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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; Shiino, 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, waxplate 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; Simons & Van Horn, 1971; Inouye, 1976; Wassersug, 1976; Kimmel & Trammell, 1981; Newman *et al.,* 1983; Taylor & Van Dyke, 1985; Sheil, 2003, 2005; Vickaryous & Hall, 2008; Maisano, 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; Meek, 1893, 1911; Shiino, 1914; Goldby, 1925; De Beer, 1937; Müller, 1967; Bellairs & Kamal, 1981; Klembara, 1991, 2005; Witmer, 1995; Lima *et al.,* 2011, 2013; Vieira *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 Iungman *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% H_2O_2 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 to 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 ($Na_2B_4O_7$ $10H_2O$; 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 DINGERKUS & 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-

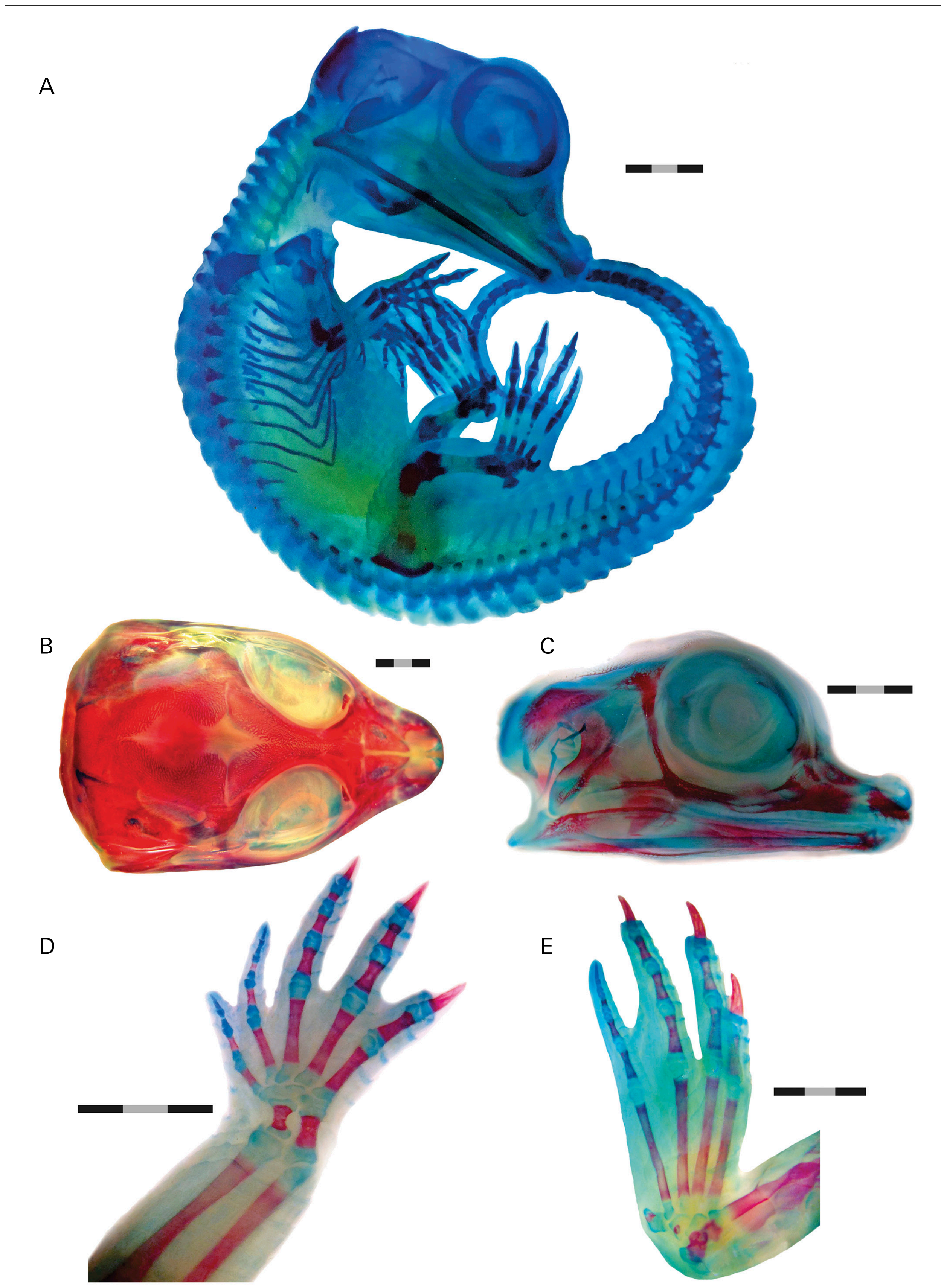


Fig. 1. Cartilages and bones of the skeleton of embryos of *Caiman* spp. Well-developed cartilages are shown in light blue color, the onset of the ossification process is in a lighter color and bones are reddish. **(A)** Right lateral view of a complete skeleton of *Caiman latirostris* of stage 23 (MLP-R.6491-CL-23-2). **(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.

