

Skeletogenesis of the pectoral girdle and forelimbs in embryos of *Caiman yacare* (Daudin, 1802) (Crocodylia, Alligatoridae)

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Abstract

Embryos of *Caiman yacare* were collected and subjected to the bone clearing and staining protocol in order to analyze the ontogenetic patterns of ossification of the pectoral girdle and forelimb skeleton. The osseous structure of the girdle and forelimbs of *C. yacare* begins to ossify starting at 30 days of incubation, with the presence of dye retention in the scapula, coracoids, humerus, radius and ulna bones. During embryonic development, the autopodio of *C. yacare* has four bones in the carpus, the radial, ulnar, pisiform and carpal distal 4+5 bone. Their ossification begins at 39 days of incubation with the radial, followed by the ulnar, and at 54 days, the pisiform and the distal carpal 4 + 5. Each mesopodio has 5 metacarpi and are present 15 phalanges, two in digits I and V, three in digits II and IV, and four in digit III (phalangeal formula 2:3:4:3:2). Ossification of the metacarpi starts at 27 days of incubation, following the sequence MCII=MCIII=MCIV>MCI>MCV. The first phalanges begin the process of ossification on day 36, continuing up to the last day of incubation. The sequence of ossification of the proximal phalanges is PPI=PPII=PPIII>PPIV=PPV, that of the medial phalanges is MPPII>MPPIII>MPdIII>MPdIV, and that of the distal phalanges is DPI>DPII>DPIII>DPV>DPdIV. The ontogenetic pattern of the bones of the forepaw of *C. yacare* generally differs from that of other reptiles, although there are some similarities.

Keywords: alizarin, morphology, ossification, Pantanal caiman, reptiles.

1 Introduction

The internal and articulated structure of vertebrates is unique in the animal kingdom, and the skeletal elements are highly diverse in their morphology and architecture (WHITE, HERSHEY, MOSS et al., 2003; HILDEBRAND and GOSLOW, 2006). The anatomy is conservative to the point that its general pattern shows the phylogenetic lines of the tetrapods. Wide variations have superimposed the general pattern that evolved gradually, following an intricate system of characteristics in a dynamic and often poorly defined pattern, although the skeleton has been sufficiently plastic to respond to the particular habits of each animal (BURKE and ALBERCH, 1985; SHUBIN and ALBERCH, 1986; WITHERS, 1992; COATES, 1994; BLOB, 2000; FABREZI, ABDALA and OLIVER, 2007).

The ossification models of the skeleton of reptiles are known, but initial studies have provided data about this pattern in only a few animals, mainly Testudines (SANCHEZ-VILLAGRA, MÜLLER, SHEIL et al., 2009; SHEIL, 2003a, b; SHEIL and GREENBAUM, 2005; VIEIRA and SANTOS, 2007; VIEIRA, SANTOS, LIMA et al., 2009; RIEPPEL, 1993a,b). Fore and hind paws are difficult to study onto- and phylogenetically in view of the large variability of phenotypes with discrete and similar development among the vertebrates. These anatomical elements are complex components that have undergone innumerable variations

along the evolutionary path, particularly with regard to the mode of locomotion (HINCHIFFE, 1994; SANCHEZ-VILLAGRA, MITGUTSCH, NAGASHIMA et al., 2007).

Study the early ontogeny may be crucial for revealing important aspects of the structural basis of the organism, and the embryo development of reptiles and other vertebrates merits attention for various reasons. Description of the ontogeny and ontogenetic transformations is essential for understanding the developmental pattern. Several segments of science have focused on studying the most diverse and varied organisms of the planet, making important contributions to the body of knowledge about the anatomy, evolution, phylogeny and embryology of, for example, the locomotor apparatus in vertebrates. To this end, numerous studies have described the ontogeny of the skeleton of various animals such as *Alligator mississippiensis* (RIEPEL, 1993b; TUMARKIN-DERATZIAN, VANN and DODSON, 2007) *Lacerta agilis exigua* (RIEPEL, 1994b) *Discoglossus pictus* (FABREZI, 1992, 1993) and *Podocnemis expansa* (SANTOS and VIEIRA, 2007; VIEIRA and SANTOS, 2007; VIEIRA, SANTOS, LIMA et al., 2009). However, few studies have reported the anatomy and development of Brazilian crocodylians.

