Regular Paper

SEQUENCE OF METACARPAL AND PHALANGEAL BONE FORMATION IN EMBRYOS OF *PODOCNEMIS EXPANSA* SCHWEIGGER, 1812 (TESTUDINES, PODOCNEMIDIDAE) STAINED WITH ALIZARIN RED S

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ABSTRACT

Twenty-four embryos were collected to investigate the sequence of metacarpal and phalangeal bone formation in the various stages of prenatal development of *Podocnemis expansa*, starting from the 18th day of natural incubation. Biometric measurements were taken and the embryos were subjected to the diaphanization technique with bone staining, followed by the Davis and Gore method. Each paw has five metacarpi (M) and 14 phalanges, two on the first finger and three on each of the other fingers, with a phalangeal formula of 2:3:3:3:3. Retention of the stain in the metacarpi occurs in the following order: M III > M II = M IV > M I > M V. In stage 20, it is evident that, in all the metacarpi, the ossification centers progress towards the epiphyses. The sequence of stain retention in the distal phalanges (DP) occurs in the order: DP III > DP II > DP IV > DP I > DP V. In the medial phalanges (MP), the stain retention sequence is: MP III > MP IV > MP II > MP V. Finally, the proximal phalanges (PP) retain staining in the following sequence: PP I > PP III > PP IV > PP II > PP V. The differences and similarities in the ossification synchronization of *Podocnemis expansa* and comparable species are evident. Thus, there is no osteogenetic pattern common to all chelonians, due to variations in the initial site of bone formation and the sequence of ossification.

Key Words: skeleton, bone, stain, *Podocnemis expansa*, reptiles.

INTRODUCTION

Animal skeletons consist of a set of live structures that grow, adapt and repair themselves. Bone tissue is present in almost all the regions of the body, and individual skeletal characteristics are highly diverse in terms of morphology and tissue architecture [32]. Most reptile bones are formed and grow by endochondral ossification, a process characterized by intermediary cartilage. The pattern of events that make up this model involves a process of several phases in which prechondrogenic mesenchymal cells form condensations before differentiating into chondroblasts [18].

According to Tagariello et al. [30], each of these stages, from condensation transition to differentiation, is characterized by specific temporal patterns and various changes occur in predictable periods. Disturbances in this highly harmonious series of unfolding events in cartilage and bone development, growth and homeostasis inevitably result in defects of the skeleton [3].

For Fritsch [7] and Gray et al. [8], knowledge of the biological criterion for the sequence of bone formation in predictable sites and times is of great clinical interest. Bones are subject to numerous pathological alterations that hinder their normal function of support and movement. These disorders are targets of preventive and surgical medicine, and their correction requires support by basic science [10].

Numerous studies of chelonians have identified biotic and abiotic factors during embryogenesis [2,15,6]. However, most of these works have mainly focused on the external morphology [4, 9.31], and few authors have supplied data describing the sequence of bone formation of the skeleton (21,27].

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Studies have also been dedicated to other reptiles and amphibians. These include the contributions of Rieppel [22], Rieppel [23] and Rieppel [24], who studied the skeletal ossification of *Alligator mississippiensis*, *Chamaeleo hoehnelii* and *Gehyra oceanica*, respectively, and those of Pugener and Maglia [19] and Perotti [17], who investigated the skeletal development of Discoglossus sardus and Leptodactylus chaquensis. These reports may offer useful comparative data to clarify the phylogenetic placement of chelonians among other reptiles and amniotes.

Podocnemis expansa, popularly called the giant Amazon turtle, is widely distributed in the Amazon basin [13]. According to Alho et al. [1], this species is the largest freshwater chelonian of South America and is currently zootechnically considered one of the most widely exploited wild animals [26].

Although some studies have focused on aspects of pre- and post-natal bone development in chelonians [6,33], the literature still lacks reports about the sequence of bone formation of the *P. expansa* members.

The use of alizarin red S dye has become universal in bone staining due to its selective properties. Based on microspectroscopy, Moriguchi et al. [12] explained the adsorption mechanism of bone staining with this dye, showing its efficiency in the detection of calcium deposits in the stain reaction.

The purpose of this study was to research the sequence of formation of bone elements that make up part of the paw segment, more specifically the metacarpal and phalangeal bones in the distinct phases of pre-natal development of P. expansa.

