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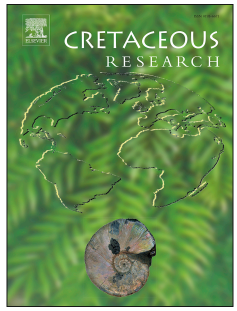

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Long bone histology of a eusuchian crocodyliform from the Upper Cretaceous of Spain: implications for growth strategy in extinct crocodiles.

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The long bone histology of a Late Cretaceous eusuchian crocodyliform from the Iberian Peninsula reveals clear variations in the cortical structure which reflects changes in the speed of bone deposition (i. e. skeletal growth) related to ontogeny. The presence of secondary woven-fibered bone tissue in the perimedullar region of the cortex, and the existence of an external fundamental system in the most external periostic cortex, which is a proxy for somatic maturity and effective cessation of growth, challenges the former idea that the growth strategy of extinct crocodylians fit in the typical ectotherm condition, according to which these animals grew slowly during life under an indeterminate growth strategy. The analysed specimen lived for a minimum of 16 years and the highest preserved apposition rates took place in an advanced ontogenetic stage. The study suggests that the general aspects of the modern crocodylian growth strategy were already in place in some lineages by the Cretaceous.

Keywords: bone histology, Eusuchia, ontogeny, Late Cretaceous, Laño Quarry, Spain.

1. Introduction

Fossil crocodiles are, among the archosaurs, the great forgotten of the histological research. Whilst ornithosuchians (pterosaurs, birds and non-avian dinosaurs) have been profusely studied, and a great number of research articles and reviews have seen the light of day in the last decades (see references in Chinsamy and Hillenius, 2004; Padian and Horner, 2004; Erickson, 2005, 2014; Cubo et al., 2012; Reid, 2012; Houssaye, 2014), extinct crocodylians remained since last years as an understudied group (see exhaustive revisions in Klein et al., 2009; Woodward et al., 2014; Sayão et al., 2016). Even other pseudosuchians, their closest relatives, and a number of non-arcosaurian reptiles, such as the extinct synapsids ("mammal-like" reptiles) and many groups of "marine reptiles", have been more profusely studied (Ricqlès et al., 2003, 2008; Salgado et al., 2007; Houssaye et al 2008, 2013, 2014; Botha-Brink and Smith 2011; Talevi and Fernández, 2012, 2015; Talevi et al., 2011, 2012; Chinsamy-Turan, 2012 and references therein; Houssaye, 2013 and references therein)

Possibly, this is due to the fact that living crocodiles are a well-known group, widely represented nowadays. Most of the generalities of extant crocodylians (feeding habits, metabolic and physiological traits, growth regimes, social and reproductive behaviour, etc.) can be unconsciously extended to most of their extinct relatives. In contrast, palaeohistological analyses are perhaps the only way to infer certain palaeobiological traits in exclusively extinct groups, such as non-avian dinosaurs, pterosaurs, "mammal-like" and aquatic reptiles.

Traditionally, extinct and living crocodylians have been considered altogether as a group that exemplifies the "typical" ectothermic reptilian condition (Ricqlès et al., 2003): unlike endotherms, extant reptiles tend to exhibit low metabolic rates, slow and intermittent, seasonally induced depositional patterns, and indeterminate growth strategies (Lance, 2003). Consequently, crocodylian bones should exhibit only slow-forming somatic tissues such as parallel-fibred and lamellar bone, and not fast-growing tissues (i. e., fibro-lamellar), except during the earliest stages of growth, when they exhibit the highest apposition rates (Chabreck and Joanen, 1979) and those well-fed captive animals with constantly food supply (Padian et al., 2004; Chinsamy and Hillenius, 2004). Classical histological studies of fossil crocodiles supported these assumptions (Buffrénil, 1980; Buffrénil and Buffetaut, 1981). In this sense, an analysis of *Deinosuchus* osteoderms demonstrated that the "terror crocodile" grew cyclically at rates comparable to that of modern crocodylians, maintaining intermittent growth throughout life (Erickson and Brochu, 1999; Schwimmer, 2002).

