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Microstructural features of the femur in early ophiacodontids: A reappraisal of ancestral habitat use and lifestyle of amniotes



Caractères microstructuraux du fémur chez les premiers ophiacodontes : réexamen du mode de vie ancestral des amniotes

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ABSTRACT

Ophiacodontids have long been considered the basalmost synapsids, and to have retained a fairly aquatic, piscivorous lifestyle typical of stem-amniotes. A restudy of their bone histology and microanatomy shows that *Clepsydropus collettii*, a Late Carboniferous ophiacodontid, has a thin, compact cortex and lacks a medullary spongiosa, two features that suggest a truly terrestrial lifestyle. The Early Permian *Ophiacodon uniformis* has a thicker cortex with a few resorption cavities and bone trabeculae surrounding the free medullary cavity. An inference model yields a terrestrial lifestyle for both taxa, though *O. uniformis* may have been slightly more aquatic (possibly amphibious) than *C. collettii*. However, an optimization of inferred lifestyle of other early stegocephalians (based on bone microanatomy) suggests that the first amniotes were terrestrial. The potentially amphibious lifestyle of *O. uniformis*, though not supported by our inference model, would thus be secondary. Histological features of femoral cortices in these two taxa closely resemble those previously described in extant species of large varanids and teids. This similarity, along with other comparative elements, is discussed in reference to the possible growth patterns and life history traits of *Clepsydropus* and *O. uniformis*.

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R É S U M É

Les ophiacodontidés ont longtemps été considérés comme les synapsidés les plus basaux et on leur a longtemps attribué le mode de vie aquatique et le régime piscivore typique des premiers amniotes-souches. Cette étude de micro-anatomie et d'histologie osseuse montre que le fémur de *Clepsydropus collettii*, un taxon du Carbonifère supérieur, possède un cortex compact peu épais et est dépourvu de spongiosa médullaire, deux caractères indicatifs d'un mode de vie franchement terrestre. *Ophiacodon uniformis*, du Permien inférieur, a un cortex plus épais avec quelques lacunes de résorption, ainsi que des travées osseuses autour de la cavité médullaire. L'usage d'un modèle d'inférence conduit à attribuer un mode de vie terrestre à ces deux taxons, encore que *O. uniformis* ait pu être un peu plus aquatique (peut-être

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amphibie) que *C. collettii*. Cependant, l'optimisation des inférences de mode de vie effectuées chez d'autres stégocéphales en référence à la micro-anatomie de leurs os suggère que les premiers amniotes étaient terrestres. D'éventuelles mœurs amphibies chez *Ophiacodon* (bien qu'elles ne soient pas confirmées par l'emploi de nos modèles d'inférence) seraient donc secondaires. Les caractères histologiques du fémur dans les deux taxons étudiés ici rappellent fortement ceux attribués dans la littérature aux grands varanidés et téidés. Cette similitude, ainsi que d'autres éléments comparatifs, est discutée en référence aux modèles de croissance et aux traits d'histoire de vie qui pourraient être attribués à *Clepsydraps* et à *O. uniformis*.

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1. Introduction

Extant amniotes include mostly terrestrial taxa, but does this reflect the initial amniotic condition? The amniotic egg is usually viewed as a useful, though not necessary adaptation to a terrestrial lifestyle (Skulan, 2000). However, even if we provisionally accept the current wisdom that the amniotic egg is a terrestrial adaptation, this does not necessarily imply that the first amniotes were terrestrial for their entire life cycle. Indeed, Romer (1957, 1958) thought that the first amniotes were still amphibious, perhaps even mostly aquatic taxa, and that they laid their eggs on land because it was a safer environment, with less predator pressure, than the aquatic environment. Even today, in at least some environments, there are apparently advantages in laying eggs outside water because some amphibians have adapted to lay eggs in this way (Martin and Carter, 2013). The benefit of laying eggs on land would have increased the farther we go back in time because there were fewer terrestrial predators back then (Laurin, 2010; Romer, 1957, 1958). Laying terrestrial eggs is also advantageous in alpine environments, in which eggs risk being swept away by the fast current, and this has led to the suggestion that this hazard may have been the selective pressure that led to the appearance of the amniotic egg (Goin and Goin, 1962). Although these considerations suggest that the amniotic egg is obviously optimized for terrestrial reproduction, they do not necessarily imply that the first amniotes were terrestrial animals.

What does the fossil record tell us about the lifestyle of the earliest amniotes? Ichnological evidence suggests that some Carboniferous stegocephalians ventured onto land (Keighley et al., 2008), even though this does not rule out the possibility that these taxa were still largely amphibious, if not mostly aquatic. Furthermore, trackways can be deposited underwater (Brand, 1992; Brand and Tang, 1991), so it is not obvious how reliable such ichnological data are to infer habitat use in long-extinct taxa. This interpretation is supported by the recent discovery of Middle Devonian stegocephalian trackways, probably produced by a taxon that was still largely aquatic (Niedzwiedzki et al., 2010). The oldest known unequivocal amniote skeletal remains are from Joggins, Nova Scotia (Carroll, 1964), and date back to the Early Pennsylvanian, about 315–316 Ma (Calder et al., 2005), Late Bashkirian to Early Moscovian (Gradstein et al., 2012). Most stegocephalians found in Joggins may have been terrestrial, based on their morphology, but the fact that most fossils were preserved in tree stumps

raises the possibility that the Joggins thanatocoenosis is a biased sample of the biocoenosis that it represents.

The scant skeletal remains found in Joggins, combined with unfavorable preservation (appendicular long bones are strongly flattened; ML, personal observation) has so far prevented the use of other types of data, such as bone microanatomy, to corroborate previous interpretations about the lifestyle of the stegocephalians found in Joggins. However, long bone microanatomy of slightly more recent, Permo–Carboniferous stegocephalians, including amniotes, has started yielding clues about the lifestyle of the first amniotes (Canoville and Laurin, 2010; Germain and Laurin, 2005; Huttenlocker and Rega, 2012; Kriloff et al., 2008; Laurin et al., 2004; Quémeneur et al., 2013). Among Permo–Carboniferous amniotes, ophiacodontids are especially relevant to assess the primitive amniotic condition, for two reasons. First, Romer (1961) viewed ophiacodontids as aquatic to amphibious, piscivorous taxa. Second, ophiacodontids are historically important: following Romer (1961), they have indeed been supposed to be the basalmost synapsids (in fact, Romer considered them to be the ancestral stock of other synapsids), a viewpoint that was upheld much more recently by Carroll (1988). Several more recent explicit phylogenetic analyses (Laurin, 1993; Reisz, 1986; Reisz et al., 1992) have not supported this conclusion, and placed ophiacodontids among eupelycosours, though Benson (2012: fig. 2C) found some support for a very basal position of this taxon. Ophiacodontids thus formed a cornerstone of Romer's scenario about the origin of amniotes, along with the apparently amphibious to aquatic diadectomorph *Limnoscelis paludis* (Romer, 1946), and they remain important to assess the primitive lifestyle of amniotes and synapsids even under recent phylogenies.