Here, we provide a pattern of developmental events of the bones of the pectoral girdle and forelimbs of embryos

of *Caiman yacare*. The purpose of this study is to describe the early development, with particular attention given to the morphology of the structures, considering the importance of developmental data in the interpretation of new fossils documenting early crocodylian evolution, our study also delivers relevant data for future comparative analyses.

2 Material and methods

2.1 Collection of embryos and descriptive morphology

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 have been authorized by the Research Committee of Ethics in Animals by Federal University of Uberlandia under permit number CEUA 032/09.

The eggs, which were kept in a semi-artificial incubation system at an average temperature of 32.3 °C, were collected randomly from the first day of natural incubation up to the moment of hatching. The regular intervals to sample as much of the ontogenetic sequence as possible. Each specimen was fixed in 10% formalin and cleared and stained for bone following standard procedures 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 onset of ossification was considered to be the earliest developmental stage at which calcification was apparent in a particular element and was recognized by

the retention of Alizarine Red stain. This generally was excellent; however, in some cases early bone formation was not indicated by Alizarin Red stain, but rather by appearance as a faint structure with a distinct surface texture (RIEPEL, 1993a; SHEIL, 2003a).

For the comparison of ossification across species presented here, only the relative timing or sequences are important, and for that reasons the differences in technical approaches across studies do not affect our study.

2.2 Comparisons of chondrification and ossification sequence through ontogeny

They were examined and scored for the presence or absence of individual skeletal elements to elucidate the ontogenetic sequence of ossification events for the skeleton.

We compared our data for *C. yacare* with those additional taxa obtained from the literature. The evolutionary relationship of turtles to other amniotes, principally among the reptiles, remains controversial (RIEPEL, 1995, 2004; RIEPEL and DE BRAGA, 1996; MEYER and ZARDOYA, 2003; MÜLLER, 2003; WERNEBURG and SÁNCHEZ-VILLAGRA, 2009). Several recent studies (ZARDOYA and MEYER, 2001; JIANG, CASTOE, AUSTIN et al., 2007) have supported the hypothesis that turtles are the sistergroup of Archosauromorpha. Though exist this controversial, the comparisons between this group were done because the poorly literature about this characteristic in crocodylians.

3 Results

The osseous structure of the pectoral girdle of *C. yacare* is composed of the coracoid (Co) and the scapula (Sc), while

