence after the first hatching day. Each specimen was euthanized with an overdose of intravenous sodium thiopental (50mg/Kg), fixed in formalin solution 10%, cleared and the bones and cartilages stained with Alcian Blue and Alizarina red S, respectively (Davis & Gore 1936, Digenkus & Uhler 1977, **Vieira & Santos 2007**). For the coloring of the cartilages, the steps corresponded in the fixing for neutral formaldehyde (10%), washing in distilled water for 72 hours, staining in Alcian Blue solution for 48 hours, hydration in growing series of ethanol solution and transferring for saturated solution of Sodium Borate. Afterwards, it was made the coloring of the bones through cleaning by potassium hydroxide (KOH 2%), coloring of the bones by Alizarin red S and conservation of the material in glycerin.

The specimens were examined in a stereoscopic microscope linked to an image capture system and the presence or absence of each of the bone elements was registered to determine the ontogenetic sequence of the ossification events. For comparison of the ossification pattern among the species shown here, only the relative sequence was important, and because of this, the differences in the technical approaches through the studies were not a problem in the investigation. We examined the development of the shell of the *P. expansa* in every stage, but the beginning of the ossification was verified from stage 16 on. Some embryos were also dehydrated, cleared and included in paraffin following the basic histology protocol with coloring of Hematoxylin and Eosin, making sagittal and frontal cuts with variable width of 6 to 14 µm. Because of the advance in the ossification process some samples (stage 26 and six days after hatching) were decalcified with nitric acid 1,5% during 72 hours, with three daily solution changes being made (Presnell & Schreibman 1997). The cuts were examined and photographed with the help of a microscope linked to an image capture system, aiming to confirm the presence of ossification centers and its developments, as well as allowing the analysis of the relationship between the endo and exoskeleton elements during ontogeny.

We compare our data for *P. expansa* with other additional taxa obtained in literature, like other Pleurodira (*P. unifilis* [Lima et al. 2011]); (*Emydura subglobosa, Pelusios sinuatus, Pelomedusa subrufa* [Scheyer et al. 2008]), (*Phrynops hilarii* [Bona & Alcalde 2009]) and Cryptodira (*Apalone spinifera* [Sheil 2003]); (*Chelydra serpentina* [Rieppel 1993, Sheil & Greenbaum 2005]); (*Macrochelys temminckii* [Sheil 2003]); (*Trachemys scripta* [Gilbert et al. 2001]); (*Pelodiscus sinensis* [Sánchez-Villagra et al. 2009]) and (*Trionyx sinensis* [Cherepanov 1995]).

RESULTS

Carapace osteology

Neural. The first neural bone is lengthened from skull to tail and narrowed laterally when compared to the others. The cranial margin articulates with the nuchal being lightly narrower than the tail margin. All the lateral margin extension articulates itself with the first pair of costals. The tail face contacts the second neural. In the other ones, the cranial lateral margin of each plate articulates itself with the cranial coastal, while the lateral tail face contacts the respective costal (Fig. 1b). Neural 2 and 3 are much longer

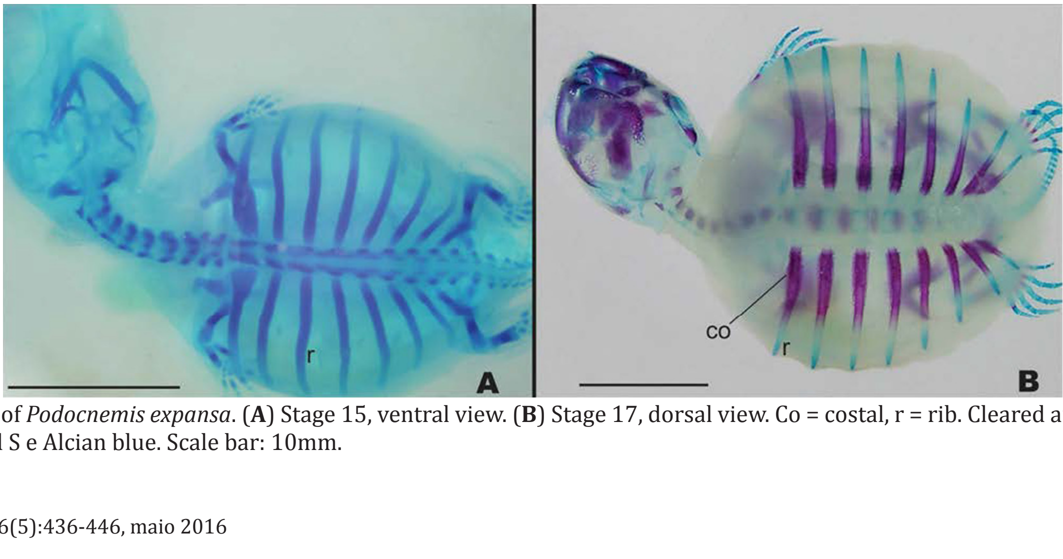

Fig.1. Embryos of *Podocnemis expansa*. (**A**) Stage 15, ventral view. (**B**) Stage 17, dorsal view. Co = costal, r = rib. Cleared and stained with Alizarin red S e Alcian blue. Scale bar: 10mm.

than wider, assuming hexagonal shape. The cranial lateral face is reduced when compared to the caudal lateral face. In general, neurals 4, 5 and 6 are also hexagonal, but cranial caudal shorter. Neural 7 is the last one in the series and has pentagonal shape (Fig.2e).

