A clearing-and-staining procedure for the study of the chondrocranium and other aspects of skeletal development in crocodilian embryos

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Abstract
Skull development has been of particular interest to crocodilian researchers, largely because their highly derived skulls have obscured homology of key phylogenetic characters. The chondrocranium has been of particular interest given its role in providing the substrate for endochondral ossification and the scaffold for dermal (intramembranous) ossification. Development of the skeleton in general and chondrocranium in particular has been studied via histology and contrast-enhanced computed microtomography (microCT), but clearing and staining of whole-mount specimens remains a relatively rapid and cost-effective means of generating adequate sample sizes. Historically, there have been many protocols for clearing and staining vertebrate skeletons that produce striking specimens with bluish cartilage and reddish bone within a relatively transparent body. However, application of this technique to crocodilians has been poorly described and standardized. Crocodylia is one of only two extant clades of Archosauria (Aves being the other), and thus the study of the development of the elements of crocodilian skeletons is crucial for evolutionary and paleontological studies. In this contribution, we describe a precise procedure for clearing and staining crocodilian embryos and young post-hatchlings, focusing on three species: Alligator mississippiensis, Caiman latirostris, and C. yacare. In brief, the steps include: initial preparation, bleaching, fixation, dehydration, cartilage and bone staining, clearing (with 0.5% KOH/glycerol series and enzymatic treatment), and storage. Using these procedures, we obtained specimens that provided clear discrimination of bony and cartilaginous anatomy, demonstrating the efficacy of this protocol for crocodilians, particularly with regard to elucidating the structure of the chondrocranium, which is illustrated here for three species.

Key words
Alligator mississippiensis; Caiman latirostris; Caiman yacare; embryonic stages; ontogeny.

Introduction
The study of the cartilaginous skull (i.e., chondrocranium) in crocodilians has been a focus of attention for many years. The first investigations date to the 19th and early 20th centuries (Rathke, 1866; Parker, 1883; Shinoh, 1914; Goldby, 1925; Bertau, 1935), and even more recent researchers continue to tackle this issue (Müller, 1967; Klembara, 1991, 2005; Witmer, 1995; Fernandez Blanco, 2019). Understanding the morphogenesis of the embryonic and adult skull is critical for a number of reasons. For example, developmental information is important for the interpretation of morphological features used in crocodilian systematics. Crocodylia comprises a clade of archosaurian reptiles closely related to extant birds and to extinct groups such as nonavian dinosaurs and pterosaurs. As in most clades, the skull is the main portion of the skeleton from which anatomical traits are extracted and then used in phylogenetic analyses. Most diagnoses of extinct and extant crocodilian species are
built on skeletal characters, mainly cranial features, and phylogenetic studies rely heavily on osteological evidence. Developmental information provides important tests of hypotheses of character homology (Patterson, 1982), which is especially important for crocodilians because many aspects of their skull structure are highly derived and difficult to compare with other clades. As a result, many paleontological studies use the information coming from the development of extant crocodilian species to make inferences about the fossil record (e.g., Witmer, 1995, 1997; Buscalioni et al., 1997; Abramyan et al., 2013; Bona et al., 2017; Foth et al., 2017; Morris et al., 2019; Fernandez Blanco et al., 2020). Consequently, an accurate interpretation of each adult skeletal element from pre-hatching stages is critical for the reconstruction of the evolutionary and biogeographic history of the group. The chondrocranium forms the substrate for the ossification of the definitive endochondral bones, as well as the scaffolding for the ossification of dermal (intramembranous) bones, and thus it holds a special place for interpreting the development and homology of crocodilian skull elements, which again are sometimes highly transformed relative to other clades.