Nevertheless, recent osteohistological and physiological studies conducted on modern crocodylians are gradually changing this picture. Nowadays, it is assumed that fast growing bone tissues can be formed not only in captive animals, but also in wild juveniles (Horner et al., 2001; Ricqlès et al., 2003; Padian et al., 2004; Woodward et al., 2014), in wild adults for brief periods (Reid,1984, 1997; Chinsamy and Hillenius 2004; Woodward et al., 2014), and even in individuals without optimal health conditions (Tumarkin-Deratzian, 2007). Furthermore, the crocodylians' indeterminate growth strategy has also been recently questioned with the discovery of histological signals of the effective cessation of growth, the so-called external fundamental system (EFS), in bones of captive American alligators and in Paleocene neosuchians (Klein et al., 2009; Woodward et al., 2011; Andrade and Sayão, 2014), confirming possibly determinate growth patterns, at least in a number of taxa of this group of ectotherms (see Andrade et al., 2015, and Sayão et al., 2016 for a review).

In this work, we document the presence of the typical lamellar zonal bone tissue in a limb bone of a Late Cretaceous eusuchian crocodyliform with evidence of the cessation of growth when the animal reached skeletal maturity, associated with the deposition of endosteal continuous parallel and fast-growing woven-fibered bone tissues. These changes in osteohistological organisation define distinct stages in the life history of the animal.

2. Material and methods

An almost complete left eusuchian humerus MCNA- L1A481 (?*Acynodon* sp.) from the upper Campanian fluvial deposits of the Laño Quarry, northern Spain (Buscalioni et al., 1999; Pereda-Suberbiola et al., 2015) was selected for osteohistological study.

The Laño Quarry (Fig. 1C) is a disused sand quarry placed in the southern part of the Basque-Cantabrian basin (Fig.1A-B) where crops out an upper Campanian- lower Maastrichtian continental to shallow marine succession composed mostly of fluviodeltaic mudstones and sandstones (Corral et al., 2016). The site constitutes a noteworthy Upper Cretaceous fossil locality which has yielded a diverse vertebrate fauna composed of actinopterygians, amphibians, squamates, chelonians, crocodyliforms, dinosaurs, pterosaurs and mammals (Pereda-Suberbiola et al., 2015). Crocodyliforms are represented by the small-sized *Acynodon iberoccitanus* and the robust and considerably larger *Musturzabalsuchus buffetauti* (Buscalioni et al., 1997, 1999).

The humerus MCNA L1A481 lacks the distal condylar region and is partially covered by ferruginous crusts over its proximal end (Fig. 2), as is usual in Laño vertebrate fossils (Elorza et al., 1999; Pereda-Suberbiola et al., 2015). The bone, despite its small size (preserved length of 88 mm and midshaft diameter of 12-14 mm), is relatively robust, as often seen in *Acynodon* species (Delfino et al., 2008), and exhibits an expanded proximal end with a convex contour and a curved shaft, which is roughly circular in cross-section. The deltopectoral crest reaches its maximum extension at the proximal third of the humerus length. The longitudinal axis of the shaft is sigmoidal, with a pronounced posterior curvature on the proximal area. This morphology, together with other features (i.e., concave profile of the deltopectoral crest in lateral view, inner tuberosity forming an oblique articulating plane relative to the humeral head), is consistent with that of other two eusuchian humeri recovered from the Laño site (MCNA 7520, 7521; see Buscalioni et al., 1999). These humeri and other postcranial remains have been tentatively referred to as Eusuchia indet. as they were found as disarticulated elements, not directly associated with cranial bones neither of *Acynodon iberoccitanus* nor *Musturzabalsuchus buffetauti* (Buscalioni et al., 1999). Supposedly, the humerus MCNA L1A481 may belong to one of these taxa, most probably to *Acynodon* because of its morphological traits and the small size for an adult bone. *Acynodon* was a small-sized breviorostrine eusuchian with heterodont dentition, of about one metre long, whose fossil remains, mainly teeth, are common at Campanian-Maastrichtian localities (Delfino et al., 2008; Martin and Delfino, 2010). *Acynodon* has been considered as a basal member of the Globidonta within the Alligatoroidea, but an alternative phylogenetic hypothesis places it within the Hylaeochampsidae, a clade of non-Crocodylia eusuchians exclusively composed of European Cretaceous forms (see Csiki-Sava et al., 2015 and references therein).