MATERIAL AND METHODS

Twenty-four embryos of *Podocnemis expansa* Schweigger, 1812 (Testudines, Podocnemididae) were collected during the spawning period of September 2005 in the reproduction area protected by IBAMA/RAN (Center for Reptile and Amphibian Conservation and Handling) on the beaches of the Araguaia River in the state of Goiás, Brazil, in the region called Remansão (13º 20' 38.7" S and 50º 38' 05.7" W), under permit no. 117/2005-IBAMA/RAN.

Specimens (eggs) were collected at random from an arbitrarily selected nest, starting on the 18th day of natural incubation (stage 16) up to hatching. The embryonic development stages were named according to the external morphological criteria described by Danni et al. [4] for *P. expansa*.

The embryos were removed from the eggs by cutting the shell with surgical scissors, isolating them from the vitellus and from their membranous sacs. The biometrics of all the embryos were recorded (Table 1), using metal calipers (125 MEB-6/150, Starret) with a precision of 0.05 mm and an analytical balance (AND HR-120, Gravimeta) with a precision of 0.1 gram. The recorded parameters were: carapace length (CL), carapace width (CW), plastron length (PL), plastron width (PW), and body weight (BW), following the method described by Malvásio et al. [11].

Each embryo was subjected to the technique of diaphanization by potassium hydroxide (KOH) with bone staining by alizarin red S, according to Davis and Gore [5]. This work was carried out in the Wild Animal Research Laboratory (LAPAS) of the Federal University of Uberlândia (UFU), Faculty of Veterinary Medicine.

The presence of bone formation centers and the distinct stages of metacarpal and phalangeal bone development (Table 1) were analyzed under a stereoscopic microscope (SZX 12, Olympus) to which a camera was attached to capture images (Figure 2). A schematic diagram was also drawn up of the paw bones of Podocnemis expansa (Figure 1).

RESULTS

The description of the events involved in the metacarpal and phalangeal bone formation was based on anatomical preparations using *P. expansa* embryos from stage 18 through 24 (Figure 2B, C, D, E, and F).

The skeletal anatomy of the P. expansa paw displays three regions: the carpus, the metacarpus and the phalanges. This document describes the latter two.

The metacarpi consist of five bone elements, conventionally numbered in the median-lateral direction of the metacarpi I, II, III, IV and V. These bones are long, with a distal head and an expanded proximal base, which articulate with the proximal phalanges and with the distal row of the carpus, respectively.

Each paw has 14 phalanges, two on the first digit and three on each of the other digits, with a phalangeal formula of 2:3:3:3:3. The proximal phalanges are shorter, except for digit I, which follows the pattern of the median phalanges. The distal phalanges are cone-shaped, ending in a pointed tip

