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Full Length Research Paper

Skeletogenesis of the pelvic girdle and pelvic members in embryos of *Caiman yacare* (Crocodylia: Alligatoridae)

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***Caiman yacare* embryos were collected and subjected to the bone cleared and Alizarin staining to analyze the ontogenic patterns of the skeletal ossification of the pelvic girdle and members. Ossification of *C. yacare* pelvis begins at 36 days of incubation. The femur, tibia and fibula present simultaneous stain retention at 30 days. It has four tarsal bones, the calcaneus, the talus, distal III and distal IV. Their ossification starts with the calcaneus from the 40th day of incubation, followed by the talus. At 54 days, the distal tarsal IV is conspicuous, as is the outline of the distal III. Each foot has five metatarsi (MT) and 13 phalanges (phalangeal formula 2:3:4:4). Ossification of the metatarsi begins at 36 days and follows the sequence MTI=MTII=MTIII=MTIV>MTV. The first phalanges begin the ossification process on the 36th day and continue up to the last day of incubation. The sequence of ossification of the proximal phalanges is PPI=PPII=PPIII>PPIV, while that of the medial phalanges is MPII> MPpIII>MDpIII>MPpIV>MDpIV and that of the distal phalanges is DPI>DPII>DPIII>DPIV, and the ontogenic pattern of the bones of the pelvic girdle and members of *C. yacare* differs from that of other reptiles, albeit with a few similarities.**

Key words: Alizarin, morphology, ossification, pantanal caiman, reptiles.

INTRODUCTION

Vertebrates have a unique skeleton that is composed of a set of plastic structures that are able to grow, adapt and repair themselves due to the wide morphological and architectural diversity of the skeletal tissue (White et al., 2003; Hildebrand and Goslow, 2006). This trait is conservative to the point that its general pattern displays in the tetrapods lineages. Its evolutionary pattern also enables this structure to respond to the particular habits of each species (Withers, 1992).

In some reptiles, the ossification patterns are well known. Several anatomical studies of the skeletons of reptiles have been conducted, as demonstrated by the contributions of Rieppel (1993a, b, 1994a), Sheil (2003b) and Vieira and Santos (2007), in the study of the skeletal ossification of *Alligator mississippiensis*, *Chamaeleo hoehnelii*, *Macrochelys teminckii* and *Podocnemis expansa,*. In these animals, endochondral ossification is the main process governing the formation of leg bones, which derive from a cartilaginous base (Shubin and Alberch, 1986); however the sequence of temporal, dynamic and specific events, in which it occurs are poorly defined (Burke and Alberch, 1985; White et al., 2003; Fabrezi et al., 2007). Müller and Alberch (1990) suggested the existence of several hypotheses based on anatomical studies of the skeleton in crocodilians that have undergone diverse changes during the evolutionary process, especially with regard to the number of phalanges and carpal and tarsal bones, many of these changes due to the characteristics of organisms which are seen as mechanical devices to maintain their biomechanics and function, alter their shape (Pounds et al., 1983).

In view of the paucity of studies on anatomy and development of crocodilians, the main goal of this work was to describe the bones development of the pelvic girdle and pelvic members of *Caiman yacare* (Daudin, 1802) (Crocodylia, Alligatoridae) embryos.

MATERIALS AND METHODS

Twenty eggs of *C. yacare* were collected during the egg-laying period in January 2007, in the nesting area of the Pousada das Araras Farm, in Aquidauana, Mato Grosso do Sul, Brazil, under permit number 021/2007 IBAMA/RAN. The experiment has been authorized by the Research Committee of Ethics in Animals by Federal University of Uberlandia under permit number CEUA 032/09.

The eggs were kept in a semi-artificial incubation system at temperature of 32.3°C (± 0.5) and were collected randomly from the first day of natural incubation up to the moment of hatching in regular intervals to 3 days. Each embryo was fixed in 10% formalin and cleared and stained for bone with Alizarin red S (Davis and Gore, 1936). The bones were then subjected to clearing with potassium hydroxide (KOH 2%), bone staining with Alizarin red S, and conservation of the material in glycerin. The cleared and stained procedures in this study are consistent with previous anatomy studies in reptiles. The onset of ossification was considered in the earliest developmental stage at which calcification was apparent in a particular element and was recognized by the retention of Alizarine Red stain.

The embryos were examined and scored for the presence or absence of individual skeletal elements to elucidate the ontogenetic sequence of ossification events for the skeleton. In some cases early bone formation was not indicated by Alizarin Red stain, rather by appearance as a faint structure with a distinct surface texture (Rieppel, 1993a). The presence of ossification centers was analyzed in a stereoscopic microscope coupled to an image capturing system.