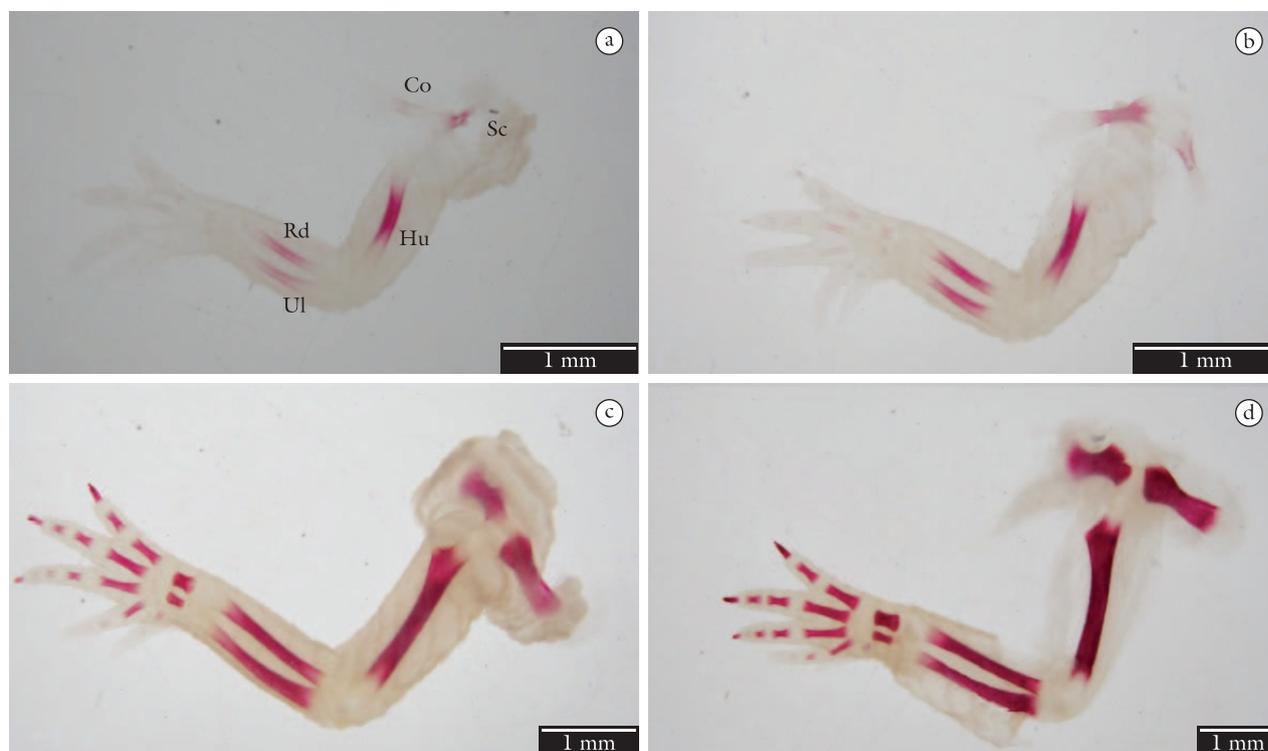


Figure 1. Photographs of the pectoral girdle and forelimbs of *C. yacare* at 36 days (a), 39 days (b), 51 days (c) and 57 days (d). Coracoid (Co), scapula (Sc), humerus (Hu), radius (Rd) and ulna (Ul). Palmar view. Bar: 1 mm.

the forelimbs consist of the humerus (Hu), radius (Rd) and ulna (Ul). Ossification begins from day 30 of incubation, with the presence of dye retention in the middle portion of these bones, by perichondral ossification. In 51 day the process is in a very advanced stage, showing a well-defined outline (Figure 1).

The manus of *C. yacare* comprises the following parts: carpus, metacarpi and phalanges (Figure 2). The carpus has four bones: the radial carpal (RC), the ulnar carpal (UC), the pisiform carpal (PC) and the carpal distal 4 + 5 (DC). By day 39 of incubation an ossification center is present in the radial carpal (Figures 3d, e, f, g and 4b, c, d), which develops at a slightly faster pace than the ulnar carpal. Both ossify from a periosteal collar. At 54 days the pisiform carpal is clearly visible, as well as the outline of the carpal distal, which becomes conspicuous on day 57 (Figures 3i, j and 4g, h).

The phalangeal formula in *C. yacare* is 2:3:4:3:2. Each forepaw has 14 phalanges, two in digits I and V, three in digits II and IV, and four in digit III (Figure 2). Ossification centers were present in MCII, MCIII and MCIV, although at 36 days MCII and MCIII showed the highest dye retention while MCIV showed the lowest (Figures 3a and 4a). MCI began ossification at 30 days but MCV only at 45 days, a phase in which the ossification process in MCI, MCII, MCIII and MCIV was already well advanced (Figures 3f and 4d). Thus, the sequence of ossification occurred as follows: MCII=MCIII=MCIV>MCI>MCV, always beginning with the diaphysis and proceeding toward the epiphyses (Figures 3 and 4).