The question, however, proves to be complex and a recent morphometric study of the *Ophiacodon* spine (Felice and Angielczyk, 2014) failed to reach a clear-cut, unambiguous conclusion on this topic. Below, we test Romer's (1957, 1958), and Felice and Angielczyk's (2014) ideas about the lifestyle of ophiacodontids, through a microanatomical study of two taxa of different geological ages: the Late Carboniferous *Clepsydraps collettii* and the Early Permian *Ophiacodon uniformis*. We hypothesize, because of the difference in geological age between both taxa (at least 15 Ma; see below), that *C. collettii* retains a lifestyle closer to that of the earliest ophiacodontids and probably of the earliest amniotes as well, than *O. uniformis*. We also take this opportunity to further describe their histology, in complement to previous descriptions by Enlow

and Brown (1957) and de Ricqlès (1974), as this can yield clues about their growth pattern and possible metabolism. Finally, we provide an update about what quantitative inference models based on bone microanatomy imply about the evolution of lifestyle. The last published synthesis of these results harks back to Canoville and Laurin (2010: fig. 8), so an update is timely.

2. Materials and methods

The specimens sectioned belong to two taxa, *Clepsydrops* and *Ophiacodon*. The *Clepsydrops* femur, Yale Peabody Museum (YPM) VP 056583 was identified in the collection as “*Clepsydrops* sp.,” but the specimen is from Vermilion County, Danville, where only *C. collettii* and *C. vinslovii* are recorded, and Reisz (1986) indicates that the latter is probably a junior synonym of *C. collettii*. Therefore, this specimen will tentatively be attributed to *C. collettii*. It is a perfectly preserved 56.8 mm long bone. The Danville locality (Illinois) is part of the McLeansboro group (Reisz, 1986), which is Middle Pennsylvanian, Late Desmoinesian, (Hatch and Affolter, 2002), equivalent to Late Moscovian to Early Kasiomovian, about 307 Ma (Davydov et al., 2012).

The *O. uniformis* femur, Carnegie Museum of Natural History, Pittsburgh, Pennsylvania (CM) Vertebrate Paleontology (VP) 9528 is also an entire bone (104.2 mm long), in excellent conservation state. It is from the Briar Creek bonebed, Admiral Formation, Artinskian (Lucas, 2006), about 288–279 Ma (Henderson et al., 2012). It is thus among the geologically oldest specimens of *O. uniformis* because this taxon is known from the Putnam formation, Early Artinskian, to the Clyde formation (Reisz, 1986), Early Kungurian (Lucas, 2006). This taxon thus ranges from about 290 Ma to about 276 Ma.

We also illustrate the *O. retroversus* radius that was used by Germain and Laurin (2005) to infer the lifestyle of *Ophiacodon*, but not illustrated in that paper. It comes from the Rattlesnake Canyon, Archer County, Admiral Formation, and is thus approximately contemporary with the *O. uniformis* whose femur we sampled. A histological and microanatomical description of the bone was published, in French, by de Ricqlès (1974), who produced the section (number 291.1.2T, in his collection). Below, we provide a brief description of this bone, for comparative purposes.

Digital pictures of mid-diaphyseal cross-sections (thickness 80 to 100 μm) of the bones were binarized to be analyzed by Bone Profiler (Girondot and Laurin, 2003), in order to obtain compactness profiles. These data were then used to obtain inferences about the lifestyle of ophiacodontids using the ternary inference model based on the femur produced by Quémeneur et al. (2013). This model requires data on snout-vent length (Supplementary On line Materials [SOM]). We do not have the exact length for the specimens used, but the literature includes many such estimates for several taxa, including *O. uniformis*, whose adult size is estimated at 666 mm snout-vent length (SVL) by Romer and Price (1940). For *C. collettii*, we did not find precise estimates in the literature, but assuming that it is indeed an ophiacodontid (Reisz, 1986), and assuming isometry between femoral mid-diaphyseal diameter and snout-vent length, we estimate that our *C. collettii*

specimen would have been about half as long as the *O. uniformis* specimen, i.e. some 333 mm SVL. Our snout-vent length estimates are thus only approximate, but we tested values $\pm 50\%$ around our estimate, and this did not change the inferred lifestyle, so the latter can be considered robust to body size errors.

Additional sagittal (longitudinal) sections in the diaphyseal and metaphyseal regions of both specimens were made to complement histological observations. The latter involved light microscopy in ordinary and polarized light at low ($\times 25$) to medium ($\times 400$) magnification using a Zeiss Axioskop microscope. All measurements of sectional dimensions were performed with the software Image J (National Institute of the Health, USA) on either microphotographs or digitalized line drawings of the sections (enlargements $\times 12$ to $\times 240$). The vocabulary used for describing the microanatomical and histological features of bone refers to Francillon-Vieillot et al. (1990), and to Prondvai et al. (2014).

3. Results

3.1. Microanatomical description

The *C. collettii* femur has a simple, nearly tubular shaft structure (Fig. 1A). Only a few, small resorption cavities can be seen, most of which are clustered close to the three short,

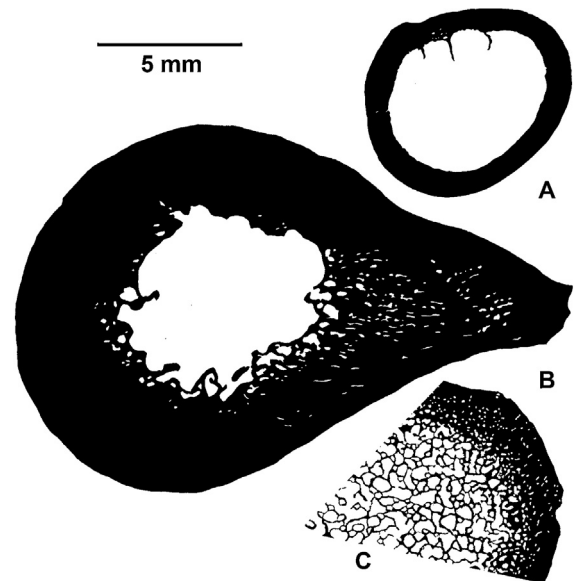


Fig. 1. Drawings showing bone microanatomy in the mid-diaphyseal region of the femur of *Clepsydrops collettii* (A) and *Ophiacodon uniformis* (B). A section in the radius of *O. retroversus* (reference 291.1.2T in A. de Ricqlès' collection) that was used for compactness profile by Germain and Laurin (2005) is also shown for comparison (C). Note the thinner cortex and simpler structure of *Clepsydrops*. These drawings were used for the Bone Profiler analysis to infer the lifestyle of these taxa.