RESULTS

Ossification of the pelvic girdle begins from Day 36 of incubation with the presence of stain retention in the ilium bone; then ossification process continues to the pube and ischium at Day 39, which reaches a very advanced stage at 51 days. At Day 30, the bones of the thigh (femur) and leg (tibia and fibula) begin the bone formation process, always starting from the diaphysis toward the epiphyses, and their rigid outline is well defined at Day 39 (Figure 1).

There are four tarsal bones: the calcaneus (Ca), talus (Ta), and the distal tarsal bones III (DTIII) and IV (DTIV). Their ossification begins on Day 48 of incubation time, with the presence of ossified centers in the calcaneus bone, which shows a slightly faster development than the talus. At Day 54, the DTIV bone is clearly evident, as is the outline of the DTIII, which becomes conspicuous at Day 57 (Figure 2).

The metatarsi (MT) consist of five bone elements conventionally classified, from medial to lateral, as MTI, MTII, MTIII, MTIV and MTV. Each foot has 13 phalanges, two in digit I, three in digit II and four in digits III and IV (phalangeal formula 2:3:4:4) (Figure 3).

Ossification of the metatarsi begins with diaphysis at Day 36 of incubation period. In this stage, ossification centers were present in MTI, MTII, MTIII and MTIV. At Day 39, the highest stain retention was expressed in MTII and MTIII and the lowest in MTIV (Figure 3). The MTV bone started ossifying at Day 51, when the other metatarsi were already at an advanced stage of ossification (Figure 3). The sequence of ossification therefore occurred as follows: MTI=MTII= MTIII=MTIV>MTV, beginning with the diaphysis and advancing toward the epiphyses (Figure 3).

The ossification process of phalanges started from Day 36 with the proximal phalanges (PP) of digits I, II and III and the distal phalange (DP) of digit I (Figure 3). At day 39, ossification started in the DP of digits II and III, the medial phalange (MP) of digit II and the medial proximal phalange (MPp) of digit III (Figure 3), without alterations to Day 42. Later, the PP of digit IV presented stain retention at Day 45 (Figure 3). The presence of ossification centers in the medial distal phalange (MDp) of digit III and the MPp of digit IV were observed at Day 48, with the MDp of digit IV present only after Day 51 (Figure 3) and the DP of digit VI only at Day 57.

The sequence of ossification of the proximal phalanges was PPI=PPII=PPIII>PPIV, while that of the medial phalanges was MPII>MPpIII>MDpIII>MPpIV>MDpIV and that of the distal phalanges DPI>DPII>DPIII>DPIV.

DISCUSSION

The ossification process starts with degeneration of the cartilage, which in the members begins with the diaphysis of the long bones (Rieppel, 1994a). In the pelvis of *Lacerta agilis exigua*, this process begins at the center of the ilium and along the lower edge of the pube, around

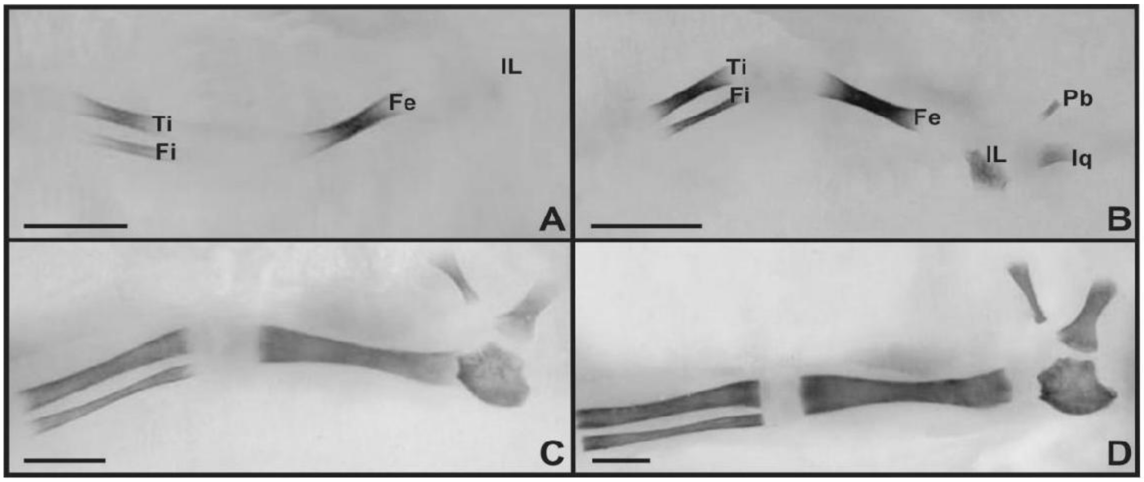

Figure 1. Photographs of the pelvic girdle and members of *C. yacare* (Daudin, 1802), at 36 days (1), 39 days (2), 51 days (3) and 57 days (4). Ilium (IL), pube (Pb), ischium (Iq), femur (Fe), tibia (Ti) and fibula (Fi). Bar: 1 mm.