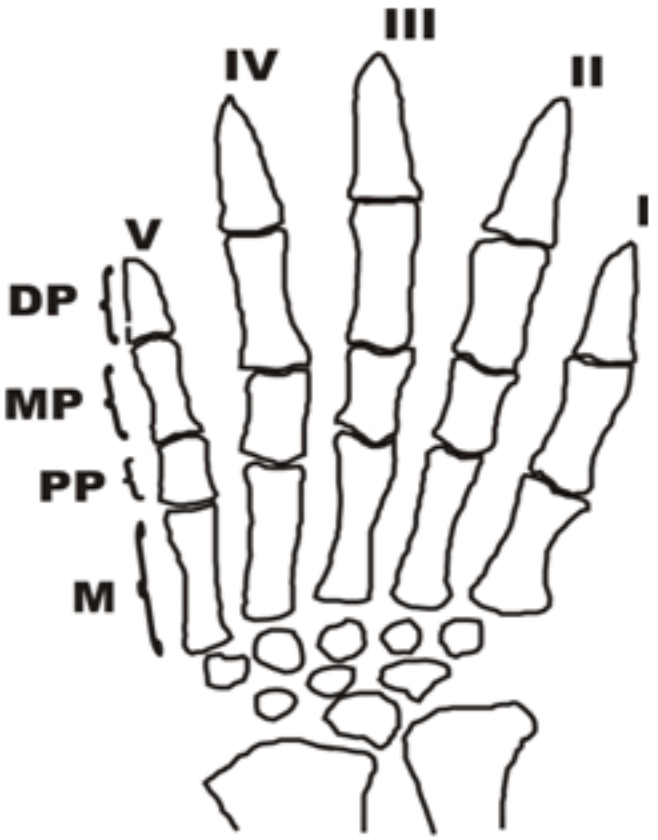

Figure 1 – Drawing of the bones of the left hind paw of Podocnemis expansa. Distal Phalanges (DP); Medial Phalanges (MP); Proximal Phalanges (PP); Metacarpi (M); Digit I (I); Digit II (II); Digit III (III); Digit IV (IV); Digit V (V).

The ossification of the metacarpi (M) becomes apparent in the diaphysis and M III displays more extensive ossification with greater retention of alizarin (Fig. 2A). This suggests that M III is the first bone to present an ossification center. In stage 18 (Fig. 2B), retention of the alizarin stain occurred in the following order: M III > M II = M IV > M I > M V. In stage 20 (Fig. 2C), it became evident that, in all the metacarpi, the ossification centers progress towards the epiphyses.

During stage 18 (Fig. 2B), the distal phalanges (DP) exhibit ossification centers in digits I to IV, with digits II and III exhibiting more advanced bone formation.