Fig. 1. Dessins montrant l'organisation micro anatomique du fémur chez *Clepsydrops collettii* (A) et *Ophiacodon uniformis* (B), ainsi que celle du radius d'*O. retroversus* (C). On remarquera que le cortex est plus fin et la structure de l'os plus simple chez *Clepsydrops*. Ces dessins ont d'abord été analysés à l'aide de Bone Profiler, et ont ensuite servi à inférer le mode de vie des trois taxons.

thin bone trabeculae that protrude into the medullary cavity. The cortex is thin and its mean thickness, 0.82 mm (i.e. 23.7% of mean diaphyseal radius), is relatively uniform.

The femur of *O. uniformis* is about twice as large as that of *C. collettii*, and has a more complex structure (Fig. 1B). Its cortex is much thicker (3.05 mm; 51% of mean diaphyseal radius), but this bone is not osteosclerotic because a well-developed medullary cavity is present, and the cortex is abundantly vascularized. Numerous small to mid-sized resorption cavities are present, especially around the medullary cavity and in the adductor crest. Several short, relatively thick trabeculae surround the medullary cavity on about half of its periphery, but none extends deep within it.

The differences evoked above are reflected by compactness profiles (Table 1 and Fig. 2); *Clepsydraps* has a much greater value of parameter *P* (the position of the inflexion point that marks the transition between cortex and medulla; Fig. 2A) than *Ophiacodon* (Fig. 2B). Both taxa have a Min value (lower asymptote) at essentially 0, and a Max value (upper asymptote) near 1, although a little lower in *Ophiacodon*, because of a denser cortical vascularization (Table 1). The value of parameter *S* (steepness of the transition between Min and Max) for *Clepsydraps* (0.012) is low, which reflects an abrupt transition between the cortex and the medulla and a fairly even thickness of the cortex. A low value of this parameter is typical of terrestrial and (to a lesser extent) amphibious taxa (supplementary on line Table 2 of Quémeneur et al., 2013). The *S* value for *Ophiacodon* (0.047), though clearly higher, remains inside the range ($0.008 \leq S \leq 0.13$) that characterizes the femur of terrestrial taxa (Quémeneur et al., 2013).

3.2. Histological features of the femora

3.2.1. *Clepsydraps*

In both cross (Fig. 3A) and sagittal (Fig. 3B) sections, the thin cortex of *Clepsydraps* femur shows a deep layer made of an osseous tissue with low, or no, birefringence, and a more peripheral layer with clear birefringence and a faint subdivision into bone lamellae. Osteocyte lacunae are ovoid or multipolar with short canaliculi in the deep layer, while those in the superficial strata are flat and have no canaliculi

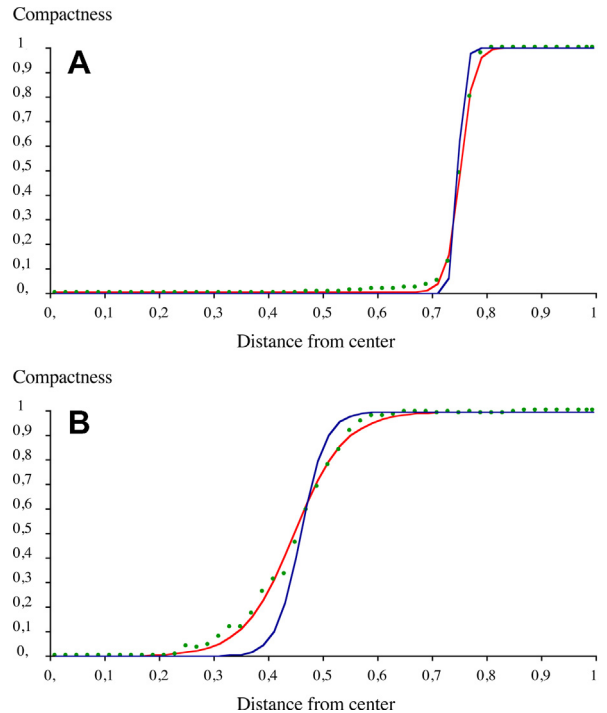


Fig. 2. (Color online.) Compactness profiles of *Clepsydraps collettii* (A) and *Ophiacodon uniformis* (B) femora. The higher value of parameter *P* in *Clepsydraps* reflects its thinner cortex. Similarly, the steeper slope at the inflexion point reflects the simpler structure of its cortex, with an abrupt transition with the medullary region. The red curve (light grey in the print version) represents the compactness profile model based on global values (the whole perimeter), whereas the blue line (darker grey in the print version) represents the profile based on angular values (the average of the values computed on 6 degree-wide sectors). For more information about this distinction, see Laurin et al. (2004).

Fig. 2. (Couleur en ligne.) Profil de compacité du fémur de *Clepsydraps collettii* (A) et d'*Ophiacodon uniformis* (B). La valeur plus élevée du paramètre *P* chez *Clepsydraps* tient à la finesse du cortex. De même, la pente plus accentuée de la courbe au point d'inflexion traduit une structure plus simple du cortex dans ce taxon avec, notamment, une limite tranchée entre cortex et médulla. La courbe rouge (gris pâle dans la version imprimée) représente le profil de compacité fondé sur les valeurs globales (du périmètre entier), alors que la courbe bleue (gris foncé dans la version imprimée) représente le profil établi à partir des valeurs angulaires (moyennes pour des secteurs de 6 degrés). Pour plus d'information sur cette distinction, voir Laurin et al. (2004).

Table 1

Compactness profile parameters of ophiacodontid femora obtained by Bone Profiler (Girondot and Laurin, 2003) version 4.5.8. Estimated snout-vent length for the two specimens analyzed: 333 mm for *Clepsydraps collettii* and 667 mm for *Ophiacodon uniformis*.

Tableau 1

Paramètres du profil de compacité obtenu par Bone Profiler (Girondot et Laurin, 2003) version 4.5.8. Longueur museau-cloaque estimée pour les deux spécimens étudiés : 333 mm pour *Clepsydraps collettii* et 667 mm pour *Ophiacodon uniformis*.

Taxon	Parameter	Mean global value	SD global value	Mean angular value	SD angular value
<i>Clepsydraps collettii</i>					
	Min	0.007	0.000	0.000	0.000
	Max	1.000	0.000	1.000	0.002
	S: 1/Slope	0.012	0.000	0.006	0.012
	P: transition	0.751	0.000	0.747	0.027
<i>Ophiacodon uniformis</i>					
	Min	0.000	0.000	0.000	0.000
	Max	0.997	0.000	0.996	0.010
	S: 1/Slope	0.047	0.000	0.023	0.017
	P: transition	0.447	0.000	0.459	0.051

SD: standard deviation.