Different approaches (e.g., traditional histology, wax-plate reconstruction, clearing and staining) have been used for the study of the chondrocranium in Crocodylia. We have developed our own investigations of Alligator mississippiensis, Caiman latirostris and C. yacare (Witmer, 1995, 1997; Fernandez Blanco, 2019) using the clearing-and-staining method. The literature shows that clearing and staining has been widely applied to species of fishes, amphibians, reptiles, birds, and mammals (e.g., Taylor, 1967; Simmons & Van Horn, 1971; Inouye, 1976; Wassersug, 1976; Kimmel & Trammell, 1981; Newman et al., 1983; Taylor & Van Dyke, 1985; Sheil, 2003, 2005; Vickaryous & Hall, 2008; Määs, 2008; Yaryhin, 2010; Di Pietro et al., 2014; Reed et al., 2019), but there is no specific protocol for crocodilian specimens. There are a few embryological studies available for some species of Crocodylia, but the clearing-and-staining technique is rarely if ever described (e.g., Rathke, 1866; Miall, 1878; Parker, 1883; Meeke, 1893, 1911; Shino, 1914; Goldsby, 1925; De Beer, 1937; Muller, 1967; Bellairs & Kamal, 1981; Klembara, 1991, 2005; Witmer, 1995; Lima et al., 2011, 2013; Vierra et al., 2018; Fernandez Blanco, 2019). Almost all of these papers provide little more than outlines of the procedures, requiring a considerable amount of subsequent experimentation and fine-tuning when implemented, potentially wasting time, money in chemical solutions, and especially, valuable specimens in failed or suboptimal attempts. Thus, a description of the precise requirements (an optimization of techniques used in previous literature) that work well with crocodilian species will be beneficial to people working with bones and cartilages of freshly acquired material of pre-hatching individuals of this clade. Moreover, it will be a significant contribution to embryological studies, which have burgeoned in recent years.

The objective of this work is to describe exactly how to efficiently produce high-quality cleared-and-stained crocodilian embryos, defining specific steps for specimen handling and demonstrating that this technique can be as reliable as others and offers some advantages over related procedures. The technique was applied to embryos of three extant species (Alligator mississippiensis, Caiman latirostris and C. yacare) and is beneficial not only for the study of chondrocranial and skull development but also the rest of the skeleton.

Materials and Methods

The total sample consisted of three embryonic ontogenetic series of 54 specimens of A. mississippiensis (stages 12–28 according to Ferguson, 1985), 37 specimens of C. latirostris and 34 of C. yacare (stages from 17/18 to 27–28 according to Jungman et al., 2008). Caiman specimens are housed in the herpetological collection of the Museo de La Plata (MLP) and were collected from nature. Alligator specimens were collected from the Rockefeller Wildlife Refuge, southwestern Louisiana, by Refuge staff as part of their routine research and census activities, and they are housed in the Ohio University Vertebrate Collection. All experiments were carried out in glass jars with nonmetallic and metallic lids indistinctly. Although sometimes glycerol tends to discolor if it contacts metallic lids (and nonmetallic lids should be used instead), lid material made no discernible difference in this study. Each specimen remained within a single jar as solutions were removed and added. Some steps required occasional agitation.

1 — Initial preparation: Specimens of Alligator mississippiensis were skinned, enucleated (removing the eyeball), eviscerated (body organs, major muscle masses and large fat bodies) and debrained. Cephalic skin must be intact if disarticulation is undesirable. Finally, specimens were rinsed in distilled or tap water (but the type of water used made no discernible difference). This step was avoided in Caiman species (see below).

2 — Bleaching: Applied only to later embryonic and post-hatching specimens of A. mississippiensis. Embryos were placed in a solution of about one part 3% H₂O₂ to nine parts 0.5% KOH for no more than 1.5–2.5 hours. Agitated frequently.

Note: Steps 1 and 2 were used in A. mississippiensis to enhance clearing, but were not necessary in the Caiman species. We present this variation in the technique to present options to other researchers.