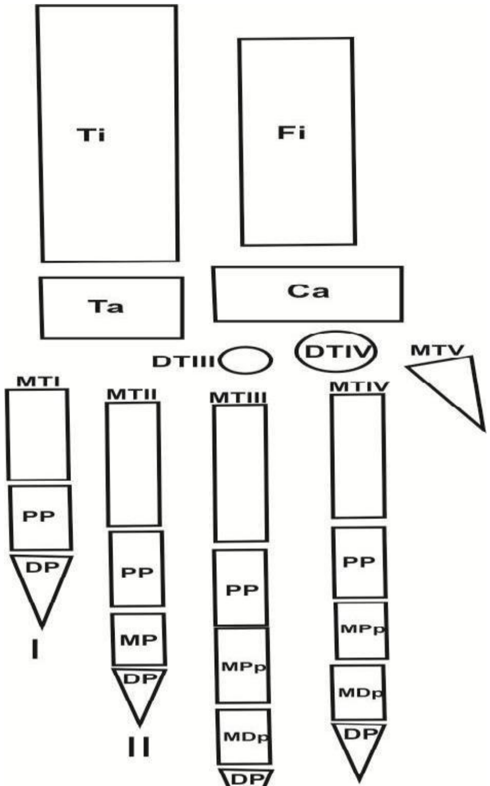

Figure 2. Schematic drawing of the foot of *C. yacare*, (Daudin, 1802). Digit I (I), digit II (II), digit III (III), digit IV (IV), tibia (Ti), fibula (Fi), tarsal calcaneus (Ca), tarsal talus (Ta), distal tarsal III (DTIII), distal tarsal IV (DTIV), metatarsi (MT), proximal phalanges (PP), medial phalange (MP), medial proximal phalanges (MPp), medial distal phalanges (MDp) and distal phalanges (DP).

the foramen, and in other species of reptiles may occur initially in the ischium (Rieppel, 1993a). This phenomenon was not observed in *C. yacare*, where stain retention occurred initially in the central region of the ilium bone, but corroborate with as has been observed *Lacerta sp.* and *C. hoehnelii* (Rieppel, 1993a), and spreading to the pube and then the ischium, as also reported by Rieppel (1993b) for *A. mississippiensis*. However, ossification in *C. yacare* begins later, starting in the posterior marginal portion of the ilium at Day 41, and by the inferior portion of the pube at Day 44, which is likely explained by the variation in the development patterns of reptiles as a function of temperature oscillations during embryogenesis.

Ossification of the pelvic membranes of *C. yacare* occurs concomitantly in the different bones (femur, tibia and fibula), always beginning in the region of the diaphysis, as in the other reptiles *L. agilis exigua, A. mississippiensis, C. serpentina, Lepidodactylus lugubris, C. hoehnelli* (Rieppel, 1992, 1993a, b, 1994a, b), differing only from a temporal standpoint. This ossification begins at Day 26 in *A. mississippiensis* (Rieppel, 1993b), and later in *C. yacare*, and in both species is preceded by ossification of the pelvic girdle. Punctual differences in the chronology, like that, could be influenced by variances in the incubation period of each species.

According to Rieppel (1993b), the tarsus of modern crocodilians is composed of a proximal row of bones, which include the tarsal calcaneus and the tarsal talus or astragalus, and a distal row with two bones called distal tarsal bones, as was observed in *C. yacare*, whose tarsus has these same four bones. However, the number of tarsal bones varies considerably among the different reptilian species (Burke and Alberch, 1985; Rieppel, 1993a, 1994a; Maisano, 2002; Crumly and Sánchez-Villagra, 2004; Fabrezi et al., 2007; Sánchez-Villagra et al., 2007; Vieira and Santos, 2007). The number reported

