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Ontogeny of the Appendicular Skeleton in *Melanosuchus niger* (Crocodylia: Alligatoridae)

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The objective of the present study was to analyze chondrogenesis and the ossification pattern of the limbs of *Melanosuchus niger* in order to contribute with possible discussions on homology and the fusion pattern of autopodial elements and phylogeny. In the *Reserva Extrativista do Lago Cuniã*, Rondônia, Brazil, six nests were marked and two eggs removed from each nest at 24-hour intervals until hatching. Embryos were cleared using KOH; bone tissue was stained with alizarin red S and cartilage with Alcian blue. Routine staining with HE was also performed. In the pectoral girdle, the scapula showed ossification centers before the coracoid process. In the pelvic girdle, the ilium and the ischium were condensed as a single cartilage, although ossification took place through two separate centers, forming distinct elements in the adult. The pubis developed from an independent cartilaginous center with free end, which reflects its function in breathing. In the initial stages, the stylopodium and the zeugopodium developed from the condensation of a Y-shaped cartilage in the limbs, and differentiation of the primary axis and digital arch were observed. The greatest changes were observed in the mesopodia. In their evolution, Crocodylia underwent a vast reduction in the number of autopodial elements as a consequence of fusions and ossification of some elements. This study shows that the chondrogenesis and ossification sequences are dissociated. Moreover, the differences between *M. niger* and other species show clear variation in the patterns for these events in Alligatoridae.

Key words: alizarin, Archosauria, development, embryo, black caiman

INTRODUCTION

Archosauria appeared in the Triassic and have survived to the present. They diversified over time, punctuated by major extinction events, producing a great diversity in terms of both morphology and the number of species, as demonstrated by an extensive fossil record (Clark, 1994). Crocodylia, together with birds, are the living representatives of this group (Benton and Clark, 1988). With respect to the skeleton, the order Crocodylia is characterized by a reduction in the number of carpal and tarsal elements, which reflects adaptations to the environment and the mode of locomotion specific to these species (Müller and Alberch, 1990). Even though the numbers of ossified elements in the autopodium of some archosaurians are the same, the chondrogenesis patterns differ considerably. In general, skeletal configurations of tetrapod limbs derive from a primitive pattern through losses and fusions of bones that may be observed in

in embryological studies (Romer, 1956; Hildebrand and Goslow, 2006; Fröbisch, 2008).

Bone and cartilage formation sequences are a source of information that has seldom been used to date. However, Maisano (2002), Sánchez-Villagra (2002), and Schoch (2006) emphasized the potential of these data in phylogenetic studies. Although these sequences are normally conserved within individual species, the factors that affect their evolution are mostly speculative, as in the effect of the ossification sequence heterochrony and morphological evolution (Sánchez-Villagra, 2002; Prochel, 2006; Sánchez-Villagra et al., 2008a).

Several groups have been conducting studies of different organisms and have provided important insights into archosaur anatomy, evolution, phylogeny, and embryology. In order to obtain such information, several studies have described the ontogeny of the skeleton in different animals (e.g., Rieppel, 1993a, 1994a, b; Sheil, 2003; Vieira et al., 2011a; Sheil and Portik, 2008; Kundrát, 2009). However, there are few studies that report bone development in Crocodylia, except for those by Müller and Alberch (1990) and Rieppel (1993b), both of which dealt with the ontogeny of *Alligator mississippiensis* skeleton, by Lima (2010), who

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described limb ossification in *Caiman yacare*, and by Larsson et al. (2010), who discussed the pentadactyl status of *Alligator mississippiensis* hands. A clearer understanding of the mechanisms of the development of tetrapod limbs enables inferences about the processes in fossil groups. Incorporation of these inferences adds perspective to the evolutionary history of tetrapod limbs.

The objectives of the present study are to describe chondrogenesis and ossification patterns of the hindlimb, forelimb, and girdles of *Melanosuchus niger*, and thereby to contribute to discussions of the evolution and homology of Crocodylia, and to improve the understanding of the connection patterns among autopodial elements, the origin of the digital arch, and the fusion of carpal and tarsal elements.

MATERIALS AND METHODS

Location and collection of the embryos

In the Reserva Extrativista do Lago Cuniã, state of Rondônia, Brazil, six nests of *Melanosuchus niger* (Spix, 1825) were marked and protected with a metal screen after oviposition between October and December 2008. Each nest was kept under natural incubation conditions, and two eggs were removed every 24 hours, starting the tenth day of development until the moment of hatching (RAN/IBAMA license number 12243-1/2007). An ontogenetic sequence with 186 samples was obtained.

Embryos were surgically removed from the eggs, killed with a thiopental overdose (50 mg/kg), and preserved in formaldehyde 3.7%. These procedures were approved by the Animal Ethics Committee from the Instituto de Ciências Biológicas at Universidade de Biológicas (UnBDOC no. 100271/2009).

Diaphanization and staining of bones and cartilages

Samples were cleared with potassium hydroxide (KOH). Bones were stained with alizarin red S and cartilages with Alcian blue following the methods by Davis and Gore (1936) and Dingerkus and Uhler (1977), with some modifications.

The specimens were analyzed with the aid of a stereomicroscope (Leica, DM 1000) equipped with a camera (Leica, Camera Software DFU Twain 6.7.0). In the present study, we used the term "ossification center" for a small area of ossification observed in each bone, recognized by means of alizarin staining, which shows the accumulation of calcium; this is not necessarily the first event in bone development.

Histology processing

Histology processing was carried out in the Laboratório de Histopatologia at Universidade Federal de Uberlândia (UFU), following a routinely generally used method (Behmer et al., 1976). From each fragment of an individual, several slides with semi-serial cuts were prepared. After that, they were stained by the routine hematoxylin and eosin method. Slides were analyzed using a binocular Olympus BX40 equipped with an Olympus OLY-200 camera. The images were captured using 5 and 10x-magnification objective lenses.

Terminology

For identification of cartilage condensations of the limbs and the ossification pattern, the terminology proposed by Burke and Alberch (1985), Shubin and Alberch (1986), and Müller and Alberch (1990) was used.

Embryonic stages were determined using external morphological characteristics, following the methods proposed by Vieira et al. (2011a).

RESULTS

Chondrogenesis of the forelimb and pectoral girdles

Before we describe chondrogenesis, it is important to emphasize that the adult *Melanosuchus niger* pectoral girdle is made up of a pair of bones, the scapula and the coracoid, as well as the interclavicle (Fig. 1A). In the forelimbs, the humerus is the stylopodium element, and the ulna and radius are the zeugopodium elements (Fig. 1B, C). *Melanosuchus niger* carpus consists of four bones: ulnare, radiale + intermedium, fused distal carpals 3 + 4 + 5, and pisiform (Fig. 1D). It has five metacarpals, numbered mediolaterally as metacarpals 1, 2, 3, 4, and 5. The phalangeal formula is 2:3:4:3:2.

In stage 10 of Vieira et al. (2011b), forelimbs started with typical developmental events also observed in other tetrapods, in which the stylopodium (humerus) and zeugopodium elements (ulna and radius) are formed in a proximal-to-distal direction, that is, elongating and branching in a Y-shape (Fig. 2A). At the beginning of stage 11, the primary axis is clearly formed. This consisted of the ulnare and distal carpal bone 4, which extended into digit IV in later stages. The cartilaginous center in the radiale (Fig. 2B) was also observed. Before the end of this stage, the two elements that form the pectoral girdle, the scapula and the coracoid, appeared as cartilaginous centers and, at the beginning of stage 13, consisted of well-defined cartilage (Fig. 5A).