The phalanges begin the ossification process on day 36 with the proximal phalanges (PP) of digits I, II and III (Figures 3c and 4a). At 39 days, ossification begins in the distal phalanx (DP) of digit I and in the medial phalanx (MP) and DP of digit II (Figures 3d and 4b). At 42 days, the DP of digit III begins the process of ossification (Figures 3e and 4c). The presence of ossification centers in the medial proximal phalanx (MPp) of digit III was observed at 45 days, as well as in the PP of digits IV and V (Figures 3f and 4d). At 48 days, the medial distal phalanx (MPd) of digit III presented dye retention (Figures 3g and 4e), while the MP of digit IV retained dye only at 51 days (Figures 3h and 4f). The DP of digit V started to ossify at 54 days (Figures 3i and 4g), and the DP of digit IV at 57 days, by which time all the osseous elements displayed a very advanced stage of ossification (Figures 3j and 4h). The sequence of ossification of the proximal phalanges was PPI=PPII=PPIII>PPIV=PPV, while that of the medial phalanges was MPPII>MPpIII>MDpIII>MPPIV and that of the distal phalanges was DPI>DPPII>DPPIII>DPV>DPPIV.

4 Discussion

In living Crocodylia, Rieppel (1993b) describes the presence of a typical digital arch developed from the bifurcation and proximal condensation of the cartilaginous axis of the member, which comprises the ulna, the ulna carpal, the distal carpal IV and digit IV. This axis curves distally and continues with the development of digits III, II and I (BURKE and ALBERCH, 1985; SHUBIN and ALBERCH, 1986). *Apalone spinifera* and *Macrochelys temminckii* (SHEIL, 2003a, b) clearly show the predominance of

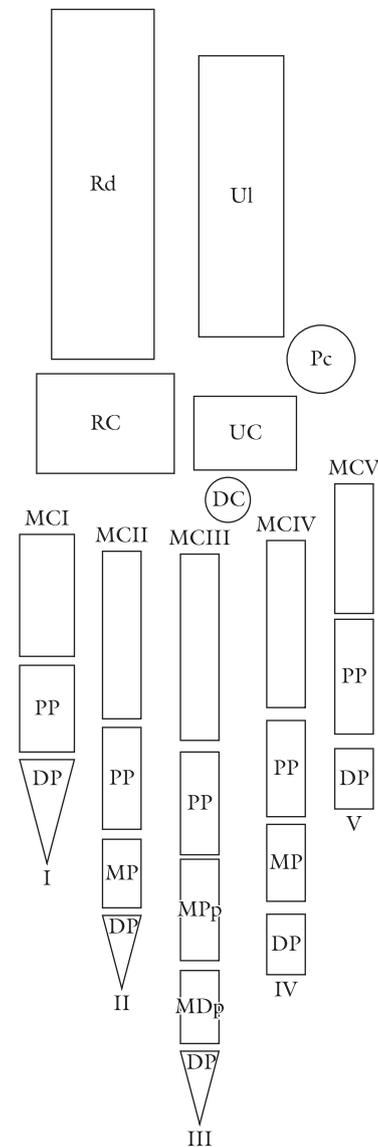


Figure 2. Schematic diagram of the forepaw of *C. yacare*. Digit I (I), digit II (II), digit III (III), digit IV (IV), digit V (V), radius (Ra), ulna (Ul), radial carpal (RC), ulnar carpal (UC), pisiform carpal (PC), carpal distal (DC), metacarpi (MC), proximal phalanges (PP), medial phalanges (MP), medial proximal phalanx (MPp), medial distal phalanx (MPd) and distal phalanges (DP).

an axis of ossification during the development of the forelimbs, which develops similarly to that of the cartilage. In *Cyrtodactylus pubisulcus* and *Tarentola mauritanica* (RIEPPPEL, 1992a), the axis is only partly visible, and the ulna carpal is the first element to ossify, followed by the distal carpal IV, and then digits III and II. In *C. yacare* are not visible predominance of any digit during ossification. The general patterns for the sequence of ossification of the bones of the pectoral girdle and forelimbs of Crocodylia and Larcertilia are similar (SHUBIN and ALBERCH 1986; HINCHLIFFE, 1994). Rieppel (1992a, 1993a, b; 1994b) reported that in *C. pubisulcus*, *C. boehmlii*, *A. mississippiensis* and *L. a. exigua*, the scapula shows an ossification center before the coracoid. In *C. yacare*, these elements already