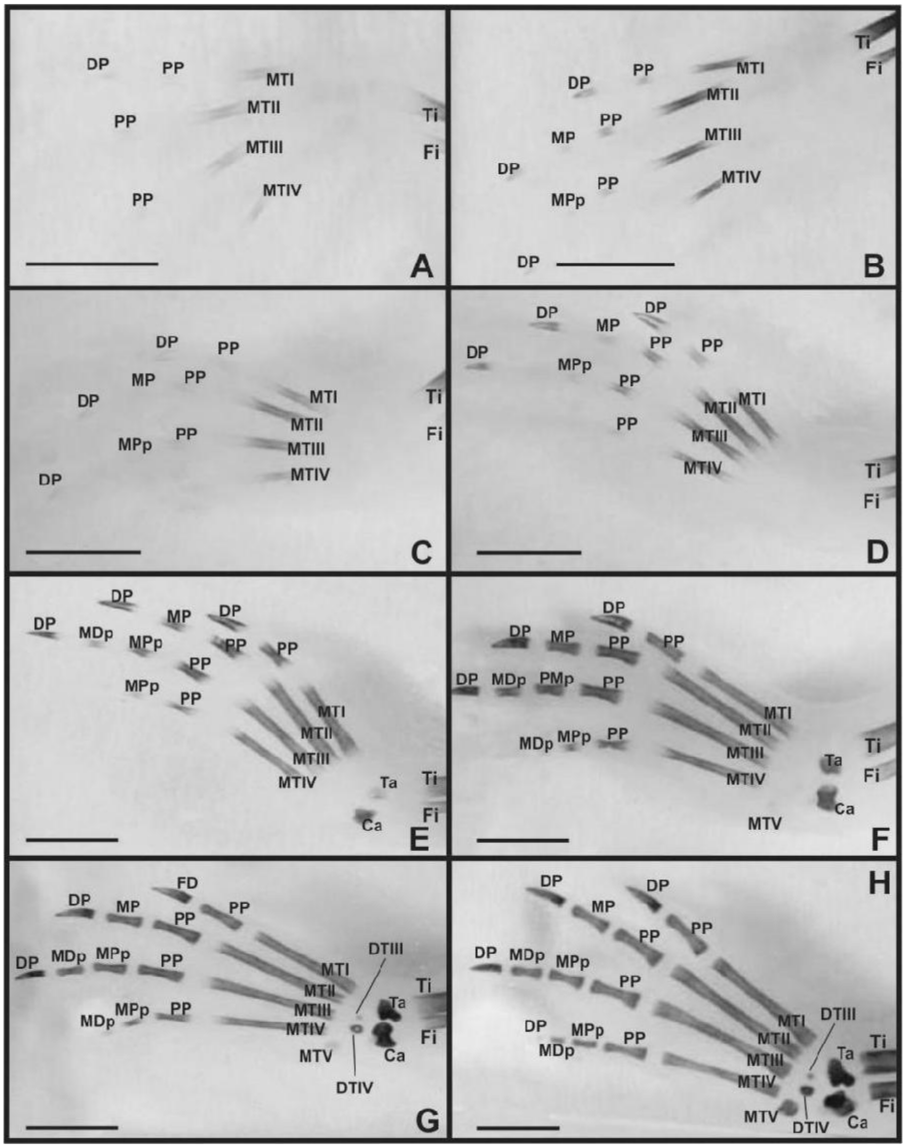

Figure 3. Photographs of the feet of *C. yacare* (Daudin, 1802), dorsal view. 36 days (6), 39 days (7), 42 days (8), 45 days (9), 48 days (10), 51 days (11), 54 days (12) and 57 days (13) of incubation. Tibia (Ti), fibula (Fi), tarsal calcaneus (Ca), tarsal talus (Ta), distal tarsal bone III (DTIII), distal tarsal bone IV (DTIV), metatarsi (MT), proximal phalanges (PP), medial phalange (MP), medial proximal phalanges (MPp), medial distal phalanges (MDp), and distal phalanges (DP). Diaphanization and staining technique (Davis and Gore, 1936). Bar: 1 mm.

by Müller and Alberch (1990) for *A. mississipiensis* is five, that is, the calcaneus, astragalus, distal carpals II, III and IV, and the distal carpal II is fibrocartilaginous, the latter not reported by Rieppel (1993b).

With regard to the variation in the number of bones in the tarsus, Müller and Alberch (1990) infer that the talus may be the fusion of other bones of the tarsus, such as the tibial and central bones, stating that the calcaneus can also be interpreted as the fibular, and that the DTIV may be the fusion of DTIV and DTV derived from the MTV. The authors also cite the probable presence of a DTIII resulting from the fusion of DTI, DTII and DTIII. In *C. yacare* only four ossified tarsal bones are present, talus, calcaneus, DTIII and DTIV.