(Fig. 3C). These characteristics suggest that the deep cortex consists of an osseous type akin to the woven-fibered type, and that more peripheral layers are made of a parallel-fibered tissue that turns into true lamellar bone towards the outer margins of the cortex. The transition between these layers is gradual, and the central part of the cortex displays characteristics between the two histological types described above. Vascularization in the deep cortex basically consists of primary osteons whose spatial density can reach 80 units/mm², and whose lumen is 25 to 30 μm in average diameter (Fig. 3A, D, E). Osteon orientation is mainly longitudinal (i.e. parallel to the long axis of the bone) and in some sectors of the cross-sections, the osteons tend to be disposed in radial files, each including 4 to 6 subsequent osteons (Fig. 3D). In other sectors, the osteons are more randomly distributed (Fig. 3A and E). Vascular density decreases progressively towards bone periphery, as also the mean diameter of canal lumen (it falls down to 15 μm). Superficial cortical layers are poorly vascularized (vascular density < 5 canals/mm²) or completely avascular in places (Fig. 3E). *Clepsydraps* femoral cortex displays neither cyclic growth marks, nor Sharpey's fibers. The deep (perimedullary) surface of the cortex displays local signs of resorption related to the widening of the medullary cavity (Fig. 3F). Conversely, in other sectors of the sections, the deep margin of the cortex is lined by a thin (50 to 150 μm in thickness) layer of endosteal, reconstructive lamellar tissue that was deposited after an episode of extensive resorption (Fig. 3D). There is no evidence of significant intracortical remodeling. The rare bone trabeculae protruding into the medullary cavity are thin (diameter 50 to 80 μm) and made of poorly remodeled parallel-fibered or lamellar bone tissues (Fig. 3G, H). None of them contains any residue of calcified cartilage.

3.2.2. *Ophiacodon*

The mid-diaphyseal sections examined here show a histological stratification of the femoral cortex into four concentric layers that are likely to reflect successive episodes of variation in the rate of periosteal bone accretion. The layers are numbered here from 1, for the deepest one, to 4 for the most superficial (Fig. 4A). Layers 1 and 3 show rather similar characteristics, and layers 2 and 4 are also histologically similar. Layers 1 and 3 are abundantly vascularized by simple vascular canals 20 to 40 μm in diameter, as also, in some sectors of the sections, by primary osteons (Fig. 4A–C, E, F). No evidence of intracortical Haversian substitution, resulting in regular secondary osteons, is noticeable in the specimen examined. However, the deepest layers of the cortex, in the vicinity of the medullary cavity, may display a few large erosion bays (Fig. 4F) whose walls were, in some cases, under reconstruction by secondary, endosteal deposits when the animal died. In layer 1, vascular density is relatively high (27 canals/mm²), and the main orientation of the canals is oblique and, to a lesser extent, longitudinal. Vascular canals are less numerous, but longer and more oblique in layer 3 (they become radial in this same layer in the adductor crest), where they tend to develop anastomoses and short ramifications (Fig. 4B, F, G). Layers 2 and 4 have a lesser vascular density (15 to 18 canals/mm²) and thinner canals

(12 to 18 μm in diameter); in addition, the most peripheral part of layer 4, on a thickness of some 300 μm , is nearly avascular (Fig. 4G). Optic opacity in layers 1 and 3 is greater than in layers 2 and 4 (Fig. 4B). This aspect is related, for a part, to a high density in osteocyte lacunae and, for another part, to the spheroid or multipolar morphology of these lacunae; conversely, the lacunae are spindle-like or flat in layers 2 and 4 (Fig. 4D). The whole cortex of the *O. uniformis* femur displays a mass birefringence (Fig. 4C and E) that reflects a relatively even orientation of collagen fibers. However, this reaction to polarized light is irregular in layers 1 and 3, especially in the region of the adductor crest. Layer 1 does not display any clear cyclic growth mark, which implies that growth would extend continuously in this whole layer (Fig. 4A, B, F). Layer 3 shows a faint and poorly defined indication of cyclic accretion (Fig. 4B, F, G). Conversely, two well-characterized *annuli* and two lines of arrested growth (LAGs) occur in layer 2, and nine to ten LAGs in layer 4 (Fig. 4F and G). The growth marks in layer 2, as also the first four LAGs in layer 4, are broadly, but evenly spaced, with intervals of 129 μm in the average in layer 2, and 110 μm to 203 μm in layer 4. The last six marks in layer 4 are tightly grouped, and have a relatively constant interval of some 26 μm indicating sustained, though very slow, growth activity during this last stage (Figs. 4G and 5). Mean apposition rate can be estimated to some 0.07 $\mu\text{m}/\text{day}$ during this last stage, which is obviously an underestimation because the presence of LAGs indicates that active growth occurred during a part of the year only. A true speed twice as high, or 0.14 $\mu\text{m}/\text{day}$, is conceivable with reference to Lance's (2003) observations revealing an active growth period of 6 months only in extant alligator populations.

On the diaphyseal face opposite to the adductor crest, the medullary cavity is lined by a thin layer of secondary lamellar bone separated from the rest of the cortex by a reversion line. The other face of the medullary cavity, under the adductor crest, is devoid of this reconstructive layer but shows signs of resorption. By the time the animal died, the expansion of the medullary cavity was thus asymmetric, with a drift in the ventral direction. There is no trace of calcified cartilage matrix within the trabeculae protruding into the medullary cavity.

3.3. Lifestyle inferences

The ternary inference model from Quémener et al. (2013) yields a terrestrial lifestyle for both *C. collettii* and *O. uniformis*. The binary model yields an amphibious to terrestrial lifestyle (SOM) for both taxa (the alternative state, aquatic, being thus excluded).