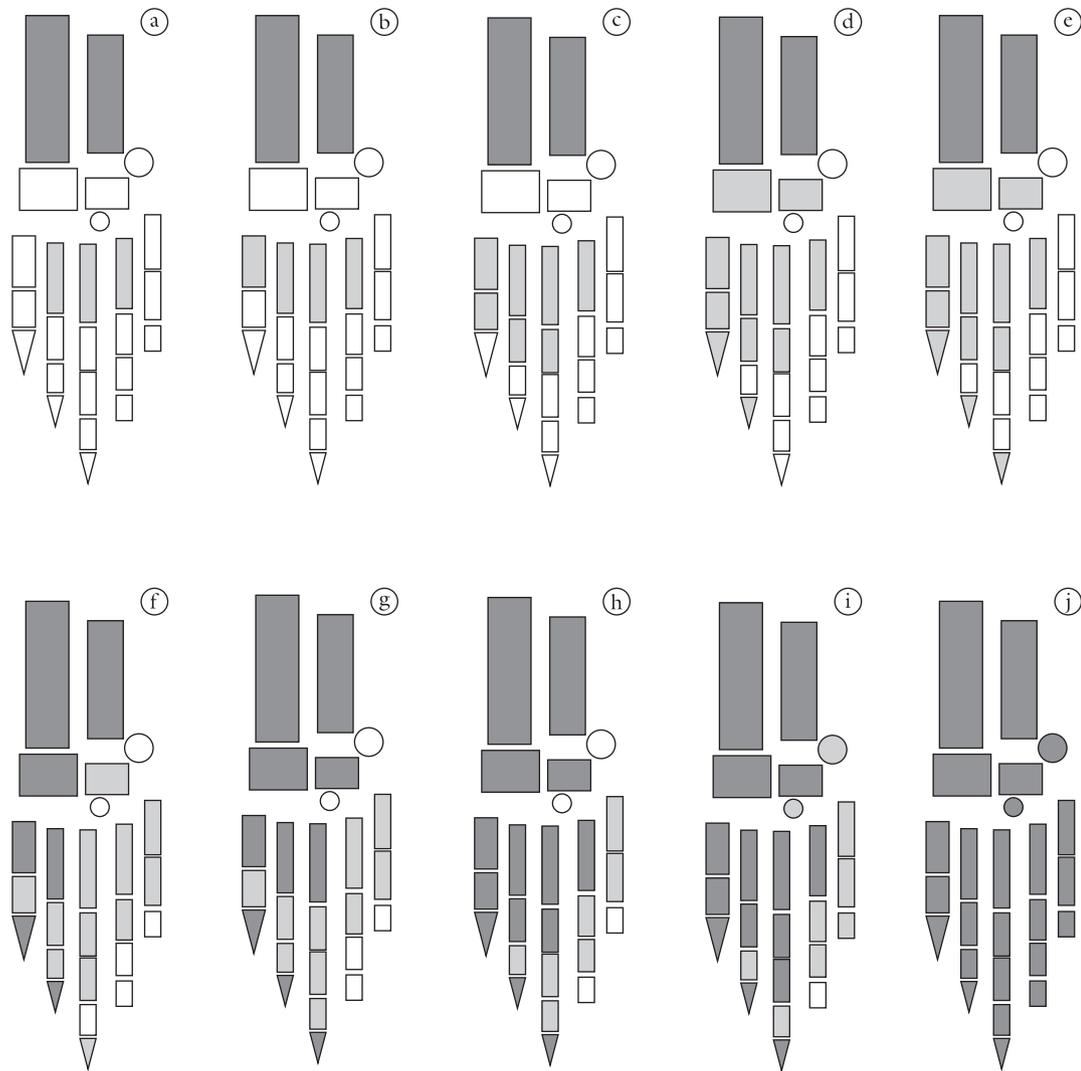


Figure 3. Schematic diagram of the sequence of ossification of the forepaw of *C. yacare*. 27 days of incubation (a), 30 days (b), 36 days (c), 39 days (d), 42 days (e), 45 days (f), 48 days (g), 51 days (h), 54 days (i) and 57 days (j).