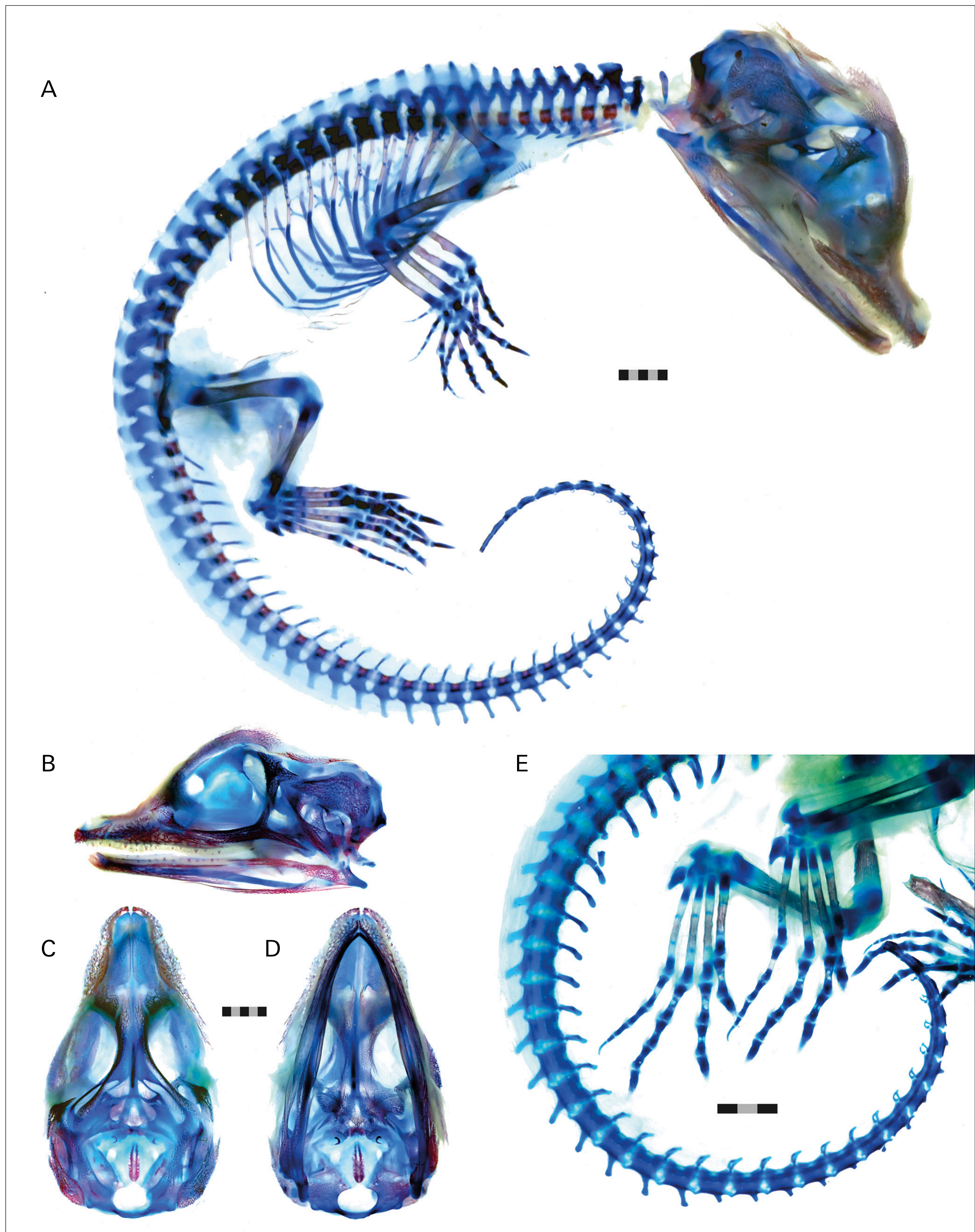