At the end of stage 11, the digital arch was composed of distal carpals (dc) 4, 3, and 2. Distal carpal 5 also appeared, but it was not part of this arch. Besides these elements, all metacarpals started to form, with metacarpal V being the last one to appear. Phalanges of digits I–IV showed very clear centers of chondrogenesis. The pisiform was also visible, and appeared as a new lateral condensation with the ulnare (Fig. 2C and D).

Phalanx 1 of digit V began its chondrogenesis in stage 12, as did phalanges 2 of digits I–III. In this stage, the numbers of phalangeal cartilages was 2:2:2:1:1. The chondrogenesis center of the centrale appeared distal to the radiale. This center appeared when the other elements of the carpus had already started to be formed (Fig. 2D). At the beginning of stage 13, phalanges 3 of digits II and III, and phalanx 2 of digit IV showed highly visible centers of chondrogenesis. The phalangeal formula in this stage was 2:3:3:2:1.

In stage 14, phalanx 3 of digit IV, phalanx 2 of digit V, and phalanx 4 of digit III were observed, yielding the phalangeal formula 2:3:4:3:2. In this stage, distal carpals 4 and 5 started to be fused with each other. The centrale appeared to be more robust and lied distal to the radiale (Figs. 2F and 4A).

Distal carpal bone 3 started to fuse with distal carpal bones 4 and 5, forming a single and large carpal element, dc 5 + 4 + 3, in stage 16 (Figs. 3A and 4C, D). The centrale got closer to these fused element and to distal carpal bone 2, but did not fuse with them (Figs. 3A, 4B–D). Phalanx 4 in digit IV also appeared, yielding the phalangeal formula 2:3:4:4:2.

Phalanx 3 of digit V appeared at the beginning of stage 18, yielding phalangeal formula 2:3:4:4:3. This phalanx was transitory, because it did not become ossified in the adult (Fig. 3B). In stage 19, phalanx 5 of digit IV appeared with

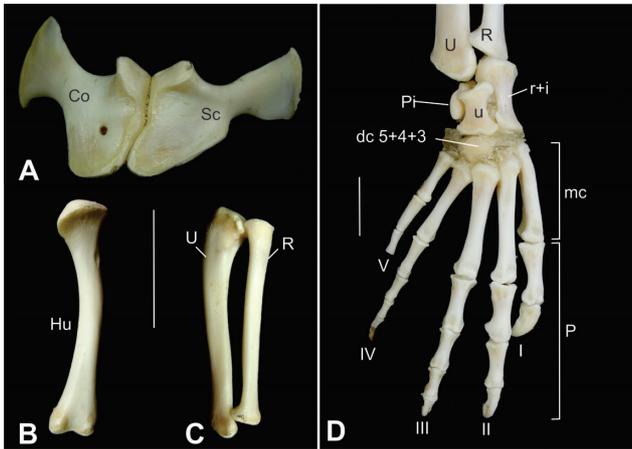


Fig. 1. Forelimb and pectoral girdle of adult *M. niger* in lateral (A), cranial (B, C) and dorsal (D) view. Abbreviations: dc5 + 4 + 3, fused distal carpals 5 + 4 + 3; Co, coracoid; Sc, scapula; P, phalanges; I–V, digits I–V; mc, metacarpals; Pi, pisiform; R, radius; r + i, radiale + intermedium; u, ulnare; U, ulna; Hu, humerus. Scale: 30 mm.

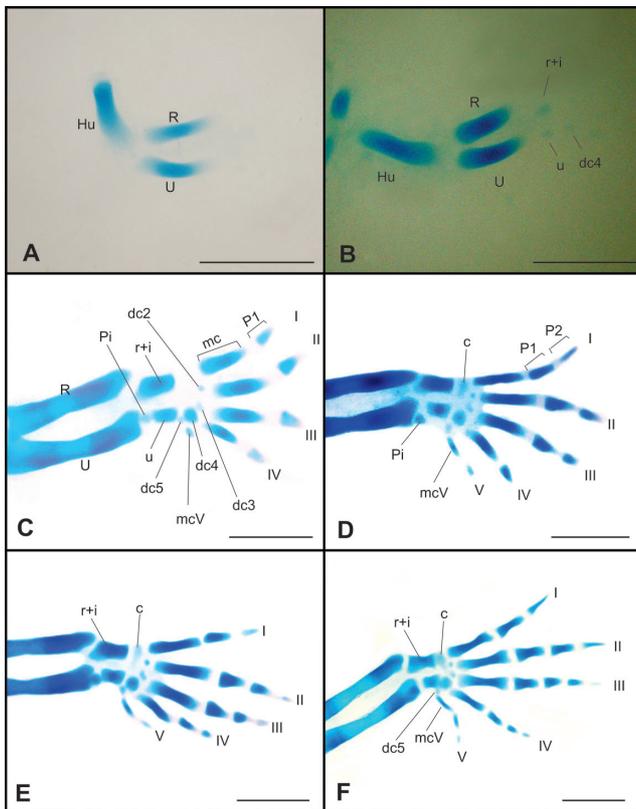


Fig. 2. Hand of *M. niger* embryos, dorsal view. (A) stage 10, (B) beginning of stage 11, (C) end of stage 11, (D) stage 12, (E) beginning of stage 13, (F) stage 14. Abbreviations: c, centrale; dc 2–5, distal carpals 2–5; I–V, digits I–V; P 1–2, phalanges 1–2; mc, metacarpals; Pi, pisiform; R, radius; r + i, radiale + intermedium; U, ulna; u, ulnare; HU, humerus. Diaphanized with KOH and stained with Alizarin red S and Alcian Blue. Scale: 2 mm.

very light staining (Fig. 3D), yielding the phalangeal formula 2:3:4:5:3. This phalanx started to fuse with phalanx 4 at the beginning of stage 20, and reduced the phalangeal formula

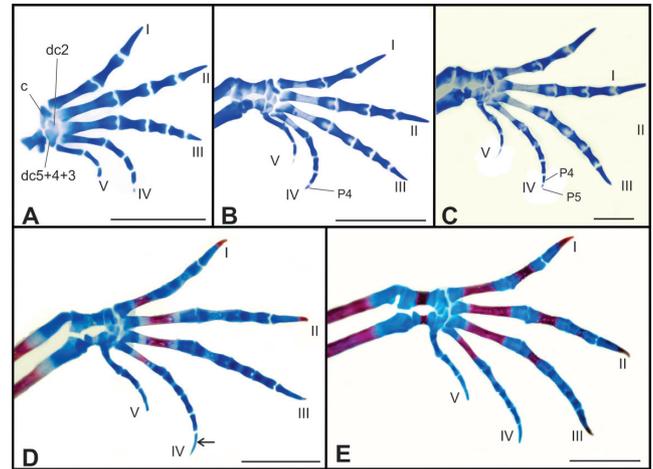


Fig. 3. Hand of *M. niger* embryos, dorsal view. (A) stage 16, (B) beginning of stage 18, (C) end of stage 22, (D) beginning of stage 24. Abbreviations: c, centrale; dc 2, distal carpal 2; dc5 + 4 + 3, fusion of distal carpals 5 + 4 + 3; P4, phalange 4; P5, phalanges 5, I–V, digits I–V; arrow, fusion of the phalanx. Diaphanized with KOH and stained with Alizarin red S and Alcian Blue. Scale: 2 mm.

to 2:3:4:4:3. At the end of stage 22, phalanx 4 was completely fused with phalanx 5 (Fig. 3E). Soon, phalanx 3 of digit V started to fuse with phalanx 2 of the same digit in stage 23. This fusion was only complete at the beginning of stage 24, yielding the final cartilaginous phalangeal formula 2:3:4:4:2 (Fig. 3F).