In *A. mississipiensis* and *C. yacare*, this bone develops after the calcaneus and the sequence of DTIV>DTIII is the same. In general, in these species and in *Lacerta*, ossification of the tarsus is faster than that of the carpus (Rieppel, 1993b), while *C. yacare* presents a shorter period for the formation of these bones, since its ossification begins after the carpus and ends concomitantly.

In *Lacerta vivipara,* the talus is the only tarsal bone to ossify in the embryonic phase. On the other hand, in *L. agilis exigua* the astragalus, calcaneus and distal tarsals III and IV ossify in this period, which is an example of intraspecific variation, for which the most reasonable explanation is possible environmental or even embryological causes (Rieppel, 1992, 1994b), which may also occur in crocodilians like *A. mississippienssis* and *C. yacare*.

Rieppel (1993a) suggests that despite numerous reports that document the contribution of multiple condensations to the formation of the cartilaginous astragalus during limb development, this element is not homologous to the same elements in amphibian grade tetrapods. As a result of the fusion of the cartilaginous condensations in a single element, he proposes an ontogenetic repatterning for the astragalus, which is evidenced by its ossification from a single center that blurs the equivalences with the tarsus of non-amniote tetrapods (Romer, 1956). Furthermore, according to Rieppel (1993a), the fossil material of basal amniotes gives no support for this fusion. In a previous analysis of the skeletal development of *M. niger*, that elements are possibly formed by fusion of the tibial and intermedium, this data are not observed in this study with *C. yacare*.

These anatomical variations are traditionally fusions, prevailing explanation for the reduction of elements do occur, but do not seem to represent the prevailing mode of transformation (Müller and Alberch, 1990). Therefore, the non-ossification of the any distal tarsal represents a primitive condition for Crocodylomorpha (Colbert and Mook, 1951; Romer, 1956; Nash, 1975). For all Archorauria, the presence of only two distal tarsal ossification is a primitive condition.

Ossification as well as the phalangeal formula varies considerably in the group of reptilians. According to Hildebrand and Glosow (2006), the generic phalangeal formula for the feet of reptiles is 2:3:4:5:4, a pattern that was not found in *C. yacare*, since it has only four phalanges in digit IV and lacks digit V. Müller and Alberch (1990) and Rieppel (1993b) found 2:3:4:5 for *A. mississipiensis*. These authors state there are several particularities during the ossification of *A. mississipiensis* when one considers the presence of cartilaginous phalanges, unlike what was found for *C. yacare*, 2:3:4:4.

For *A. mississipiensis*, Müller and Alberch (1990) and Rieppel (1993b) report the presence of five digits, although it should be noted that, according to the standards of the International Committee on Veterinary Gross Anatomical Nomenclature (2005), only appendices of the paws or feet that have phalanges are considered digits, which is not the case in most crocodilians, as is the case of *C. yacare*, which has phalanges in four of the five metatarsi. Müller and Alberch (1990) also state that the MTV is the only remnant of the fifth digit, and that during the embryonic period the other metatarsi have digital expansions which, through specific events, form the phalanges of the other digits. Thus, it is found that, in crocodilian with such characteristics that it is correct to present the foot with only four digits, although it has five metatarsi.

The pentadactyl ground state in the pes of birds and Alligator indicates such a state was most parsimoniously present at Archosauria. The pentadactyl ground state is also likely the plesiomorphic condition for all modern tetrapods, although we are currently surveying a broader set of taxa to confirm this. Developmental evolution of digital reduction in theropods now shifts back to an argument centered on the discrepancy between the avian and crocodilians digits within the framework of a pentadactyl ground state. Although a homeotic transformation has been proposed (Wagner and Gauthier, 1999) and supported by skeletogenic condensation patterning (Larsson and Wagner, 2002) and Hox expression patterns (Vargas et al., 2008) more experimental work needs to be done to further this hypothesis and fully reject an argument of evolutionary convergence (Larsson et al. 2010).

The bone anatomy of the pelvic members of *C. yacare* is similar to that of the other crocodilians reported in the literature, as are the ossification patterns of the pelvic girdle, thigh and leg. The tarsus has four bones, which indicates that in these there is also a tendency for reduction of the foot's skeletal elements. The sequence of ossification of the foot varies chronologically in relation to the other species, and the ontogenic pattern of *C. yacare* differs from that of other reptiles.

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