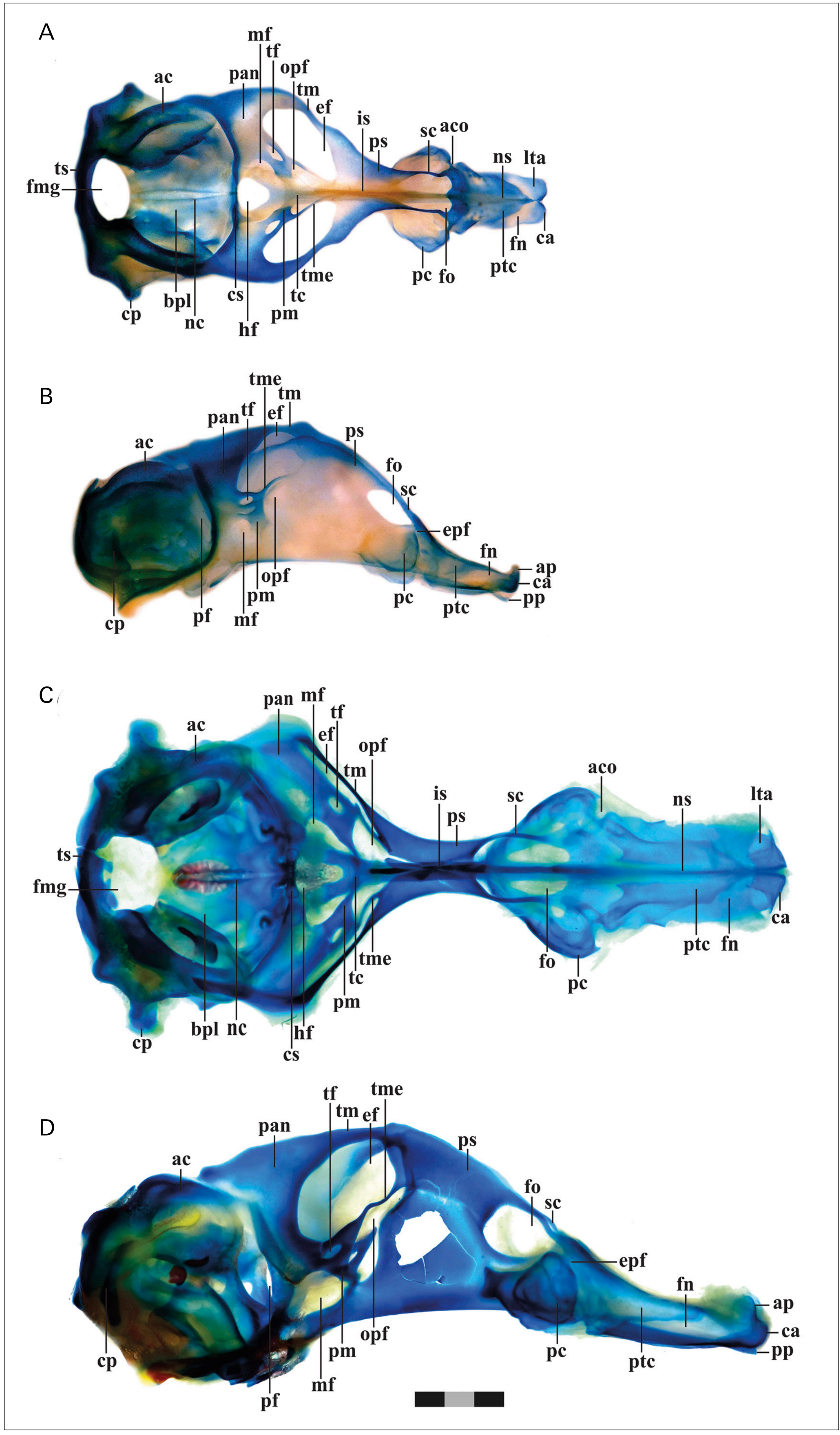