Forelimb and pectoral girdle ossification pattern

In stage 21, the scapula had an ossification center and, at the end of this stage, so did the coracoid (Fig. 5B). Alizarin red S staining was observed in the diaphyses of the humerus, radius, and ulna at the start of stage 18, indicating the beginning of the perichondral ossification process. The sequence of ossification among these stylopodial and zeugopodial elements was not clear (Fig. 6A). Staining gradually advanced towards the epiphyses in these elements, and showed a well-defined outline, very similar to that of adult bones in stage 28 (Fig. 7F).

Ossification of metacarpals (mc) started in the diaphysis of mc II and III, as shown by discrete alizarin staining in stage 21 (Fig. 6B). Mc I and IV started their ossification in stage 22. In the same embryo, ossification centers also appeared in phalanx 2 of digit I and phalanx 3 of digit II. At the end of this stage, only a discreet ossification center was observed in the radiale + intermedium, and another in the ulnare among carpal bones (Fig. 6C), both of which intensified in later stages (Fig. 7C).

At the beginning of stage 24, ossification centers were observed in phalanges 1 of digits I through III, and a discreet ossification center in phalanx 4 of digit III (Fig. 6E). Slight signs of alizarin staining in metacarpal V and phalanx 1 of digit IV were also observed, which became more intense later in stage 24. Besides these ossification centers, alizarin staining was observed in phalanx 1 of digit IV, and phalanges 2 of digits II and III, appearing more discreet in the latter digit (Fig. 6F). Phalanx 3 of digit III showed discreet alizarin

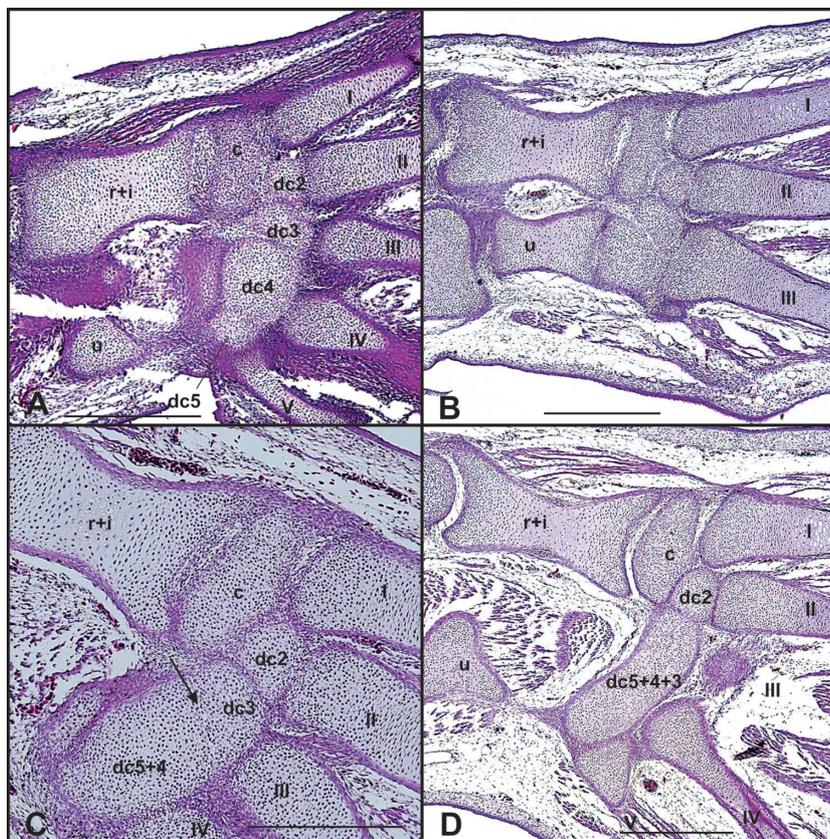


Fig. 4. Histological sections of the hand of *M. niger*. (A) beginning of stage 14, (B) end of stage 14, (C) stage 15, (D) beginning of stage 18. Abbreviations: c, centrale; dc2–5, distal carpals 2–5; I–V, digits I–V; r + i, radiale + intermedium; u, ulnare; arrow, site of the condensation. Staining: Hematoxylin and eosin. Scale: 0.5 mm.

V showed ossification centers in later stage 25; in the carpus, an ossification center was observed in the pisiform (Fig. 7C). In stage 26, dc 5 + 4 + 3 showed a single ossification center; this was the last element of the carpus to start the ossification process (Fig. 7E).

Intraspecific variations were observed in some embryos beginning in stage 25. Most of the embryos showed ossification in all the elements of the carpus and, only later, in phalanx 4 of digit IV (Fig. 7C and E). In six of 15 specimens, this phalanx showed an ossification center first (Fig. 7D).

As a consequence of the events in the morphogenetic series described above, the carpus in embryos of *M. niger* was made up of three proximal elements, i.e., the radiale + intermedium, the ulnare and the pisiform, and five distal elements: the centrale, and distal carpals 2, 3, 4, and 5. Between the end of the embryonic period and recent hatchlings, carpal elements underwent extensive remodeling, fusions and ossifications to yield the adult pattern. That is, ossification was complete in the radiale + intermedium, the ulnare, and the pisiform. Distal carpals 3, 4, and 5 also fused and ossified with only one ossification center, but an extensive cartilaginous cover was maintained; distal carpal 2 contributed to this cover, and did not ossify. The centrale did not ossify, either, becoming more fibrocartilaginous.

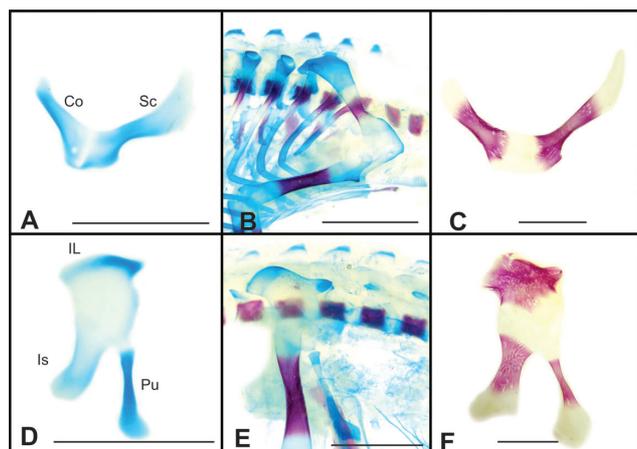


Fig. 5. Forelimb (A–C) and hindlimb (D–F) of *M. niger* embryos. (A) ventral view, beginning of stage 13, (B) lateral view, end of stage 21, (C) ventral view, end of stage 24, (D) lateral view, beginning of stage 13, (E) lateral view, end of stage 21, (F) lateral view, end of stage 24. Abbreviations: Co, coracoid; Ss, scapula; IL, ilium; Is, ischium; Pu, pubis. Diaphanized with KOH and stained with Alizarin red S and Alcian Blue. Scale: 5 mm.

staining at the end of stage 24 (Fig. 7A). Phalanx 1 of digit V did so as late as at the beginning of stage 25 (Fig. 7B).