presented marked ossification centers on day 30, although dye retention was stronger in the scapula.

The skeletal elements of the arm and forearm of *A. mississippiensis* begin to ossify at 27 days, a process that is strongly marked in the radius and ulna. In *C. yacare*, the presence of ossification centers in these bones also occurs concomitantly, as it does in *L. agilis exigua* (RIEPEL, 1994b). However, the pattern is inverse, with the humerus ossifying at a faster pace than the other bones, while in *G. oceanica* (RIEPEL, 1994a) ossification of the members begins before the elements of the pectoral girdle.

The carpus of living Crocodylia is composed of a proximal row of carpal bones comprising the ulnar, radial, and pisiform bone, and a distal row comprising the carpal distal bones, although some of these are cartilaginous (RIEPEL, 1993b). However, the number of carpal bones varies significantly among the different species of reptiles (BURKE and ALBERCH, 1985; RIEPEL, 1993a, 1994a; MAISANO, 2002; CRUMLY and SÁNCHEZ-VILLAGRA, 2004; FABREZI, ABDALA and OLIVER, 2007; SÁNCHEZ-VILLAGRA, WINKLER and WURST,

2007; VIEIRA and SANTOS, 2007), although the drastic reduction in the number of carpal bones is a characteristic of living Crocodylia (SHUBIN and ALBERCH, 1986).

The lacertilians present four to eleven bones in the carpus, while the Testudines have eight to ten bones. The carpus of *A. mississippiensis* comprises four ossified elements (MÜLLER and ALBERCH, 1990; RIEPEL, 1993a; BUSCALIONI, ORTEGA, RASSIKIN-GUTMAN et al., 1997), as does that of *Caiman crocodilus* (FABREZI, ABDALA and OLIVER, 2007) and *C. yacare*, with three bones in the proximal row and one in the distal row. The presence of only one distal bone in the ossified carpus of living Crocodylia, representing the distal carpal 4 + 5, is the most recent trait and indicates an apomorphic condition (BUSCALIONI, ORTEGA, RASSIKIN-GUTMAN et al., 1997) such as that presented by *A. mississippiensis* (MÜLLER and ALBERCH, 1990) and *C. yacare*. In the carpus of *A. mississippiensis*, Müller and Alberch (1990) also observed the presence of two other cartilaginous elements in adult life. The presence of elongated proximal bones of the carpus (radial and ulnar) and a radial that is more