Costal. The first costal is cranial caudally bigger in comparison to the other ones. Its cranial lateral margin is curved and articulates itself with the nuchal, cranial medially and peripheral 1, 2, 3 and 4 laterally. The medial margin articulates itself with neural 1 and 2. In general, its morphology does not vary between the plates. The medial margin of costals 2 to 6 also articulate themselves with the neurals, while costal 7 articulates itself with neural 7 and with the counter lateral costal. The costal 8 contacts medially the counter lateral and the suprapygal.

Ribs. there are 11 pairs of ribs, which contribute to the development of costals (Fig.1a, b). These bones are lengthened, being merged with the costal, except in the parasagittal region, next to the median sagittal line, where the ribs curve themselves ventrally and articulate with the vertebral body.

Nuchal. *P. expansa* has 1 nuchal bone with trapezoidal shape. The cranial margin contributes with the formation of the cranial border of the carapace. The caudal margin articulates with the first pair of costal and the neural. The lateral margins articulate with the first pair of peripheral (Fig.2e, f).

Suprapygal. It is an odd plate with triangular shape. Cranially articulates itself with the caudal margin of the last pair of costals. Caudally articulates with the pygal and the eleventh pair of peripheral (Fig.3c).

Pygal. This one contributes to the formation of the caudal border of the carapace. Cranially, articulates itself with the caudal margin of the suprapygal and laterally articulates with the eleventh pair of peripheral (Fig.3c).