Phalanges 2, 3, and 4 of digit IV, and phalanx 2 of digit

Chondrogenesis of the hindlimb and pelvic girdle

Before description of the chondrogenic sequence, it is important to emphasize that, in the adult *M. niger*, the pelvic girdle is made up of three bones: ilium, ischium, and pubis (Fig. 8A). The stylopodium consists of the femur, and the zeugopodium of the tibia and the fibula (Fig. 8B and C). *Melanosuchus niger* tarsus consists of four bones: fused intermedium + centrale, fibulare, distal tarsal 3, and distal tarsal 4. It has four long metatarsals I, II, III and IV, with metatarsal II and III being relatively longer than the others. Metatarsal V is a very small bone and is morphologically different from the other metatarsals. No phalanges articulate with it, and it is placed laterally to digit IV, with a large articulation area with distal tarsal 4. The feet have a phalangeal formula 2:3:4:4 (Fig. 8D).

The first cartilaginous condensation appeared at the end of stage 7. At this stage, the accumulation of cartilaginous condensation, marked in blue, was Y-shaped. The proximal part corresponds to the stylopodium (femur) and the distal branches, to the zeugopodium elements (fibula and tibia). At the end of stage 8, clear cartilaginous outlines of the femur, tibia and fibula, separated from one other, were observed (Fig. 10A).

At the end of stage 9, in the distal end of the fibula, the fibulare and distal tarsal 4 appeared, with discrete staining by Alcian blue, starting to form the primary axis of this limb

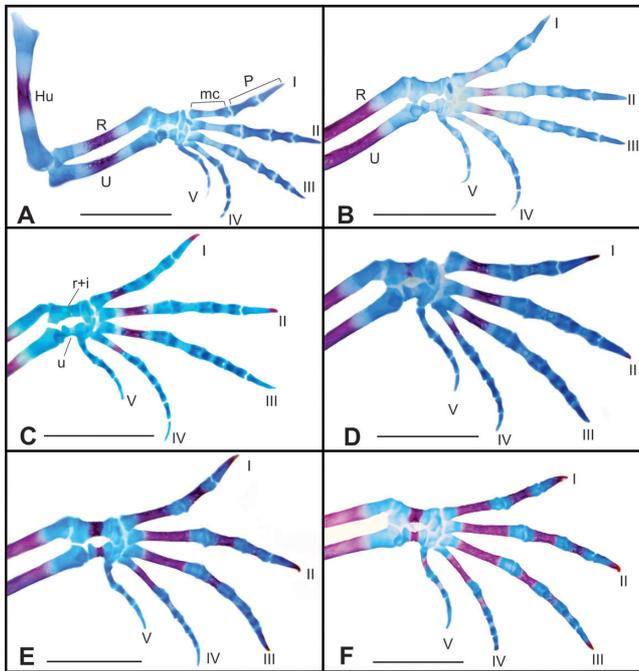


Fig. 6. Hand of *M. niger* embryos, dorsal view. (A) beginning of stage 18, (B) stage 21, (C) end of stage 22, (D) stage 23, (E) beginning of stage 24, (F) later stage 24. Abbreviations: P, phalanges; I–V, digits I–V; mc, metacarpals; U, ulna; u, ulnare; Hu, humerus; R, radius; r + i, carpal radiale + intermedium. Diaphanized with KOH and stained with Alizarin red S and Alcian Blue. Scale: 5 mm.

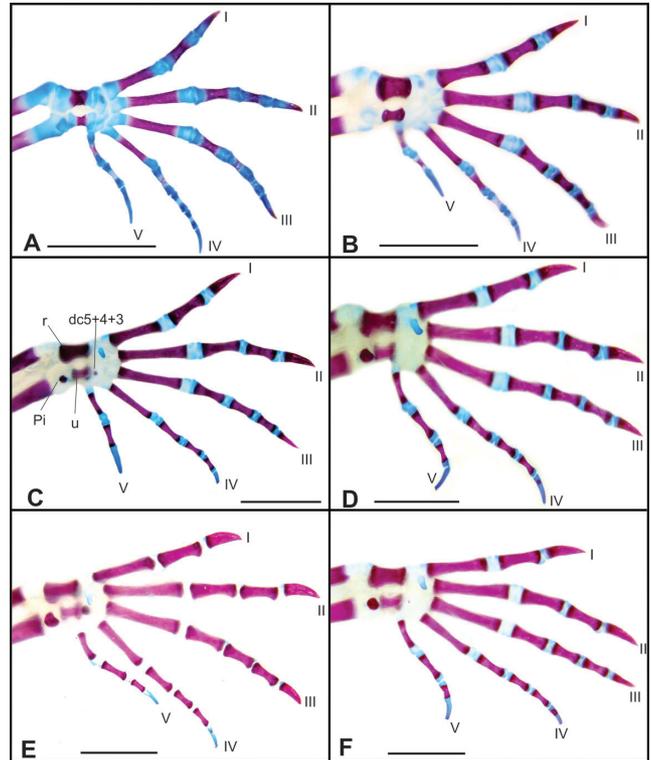


Fig. 7. Hand of *M. niger* embryos, dorsal view. (A) end of stage 24, (B) beginning of stage 25, (C) later stage 25, (D) end of stage 25, (E) beginning of stage 26, (F) stage 28. Abbreviations: I–V, digits I–V; dc 5 + 4 + 3, fusion of distal carpals 5, 4 and 3; Pi, pisiform; r + i, radiale + intermedium; u, ulnare. Diaphanized with KOH and stained with Alizarin red S and Alcian Blue. Scale: 5 mm.

(Fig. 10B). In stage 10, metatarsals III and IV appeared as a prolongation of this axis (Fig. 10C). Still in this stage, intermedium formation began to be observed between the distal ends of the fibula and tibia, besides a discrete indication of the centrale, although the image did not show it clearly (Fig. 10C).

At the beginning of stage 11, an indication of distal tarsal 3, without an apparent link with distal carpal 4 was observed, showing the initial formation of the digital arc, at the end of stage 11 (Fig. 10D). The intermedium showed to be more robust, prolonging towards the distal end of the tibia. In this stage, the intermedium started to fuse with the centrale to form the intermedium + centrale (Figs. 9 and 10D, F). Besides this element, the metatarsals were also formed, including atypical metatarsal V and phalange 1 of all the digits. Phalange 2 also was found in digits II, III, and IV. In this stage, in the pre-axial/post-axial direction, the phalangeal formula was 1:2:2:2:0 (Fig. 10D). We observed that these three elements formed the pelvic girdle, pubis, ilium and ischium, with defined cartilage morphogenesis. The ilium and the ischium developed as a single cartilaginous condensation.

In stage 12, the only new events were the appearances of phalanx 2 of digit I and phalanx 3 of digit III, with very light staining (Fig. 10E). Phalanges 3 of digits II and III appeared at the beginning of stage 13. In addition, distal tarsal 2 also appeared (Fig. 10F). Phalanx 4 of digit III appeared at the end of stage 14 (Fig. 10G).