Transverse sections of the bone were taken from the middle and distal parts of the humeral diaphysis. Samples were embedded in polyester resin (Technovit® 4004), thin-sectioned with a precision saw, mounted on glass slides, ground and polished with silicon carbide paper grits. Photographs of the thin sections were taken using a petrographic microscope (Olympus BXTR BX40) equipped with a digital camera (Sony Cybershot ™ QX-100). Captured images were edited with Adobe© Photoshop CS5©.

The histological description is discussed in detail, from the endosteal margin to the periosteal surface. Descriptive terminology of the microstructure follows that of Francillon-Vieillot et al. (1990) and posterior works.

Estimates of animal size (in percentage) were obtained relating measurements of growth rings diameters to the diaphyseal diameter, considering that the humerus belongs to a full-grown adult.

Institutional abbreviations: MCNA, Museo de Ciencia Naturales de Alava/Arabako Natur Zientzien Museoa, Vitoria-Gasteiz (Spain).

3. Description

The midshaft cross section of the humerus shows a thick cortical region composed mostly of compact primary bone, surrounding an empty medullary cavity slightly offset from the centre of the bone by lateral drift. The entire medullary cavity is lined by a thin inner circumferential layer (ICL) of endosteal lamellar bone (Fig. 3A).

A histological examination of the cortex reveals clear changes in the organisation of the bone tissue microstructure. The cortex exhibits the lamellar-zonal tissue organization usual in crocodylian appendicular bones (Fig. 3A), composed mostly of parallel-fibred bone tissue organised in zones and annuli (Lee, 2004; Woodward et al., 2014). Bone apposition rates seem to be greater on the ventrolateral side of the humerus, producing a thickening of the zones and annuli with respect to the dorsal side.

The innermost primary cortex has been partially obliterated due to expansion of the medullary cavity during growth, and by the deposition of secondary bone tissue in one side of the perimedullary region (Fig. 3A). Consequently, as usual, the histological record of the earlier stages of growth is missing. Nevertheless, in the perimedullar region of the cortex there is a narrow area of primary, parallel-fibred lamellar tissue, almost avascular, which preserves at least five closely spaced depositional cycles of zones and annuli. Some annuli split locally. Here, vascularity is almost absent, but when present, is composed of simple vascular canals, longitudinally oriented. The osteocyte lacunae are organised into circumferential layers, running parallel to the bone fibers. Taking into account that growth rings are annual, the bone deposition rate during this early period is very low, as denoted by the reduced spacing between consecutive growth marks. At this stage of the life history of the animal, shaft diameter represents an individual between 34% and 52% of adult size. This initial slow-growing region ends with a set of three-to-five densely packed growth lines, visible as a darkened band (Fig. 3A, B), whereupon the cortex continues outwardly with a better vascularised region of lamellar-zonal tissue, composed of wide concentric zones (9) and annuli (Fig. 3A, C). In this part of the cortex, the zones are much wider, and the vascular canals are relatively abundant compared to the inner cortex, indicating an increasing rate of bone deposition. Vascular canals are mostly simple vascular canals, with occasional reticular anastomoses. Collagen fibre organisation ranges from parallel to lamellar towards the outer third of the cortex, and vascularity gradually changes from longitudinal to radial towards the bone periphery (Fig. 3A, C). Osteocyte density and organisation do not vary significantly with respect to the innermost part of the cortex. Sharpey's fibres are irregularly distributed throughout the cortex. Approaching the periosteal surface, the spacing between consecutives annuli decreases gradually, accompanied by a noticeable reduction in vascular density, which marks a significant slowing down in growth rate, when somatic maturity is neared. Lastly, the most external part of the cortex exhibits a nearly avascular layer of lamellar bone provided with tightly packed growth marks (Fig.