3 — Fixation: This step was applied slightly differently in the fresh material of the three species. Caiman specimens were fixed in 4% formaldehyde (prepared from 40% pure formalin – stabilized with methanol – plus distilled water) with a saturated solution of calcium carbon-
ate as a buffer. They were fixed before staining, and some specimens remained in this fixative for more than one year. The *Alligator* sample was fixed with 37% formaldehyde for between two and five days, depending on size, and agitated occasionally. To remove the formalin after fixation, *Alligator* specimens were washed two or three days in running (or several changes of) tap or distilled water followed by one day of several changes of distilled water before further processing.

*Note:* The absence of washing in *Caiman* specimens did not influence the clearing-and-staining process.

4 — **Dehydration:** *Caiman* embryos were taken to 96% ethanol via a graded series of 15% ethanol, 40% ethanol, 70% ethanol, and finally 96% ethanol, spending 4 hours in each solution. *Alligator* embryos followed a similar process but using 95% ethanol and spending 2–4 hours in each solution. Additionally, *Alligator* specimens were transferred to acetone for two to three days to remove fat deposits. Agitating occasionally. This dehydration step avoids the loss of water and calcium from tissues and skeletons respectively due to the action of the acetic acid in the next step (Cartilage staining).

5 — **Cartilage staining:** This step was identical in the three species. Embryos were transfer to a solution of 11 mg Alcian blue, 77.5 ml 96% ethanol, and 22.5 ml acetic acid. They remained there for a time equivalent to 1.5–2 times (in hours) the age of the specimen (in days), up to 48 hours total. To assure complete staining, occasional frequent agitation was necessary to shift the specimen to avoid continuous contact of the same area of the embryo with the glass of the vial.

6 — **Dehydration:** Specimens were dehydrated for 24 to 48 hours depending on their size in a 96% ethanol solution (or 95% ethanol for *A. mississippiensis*; the percent difference results simply from differences in how stock solutions were supplied in the authors’ different countries), changing to fresh ethanol every 12 hours (i.e., one to three times).

7 — **Enzymatic clearing:** This step was only used in larger specimens of *A. mississippiensis*. Specimens were taken from ethanol to distilled water through a graded ethanol series: 70% ethanol, 40% ethanol, 15% ethanol, distilled water. They remained at each stage of the series for 2–4 hours or about twice the time it took for the specimen to sink. The following enzyme solution was prepared fresh: 30 ml of saturated aqueous sodium tetraborate (Na₈B₁₂O₁₉·10H₂O; about 4 g of sodium tetraborate will saturate 100 ml of distilled water), 70 ml of distilled water, and 1 g of 4 × pancreatin (following Taylor (1967) and Dingerskus & Uhler (1977); pancreatin contains trypsin and other enzymes). Enzyme solution was added to the specimen vials and changed every four or five days. Vials were kept in 37°C water bath until much of the skeleton was visible. They were rinsed in a couple of changes of distilled water.

*Note:* The absence of this step in *Caiman* species and small specimens of *Alligator* did not influence the clearing-and-staining process.

8 — **Bone staining:** Both *Alligator* and *Caiman* specimens were placed in a solution of alizarin red S (15 drops of 0.1% aqueous alizarin red S in the case of *A. mississippiensis*, and a small amount of alizarin red S powder in *Caiman* species, until a deep purple color is obtained) in 100 ml of 0.5% KOH. Specimens were kept in this solution until bone tissue reached a deep red color, depending mostly on the size of the specimen. Most specimens spent between 24 and 48 hours in the alizarin solution.

*Note:* The potentially variable amount of alizarin red S powder in *Caiman* species had little effect on the final staining results.

9 — **Clearing:** Final clearing was achieved by taking the specimens to glycerol through a graded 0.5% KOH/glycerol series (i.e., 3:1, 1:1, 1:3, and pure glycerol). Specimens stayed in each stage of the series for three to five days depending on their size and the amount of clearing required. KOH could be replaced by distilled water in very young embryos, and larger embryos and post-hatching specimens could require higher concentrations of KOH (e.g., 1% or 2% KOH).