Fig. 2. Cartilages (light blue) and bones (reddish) of the skeleton of embryos of *Alligator mississippiensis*. Complete skeleton **(A)** in right lateral view and skull in **(B)** left lateral view, **(C)** dorsal view, and **(D)** ventral view of an embryo (OUVC 10167) of stage 23; scale bars = 5 mm. Hindlimbs **(E)** of an embryo (OUVC 10175) of stage 24; scale bar = 3 mm.

dilians, and it will likely be useful for other crocodilian genera (e.g., *Crocodylus*, *Gavialis*) or even other sauropsid 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) or Klembara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(1991) employed, which are time-consuming technique. Furthermore, the tridimensional structure is preserved in cleared-and-stained specimens whereas it is lost using serial histological sections. Additionally, it is a relatively inexpensive process as chemical products are not usually costly. Some researchers are now doing contrast-enhanced microCT (Tesařová *et al*., 2019) which is a valuable new option, but it requires much higher costs on a per-specimen basis, and visualization of cartilage can be compromised by inadequate binding of some stains (e.g., Lugol's iodine) to hyaline cartilage (Gignac *et al.,* 2016). Finally, and most important, clearing and staining can be done in any lab without the utilization of any expensive machine, unlike microCT and histology.

Some comments on different methodological steps can be pointed out. (1) Although *Alligator* specimens were eviscerated and bleached, it was not found to be necessary for *Caiman*. Neither of the two *Caiman* species was skinned but all caiman specimens exhibited adequately cleared tissues. (2) Time spent in each alcohol solution of the first dehydration step could slightly vary without influencing the process. (3) Quantities in the cartilage staining step could also be slightly modified (3 – 4 ml/mg). (4) During the second dehydration step, the changing to a fresh ethanol solution is not completely necessary but it is advisable in that replacing the previous somewhat hydrated solution with fresh ethanol enhances dehydration which in turn decreases fading of the Alcian blue staining (Wassersug, 1976). (5) Times given for any step are an average, and precise timing is not critical. (6) Steps that require agitation may damage fragile embryos if it is too vigorous. Transferring individuals from solution to solution is also potentially dangerous for specimens. Small artist paintbrushes were sometimes helpful in safely manipulating delicate specimens.

Some workers reported difficulty achieving good results in clearing embryos that had been stored in formaldehyde or ethanol solutions for many years (Taylor, 1967; Wassersug, 1976). However, we did not detect an issue with the length of storage in formaldehyde in the present study (some of them continued there for more than one year). Likewise, the finished cleared-and-stained specimens prepared with these methods have proven to be very stable over time (e.g., the photographs of the alligator embryos in Figure 2 were made 30 years after the specimens were generated using the current protocol).

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

Abramyan, J., Leung, K. J-M. & Richman, J. M. (2013). Divergent palate morphology in turtles and birds correlates with differences in proliferation and BMP2 expression during embryonic development. *Journal of Experimental Zoology, Part B-Molecular and Developmental Evolution*, **322B**, 73 – 85.