At the beginning of stage 18, the last cartilaginous element of the hindlimb, phalanx of digit IV appeared with very

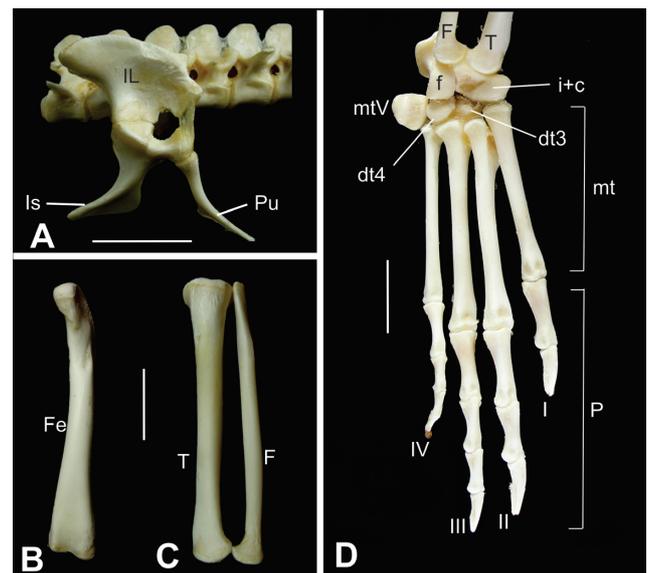


Fig. 8. Hindlimb and right pelvic girdle of *Melanosuchus niger* in lateral (A), cranial (B, C) and dorsal (D) view. Abbreviations: dt3–4, distal tarsals 3 and 4; F, fibula; f, fibulare; Fe, femur; P, phalanges; I–V, digits I–V; i + c, intermedium + centrale; IL, ilium; Is, ischium; mtV, metatarsal V; Pu, pubis; T, tibia. Scale: 30 mm.

light staining, yielding the final cartilaginous phalangeal formula 2:3:4:5:0 (Fig. 10H).

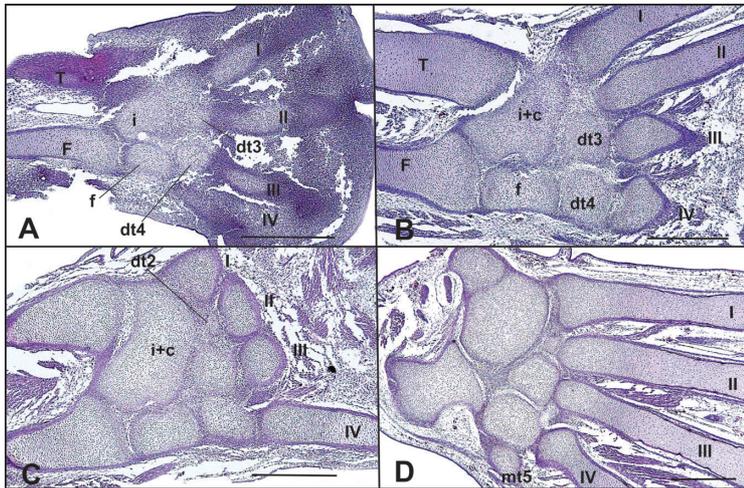


Fig. 9. Histological sections of the feet of *M. niger*. (A) end of stage 11, (B) beginning of stage 13, (C) end of stage 13, (D) end of stage 16. Abbreviations: dt2–4, distal tarsals 2–4; F, fibula, f, fibulare; i, intermedium; i + c, intermedium + centrale; I–V, digits I–V; mt5, metatarsal 5; T, tibia. Staining: Hematoxylin and eosin. Scale: 0.5 mm.

Hindlimb and pelvic girdle ossification pattern

The elements of the pelvic girdle started to ossify at the end of stage 21, with a minuscule ossification center appeared at the pre-acetabular process of the ilium. At the end of this stage, ossification centers also appeared in the ischium and the pubis (Fig. 5E). The ossification of the stylopodium (femur) and zeugopodium elements (tibia and fibula) appeared as simultaneous alizarin staining, for the first time, at the end of stage 18 (Fig. 11A). Bone formation always began at the diaphyses, starting as perichondral bones, and progressed towards the epiphyses. In stage 28, they showed a well-defined outline of the bones similar to adults.

Metatarsal ossification (mt) started in the diaphysis at the end of stage 19, with staining of mt II and III, followed by mt I and IV, at the beginning of stage 20 (Fig. 11B). Atypical metatarsal V ossified much later, together with elements of the tarsus, at the beginning of stage 25, a period when the other metatarsals were already very advanced in the process (Fig. 11H).

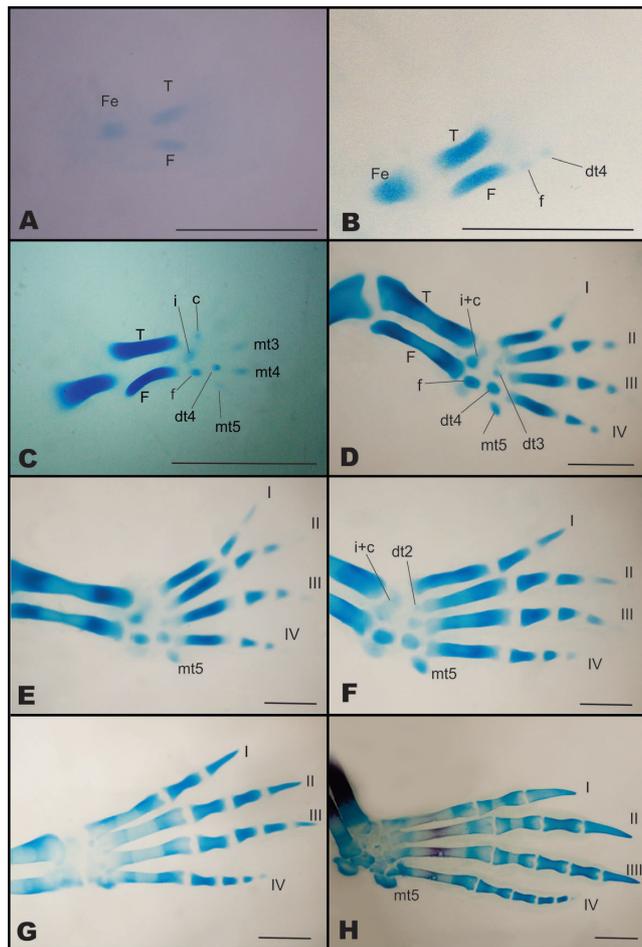


Fig. 10. Feet of *M. niger* embryos, dorsal view. (A) end of stage 8, (B) end of stage 9, (C) beginning of stage 10, (D) end of stage 11, (E) stage 12, (F) beginning of stage 13, (G) end of stage 14, (H) beginning of stage 18. Abbreviations: dt2–4, distal tarsals 2–4; Fe, femur; F, fibula; f, fibulare; I–V, digits I–V; i, intermedium; i + c, intermedium + centrale; mt5, metatarsal 5; T, tibia. Diaphanized with KOH and stained with Alizarin red S and Alcian Blue. Scale: 2 mm.