4. Discussion

4.1. Comparative data on bone histology

To our knowledge, long bone structure in *Clepsydraps* has never been described. Conversely, descriptions of the histological features of *Ophiacodon* long bones have been published by Enlow and Brown (1957), about a rib and an undetermined long bone, and de Ricqlès (1974) about

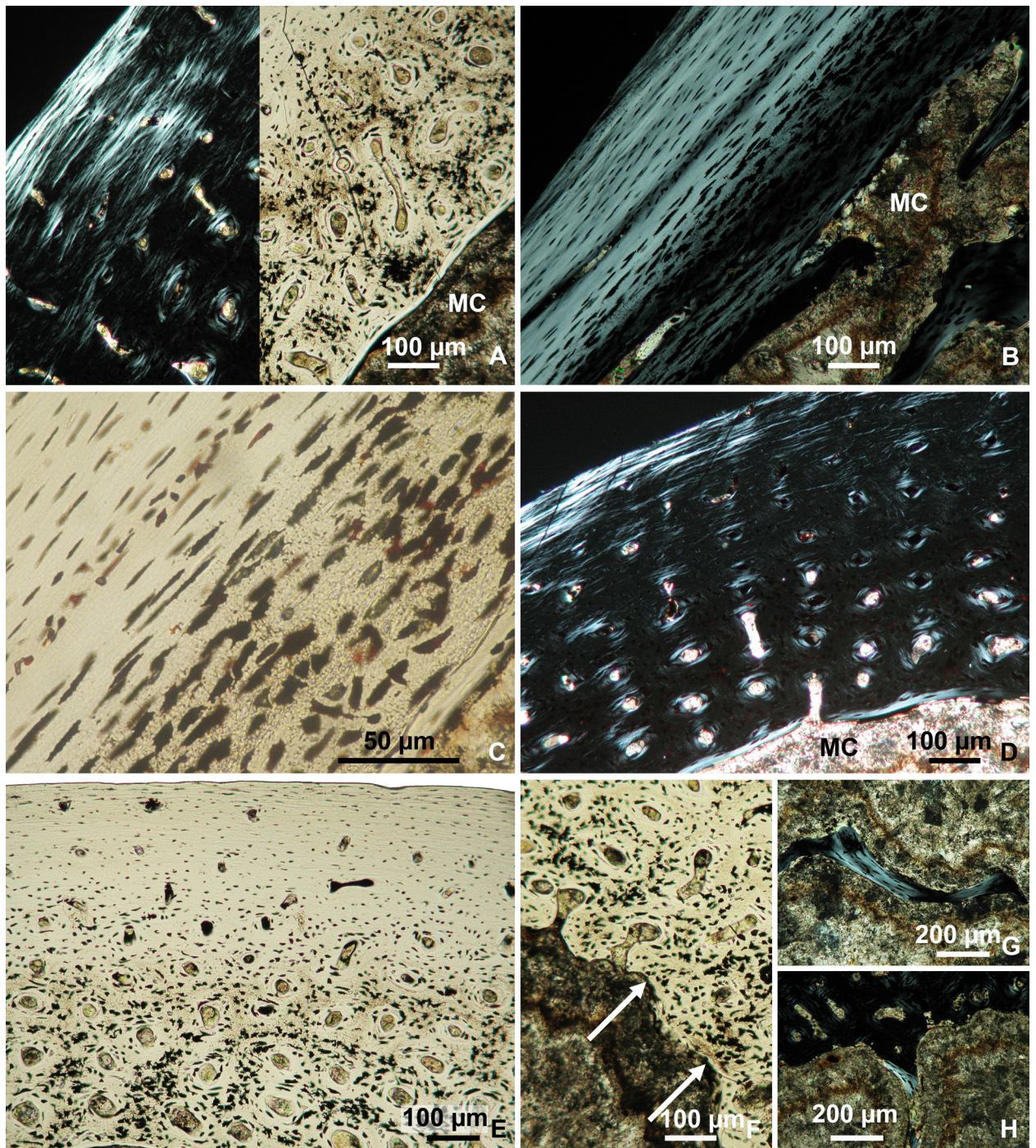


Fig. 3. Histology of a *Clepsydrops collettii* femur. A. General aspect of the cortex in cross-sections. The left half: polarized light, right half: ordinary transmitted light. The cortex is nearly monorefringent in depth, and progressively turns to birefringent towards its periphery. B. Aspect of the femoral cortex in a longitudinal section. Polarized, transmitted light. Same comments as for the previous figure. C. Morphology of cell lacunae in the deep (lower right half) and superficial (upper left half) parts of the cortex. In depth, osteocyte lacunae are ovoid with short canaliculi; they are flat without canaliculi in peripheral layers. D. Longitudinal primary osteons disposed in radial rows in the deep cortex. Polarized transmitted light. E. Progressive change in the vascular supply of the bone from depth to periphery. Deep vascularization is composed of numerous primary osteons with a wide lumen. Superficial vascularization is meager, with fewer thin-diameter simple vascular canals. F. Local, perimedullar resorption (arrows) in the femur. G and H. Moderately remodeled bone trabeculae protruding into the medullary cavity (MC).

Fig. 3. Histologie du fémur de *Clepsydrops collettii*. A. Aspect général du cortex en coupe transversale. La moitié gauche est vue en lumière polarisée, la droite en lumière ordinaire transmise. Le cortex est quasi monoréfringent en profondeur, et devient progressivement biréfringent vers la périphérie. B. Aspect du cortex fémoral en section longitudinale. Lumière polarisée transmise. Voir les commentaires de la partie A. C. Morphologie des lacunes ostéocytaires dans les parties profonde (moitié droite) et superficielle (moitié gauche) du cortex. En profondeur, les logettes ostéocytaires sont ovoïdes et montrent de courts

a radius, in *O. retroversus* (see also the recent synthesis by Huttenlocker and Rega, 2012). These studies had convergent results: *O. retroversus* bone cortices are basically composed of an abundantly vascularized (simple vascular canals and primary osteons) parallel-fibered tissue that tends to turn into true lamellar bone towards cortical periphery. Such a structure is in good agreement with the results of the present study. Some differences nevertheless distinguish the femur of *O. uniformis* studied here from the bones of *O. retroversus*. The latter display only longitudinal non-anastomosed vascular canals, whereas our specimen also displays oblique and radial canals that are frequently anastomosed. The density of vascular canals, as also their orientation, can vary between the bones of a single skeleton, or even between the different sectors of a single section (e.g., de Buffrénil et al., 2008, and the results of the present study); a difference in these features between, for example, the femur and the radius is therefore unsurprising when two distinct taxa are considered. Moreover, the unidentified long bone of *O. retroversus* described by Enlow has apparently a greater density of primary osteons than the femur of *O. uniformis* and, along with the radius described by de Ricqlès (1974), it contains some secondary osteons (Haversian substitution) that do not occur in *O. uniformis*. Finally, the radius of *O. retroversus* displays no free medullary cavity (Fig. 1C). The latter is occupied by a continuous network of trabeculae, a situation quite distinct from the femoral diaphysis of *O. uniformis*. It seems possible that Ricqlès' radius section (number 291.1.2T in this author's collection) was not sampled exactly at the mid-diaphyseal level. Indeed, this section measures 18 by 11 mm in diameter. Comparing with figure 46A and B (ventral and medial views, respectively) of the *O. retroversus* radius in Romer and Price (1940), these proportions best match the distal metaphysis; in the mid-diaphyseal region, the difference between perpendicular diameters is much smaller.

Among other basal synapsids, the general histological type observed in *O. uniformis* femur is encountered also in the contemporary (Early Permian) sphenacodontids, *Sphenacodon* spp. (Huttenlocker and Rega, 2012) and *Dimetrodon natalis* (Shelton et al., 2013; see also Huttenlocker et al., 2010).