Bellairs, A. D. A. & Kamal, A. M. (1981). The chondrocranium and the development of the skull in recent reptiles, pp. 1 – 264 *in:* Gans, C., Billett, F. & Maderson, P. F. A. (eds) *Biology of the Reptilia*. Vol. 11. New York, Academic Press.

Bertau, M. (1935). Zur Entwicklungsgeschichte des Geruchsorgans der Krokodile. *Zeitschrift für Anatomie und Entwicklungsgeschichte*, **104,** 168 – 202.

Bona, P., Fernandez Blanco, M. V., Scheyer, T. M. & Foth, C. (2017). Shedding light on the taxonomic diversity of the South American Miocene caimans: the status of *Melanosuchus fisheri* (Crocodylia, Alligatoroidea). *Ameghiniana*, **54**, 681 – 687.

Buscalioni, A. D., Ortega, F., Rasskin-Gutman, D. & Pérez-Moreno, B. P. (1997). Loss of carpal elements in crocodilian limb evolution: morphogenetic model corroborated by palaeobiological data. *Biological Journal of the Linnean Society*, **62**, 133 – 144.

← **Fig. 3.** Chondrocrania of *Alligator mississippiensis* and *Caiman* spp. with surrounding dermal bones removed showing cartilages (light blue) and ossifying areas (reddish). **(A)** Dorsal view of the stage 22 chondrocranium of *Caiman yacare* (MLP-R.6490-CY-22-1). **(B)** Right lateral view of the stage 22 chondrocranium of *Caiman latirostris* (MLP-R.6491-CL-22-4). Dorsal **(C)** and right lateral **(D)** views of the stage 24 chondrocranium of *Alligator mississippiensis* (OUVC 10175). Scale bar = 3 mm. **Abbreviations**: **ac**, auditory capsule; **aco**, aditus conchae; **ap**, alar process; **bpl**, basal plate; **ca**, cupola anterior; **cp**, crista parotica; **cs**, crista sellaris; **ef**, epioptic fenestra; **epf**, epiphanial foramen; **fmg**, foramen magnum; **fn**, fenestra narina; **fo**, fenestra olfactoria; **hf**, hypophysial fenestra; **is**, interorbital septum; **lta**, lamina transversalis anterior; **mf**, metoptic fenestra; **nc**, notochordal canal; **ns**, nasal septum; **opf**, optic fenestra; **pan**, pila antotica; **pc**, paranasal cartilage; **pf**, prootic fenestra; **pm**, pila metoptica; **pp**, prenasal process; **ps**, planum supraseptale; **ptc**, parietotectal cartilage; **sc**, sphenethmoid commissure; **tc**, trabecula communis; **tf**, trochlear foramen; **tm**, taenia marginalis; **tme**, taenia medialis; **ts**, tectum synoticum.

De Beer, G. R. (1937). *The development of the Vertebrate Skull.* Chicago & London, The University of Chicago Press.

Dingerkus, G. & Uhler, L. D. (1977). Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. *Stain Technology*, **52**, 229–232.

Di Pietro, D. O., Alcalde, L. & Williams, J. D. (2014). Nasal cartilages, hyobranchial apparatus, larynx, and glottal tubes in four species of *Hydropsini* (Serpentes: Dipsadidae: Xenodontinae). *Vertebrate Zoology*, **64**, 103–111.

Ferguson, M. W. J. (1985). Reproductive biology and embryology of the crocodilians, pp. 329–491 *in*: Gans, C., Billett, F. & Maderson, P. F. A. (eds) *Biology of the Reptilia*. *Vol. 14*. New York, Academic Press.

Fernandez Blanco, M. V. (2019). Development of the chondocranium of two caiman species, *Caiman latirostris* and *Caiman yacare*. *Journal of Anatomy*, **234**, 899–916.

Fernandez Blanco, M. V., Ezcurra, M. D. & Bona, P. (2020). New embryological and palaeontological evidence sheds light on the evolution of the archosauromorph ankle. *Scientific Reports*, **10**, 1–8.