Peripheral: There are 11 pairs of peripheral which ar-

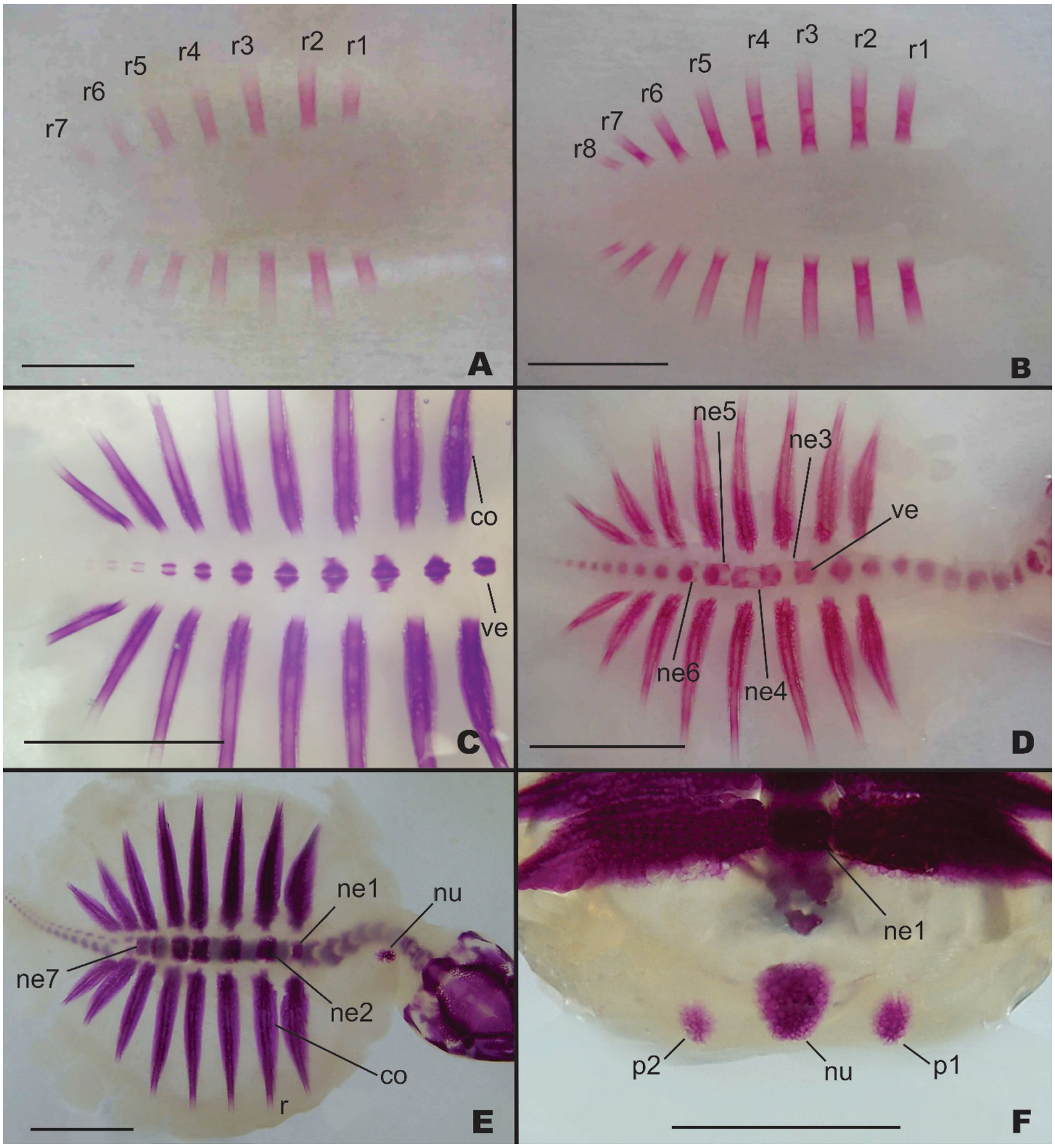

Fig.2. Embryos of *Podocnemis expansa*, dorsal view. (**A**) Stage 16. (**B**) Stage 17. (**C**) Stage 19. (**D**) Stage 20. (**E**) Stage 21. (**F**) Stage 23. R = rib, co = costal. Ve = vertebrae, ne = neural, nu = nuchal, p = peripheral. Cleared and stained with Alizarin red S. Scale bar: 10mm.

ticulate with their corresponding cranial and caudally, and medially with the costal. The first pair of peripheral articulate with the nuchal and the last pair with the pygal and suprapygal. These form the lateral border of the carapace (Fig.2f, 3c-e).

There were not found intra-specific variations in the analyzed specimens, although we believe that these could occasionally happen and that the limited number of skeletons available for evaluation made this investigation impracticable.

Carapace development

The ribs and the neural arches of the vertebrae were marked by the blue staining with Alcian, indicating that in these elements there is ossification of the endochondral type (Fig.1a), fact confirmed by histologic evaluation (Fig.4a-c). The ribs are the first elements to develop, still in stage 15 from a cartilaginous mold (Fig.1a, b). In stage 16 there are centers of ossification from the second to the seventh pair of ribs, with the three last pairs presenting lower degree of retention of the dye alizarin (Fig.2a, b). In the beginning of stage 17 the eighth pair of ribs already shows centers of ossification and in the stage 23 the first pair shows ossification centers.

All the ribs are circular in sagittal section. From stage 16 on it is possible to observe the presence of a slender pe-