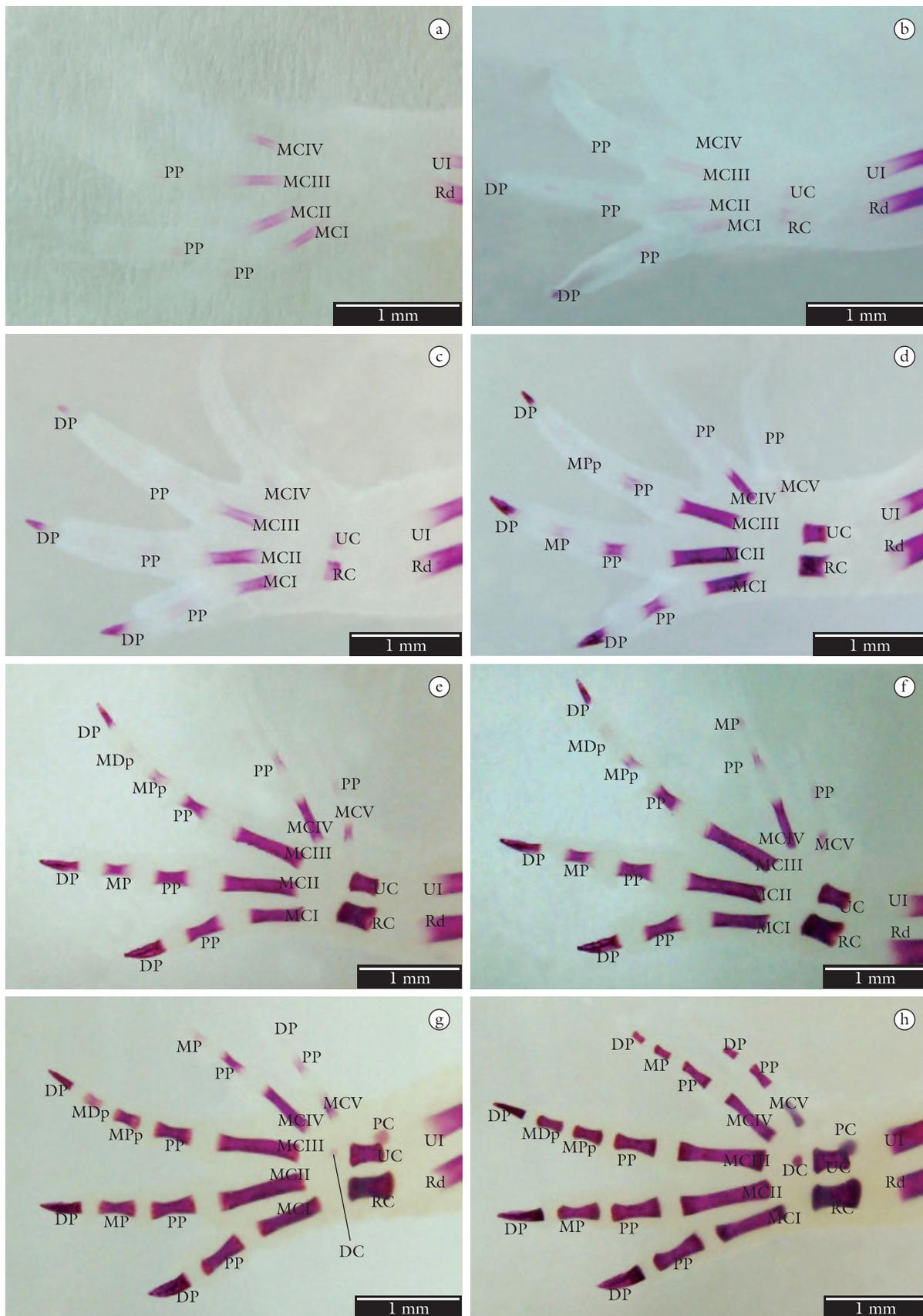


Figure 4. Photographs of the forepaw of *C. yacare*. 36 days of incubation (a), 39 days (b), 42 days (c), 45 days (d), 48 days (e), 51 days (f), 54 days (g) and 57 days (h). Radial carpal (RC), ulnar carpal (UC), pisiform carpal (PC), distal carpal (DC), metacarpi (MC), proximal phalanges (PP), medial phalanges (MP), medial proximal phalanx (MPp), medial distal phalanx (MPd) and distal phalanges (DP). Palmar view. Bar: 1 mm.

developed than the ulnar is one of the synapomorphies of the Crocodylia (BENTON and CLARK, 1988).

The elements of the carpus of *Hemiergis* and *C. pubisulcus* (RIEPEL, 1992a) only begin to ossify after hatching, starting with the ulnar, followed by the DC III and then the DC IV, but only after the beginning of ossification of the tarsus, as has been reported for other lizards (RIEPEL, 1992b, 1993a, 1994a).