Foth, C., Fernandez Blanco, M. V., Bona, P. & Scheyer, T. M. (2017). Cranial shape variation in jacarean caimanines (Crocodylia, Alligatoroidea) and its implications in the taxonomic status of extinct species: the case of *Melanosuchus fisheri*. *Journal of Morphology*, **279**, 259–273.

Gignac, P. M., Kley, N. J., Clarke, J. A., Colbert, M. W., Morhardt, A. C., Cerio, D. G., Cost, I. N., Cox, P. G., Daza, J. D., Early, C. M., Echols, M. S., Henkelman, R. M., Herdina, A. N., Holliday, C. M., Li, Z., Mahlow, K., Merchant, S., Müller, J., Orsbon, C. P., Paluh, D. J., Thies, M. L., Tsai, H. P., & Witmer, L. M. (2016). Diffusible iodine-based contrast-enhanced computed tomography (diceCT): an emerging tool for rapid, high-resolution, 3-D imaging of metazoan soft tissues. *Journal of Anatomy*, **228,** 889–909.

Goldby, F. (1925). The development of the columella auris in the Crocodilia. *Journal of Anatomy*, **59**, 301–325.

Inouye, M. (1976). Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red S. *Official Journal of Congeital Anomalies Research Association of Japan*, **16**, 171–173.

Iungman, J., Piña, C. I. & Siroski, P. (2008). Embryological development of *Caiman latirostris* (Crocodylia: Alligatoridae). *Genesis*, **46**, 401–417.

Kimmel, C. A. & Trammell, C. (1981). A rapid procedure for routine double staining of cartilage and bone in fetal and adult animals. *Stain Technology*, **56**, 271–273.

Klembara, J. (1991). The cranial anatomy of early ontogenetic stages of *Alligator mississippiensis* (Daudin, 1802) and the significance of some of its cranial structures for the evolution of tetrapods. *Palaeontographica, Abteilung A*, **215**, 103–171.

Klembara, J. (2005). Ontogeny of the partial secondary wall of the otoccipital region of the endocranium in prehatching *Alligator mississippiensis* (Archosauria, Crocodylia). *Journal of Morphology*, **266**, 319–330.

Lima, F. C., Gonccedil, L., Souto, C. N., De Simone, S. B. S., De Araujo Martins, C. & Coutinho, M. E. (2013). Skeletogenesis of the pelvic girdle and pelvic members in embryos of *Caiman yacare* (Crocodylia: Alligatoridae). *Scientific Research and Essays*, **8**, 2148–2153.

Lima, F. C., Vieira, L. G., Santos, A. L. Q. & Coutinho, M. E. (2011). Sequência de ossificação do sincrânio e hioide em embriões de *Caiman yacare* (Crocodylia, Alligatoridae). *Iheringia Serie Zoologia*, **101**, 161–172.

Maisano, J. A. (2008). A protocol for clearing and double-staining squamate specimens. *Herpetological Review*, **39**, 52–54.

Meek, A. (1893). On the occurrence of a Jacobson's organ, with notes on development of nasal cavity, lachrymal duct, and harderian gland in *Crocodilus porosus*. *Journal of Anatomy and Physiology*, **27**, 151–160.

Meek, A. (1911). On the morphogenesis of the head of the crocodile (*Crocodilus porosus*). *Journal of Anatomy and Physiology*, **45**, 357–377.

Miall, L. C. (1878). *The skull of the Crocodile. A manual for students. Studies in comparative Anatomy I.* London, MacMillan.

Morris, Z. S., Vliet, K. A., Abzhanov, A. & Pierce, S. E. (2019). Heterochronic shifts and conserved embryonic shape underlie crocodylian craniofacial disparity and convergence. *Proceedings of the Royal Society B: Biological Sciences*, **286**, 20182389.