As compared to extant species, the histological structure of the femoral cortex of *Clepsydropus* looks remarkably similar to that of large varanids (e.g., *Varanus niloticus*: de Buffrénil and Castanet, 2000). In both cases, bone matrix is of a parallel-fibered tissue type that tends to turn into the woven-fibered type in depth, and to the lamellar type in periphery, with an abundant network of primary osteons and simple vascular canals. Consistent with the many experimental data that link the histological structure of primary bone deposits with their accretion rate (Castanet et al., 1996; de Margerie et al., 2002, 2004), this

resemblance would mean that the general dynamics of the radial growth of *Clepsydropus* femur was comparable to that of, e.g., the Nile monitor. In ectothermic tetrapods, growth curves derived from the rate of cortical accretion (radial growth of the bones) proved to be reliable indicators of the general growth pattern of an individual (Castanet and Baez, 1991). Therefore, the similarity in bone structure between *Clepsydropus* and large varanids may indicate relatively comparable growth speeds for the body as a whole (cf. de Buffrénil and Hémerly, 2002, 2007 for *V. niloticus* growth curve). Two differences nevertheless exist between *Clepsydropus* and large varanids. The first is the frequent occurrence of annual growth marks (LAGs or *annuli*) in varanids, whereas such marks are not observed in *Clepsydropus*. This situation may be explained by three distinct, mutually exclusive hypotheses:

- *Clepsydropus* had a continuous, non-cyclic, growth;
- *Clepsydropus* had a cyclic growth, but our specimen is from an animal that died before the end of its first year;
- some growth marks were once present in deep cortical layers, but were eroded by the expansion of the medullary cavity.

All three hypotheses are possible, though the first one (non-cyclic growth) would designate *Clepsydropus* as an exception among ectothermic tetrapods. The presence of peripheral lamellar tissue in bone cortex indicates that the growth rate of our specimen was strongly decreasing, a sign of possible somatic maturity. The latter could have occurred either before, or after, the age of 1 year, a question that cannot be settled with the data presently available. In some heavily exploited populations of Nile monitors, males and females reach an SVL 35 to 40 cm, along with sexual maturity, by the age of 1 year (de Buffrénil and Hémerly, 2002, 2007). The hypothetical attribution of similar life history traits to *Clepsydropus* is thus by no means unrealistic. The second difference is relative to the orientation of vascular canals: they are mainly longitudinal in *Clepsydropus*, whereas oblique or radial canals are frequently observed in varanid bones. However, this characteristic is fairly variable between bones, individuals or varanid populations (de Buffrénil and Castanet, 2000; de Buffrénil et al., 2008), and the possible occurrence of similar variability in *Clepsydropus* cannot be discarded. An interesting comparative element is that monitors species less than 460 mm in adult snout-vent length (SVL) have avascular femoral cortices, as is the case for most other squamates (Cubo et al., 2014; de Buffrénil et al., 2008). The snout-vent length of our specimen was estimated at 333 mm, and histological observations suggest that this individual was an adult approaching the end of its somatic growth. If so, its size was far below the threshold for bone vascularization observed in varanids. Considering the general relationship

canalicules ; elles sont plates et sans canalicules dans les couches périphériques. D. Ostéons primaires longitudinaux disposés en rangées radiales dans le cortex profond. Lumière polarisée transmise. E. Changement progressif de la vascularisation osseuse de la profondeur vers la périphérie. La vascularisation profonde est formée de nombreux ostéons primaires à large lumière. La vascularisation superficielle est plus clairsemée, avec des canaux vasculaires simples de petit diamètre. F. Résorption périmédullaire locale (flèches) dans le fémur. G et H. Travées modérément remaniées faisant saillie dans la cavité médullaire (MC).