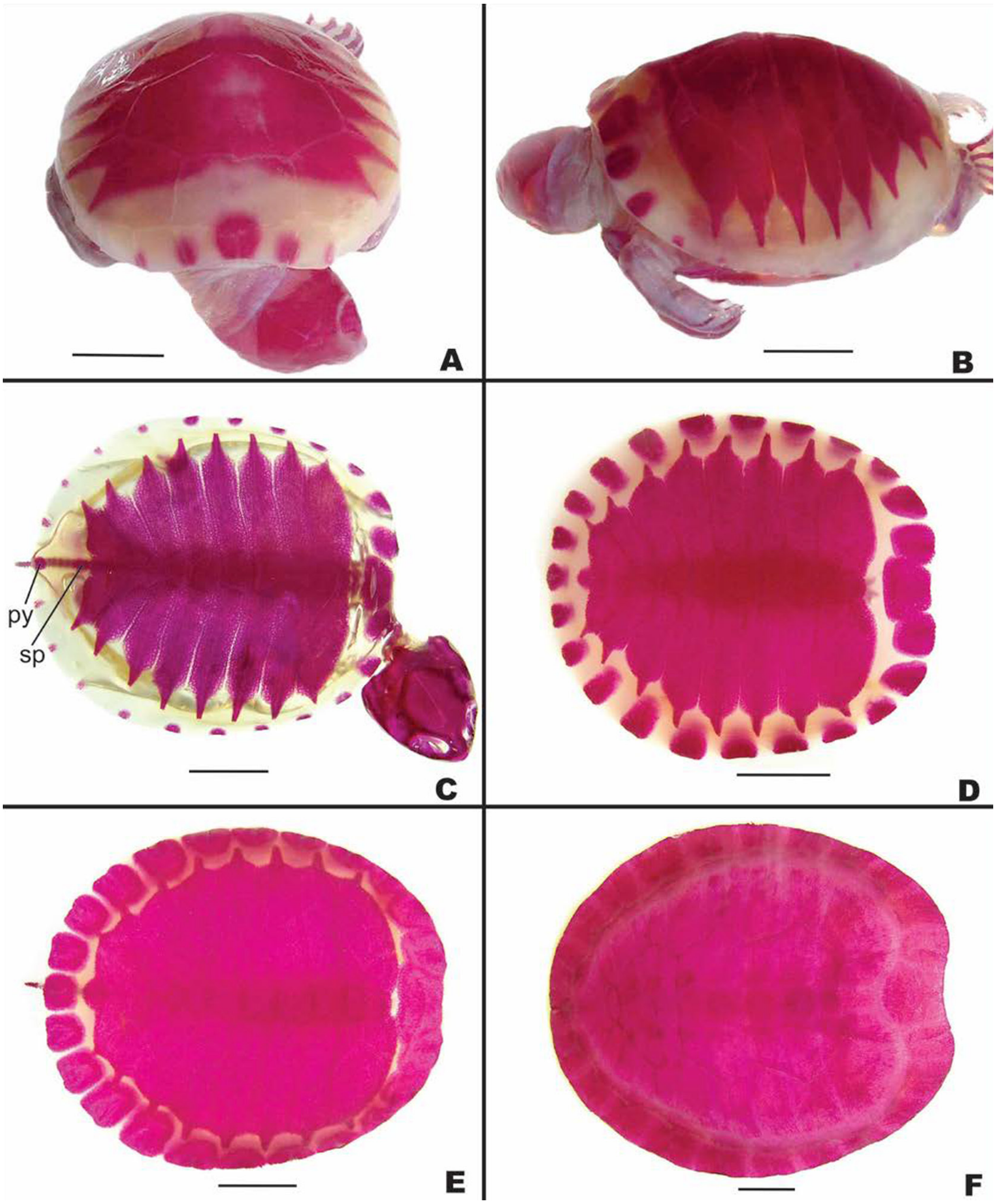

Fig.3. Embryos and hatchlings of *Podocnemis expansa*, dorsal view. (**A**) Stage 25. (**B**) Stage 26. (**C**) 5th posthatching day. (**D**) 18th posthatching day. (**E**) 43th posthatching day. (**F**) 46th posthatching day. Sp = supra-pygal, py = pygal. Cleared and stained with Alizarin red S. Scale bar: 10mm.

riosteal collar and fully formed, consisting in a thin layer of cellular tissue and other fibrous layer. The presence of osteocytes in the periosteal collar indicates the beginning of the ossification process, which occurs initially in the median and dorsal portions of the ribs (Fig.4a-c, f).

The ribs orientate the development of the ossification centers of the costal bones, there existing the correspondence of 1:1 between these elements (only for ribs from 2 to 9). The costals develop themselves as small projections of the external surface of the periosteal collar of the ribs, forming bone spikes. From both sides of the collar such bone projections emerge and guide themselves, predominantly, ventral and horizontally (Fig.2e, 3c-d, 4b-e).

By the end of stage 19, the bones expansions begin in the most cranial ribs, which orient the development of the costal bones (Fig.4c). There are bilateral projections of the periosteal collar of the ribs, although they do not show the same formation features. The bone spikes form from the mesenchymal tissue adjacent to the rib, where it is possible to observe a big amount of collagen tissue fibers which detached from the ribs, probable an artifact of the histologic technique, although there is some collagen tissue in the matrix.