Müller, F. (1967). Zur embryonalen Kopfentwicklung von *Crocodylus cataphractus* Cuvier. *Revue Suisse de Zoologie*, **74**, 189–294.

Newman, Jr. S. M., Dugan, T. S. & Dumont, J. N. (1983). A rapid and simple procedure for visualization of amphibian skeletons for teratological studies. *Transactions of the American Microscopical Society*, **102**, 300–305.

Parker, W. K. (1883). On the structure and development of the skull in the Crocodilia. *Journal of Zoology*, **11**, 263–310.

Patterson, C. (1982). Morphological characters and homology, pp. 21–74 in: Joysey, K. A. & Friday, A. E. (eds) *Problems of Phylogenetic Reconstruction*. New York, Academic Press.

Rathke, H. (1866). *Untersuchungen über die Entwickelung und den Körperbau der Krokodile*. Braunschweig, Friedrich Vieweg und Sohn.

Reed, R. B., Kendall, C. R., Tsangarides, N. & Evans, H. E. (2019). Clearing and staining small vertebrates for demonstrating ossification of the skeletal system using the KOH and glycerine clearing method. *Anatomia, Histologia, Embryologia*, **48**, 605–608.

Sheil, C. A. (2003). Osteology and skeletal development of *Apalone spinifera* (Reptilia: Testudines: Trionychidae). *Journal of Morphology*, **256**, 42–78.

Sheil, C. A. (2005). Skeletal development of *Macrochelys temminckii* (Reptilia: Testudines: Chelydridae). *Journal of Morphology*, **263**, 71–106.

Shiino, K. (1914). Studien zur Kenntnis des Wirbeltierkopfes. *Anatomy and Embryology*, **50**, 253–382.

Simons, E. V. & Van Horn, J. R. (1971). A new procedure for whole-mount alcian blue staining of the cartilaginous skeleton of the chicken embryos, adapted to the clearing procedure in potassium hydroxide. *Acta Morphologica Neerlando-Scandinavica*, **8**, 281–292.

Taylor, W. R. (1967). An enzyme method of clearing and staining small vertebrates. *Proceedings of the United States National Museum*, **122**, 1–17.

Taylor, W. R. & Van Dyke, G. C. (1985). Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. *Cybium*, **9**, 107–109.

Tesařová, M., Heude, E., Comai, G., Zikmund, T., Kaucká, M., Adameyko, I., Tajbakhsh, S. & Kaiser, J. (2019). An interactive and intuitive visualisation method for X-ray computed tomography data of biological samples in 3D Portable Document Format. *Scientific Reports*, **9**, 14896.

Vickaryous, M. K. & Hall, B. K. (2008). Development of the dermal skeleton in *Alligator mississippiensis* (Archosauria, Crocodylia) with comments on the homology of osteoderms. *Journal of Morphology*, **269**, 398–422.

Vieira, L. G., Lima, F. C., Mendonôa, S. H. S. T., Menezes, L. T., Hirano, L. Q. L. & Santos, A. L. Q. (2018). Ontogeny of the Postcranial Axial Skeleton of *Melanosuchus niger* (Crocodylia, Alligatoridae). *The Anatomical Record*, **301**, 607–623.

Wassersug, R. J. (1976). A procedure for differential staining of cartilage and bone in whole formalin-fixed vertebrates. *Stain Technology*, **51**, 131–134.

Witmer, L. M. (1995). Homology of facial structures in extant archosaurs (birds and crocodilians), with special reference to paranasal pneumaticity and nasal conchae. *Journal of Morphology*, **225**, 269–327.

Witmer, L. M. (1997). The evolution of the antorbital cavity of archosaurs: a study in soft-tissue reconstruction in the fossil record with an analysis of the function of pneumaticity. *Journal of Vertebrate Paleontology*, **17**, 1–73.

Yaryhin, O. (2010). Development of pterygoquadrate complex in the embryogenesis of sand lizard, *Lacerta agilis* (Reptilia, Squamata). *Vestnik Zoologii*, **44**, 107–114.