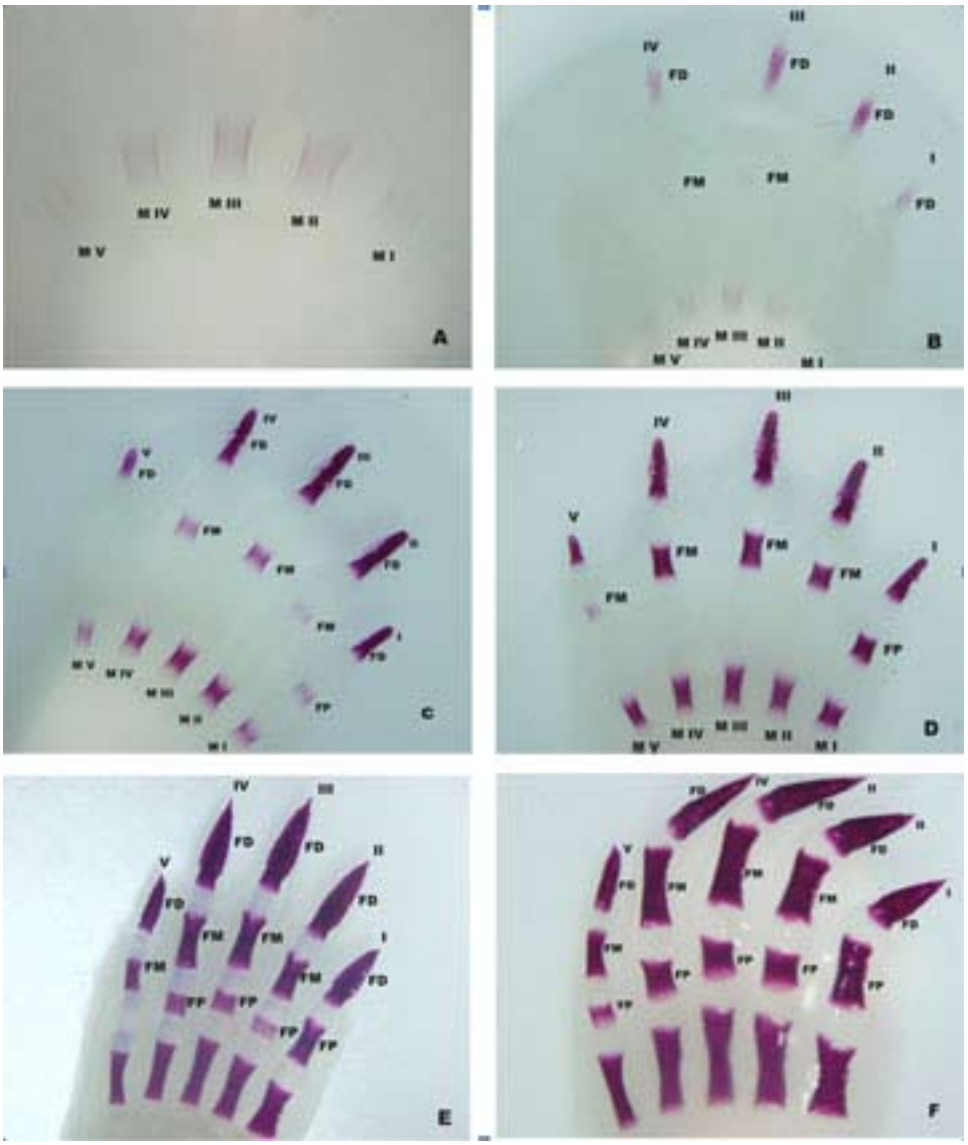

Figure 2 - Photographs of paws of *Podocnemis expansa* embryos, dorsal view. A and B - stange 20: Development of the metacarpi and distal phalanges; C and D - stange 20, 21: Development of the medial phalanges E and F - stage 22, 23: Development of the proximal phalanges F - stage 23: all the centers of bone formation. Distal Phalagens (DP); Medial Phalagens (MP); Proximal Phalanges (PP); Metacarpi (M); Digit I (I); Digit II (II); Digit III (III); Digit IV (IV); Digit V (V). Diaphanization by KOH and staining with Alizarin red S. Magnification: (A) 40 x, (B, C, D e E, F) 16 x, (E) 12,5 x.

Ossification of all the distal phalanges was only found at stage 20 (Fig. 2C). Therefore, the sequence of stain retention in the distal phalanges occurs in the following order: DP III > DP II > DP IV > DP I > DP V.

Table 1 – Mean ± SD of length (L) and width (W) of the carapace and plastron, height, cranium-to-tail length (CT) and weight of *Podocnemis expansa* embryos in stages 18 to 24.

Stage	Carapace		Plastron		Height	CT	Weight
	Length	Width	Length	Width			
18	18,45 ±1,28	15,26 ±1,87	11,98 ±2,14	9,05 ±1,37	8,12 ±1,18	36,68 ±1,41	4,03 ±1,08
19	19,76 ±1,36	16,16 ±1,30	13.31 ±0,02	10,78 ±0,54	9,95 ±0,52	37,85 ±1,25	4,52 ±0,23
20	19,99 ±1,16	17,05 ±0,96	14,18 ±0,56	11,85 ±0,87	10,05 ±0,96	39,25 ±1,06	4,97 ±0,35
21	30,51 ±1,01	26,51 ±1,22	22,96 ±1,24	21,75 ±1,60	14,30 ±0,74	42,80 ±0,96	12,98 ±1,05
22	36,42 ±0,45	32,60 ±0,65	27,23 ±1,12	24,13 ±3,64	17,61 ±0,63	49,80 ±1,23	20,02 ±0,47
23	37,95 ±0,65	36,11 ±0,02	26,50 ±1,21	25,31 ±13	20,18 ±0,28	52,90 ±1,42	23,83 ±0,61
24	38,55 ±0,62	37,25 ±1,39	27,89 ±1,97	25,02 ±0,89	21,48 ±1,65	54,11 ±1,81	25,23 ±1,87

Table 2 – Sequence of ossification of *Podocnemis expansa* metacarpal and phalangeal bones in stages 18 to 24. Distal Phalanges (DP); Medial Phalanges (MP); Proximal Phalanges (PP); Metacarpi (M); Digit I (I); Digit II (II); Digit III (III); Digit IV (IV); and Digit V (V).

Stage		18	20	21	23	24
I	M	X	X	X	X	X
	PP		X	X	X	X
	DP	X	X	X	X	X
II	M	X	X	X	X	X
	PP				X	X
	MP		X	X	X	X
	DP	X	X	X	X	X
III	M	X	X	X	X	X
	PP				X	X
	MP	X	X	X	X	X
	DP	X	X	X	X	X
IV	M	X	X	X	X	X
	PP				X	X
	MP	X	X	X	X	X
	DP	X	X	X	X	X
V	M	X	X	X	X	X
	PP					X
	MP			X	X	X
	DP		X	X	X	X

The ossification centers of the median phalanges (MP) first appear in digit III at stage 18 (Fig. 2B), followed by digits IV and II at stage 20 (Fig. 2C). At the same time, the presence of an ossification center was observed in the proximal phalanx of digit I. The median phalanx of digit V displays an ossification center at stage 21 (Fig. 2D). The degree of bone formation occurs thus: MP III > MP IV > MP II > MP V.