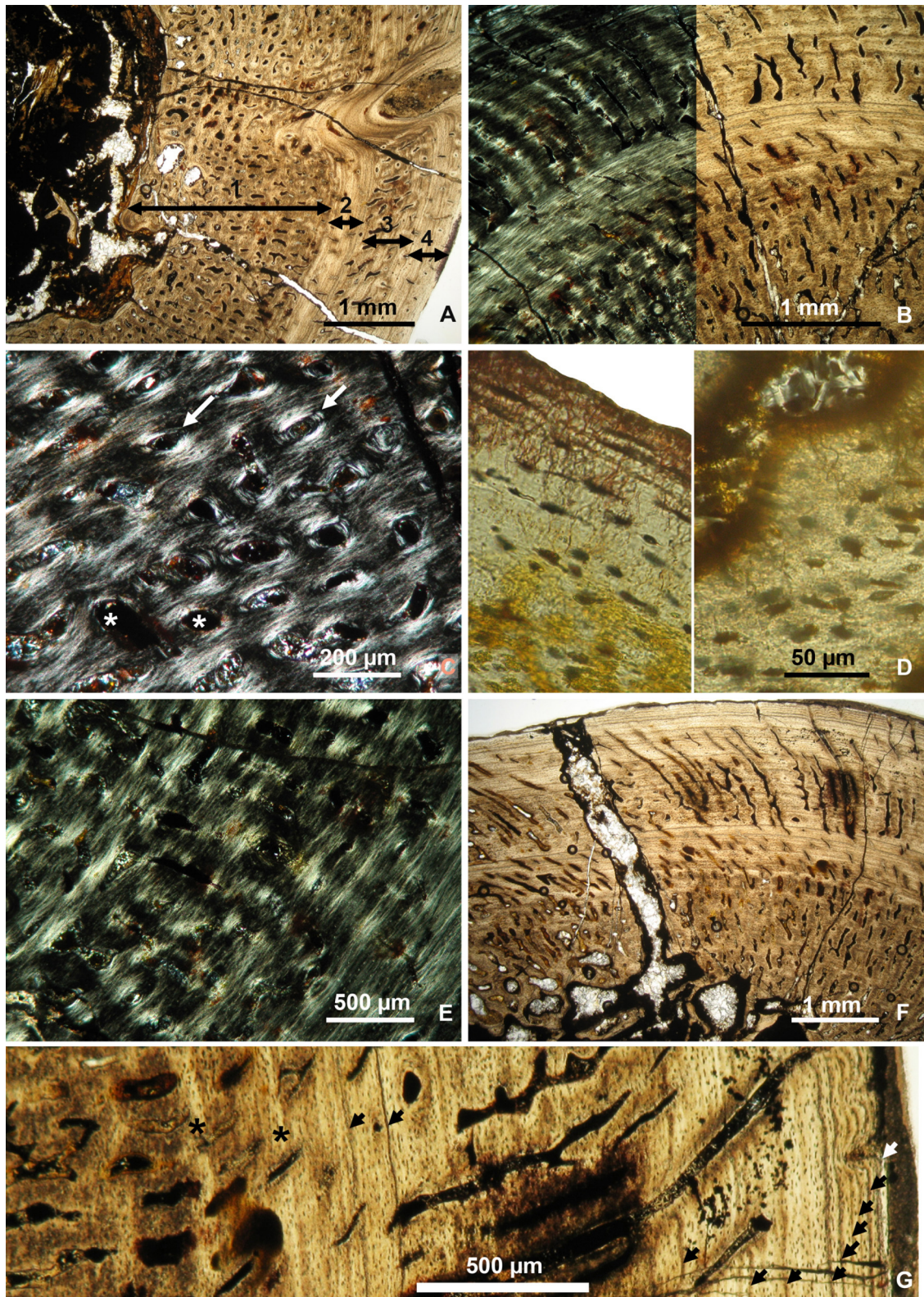


Fig. 4. Histology of an *Ophiacodon uniformis* femur. A. General view of the cortex showing stratification into four broad layers numbered 1 to 4. B. Differences in vascularization and birefringence properties between, on the one hand, layers 1 and 3 and, on the other hand, layers 2 and 4. Layers 1 and 3 are abundantly vascularized by large oblique and longitudinal simple canals, while layers 2 and 4 are less vascularized by scarce and thin canals. C. Simple vascular canals (asterisks) and primary osteons (arrows) in the cortex. Bone matrix is of a rough parallel-fibered type and contains many longitudinal vascular canals.

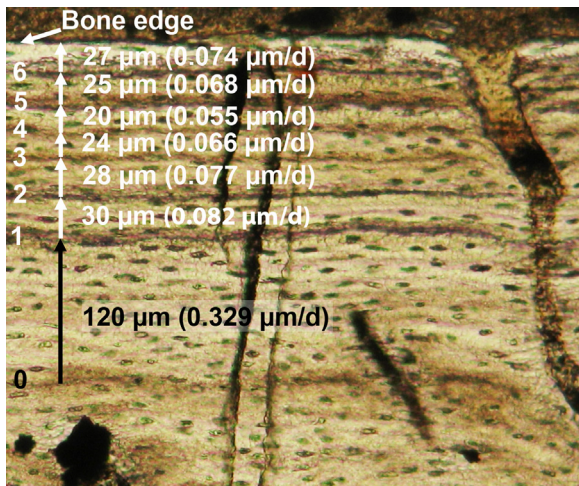


Fig. 5. (Color online.) Sub-periosteal apposition rates during the last six growth cycles of the *Ophiacodon* specimen. The thickness of the bone layer deposited during each cycle (designated by an arrow), as well as the cortical apposition rate (cycles are supposed to have an annual periodicity), suddenly collapse after the accretion of cycle 0. This abrupt decrease in growth can be interpreted either as consecutive of the onset of sexual maturity, or as an indication of the end of somatic fast growth, cycles 1–6 then forming an external fundamental system (also called external circumferential lamellae).

Fig. 5. (Couleur en ligne.) Vitesse d'accrétion sous-périostique durant les six derniers cycles de croissance chez le spécimen d'*Ophiacodon*. L'épaisseur de la couche osseuse déposée lors de chaque cycle (désignée ici par une flèche), ainsi que la vitesse d'apposition (les cycles sont supposés de périodicité annuelle), s'effondrent brusquement après l'accrétion du cycle 0. Cette décroissance rapide peut être vue soit comme une conséquence de l'accession de l'animal à la maturité sexuelle, soit comme une indication de la fin de la croissance somatique rapide, les cycles 1 à 6 représentant alors un système fondamental externe.

between bone vascular supply and growth speed (Castanet et al., 1996; de Margerie et al., 2002, 2004), this may mean that the *Clepsydraps* growth rate was comparable to that of larger monitors, and was thus faster than that of the varanids of comparable adult size. This possibility seems all the more plausible that the deepest (i.e. ontogenetically earliest) parts of *Clepsydraps* cortex, which were obviously deposited at a higher rate than the more peripheral parts, were eroded by perimedullar resorption and are thus not observed in our section. Permo-Carboniferous synapsids had primitive epiphyses (sensu Haines, 1942, 1969; i.e. epiphyses devoid of secondary ossification centers); the growth of *Clepsydraps* could thus have been potentially unlimited, though slow in advanced ontogenetic stages.

Recent studies dealing with, e.g., crocodylians (Woodward et al., 2011; see also de Ricqlès et al., 2003) nevertheless show that primitive epiphyses are not necessarily indicative of unlimited growth, and that the taxa having this kind of epiphyses actually reach an asymptotic length. However, the latter is delayed and may not be attained before death of most individuals of a given population. For some authors (e.g., Lee et al., 2013), true unlimited growth is likely to be a fictive concept (see also Congdon et al., 2013, on this topic), at least among vertebrates.

The histological traits of bone cortices in *Ophiacodon* are also closer to those observed in varanids and some teids of large size (e.g., *Tupinambis*: Cubo et al., 2014), than to those of all other extant ectothermic amniotes. However, the occurrence of ramified, anastomosed vascular canals is unknown in varanids. This feature suggests that growth rate in *Ophiacodon* was somewhat faster than in extant large squamates (see on this topic Castanet et al., 1996).

Growth rates in the *Ophiacodon* femur studied here was submitted to both cyclic and accidental fluctuations. Histological observations indicate that layers 1 and 3 formed during episodes of fast growth, and layers 2 and 4 during phases of slower growth. These fluctuations are unlikely to result from regular, cyclic processes because layers 2 and 4 (and to a lesser extent layer 3) are themselves subdivided into thinner layers defined by typical cyclic growth marks, the *annuli* and the LAGs that are known to have a basic annual periodicity in most tetrapods (Castanet et al., 1977, 1993). If such marks were indeed annual in our *Ophiacodon* specimen, it can be hypothesized that, after an initial active, most likely juvenile, growth stage of unknown duration (accretion of layer 1), the growth of the femur experienced a much lower rate during 4 years (accretion of two *annuli* and two LAGs in layer 2). A new phase of relatively fast growth succeeded during several years (layer 3), before being itself followed by a final episode of slow (base of layer 4) or very slow (last six cycles) growth that lasted at least about 10 years and ended with the death of the animal. This interpretation suggests that *Ophiacodon* growth presented regular, most probably annual, cycles, in addition to being sensitive to stochastic events likely to reflect environmental variations. These events were able to provoke either a sudden increase (layer 3) in a growth activity that had been basically reduced after the deposit of layer 1, or a double decrease (layers 2 and 4) in an otherwise sustained growth activity evidenced by layer 3. Both alternatives have specific bearings on the general interpretation of *Ophiacodon* growth, as developed below.