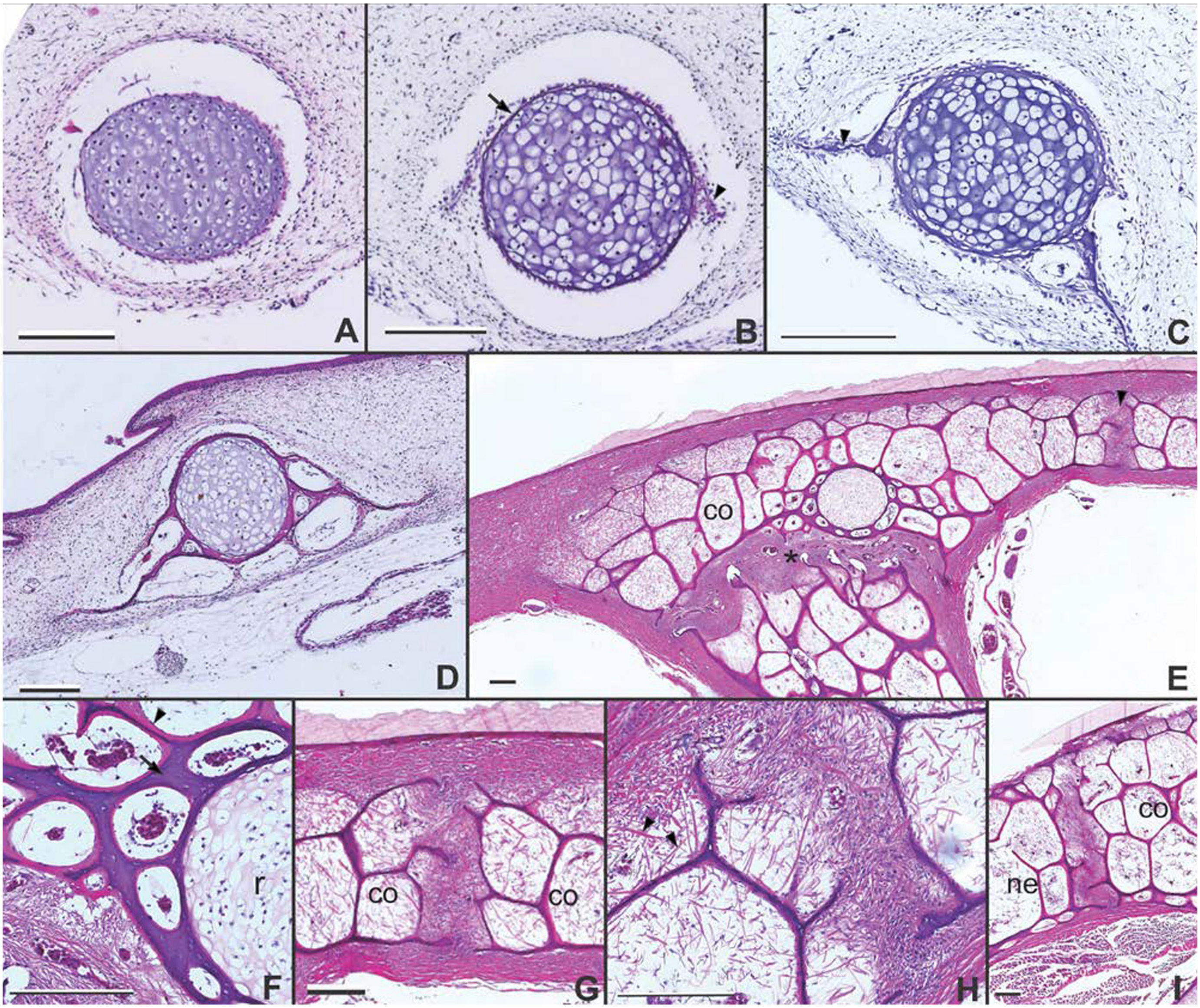

Fig.4. Histological studies of the carapace formation in *Podocnemis expansa*. Slices of the carapace stained with Hematoxylin and Eosin. (**A**) Sagittal sections of the ribs in stage 16, (**B**) stage 18, and (**C**) stage 19. 20x. In A shows a cartilaginous rib. B and C show a rib still containing cartilaginous portions and the beginning of the formation of the periosteal collar (arrow). The arrowhead in B and C points the mesenchymal condensations. C and D (10x) the sections show the development of the costal with lateral growth of the trabecular bony projections (**D**) starting from the periosteal collar of the rib (arrowhead). (**E**) Costal-rib section panoramic view (4x). This section shows the rib still composed of cartilage with expansion, very trabeculary, of the respective costal. * Shows the conective tissue under the costal bone. The arrowhead indicates the contact area between costal bones (detail in **G** - 20x). In **F** (40x), primary or old bone (arrow) and secondary or early bone (arrowhead) are formed during carapace ontogeny. **H** (40x) shows detail of the collagenous fibers (arrowheads) in the medullary cavity of the costal. **I** (10x): Section shows the contact of the trabecular projections of the neural and costal. Scale bar: 200μm.

In stage 21 the projection of costal bones is conspicuous and advances bilaterally in direction of the neighbor costal plate, remaining the rib structure, still cartilaginous, among the costal bone plates (Fig.4d). Conjunctive tissues sit between the costal plates, which in this stage still do not contact each other (Fig.4e, g). In stage 23 it is possible to perceive that this ossification progresses in a non-uniform way and in this same stage the most cranial costal bones begin to merge, in the mediolateral way (Fig.2f, 4g). In the same embryo it is possible to evaluate, in different stages, the formation of costal bones, since the formation of the periosteal collar in the ribs to the appearing and growing of the osseous expansions, from the formation of trabeculae in the mesenchyme to the formation of coastal plates (Fig.4i). A small osseous spike is continuous to the periosteal collar and develops itself in foreseen anastomosis with trabecular bone, resulting in the aspect of the costal bone. During the development of the spikes, they are surrounded by mesenchymal condensations and the cavities present in the skeleton are filled with fat tissues and bone narrow, besides fragments of collagen that unbounded from the spikes. Still in stage 26 it is not possible to investigate with the cleared embryos, stained and also with the histological slices the contact of the costal plates. The trabeculae still did not contact each other and between them there is a huge amount of mesenchymal tissue (Fig.4h).