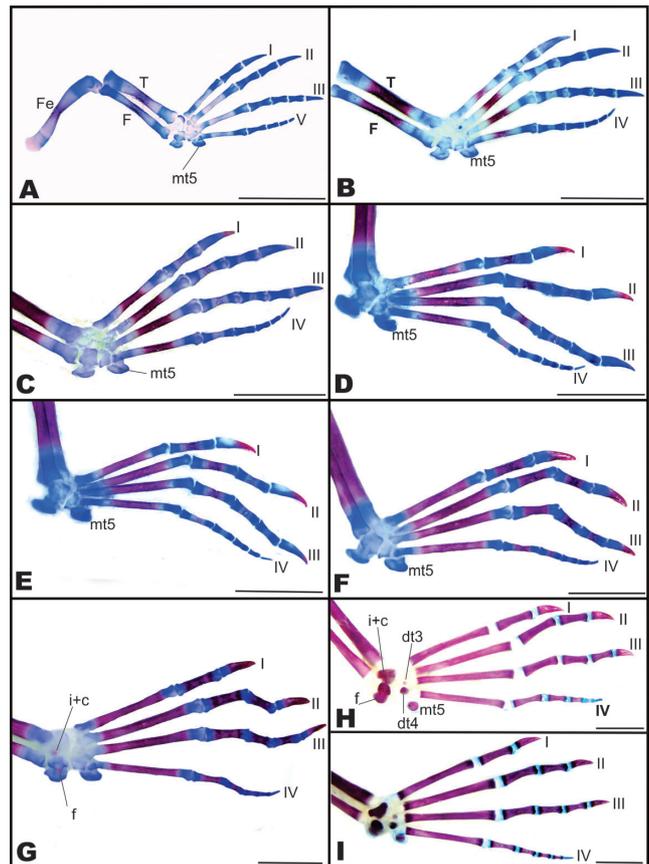


Fig. 11. Feet of *M. niger* embryos, dorsal view. (A) end of stage 18, (B) end of stage 19, (C) end of stage 21, (D) beginning of stage 22, (E) end of stage 22, (F) beginning of stage 24, (G) end of stage 24, (H) beginning of stage 25, (I) beginning of stage 26. Abbreviations: dt3–4, distal tarsals 3–4; Fe, femur; F, fibula; f, fibulare; I–IV, digits I–IV; i + c, intermedium + centrale; mt5, metatarsal 5; T, tibia. Diaphanized with KOH and stained with Alizarin red S and Alcian Blue. Scale: 5 mm.

Among the phalanges, ossification was apparent only in phalanx 2 of digit I at the end of stage 21 (Fig. 11C). Discreet disappearance of the Alcian blue staining in phalanx 1 of all digits was observed, but without alizarin staining. These phalanges only showed staining at the beginning of stage 22. In addition, phalanx 3 of digit II showed intense alizarin staining (Fig. 11D). There were slight signs of alizarin staining in phalanges 2 of digits II and III (Fig. 11D). At the end of stage 22, the only new event was the appearance of the ossification center in phalanx 3 of digit II (Fig. 11E). At the beginning of stage 24, ossification centers appeared phalanges 2 of digits II and III. In addition, phalanx 2 of digit IV and phalanx 3 of digit III also had ossification centers. At the end of this stage, slight signs of alizarin staining were observed in phalanx 3 of digit IV. In the tarsal elements, there was a discrete ossification center in the fibulare, and another in the intermedium (Fig. 11F and G).

Distal tarsal 4 and metatarsal V showed ossification centers at the beginning of stage 25 (Fig. 11H). At the end of this stage, distal tarsal 3 appeared, the ossification process that was intensified only in the beginning of stage 26 (Fig. 11I). Signs of ossification in phalanx 4 of digit IV were also observed, although the ossification process became evident in the following stage. Therefore, the stage 27 embryo had the phalangeal formula of the adult, 2:3:4:5.

There were five tarsal elements in the embryo: intermedium + centrale, fibulare, and distal tarsals 4, 3, and 2. Similar to the carpus, the juvenile elements of the tarsus underwent extensive remodeling and changes in size to yield the adult form. The intermedium + centrale, fibulare, distal tarsals 4 and 3 were ossified. Distal tarsal 2 became fibrocartilaginous and remained intimately associated with distal tarsal 3 and the base of metatarsal I. Phalanx 5 of digit IV remained cartilaginous and became smaller in the adult.

DISCUSSION

Pectoral and pelvic girdles

In ancestral archosaurs, according with Clark (1994), the scapula is larger than the coracoid, and this is a primitive condition. In fact, most known basal taxa of Mesoeucrocodylia have a scapula that is longer than the coracoid. The difference in length between these bones is intermediate in Crocodyliformes including extant Crocodylia, as their scapula is only slightly longer than the coracoid. In *M. niger*, the scapula showed ossification centers before the coracoid did, and the initial difference in length between these two bones was maintained during development. However, in adult individuals, these bones did not differ significantly.

In the pelvic girdle, the ilium and ischium developed from a single cartilaginous condensation in *M. niger*, in spite of the fact that ossification started in two ossification centers and formed, distinct elements that are strongly connected by a fibrous joint, in adult. This characteristic aids the breathing process in a way that the lack of participation of the pubis in *M. niger* and other living Crocodylia in the formation of the acetabulum enables dorsoventral movements and, consequently, lung expansion (Farmer and Carrier, 2000). As the rotation of the pubis enables the expansion of the abdominal cavity and provides space for the movement of the visceral caudal during air intake, it is possible that the evolution of the pubis in Crocodylia reflects a tendency for aquatic hab-

its, such as longer submersion.

Limbs

Our observations on the development of *M. niger* limbs were consistent with the pattern reported in other tetrapods (Shubin and Alberch, 1986), and may be comparable with those of similar studies carried out in other taxa. However, we did identify some differences, and we explore their significance in the following paragraphs.

Primary axis and digital arch

Past analyses of ontogeny have shown the notable cartilage morphogenesis in tetrapods. Cartilage morphogenesis precedes the development of the limb skeleton and is invariable among amniote classes (Hinchliffe and Hecht, 1984; Shubin and Alberch, 1986). Shubin and Alberch (1986) discussed the origin and organization of the limbs, proposed a pattern for this morphogenesis, and defined the plan for tetrapod limbs. These authors described the development of the skeleton based on spatial connections, where the first skeletal condensations appear proximally, and other condensations take place by segmentation and branching, thus forming the primary axis.

Müller and Alberch (1990) concluded that the primary axis is the most prominent and invariable characteristic in limb development. A similar pattern was found in *M. niger*, as well in other archosaurs, such as *Alligator mississippiensis* (Müller and Alberch, 1990), *Struthio camelus* (Kundrát, 2009), and in *Gallus gallus* (Burke and Feduccia, 1997). The same pattern is also followed in anurans (Fabrezi and Alberch, 1996), lizards (Blanco and Alberch, 1992), and Testudines (Sanchez-Villagra et al., 2008, 2009; Sheil and Portik, 2008; Vieira et al., 2011b).

Another morphogenetic pattern, the digital arch, is also invariable during development (Sheil and Portik, 2008). In *A. mississippiensis*, this arch originates as a branch of the primary axis, consisting of distal carpals/tarsals 4, 3 and 2; the corresponding metacarpals/metatarsals appear as a result of the sequence of branching events that follow the development of this arch (Muller and Alberch, 1990). Studies by these authors showed results similar to those of Kundrát (2009) for *S. camelus*, who emphasized that distal carpal 3 and 2 developed from a single cartilaginous focus and, in later stages, fused with distal carpal 4, forming a single element, distal carpal 4 + 3 + 2. The analyses carried out in *M. niger* confirmed that this arch appeared after the primary axis. However, the branching pattern in the origin of these elements is unclear, different from what was reported by several authors for different taxa (Burke and Alberch, 1985; Shubin and Alberch, 1986; Müller and Alberch, 1990; Fabrezi et al., 2007). Staining and histological processing indicated that there were independent condensations in all distal carpals in *M. niger*, demonstrating that much remains to be studied on limb development. According to Hinchliffe (2002), and Sheil and Portik (2008), before it is possible to determine a complete outlook of limb evolution, it will be important to understand the exact patterns of formation in known taxa.

Radiale + intermedium and centrale morphogenesis

In *M. niger*, no focus of chondrogenesis was observed

in the intermedium. Only the radiale was clearly seen. However, Hinchliffe and Hecht (1984), Müller and Alberch (1990), and Burke and Feduccia (1997), reported the presence of an embryonic condensation that represented the fusion of the intermedium with the condensation of the radiale in Crocodylia and birds. In Testudines, in contrast, these elements develop from individual foci of chondrogenesis (Burke and Alberch, 1985; Sheil, 2003, 2005). Mathur and Goel (1976) described the intermedium as a transitory cartilage that disappears during development in *Calotes versicolor*. Leal et al. (2010) suggested that the intermedium may appear in some lizard species during embryonic development, in spite of its absence in the carpus of adult specimens. Fabrezi et al. (2007) analyzed several lizard taxa and concluded that more detailed analyses of limb development should be carried out to confirm the identity of this element. Given these results, it is important that more studies on the formation of these elements are undertaken before it is stated that there is a conserved pattern in amniotes.