10 — **Storage:** Embryos were stored in fresh and pure glycerol. Some thymol crystals were added to inhibit mold and bacteria in the *A. mississippiensis* sample. Thymol was not added to the *Caiman* sample due to availability issues, but mold has not been observed. All specimens were stored in the dark to prevent diminution of the stain intensity by exposure to light.

**Results and Discussion**

The technique employed here has proven to be very effective for crocodilian embryos because almost all cartilages and bones could be clearly seen both in *Alligator* and *Caiman* (Fig. 1 and 2), revealing broad similarities in their chondrocrania (Fig. 3). Although there were some elements in early ontogenetic stages (e.g., some distal carpal elements) that, due to weak chondrification or ossification, were difficult to detect because they had indistinct edges, most could be fully distinguished. Furthermore, there were not significant staining differences among crocodilian species as all cartilages and bones acquired the same tone. This finding demonstrates that our protocol works across species and even along ontogeny, allowing inter- and intraspecific comparisons. It can be seen that the protocol described in this study is entirely adequate for crocodilian embryos in general, providing excellent results in somewhat phylogenetically distant species (*Alligator* and *Caiman*). This work provides a strong foundation for future studies as there is no previously well-described protocol specific to croco-
Fig. 1. Cartilages and bones of the skeleton of embryos of *Caiman* spp. Well-developed cartilages are shown in light blue color, the onset of the ossification process is in a lighter color and bones are reddish. (A) Right lateral view of a complete skeleton of *Caiman latirostris* of stage 23 (MLP-R.6491-CL-23-2). (B) Dorsal view of an almost completely ossified skull of *Caiman latirostris* of stage 27–28 (MLP-R.6491-CL-27-28-2). (C) Right lateral view of the skull of *Caiman yacare* of stage 23 (MLP-R.6490-CY-23-2). (D) Dorsal view of the left forelimb and (E) hindlimb of *Caiman latirostris* of stage 27–28 (MLP-R.6491-CL-27-28-5). Scale bars = 3 mm.
dilians, and it will likely be useful for other crocodilian genera (e.g., Crocodylus, Gavialis) or even other sauropсид species (e.g., turtles, squamates, birds). Moreover, some advantages of the application of this technique over others can be pointed out. For example, a large number of specimens can be processed relatively rapidly, certainly in comparison with traditional histology or wax-plate reconstruction, such as Shiino (1914) of Klembara

Fig. 2. Cartilages (light blue) and bones (reddish) of the skeleton of embryos of Alligator mississippiensis. Complete skeleton (A) in right lateral view and skull in (B) left lateral view, (C) dorsal view, and (D) ventral view of an embryo (OUVC 10167) of stage 23; scale bars = 5 mm. Hindlimbs (E) of an embryo (OUVC 10175) of stage 24; scale bar = 3 mm.
Finally, the method is not particularly rapid but it produces high-quality specimens that clearly discriminate bony and cartilaginous components. As a result, cleared-and-stained crocodilian embryos allow identifying and studying every single cartilaginous and osseous element of the skeleton to assist in establishing their identities (homologies) in post-hatching individuals.

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References


Abbreviations: ac, auditory capsule; ad, aditus conchae; ap, alar process; bpl, basilar plate; ca, cupola anterior; cp, crista parotica; cs, crista sellaris; ef, epiotic fenestra; epf, epiphygal foramen; fmg, foramen magnum; fn, fenestra narina; fo, fenestra olfactoria; hf, hypophysial fenestra; is, interorbital septum; lt, lamina transversalis anterior; mf, metoptic fenestra; nc, notochordal canal; ns, nasal septum; of, optic fenestra; pan, pila antotica; pc, paranasal cartilage; pf, proptic fenestra; pm, pila metotica; pp, prenasal process; ps, planum supraseptale; ptc, parietotetral cartilage; sc, sphenethmoid commissure; sc, trabecula communis; sf, trochelear foramen; tm, taenia marginalis; tme, taenia medialis; ts, tectum synoticum.


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