The costal bones develop themselves by intramembranous ossification, being so the carapace composed by endochondral axial skeleton (ribs) and by dermic skeleton (costal plates). The costal bones form themselves associated to the periosteal collar structure of the ribs, by expansions in the shape of trabeculae. Dorsal to the neural arches, the neural plates begin to form. Small osseous spikes grow dorsal and laterally over the dermic tissue above the periosteal collar of the neural arch. Each spike is not overlaid by periosteum and these merge in a trabecular tissue. In stage 20, there are visible centers of ossification in the neural bones 3 to 6. In stage 21, all neural bones and nuchal bone show ossification centers. In stage 23, the first pair of peripheral bones show ossification centers (Fig.3d, e).

In stage 25, the second pair of peripheral bones show ossification centers, followed by the third to the eighth pair of peripheral bones in stage 26 (Fig.3c-f). In this same stage, ossification centers are still observed in the suprapygal and pygal bones (Fig.3c). From the ninth to the eleventh pair of peripheral bones, the ossification centers surge only five days after hatching. The approaching of the bones, which form the rigid armor of the carapace, only occurs after 46 days from hatching (Fig.3f). The ossification in the lateral region of the carapace takes longer and forms fontanelles between the costal bones and the peripheral bones. The osseous development of the carapace occurs, at the same time, in the skull-tail and mediolateral directions.

In contrast with the neural and costal bones, the nuchal bone is not directly associates to the development of the endoskeleton, although its ossification occurs also in an intramembranous way (Fig.3f). First of all, this bone forms itself by means of a narrow layer of condensed cells below the dermis, before deposit of calcium. The deposit of calcium, as a positive evidence of coloring with alizarin is reported in stage 21. Afterwards, in stage 22, it is possible to see, in section, a median bar of ossified trabeculae above the level of the two last cervical vertebrae. This posterior expansion of dermic bone forms the main portion of the nuchal.

DISCUSSION

The anatomy of the carapace is variable among the Pleurodira (França & Langer 2005). Broin (2000) suggests that the general morphology can vary ontogenetically from rounded in youths to lengthened in adults of Podocnemididae. *Podocnemis unifilis* (Lima et al. 2011) and *P. expansa* present very similar anatomy, with the presence of 7 neural bones, 8 costal, 11 pairs of peripheral, 1 nuchal bone, 1 pygal and 1 suprapygal. In *Apalone spinifera* (Sheil 2003) the carapace has 9 pairs of ribs and 22 dermic plates that project themselves dorsally, being the distal margins of ribs 2 to 8 extended beyond the lateral margins of dermic bones, forming fontanelles covered by epidermic shield membranes. This feature is absent in *P. expansa*, once in this species there are no fontanelles being formed.

In the *Trionyx* genera the neural is very variable, considering the morphology and the number of elements (Gardner & Russell, 1994). Ogushi (1911) mentioned 7 neurals in *Pelodiscus sinensis*, although 1 additional plate can be present. Either way, this pre-neural plate in some specimens, located posteriorly to the nuchal (Meylan 1987), can be considered neural 1, adding up to 8 bones. The pre-neural (neural 1) and the immediately posterior plate (neural 2) can be merged (Sánchez-Villagra et al. 2009), what occurs with *Phrynops hilarii*, where there are 6 neural bones, since during the ontogeny plates 1 and 2 merge (Bona & Alcade 2009). In *P. unifilis* (Lima et al. 2011) and *P. expansa* the number of neural bones remained constant, not having any merging between plates during ontogeny, hence seven bones were formed, this being the adult conduction.

Except some peripheral bones, the development of dermic bons of the carapace of *P. expansa* occur still in the embryonary period, differently from what was show by Gilbert et al. (2001) for *T. scripta*. Although the ribs of these animals begin to ossify still in the embrionary period the dermic bones of the carapace develop after the hatching, as an appearing of small ossification centers in the margins of the most cranial ribs and an extension of bone in the nuchal region.

Cuvier (1799) and Saint-Hilaire (1818) defined the carapace simply as expansions of the ribs and vertebrae. Wiedelman (1802) maybe was the first one to attribute a double origin to this structure, being a mix of endo and exoskeleton, followed by other researchers like Carus (1834) and confirmed afterwards by Rathke (1848) and Owen (1849), besides recent investigations (Gilbert et al. 2001, Scheyer et al. 2008, Vickaryous & Sire, 2009, Lima et al. 2011).