With the exception of digit V at stage 23 (Fig. 2E), all the proximal phalanges (PP) present an ossification center. This event takes place in the following order: PP I > PP III > PP IV > PP II > PP V.

By stage 24 (Fig. 2F), all the metacarpi and phalanges are in an advanced process of embryonic bone formation. (see tables 1 and 2)

DISCUSSION

The bone diaphanization and staining technique has been used and proved efficient to reveal ossification centers, as reported by Franz-odendaal [6], Patton and Kaufman [16], Nakane and Tsudzuki [14] in their studies of bone formation in rats, C. serpentina turtles and Japanese quail.

In the present study, although the embryonic stages are given, comparisons were made among species in terms of the sequence of bone development events.

Differences and similarities are apparent in the ossification synchrony of the structural units in the paws of *Chelydra serpentina, Apalone spinifera, Macrochelys temminckii and P. expansa*. Anatomical differences in the phalangeal formulation also occur, with 2:3:3:3:3 for *P. expansa*, *C. serpentina* and *M. temminckii* and 2:3:3:4:3 for *A. spinifera*, even though they all belong

to the order Testudines.

C. serpentina, *A. spinifera*, *M. temminckii* and *P. expansa* all show a chronologically similar onset of bone formation in M I and M V.

In a new study of the skeletal development of C. serpentina, Sheil and Greenbaum [27] found evidence of intraspecific variations indicating that M IV is the first ossification center, followed by M III and then M II. These events are similar to those found in M. temminckii and different from those occurring in P. expansa.

According to Sheil [28], the ossification centers in *A. spinifera* begin to appear in M I, M III and M IV, without revealing the sequence of stain retention, although, in *P. expansa*, the greatest variation in the sequence of ossification occurred in these three bones.

The patterns of phalangeal bone formation in C. serpentina proposed by Rieppel [21] and Sheil and Greenbaum [27] are not identical at every point. However, it can be stated that in both studies, bone formation in the digits of the paw begins in the distal phalanges and proceeds in the distoproximal direction. This pattern was found in *P. expansa*, as is the case of *M. temminckii* [29], in which the ossification centers first appear in the distal phalanges of digits I to IV. Subsequently, they appear in the median phalanges of digits II to IV and then in the proximal phalanx of digit I. On the other hand, in *A. spinifera* [28], ossification of the phalanges of digits I to III progresses in the proximodistal direction.

This information indicates that in *P. expansa*, *M. temminckii* and *C. serpentina*, the pattern of bone formation of digits I to IV is similar, with the exception of digit V of *M. temminckii* [29], where bone formation is slower than in the other digits and advances in the proximodistal direction.

Details of the relative sequence of bone formation have been documented for lizards and crocodilians and comparisons among those studies have identified patterns among important lines of Reptilia.

The sequence and synchrony of bone formation in P. expansa displays both similarities to and differences from *Lacerta vivipara* [20], Lacerta agilis exígua [25] and *Alligator mississippiensis* [22] It is evident that more quantitative studies are necessary to document the natural variability of bone formation.

The phalanges of Lacerta vivipara, Lacerta agilis exígua and *Alligator mississippiensis* usually ossify in the proximodistal direction, while in P. expansa, these elements ossify in the distoproximal direction.

According to Rieppel [21], considerable attention has focused on documenting sequences of bone development during ontogeny, but relatively little is known about intraspecific variations in any taxon. In this context, one can highlight the differences in the chronology of bone formation observed among some of the bones that make up the skeletal anatomy of the *P. expansa* paw.

CONCLUSIONS

The phalangeal formula of *Podocnemis expansa* during its prenatal development is 2:3:3:3:3. The ossification of digits I, II, III, IV and V occurs in the distal-to-proximal direction.

The sequence of bone formation is: M III > M II = M IV > M I > M V; FD III > FD II > FD IV > FD I > FD V; FM III > FM IV > FM II > FM V e FP I > FP III > FP IV > FP II > FP V.

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