As for the centrale, the present study and that by Müller and Alberch (1990) in *A. mississippiensis* considered that only the centrale remained unformed in the digit arch. These authors reported that the centrale was the last element to appear. It developed in an uncommon manner because it originated from the segmentation of the radiale + intermedium, whereas Shubin and Alberch (1986) emphasized that the central elements of the carpus have a post-axial origin. In *M. niger*, we also regarded this element as centrale, due to its location in the adult. However, we do not agree that the radiale + intermedium is involved in its development.

In other reptiles, such as Testudines, Burke and Alberch (1985) also observed that the central regions of the carpus remained undifferentiated, except for the condensation of the intermedium, during the differentiation of elements in the digital arch. These authors also suggested that centrale IV appeared via a condensation inside this central region of the autopodium. Such evidence was already reported by Sanchez-Villagra et al. (2007) in *Chelonia mydas*. However, these authors only analyzed the dimensions of these elements. Different from *M. niger* and the other crocodylians, two centrales are described in Testudines (Sanchez-Villagra et al., 2007; Vieira et al., 2011b).

Intermedium + centrale morphogenesis

Similar to the case in *A. mississippiensis* (Müller and Alberch, 1990), the intermedium appeared between the distal ends of the fibula and tibia. Later on, it developed as a distinct anterior process and occupied the distal region of the tibia soon after the formation of the primary axis in *M. niger*. In this process, a secondary cartilaginous center, the centrale, appeared and remained connected with the intermedium. Therefore, the intermedium + centrale cartilage differentiated as a single chondrogenesis condensation with two foci: a proximal one (the intermedium), and a distal one (the centrale). Similar to *A. mississippiensis*, expansion of the intermedium was considered to be the centrale, due to this embryonic origin and to the region it was located.

As for the ossification process, this element was ossified from a single ossification center, although its genesis involved two foci of chondrogenesis, in the *M. niger*. The formation of these elements in lizards is similar to that of

Crocodylia (Fabrezi et al., 2007). In other reptiles, the centrale may be absent, as observed in *P. expansa* (Vieira et al., 2011b), *C. mydas*, and *C. caretta* (Sanchez-Villagra et al., 2007).

Ossification and reduction of carpal and tarsal bones

Carpal bones

Holmgren (1933), Romer (1956), and Hinchliffe (1985, 1991) sought to explain the evolution of archosaurian limb skeleton. Many of these studies were based on comparative data, and only later on, on embryological studies. According to these studies, 13 pre-condensations represent the initial condition of cartilage morphogenesis of carpal elements in Crocodylia (Steiner, 1934 *apud* Müller and Alberch, 1990). Subsequently, several fusions result in only six elements (Müller and Alberch, 1990; Buscalioni et al., 1997).

Our findings are different from some of these traditional interpretations. As a consequence of events in the morphogenetic series, stages, the carpus of *M. niger* was made up of three proximal elements (radiale + intermedium, ulnare, and pisiform), and five distal elements (centrale, and distal carpals 2, 3, 4, and 5), totaling eight condensations in the initial development stages. At a more advanced embryonic stage (stage 16), fusions, remodeling, and ossifications produced the carpus with four ossified elements and two cartilaginous elements. Therefore, fusions and lack of ossification led to the reduction in the number of these elements in *M. niger*. This characteristic is shared by other living crocodylians, such as *A. mississippiensis* (Müller and Alberch, 1990; Rieppel, 1993a; Buscalioni et al., 1997) and *Caiman crocodylus yacare* (Lima et al., 2011).

As for the ossification pattern in the carpal region, the radiale + intermedium and ulnare as the first elements to present ossification centers in *M. niger*. These are elongated elements, with the radiale longer than the ulnare. According to the Benton and Clark (1988), this characteristic is one of the synapomorphies of the Crocodylomorpha. The pisiform showed an ossification center after these elements. According to Shubin and Alberch (1986), Müller and Alberch (1990), also in *M. niger*, the pisiform appeared as a new post-axial condensation, to the side of the ulnare. It was then completely ossified, and together with the radiale + intermedium and ulnare, they are the most common findings in fossil records (Buscalioni et al., 1997).

Cartilaginous condensation of distal carpals 3 + 4 + 5 in *M. niger* and in *A. mississippiensis* (Müller and Alberch, 1990) results in a single ossification center. The other distal carpals remained cartilaginous. According to Müller and Alberch (1990), this latter element becomes more fibrocartilaginous after hatching.

Using paleobiological and embryological data, Buscalioni et al. (1997) carried out a comparative study in order to analyze the hypothesis of loss of carpal elements in Crocodylia. According to these authors, a review of extinct taxa showed no more than three ossified distal carpals. This observation was recorded in fossils of species in the Atoposauridae and Thalattosuchia families (Wellnhofer, 1971; Buscalioni et al., 1997). On the other hand, living Crocodylia represent an homogenous group in which all members of the clad have only one ossified distal carpal (Romer, 1956). These statements are coherent with our findings in *M. niger*, as well as

data on *A. mississippiensis* (Müller e Alberch, 1990) and *C. yacare* (Lima, 2010). Other reports showed the occurrence of two ossified distal carpals in *Orthosuchus stormbergi* (Nash, 1975), *Sichuanosuchus huidongensis* (Peng, 1995 *apud* Buscalioni et al., 1997), and *C. paradoxos* (Wu and Sues, 1996). As a consequence, given observations on these distal carpals in fossils of these Crocodylomorpha and the understanding of embryonic development of the hand in living Crocodylia, such as *M. niger*, *A. mississippiensis* and *C. yacare*, it is possible that the absence, because of lack of formation, of distal carpal 1 in living Crocodylia may be extended to other Crocodyliformes. That the only condensation between these elements involves distal carpals 3, 4 and 5, and distal carpal 2 is kept as an independent element throughout the evolutionary history of the group.

In addition, based on the distribution of 2–3 distal carpals in the crocodyliformes phylogeny, we agree with Buscalioni et al. (1997), that reduction in the number of ossified distal carpals occurred throughout the phylogeny of the clade. The presence of three ossified elements, as found in Atoposauridae and Thalattosuchia, may represent the plesiomorphic condition of the clade, and the presence of a single ossified distal carpal may be as an apomorphic condition acquired in Crocodylia through paedomorphosis.

In Crocodylomorpha, the identification of distal carpals has been controversial (Riff, 2007). In fossil crocodylomorpha, the number and identity of distal carpals differs among authors, making it historically difficult to identify homologies between these elements. Embryological studies of recent forms that analyze the origin and development of these elements in detail are relatively new and have been carried out in only few species.

Tarsal bones

In the region on the tarsus of *M. niger*, the intermedium + centrale and fibulare showed discreet ossification centers, simultaneously, at the end of stage 24. A different observation was recorded by Lima (2010) in *C. yacare*, and Müller and Alberch (1990) in *A. mississippiensis*, in which the fibulare showed an ossification center before the intermedium + centrale. Rieppel (1992, 1993a, 1994a, b), analyzing Lacertilia, showed that the ossification process of the intermedium + centrale preceded that of the fibulare.