4A) that resembles the external fundamental system (EFS) observed in mature specimens of other tetrapods, but rarely found in crocodiles (Klein et al, 2009; Woodward et al 2011; Andrade and Sayão, 2014) (Fig. 4B, C).

Secondary osteogenesis is especially noticeable in one quadrant of the perimedullary region, where a thick layer of endosteally formed bone cuts across part of the primary bone of the innermost cortex (Fig. 3A, D), suggesting a drift of the medullary cavity. A very faint reversal line marks the boundary with the remainder lamellar-zonal tissue of the innermost primary cortex (Fig. 5B). This endosteal tissue, secondary in origin but primary in structure, has a patchy structure: partially is built-up of woven-fibred tissue, provided with longitudinally oriented vascular canals with osteal development (i.e., primary osteons) and randomly oriented osteocyte lacunae (Fig. 3D, E), but is mostly composed of parallel-fibred bone tissue with flattened osteocyte lacunae, collectively aligned parallel to the direction of bone fibres (Fig. 3D, 5A). The fibro-lamellar bone tissue of the endosteal layer constitutes the more rapidly formed bone tissue in the examined cortex. Similar well-developed endosteal layers have been documented lining the medullary cavity in limb bones of extant crocodiles (see Woodward et al., 2014: supplemental information).

Haversian remodelling is scarce throughout the bone. Isolated secondary osteons concentrate mainly in the deep and in the inner half of the cortex. The inner cortex also contains most of the resorption cavities, indicating active Haversian reconstruction at the moment of death.

The epiphyseal part of the humerus is mostly composed of compacted coarse cancellous bone, surrounded by a thin cortex of lamellar-zonal bone with narrow cycles of zones and annuli (Fig. 3F). Spongy bone is dense, with low porosity.

4. Discussion

The distribution of bone tissue types observed in the humerus MCN-L1A481 is clearly related to ageing. This represents different developmental stages in the life story of the animal that imply changes in the rate of bone deposition. The microstructure of the primary cortex is restricted to the cycles of “fast” and slow growth of the lamellar-zonal bone pattern typical of living crocodiles (Hutton, 1986; Lee, 2004; Woodward et al., 2014).

The oldest bone tissue preserved in the humerus corresponds to an almost avascular lamellar-zonal bone, with narrow growth rings, which denotes a post-hatchling stage of extremely slow and interrupted growth, as a result of its high organisation and almost complete lack of vascularization (Huttenlocker et al., 2013). Surprisingly, this developmental stage corresponds to that of a young individual between one-third and one-half of the adult size. The multiple-LAG layer of bone deposited at the end of this period, visible as a thick annulus (Fig. 3B ,C), would represent an annual marker of growth (Freedman-Fowler and Horner, 2015) and points to a near cessation of effective growth, suggesting long-term stress, probably produced by environmental factors or nutritional deficiency (Hutton, 1986; Padian et al., 2004; Klein, 2010). After this early ontogenetic stage characterised by low rates of periosteal osteogenesis, the animal started to grow more actively for almost one decade, as suggest the presence of nine sets of wide zones and annuli of moderately to well-vascularised parallel-fibred bone. Finally, after a gradual decline of osteogenesis marked by a narrowing of growth rings, the presence of an external fundamental system (EFS) in the cortical periphery would indicate a determinate growth strategy in this taxon, suggesting a complete somatic maturity and effective cessation of growth when the animal perished.