Effectively the ribs are the first elements integrating the carapace to ossify (Vallén 1942, Kälin 1945, Suzuki 1963, Ewert 1985, Cherepanov 1997, Gilbert et al. 2001, Scheyer & Sánchez-Villagra 2007, Scheyer et al. 2008, Vickaryous & Sire 2009, Lima et al. 2011). In *T. sinensis* the ribs 3 to 7 present 2 additional centers, a proximal and a distal to the medium center. According to Gilbert et al. (2001), in *T. scripta* the ribs begin their ossification in the embrionary period, but the majority of the carapace bones only after eclosion and without uniformity, what differs meaningly to what is found for *P. unifilis* (Lima et al. 2011) and in *P. expansa*, since big part of the dermic bones start their development still in the pre-birth period, with exception of the pygal and suprapygal in *P. unifilis* and the peripheral in *P. expansa*. In *C. serpentina* and *A. spinifera*, the ribs are also the first ones to develop, the ossification is bigger in the cranial region indicating that the direction is also skull-tail (Sheil, 2003, Sheil & Greenbaum 2005).

Yntema (1968) and Cherepanov (1997) reported in several Cryptodira the beginning of the ossification of the ribs in stage 19-20, about the same period where the neural arches begin their ossification in these. In the Pleurodira *E. subglobosa; P. sinuatus, Pelomedusa subrufa* (Scheyer et al. 2008) *P. unifilis* (Lima et al. 2011) the ossification of the neural arches began in stage 22. After the beginning of the ossification of the ribs in *C. serpentina* (Gilbert et al. 2001), in *T. scripta* (Kalin 1945) in *P. unifilis* (Lima et al. 2011) and also in *P. expansa*, dermic ossification centers, which tend to guide the development of the costal bones in this direction, appear bilaterally to these bones.

The ossification of costal bones in Pleurodira (*P. unifilis, Emydura subglobosa, Pelusios sinuatus, Pelomedusa subrufa, P. expansa*) and Cryptodira (*P. sinensis, T. sinensis, T. scripta, C. serpentina*) begins with the formation and a cartilaginous bar, the precursor of the rib, overlayed by a thin layer of periostea. This one consolidates to the lateral portion of the mesenchyme of the derma tissue to form a three-dimensional spongy structure in the tissue (Vallén 1942, Kälin 1945, Suzuki 1963, Ewert 1985, Cherepanov 1997, Gilbert et al. 2001, Scheyer & Sánchez-Villagra 2007, Scheyer et al. 2008, Vickaryous & Sire 2009, Lima et al. 2011).

For these Testudines the cartilaginous matrix of the rib degenerates itself inside the periostea, what induces the formation of the costals in a layer of dermic cells (Suzuki 1963). Small osseous spikes grow laterally of the periostea of the ribs in the adjacent dermic tissue, being the periosteal involved only in the initial stage of the formation process (Kälin 1945). Afterwards, the ossification occurs for mesenchymal aggregation processes forming spongy tissue and an inconspicuous cortical layer by intramembranous ossification (Scheyer & Sánchez-Villagra 2007). In *P. expansa* it is possible to observe still an agglomerate of collagenous fibers that permeate an abundant amount of fibroblasts scattered throughout the matrix during the process of formation of trabeculae. The trabecular osseous tissue is conspicuous from stage 20 and, in embrionary stages, still does not present diploe structure, feature of adult individuals (Scheyer & Sánchez-Villagra 2007, Scheyer et al. 2007, Cebra-Thomas et al. 2005).

Even though there is a current elucidation about the origin of costal bones, some features during development are still little reported. According to Gilbert et al. (2001), the costal bones form in two steps. The first in the medium of projected bars of the periosteal and the second when these bars form trabeculae which spread throughout the dermis. Our investigation indicated that in *P. expansa*, after the cartilaginous formation of the ribs, the periostea organizes itself in the outside portion of the precursor and laterally, in parasagittal cut, it is possible to observe the condensation of mesenchymal cells from where, in a primary step, the costal expansions begin. Afterwards, such expansions project themselves in a formation of trabeculae rich in bonny cells and overlayed by periostea, inserted in a big amount of fibroblasts, collagen and mesenchymal tissue. The differences are not subtle, but present a big similarity in the morphologic relations between the dermic and endochondral ossification in the ribs and costal bones.