The development of the skeletal elements of the forepaw of *C. yacare* differs from that of other tetrapods (SHUBIN and ALBERCH, 1986; HINCHIFFE, 1994), in which the sequence of ossification follows that described for the axis of the member (BURKE and ALBERCH, 1985; SHUBIN and ALBERCH, 1986). In this member, the predominance of digit II is the most conspicuous variation, unlike *A. mississippiensis* and *Lacerta*, which present a predominance of digit III (RIEPEL, 1993b).

Ossification of the carpal bones of *A. mississippiensis* begins at 18 days with the distal carpal, followed by the radial and the pisiform at 29 days (MÜLLER and ALBERCH, 1990). The pisiform bone also tends to be one of the last elements to begin ossification in *C. serpentina*, *Lacerta* (RIEPEL, 1993a) and *C. yacare*. In contrast, in *Dermochelys*, where it is arranged differently from the other reptiles and has an active biomechanical function at the base of the forepaw, the pisiform is the first element to ossify in the carpus. This is a clear example that illustrates the variations in the pattern of ossification of different species according to the modifications and peculiarities of their skeleton. Rieppel (1992b) also infers that this situation may be associated with the type of ossification of this bone, which is probably not endochondral, like other elements of the carpus, and that the pisiform is a sesamoid bone, since it does not originate from the segmentations of the central axis of development (SHUBIN and ALBERCH, 1986; RIEPEL, 1992a).

The ontogeny of the bones of Testudines is well described in the literature. Ossification of the metacarpus in *P. expansa* (SANTOS and VIEIRA, 2007) occurs from MCV to MCI, with emphasis on dye retention by MCIII, similar to *A. mississippiensis* (RIEPEL, 1993b). The same sequence of ossification was described by Fabrezi, Abdala and Oliver (2007) in *Tupinambis merianae*, but with intense dye retention in MCIV. In both *C. yacare* and *G. oceanica* (RIEPEL, 1994a), ossification of the metacarpus begins with MCII, MCIII and MCIV and later proceeds to MCI and MCV.

In the reptile group, ossification, as well as the number of phalanges, varies considerably. According to Hildebrand and Goslow (2006), the generic phalangeal formula for the forepaws of reptiles is 2:3:4:5:3, a pattern that is very similar to the one found in *C. yacare*, differing only in the number of phalanges of digit IV, which comprises only three bones. For *A. mississippiensis*, Rieppel (1993b) described the formula 2:3:4:4:4, which is reported by Müller and Alberch (1990) with a slight variation, i.e., 2:3:4:4:3. However, these authors state that there are several particularities during the ossification of *A. mississippiensis* and that, when one takes into account the presence of cartilaginous phalanges, the correct number is 2:3:4:5:4. Nevertheless, the number still differs from that reported by Rieppel (1993b) and found in *C. yacare*, 2:3:4:3:2.

In general, ossification of the phalanges begins from the proximal elements to the distal, although the ungual phalanges are more strongly stained than the others. In *A. mississippiensis* this ossification starts at 44 days with the PP and MP of digits I and IV (RIEPEL, 1993b), while in *C. yacare* this occurs at 39 days, with the PP of digits I, II and III the first to ossify at 36 days. There is a major advance in the process of ossification of the phalanges of *A. mississippiensis* at 47 days, which was observed at 48 days in *C. yacare*, with strong dye retention by the various osseous elements. The proximal-distal direction of ossification found in *C. yacare* was similar to the pattern reported by Rieppel (1992b, 1993b, 1994b) for *L. vivipara*, *A. mississippiensis* and *L. agilis exigua*.

5 Conclusion

The anatomy, as well as the sequence of ossification of the pectoral girdle and forelimbs of *C. yacare*, presents some similarities but also differences from those reported for other reptiles. The tendency for fewer skeletal elements in the distal portions of the members is apparent, as illustrated by the reduced number of carpal bones. Differences in the chronology of ossification among the animals compared here are tentatively attributed to variations in temperature during development and the periods of incubation, which influence the chronology, albeit not the ontogenetic pattern of reptiles.

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