The secondary endosteal bone formed by lateral drift of the marrow cavity is composed of bone tissues with different apposition rates (i. e., parallel-fibred and woven-fibered tissues). In this context, the presence of small amounts of woven-fibered bone, provided with osteocyte lacunae of irregular shapes an chaotic organization, confirms that under certain conditions these animals could deposit fast-growing tissues in an advanced ontogenetic stage, far away from the earlier ontogenetic stages when growth rates are the highest, retaining the plesiomorphic archosaur capability for having high apposition rates (Woodward et al., 2014). The presence of resorption cavities in the inner primary cortex and in the endosteal layer suggests that active osteogenesis occurred in the form of osteonal remodeling by the time the animal died.

5. Conclusions

A histological analysis of a eusuchian humerus from the Laño Quarry supports that, in the Cretaceous, at least certain fossil eusuchians combined the relatively slow and cyclical growth pattern observed in pseudosuchians and basal reptiles with the determinate growth strategy typical of the ornithosuchians. Determinate growth is very rare in crocodiles and has only recently been documented in a reduced number of specimens, which supposes a new aspect of the evolutionary history of crocodylians (Klein et al., 2009; Woodward et al., 2011; Andrade and Sayão, 2014).

Excepting at the end of the animal’s lifetime, when the deposition of the EFS documents a plateau in skeletal growth, bone apposition rates were lowest in the first preserved years of its life history, when the animal should supposedly grow at higher rates. The highest yearly apposition rates correspond with the most vascularised zones deposited during the second half of the animal’s life history. The animal also had the ability to lay down small amounts of fibro-lamellar bone, a disorganised and fast growing tissue common in mammals and ornithosuchians, but scarcely documented in crocodyliforms.

In conclusion, the study provides excellent evidence that the general aspects of the modern crocodylian growth strategy were already in place by the Cretaceous at least in some lineages.

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Figure captions:

Fig. 1. Location maps and general view of the Laño Quarry, Condado de Treviño, Burgos province. A, map of the Iberian Peninsula showing the location of the studied area, in the vicinity of the city of Vitoria-Gasteiz (inset shows the position of the geological map). B, geological sketch map of the studied area showing the main stratigraphic units outcropping in the region. Star indicates the location of the fossil site. C, general view of the Laño quarry, showing the outcrop where the specimen has been collected. Modified from Pereda-Suberbiola et al. (2015).

Fig. 2. Eusuchian humerus MCNA-L1A481 from the Upper Cretaceous of the Laño Quarry. (A) Medial view. (B) Dorsal view. Arrows point the location of the transverse sections. Scale bar = 5 cms.