According to Cebra-Thomas et al. (2005), bone morphogenetic proteins (BMP) signalized during the endochondral formation of the rib are capable of inducing the formation of bone in an intramembranous way in the adjacent dermic cells. As the cells ossify, they seem to transmit the BMP signal to the nearby cells, what creates a cascade and allows other derma cells to ossify. The combination of ribs to the dermic ossification results in the formation of the costal elements. Still according to the author, the total mineralization of these structures occur in the postnatal period in several Testudines. Suzuki (1963) informed that the neural bones are the first dermic bones to show signals of ossification in the carapace of *T. scripta*. On the other hand, in *P. expansa*, the ossification of the costal bones is more advanced than the neural bones, what was also observed by Gilbert et al. (2001) in the studies with *T. scripta* and *C. serpentina*. In these species, the neural bones are formed in association with the neural spines in the thoracic vertebrae. Rieppel (1993) observed that the ossification of the costal bones is apparent before the neural bones for this species and also for *C. serpentina*, just as related to *P. uniflis* (Lima et al. 2011) and *P. expansa*. Either way, in *P. sinensis* (Sánchez-Villagra et al. 2009), the first dermal bone to initiate ossification is the nuchal bone, with dyer retention in stage 21, the ribs only in stage 22 and the costal bones in stage 24. In *A. spinifera* (Sheil 2003), the sequence initiates with the nuchal bone and afterwards the costal ones. In *C. serpentina* (Rieppel 1993) only in stage 23 the ossification centers in the nuchal and pygal appear, similar to *P. unifilis*. The nuchal plate in *E. subglobosa* (Werneburg et al. 2009) begins to ossify simultaneously with the ribs, and the costal bones are the last elements of the carapace to ossify before hatching, and the neural only in the postnatal period, uncommon fact to the other Testudines reported.

Similar to the formation of costal bones, the neural bones have origin from the endoskeleton (neural arches). The cartilaginous matrix of the neural arch also degenerates inside the periostea and induces the formation of the neural through osseous spikes in the adjacent dermic tissue (Scheyer et al. 2008, Scheyer & Sánchez-Villagra 2007). According to what was found by Goette (1899), Vallén (1942), Gilbert et al. (2001) and Cebra-Thomas et al. (2005) for Criptodira and Scheyer et al. (2008) for Pleurodira, added to the descriptions for *P. unifilis* (Lima et al. 2011) and those contained here for *P. expansa*, such structures develop in a similar way among the two groups of Testudines (Scheyer et al. 2008). In both groups, such elements develop inside a homogenous derma with a random arrangement of collagenous stripes (Schmidt 1921).

The ossification centers in the peripheral bones begin in the cranial region of the carapace. These ossification centers form the border of the carapace and expand both in direction to the peripheral adjacent bones and in direction of the costal bones, during growth, just like it happens in *P. unifilis* (Lima et al., 2011) and *P. expansa*. The pygal bone is formed after the last pair of peripheral bones, being, so, the last bone to ossify. In *C. serpentina* the peripheral bones begin to ossify after the nuchal bone (Gilbert et al., 2001).

According to Werneburg et al. (2009) the nuchal bone begins to ossify early in the Cryptodira and later in the Pleurodira, an easy conclusion to be confirmed under careful analysis of the sequence of ossification in these species. We can observe also that in the Cryptodira the nuchal, neural and costal bones are the first ones to ossify without a definite pattern among species, once, in the Pleurodira, the nuchal bone normally appears after the neural and costal. The ossification sequence of the neural plates does not differ significantly among the Pleurodira, only when compared to the Cryptodira. Vallois (1922), Shah (1963) and Herrel et al. (2008) pointed some differences in the muscles of the neck in these groups, because of the different way of retraction and movement of the neck that after a more directed analysis may indicate some relation to the ontogeny of the nuchal bone.

The ossification centers in the peripheral bones of *P. sinensis* (Sánchez-Villagra et al. 2009), *T. scripta* (Gilbert et al. 2001), *P. hilarii* (Bona & Alcalde 2009) and *E. subglobosa* (Werneburg et al. 2009) and *P. expansa* are observed only in the post natal period, different of what was reported for *C. serpentine* (Gilbert et al. 2001) and *P. unifilis* (Lima et al. 2011) where these bones present retention of the dyer still in the embrionary period. The peripheral bones present, in every reported species, a skull-tail ossification pattern, these being the last ones to ossify in *C. serpentina* (Rieppel 1993), and the pygal bone in *P. expansa*.

In *P. sinensis* (Sánchez-Villagra et al. 2009), *E. subglobosa* (Werneburg et al. 2009), *P. hilarii* (Bona & Alcalde, 2009) e *P. unifilis* (Lima et al. 2011) the suprapygal does not present ossification center in the embrionary period. The differences in the ossification sequence were observed between *P. expansa* and the other Testudines confronted, although the ribs are the first carapace forming structures to ossify in all of these.

CONCLUSIONS

Costals and neurals are plates derived from ribs and neural arches, respectively, in continuity with the periostea of the endoskeleton.

There were chronological differences in the ossification of the carapace of *Podocnemis expansa* in comparison to the other Testudines.

The first element to form was the ribs, which presented uniformity among the reported species.

The Podocnemididae *P. expansa* and *P. unifilis* share many similarities during the carapace ontogeny.

The main differences are in the chronology and may express variations because of abiotic variations that influenced the incubation period. The phylogenetic proximity of these two species may also explain such similarity.

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