Living crocodylians have three distal tarsals. Each of them appears from an independent cartilaginous condensation, called dt 2, 3 and 4, a reference to the digits to which they are articulated, and the sequence of their formation (Brinkman, 1980; Müller and Alberch, 1990). In *M. niger*, only distal tarsal 3 and 4 became ossified among these bones, as observed by Müller and Alberch (1990) in *A. mississippiensis*, and by Lima (2010) in *C. yacare*. On the other hand, distal tarsal 2 remains as a minuscule cartilaginous element, lying at the base of metatarsal 2, and remains intimately associated with distal tarsal 3 in adults of *M. niger* and *A. mississippiensis* (Müller and Albrech, 1990).

Although proximal tarsal elements are well-documented in the fossil record of Crocodylomorpha, little is known about distal tarsals because they are loosely articulated with the other bones of the feet, and may easily be misplaced, as is the case with distal carpals. However, the lack of ossification of at least one distal tarsal probably represents a primitive

condition in Crocodylomorpha, because *Terrestriusuchus*, *Orthosuchus*, and *Geosaurus* preserved only distal tarsals 3 and 4 (Romer, 1956; Nash, 1975; Crush, 1984), whereas *Protosuchus* preserved only distal tarsal 4 (Colbert and Mook, 1951).

Cartilaginous morphogenesis and digit ossification

In *M. niger*, all metapodia develop in a similar manner, with each element consisting of a small cartilaginous column. However, it was not possible to observe the sequence of events described by Müller and Alberch (1990) in *A. mississippiensis*, in which metacarpals appear as a result of a series of branching events that follows the development of the digital arch. An extensive column of cartilaginous condensation forms in the each digit and, later on, undergoes segmentation. Unfortunately, development was too rapid to determine the exact origin of the digits of *M. niger*, even with daily sample collections. We suggest that many specimens in the stages when these episodes take place need to be analyzed in intervals shorter than 24 hours to tease apart such possible events. In *M. niger*, there was no rule observed for the sequence of development of these elements. Digits I, II, and III started appearing simultaneously, followed by digits IV and V. However, digit IV was the last one to complete the chondrogenesis process.

Crocodylia feet showed a reduced digit V compared with basal forms. In *M. niger*, as well as in *A. mississippiensis*, metatarsal V is the only element of digit V (Müller and Alberch, 1990). This finding had already been mentioned in the study by Parrish (1987). According to this author, metatarsal V still underwent drastic reduction compared with the other metatarsals, an evident characteristic in the development of *M. niger*.

As for the ossification process of this region of the limbs, metapodia of several Testudines species are ossified before any other autopodium and phalangeal element. The species *M. niger*, *C. yacare* (Lima et al., 2011), and *A. mississippiensis* (Müller and Alberch, 1990) show the same pattern of Testudines species, and Frobisch (2008) emphasizes this finding. After the ossification centers appear in the metapodia of *M. niger*, ossification of the phalanges ensues. In relation to the level of ossification of *M. niger* phalanges, the overall tendency showed that it occurred in the anteroposterior direction. That is, it began in digit I and ended in digit IV (feet) and V (hands). It was also seen in the distal-proximal direction in digit I (feet and hands), proximal-distal direction in digits IV and V (hands), IV (feet). Ossification of digits II and III did not follow any of these patterns, starting rather in the distal phalanges, then in the proximal ones and finally, in the phalanges between them. The finding of more than one tendency of digit ossification is shared with other taxa.

The presence of more than one ossification pattern in the digits is shared with other tetrapod taxa.

Phalangeal formula

The phalangeal formula of hand digits in extant Crocodylia is traditionally recognized as 2:3:4:4:3 (Reese, 1915; Gegenbaur, 1864; Romer, 1956). According to Riff (2007), it is difficult to define the number of phalanges in the digits of ancestral species, since a complete set of phalanges is preserved in very few fossil specimens. Basal Crocodylomorpha,

such as *Terrestrisuchus* show, as well as living *Crocodylia*, three and four phalanges in digits II and III, respectively, and at least three phalanges in digits IV and V (Crush, 1984).

According to Müller and Alberch (1990), the phalangeal formula is 2:3:4:5:4 in adult *A. mississippiensis* counting elements that remain cartilaginous. These authors suggest that this is the correct formula applicable across all species of *Crocodylia*. We do not agree with this argument, because there is no single, well-defined phalangeal formula applicable for *Crocodylia* in general. In *M. niger*, after cartilaginous development and fusions of distal phalanges of digits IV and V, the phalangeal formula of the adult, including phalanges that do not ossify, is 2:3:4:4:2. Therefore, the number of phalanges observed at the end of the embryonic period is the same found in the adult in *M. niger*. It is important to emphasize that during development, digit IV presents 5 phalanges, as observed by Müller and Alberch (1990).

Another consequence of the absence of ossification in the extremities of phalanges IV and V of *M. niger* and other living forms, was the absence of claws in these digits, as occurs in *Atoposauridae* (Wellnhofer, 1971), *A. mississippiensis* (Müller and Alberch, 1990), *Uberabasuchus* (Vasconcellos et al., 2004) and *Stratiotosuchus* (Riff, 2007), suggesting that this is a primitive condition in *Crocodyliformes*.

As for the feet, the phalangeal formula in *M. niger* and in other living *crocodylians* is 2:3:4:5:0, including the unguals found only in digits I–III. The minuscule distal-most phalanx of digit IV continued to be cartilaginous in the adult, and the other ossified completely. The same pattern was observed by Müller and Alberch (1990) in *A. mississippiensis*. Based on the *Stratiotosuchus* material, Riff (2007) emphasized on the ossification of phalanx 5 of digit IV. This phalanx is represented, only by its proximal part, articulated to phalanx 4 of this digit, in this taxon. Although incomplete, phalanx of digit IV is certainly the smallest phalanx of the foot.

The few fossils with a known phalangeal formula show the same number of phalanges as living *crocodylians* only the ossified elements are considered. The phalangeal formula is 2:3:4:4:2 with a functional fifth digit in *Trestrisuchus* (Crush, 1984) and 2:3:4:4:0 in *Protosuchus* (Colbert and Mook, 1951). Both species show unguals only in digits I–III. Burke and Feduccia (1997) stated that the variation in the phalangeal formula is common among different taxa and that loss of digits is possible. The loss of phalanges occurs most frequently in digits I and V, as has been reported in *amphibians*, *mammals*, *lizards*, *birds*, *turtles* (Alberch and Gale, 1985), *A. mississippiensis* and *M. niger* as demonstrated in the present study. It should be emphasized that the combining information on fossils and embryonic development is essential for a better understanding of the evolutionary process in *tetrapods*.

Concluding remarks

In this study, we emphasize that the skeletal elements of *M. niger* are similar to those of other *Crocodylia*. The reduction in hand elements took place with condensations of three distal carpals, absence of cartilaginous formation in distal carpal 1, and absence of ossification of the centrale and distal carpal 2. There was an intraspecific variation in the sequence followed by ossification centers to appear in

the carpal elements. In the feet, there were no condensations, but absence of cartilaginous formation of distal carpal 1 and 5, and absence of ossification of distal carpal 2, characterizing a reduction in the elements of this region. Besides, as in other studies carried out in several groups of *reptiles* (Rieppel, 1993c; Maisano, 2002; Sheil and Greenbaum, 2005; Sánchez-Villagra et al., 2007a; Vieira et al., 2011b), incongruent chondrogenesis and ossification patterns in the limbs observed in this study indicates that these development events are separated. In summary, sequence of skeleton chondrogenesis is different from the ossification process sequence.

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