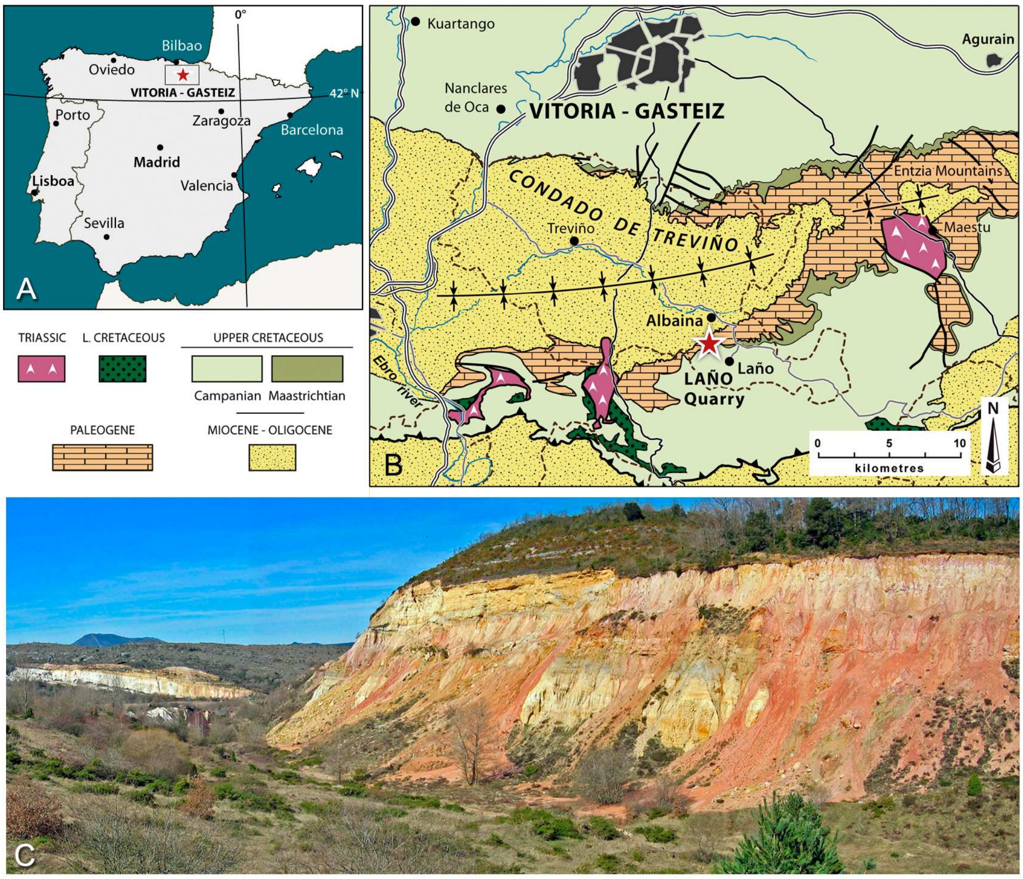
Fig. 3. Bone histology of a eusuchian humerus MCNA-L1A481 from the Upper Cretaceous of the Laño Quarry. (A) General view of the mid-diaphyseal transverse section showing the presence of a well-developed endosteal layer (yellow arrows) cutting away the innermost (older) cortex (a white line marks the extension of the endosteal bone tissue). The remainder part of the cortex, as usual in crocodyliforms, is composed of cyclical zonal bone stratified in growth rings (small arrows). (B) Enlargement of the mid-cortex showing zonal bone with annual zones and annuli (dark rings). Note the presence of a set of closely spaced growth marks (white arrows) separating the first cycles of growth rings, almost avascular, from the outer richly vascularized region composed of wide zones and annuli. Osteocyte lacunae are circularly oriented. (C) Complete mid cortical section showing the evolution of vascularity from a nearly avascular inner cortex to a higher vascularization towards

bone periphery. (D) Enlargement of the deeper cortex showing details of the thick endosteal layer, especially the presence of parallel-fibred and fibro-lamellar tissues. The presence of resorption cavities and secondary osteons denote osteonal remodeling. (E) Detail of the endosteally formed bone showing the presence of fibro-lamellar tissue. (F) General view of the distal diaphyseal section showing extensive compacted cancellous bone surrounded by a thin zonal cortex stratified as a result of the alternate deposition of zones and annuli. **Abbreviations: an**, annuli; **efs**, external fundamental system; **er**, erosion room; **flb**, fibro-lamellar bone; **icl**, internal circumferential layer; **lvc**, longitudinal vascular canal; **pfb,** parallel-fibred bone; **po**, primary osteon; **rvc**, reticular vascular canal; **ravc**, radial vascular canal; **so**, secondary osteon; **svc**, simple vascular canal. Images A-D, F: plane polarized light; E, cross-polarized light. Scale bars in A, F = 1 mm. Scale bars in B-D = 0.5 mm. Scale bars in E = 0.25 mm.

Fig. 4. External fundamental system (EFS) in diverse fossil crocodyliforms. A, MCNA-L1A481, indeterminate eusuchian humerus, Laño Quarry, Spain. B, CAV 0010-V, indeterminate Paleocene dyrosaurid rib, Poty Quarry, northeast Brazil. C, CAV 0014-V, indeterminate Paleocene dyrosaurid femur, Poty Quarry, northeast Brazil. (Images B and C modified from Andrade et al., 2015). White arrows signal the closely spaced LAGs of the EFS in A. White arrows delimited the extension of the EFS in B, C. **Abbreviations: rvc**, radial vascular canal. Scale in A-C = 0.2 mm.

Fig. 5. Bone histology of a eusuchian humerus MCNA-L1A481 from the Upper Cretaceous of the Laño Quarry. A, Enlarged view of the endosteal bone formed at the perimedullary region seen in figure 3D. Note the strong variation in orientation and shape of the bone cell lacunae in the woven-fibered respect to the lamellar tissue.

B, Detail of the faint reversal line separating the endosteally formed bone from the primary zonal bone (pink arrows). Sharpey’s fibers are abundant in the primary cortex, aligned perpendicularly to the growth rings. **Abbreviations: er**, erosion room; **pfb**, parallel-fibered bone; **po**, primary osteon; **so**, secondary osteon; **Sf,** Sharpey’s fibers; **wfb,** woven-fibered bone. Polarised light with lambda compensator. Scale in A-B = 0.25 mm. Image (A) taken under plane polarized light; image (B) taken under cross polarized light with lambda compensator.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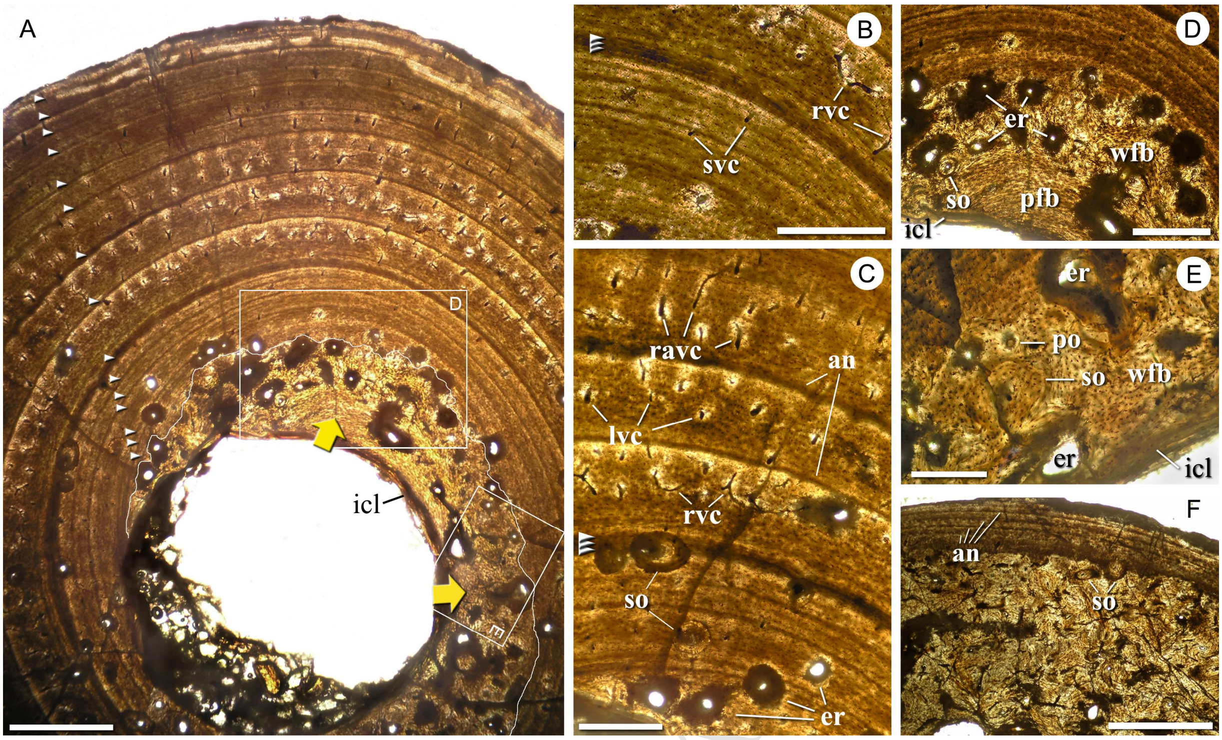
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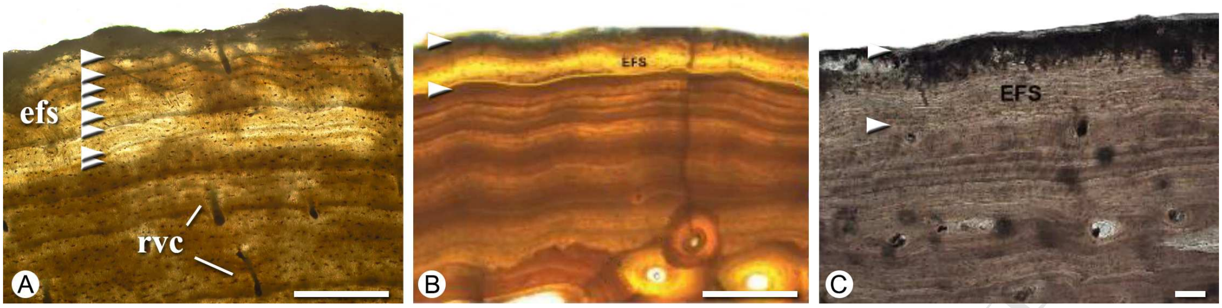
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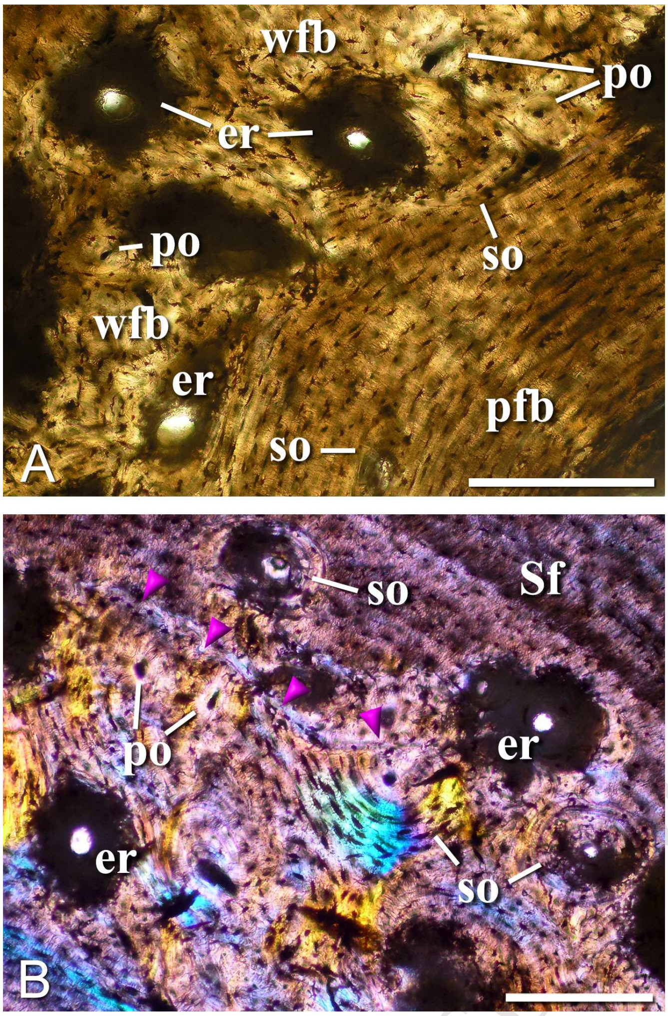
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- A Late Cretaceous crocodylian long bone has been sampled for histological study.
- The bone shows changes in bone tissue organization clearly related to changes in growth rate during ontogeny.
- Bone tissue types found indicate a determinate growth strategy.
- It is confirmed that modern crocodylian growth strategy was already in place by the Cretaceous.