D. Morphology of cell lacunae in layers 2 and 4 (left), and layers 1 and 3 (right). E. Rough mass birefringence of bone matrix in the cortex. Transmitted polarized light. F. General view of the cortex showing cyclical growth marks (*annuli* and lines of arrested growth, or LAGs) in layers 2 and 4, but no clearly characterized marks in layers 1 and 3. G. Growth mark count. Layer 2 contains two *annuli* (asterisks) and two LAGs (arrowheads), while layer 4 has 9 obvious LAGs. The last 6 marks in layer 4 are tightly grouped.

Fig. 4. Histologie du fémur d'*Ophiacodon uniformis*. A. Vue générale du cortex montrant sa stratification en quatre couches numérotées de 1 à 4. B. Différence de vascularisation et de réfringence entre, d'une part, les couches 1 et 3 et, d'autre part, les couches 2 et 4. Les couches 1 et 3 sont abondamment vascularisées par de gros canaux primaires obliques ou longitudinaux, alors que les couches 2 et 4 ne sont vascularisées que par de rares canaux de petit diamètre. C. Canaux vasculaires simples (astérisques) et ostéones primaires (flèches) dans le cortex. La matrice osseuse est, globalement, du type « à fibres parallèles » et contient de nombreux canaux vasculaires longitudinaux. D. Morphologie des lacunes ostéocytaires dans les couches 2 et 4 (à gauche) et 1 et 3 (à droite). E. Biréfringence globale de la matrice osseuse dans le cortex. Lumière polarisée transmise. F. Vue générale du cortex montrant des marques de croissance cycliques (*annuli* et lignes d'arrêt de croissance) dans les couches 2 et 4, mais également l'absence de telles marques, nettement définies, dans les couches 1 et 3. G. Dénombrement des marques de croissance. La couche 2 contient deux *annuli* (astérisques) et deux lignes d'arrêt de croissance (pointes de flèches), alors que la couche 4 compte neuf LAGs, dont les six dernières sont très resserrées.

In layer 2 and the base of layer 4, the spacing of cyclic growth marks is broad and relatively constant. The narrow spacing of LAGs that suddenly occurred in the peripheral region of layer 4 suggests that growth rate decrease was particularly abrupt and pronounced just before the last 6 years of life of the animal. This aspect can be viewed in two different ways:

- it can reflect the consequences on local bone histology of the attainment of sexual maturity, as described in many ecto-poikilothermic tetrapods (e.g., [Castanet et al., 1988, 1993](#));
- it can also be interpreted as indicative of the end of fast skeletal growth, at least for the bone and the region of this bone, considered here.

According to the first hypothesis, our *Ophiacodon* specimen may have been an adult that had already completed most of its somatic growth before the onset of sexual maturity, but was still growing at a very low, but regular rate after it. In this interpretation, sexual maturity was relatively late (more than eight years for the present specimen), as is the case for many extant “reptiles”, including, for example, lepidosaurians (*Sphenodon punctatus*; [Castanet et al., 1988](#)), crocodiles ([Webb et al., 1978](#)), etc. The alternative hypothesis would postulate that sexual maturity occurred after layer 1 (whatever its duration), and that growth decreased consequently with a short resumption corresponding to the formation of layer 3. The last six (closely spaced) LAGs in layer 4 would then represent an “external fundamental system” (EFS) characteristic of the end of skeletal development in the amniotes, including taxa like the pseudosuchians that are supposed to have potentially unlimited growth ([de Ricqlès et al., 2003](#); [Woodward et al., 2011](#)).

Whatever the hypothesis considered, this study shows that growth activity in our specimen had fallen to a very low, residual level 6 years before the death of the animal. An accretion rate of some 0.14 $\mu\text{m}/\text{day}$, at most, is indeed close to or slightly below the range of accretion speeds (0.2 to 2.8 $\mu\text{m}/\text{day}$) that defines the slowest bone deposits of lamellar tissue ([de Margerie et al., 2002](#)). The timing of this quasi-cessation of growth, as compared to the epoch of sexual maturity is an important question for interpreting *Ophiacodon* life history traits. However, settling this problem properly would require a growth series of *Ophiacodon* bones, a paleontological material still lacking. In addition, some uncertainty persists in literature about *Ophiacodon* adult size (reviewed in [Huttenlocker and Rega, 2012](#)). This study also suggests that the overall course of *Ophiacodon* growth differed from that of *Clepsydrops*, in which growth seems to have decelerated more gradually by the end of somatic development.

4.2. Bone compactness profile and lifestyle inference

The terrestrial lifestyle of both *C. collettii* and *O. uniformis*, suggested by the inference model of [Quémeneur et al. \(2013\)](#) could be viewed as surprising (though not implausible), considering the ancient and widespread opinion that *Ophiacodon* was semi-aquatic.

For *C. collettii*, this inference appears intuitively correct, considering the clear tubular structure (a fairly thin, compact cortex and a large, free medullary cavity) of the femur in this taxon. The transition between cortex and medulla is particularly sharp, given that there are only three bone trabeculae in the section sampled at mid-shaft. This type of bone architecture is characteristic of terrestrial tetrapods ([Laurin et al., 2011](#)). For *O. uniformis*, we are somewhat less confident in the reliability of the inferred terrestrial lifestyle because the much thicker cortex might also reflect an amphibious lifestyle, rather than a truly terrestrial one. However, considering the greater geological age of *C. collettii* (this species is 15–28 Ma older than *O. uniformis*), if *O. uniformis* was amphibious, this would most likely have resulted from a return to a more aquatic lifestyle, rather than a retention of a primitive link to the aquatic environment as [Romer \(1957, 1958\)](#) hypothesized. Our interpretation is also strengthened by the recent terrestrial lifestyle inference of *Dimetrodon* based on microanatomy ([Kriloff et al., 2008](#)). Indeed, an optimization of lifestyle in early amniotes based on the inferences obtained so far from bone microanatomy and our quantitative inference models suggest that the first synapsids were terrestrial ([Fig. 6](#)). A few problems remain, however. Chiefly among these is our previous inference of a fairly aquatic lifestyle for *Ophiacodon* based on Ricqlès' radius section number 291.1.2T ([Germain and Laurin, 2005](#)). However, as mentioned by [Germain and Laurin \(2005\)](#), the *Ophiacodon* radius was located by the model well outside the distribution polygons, so we had already expressed reservations about that inference. Since the radius section used in that study is likely to have been sampled at some distance from the mid-diaphyseal plane towards the distal metaphysis, this would explain the abundance of spongy bone in the medullary region which led to the inference of a fairly aquatic lifestyle for that taxon. Another potential explanation is interspecific variability, given that the radius is from *O. retroversus* and the femur, from *O. uniformis*. [Ricqlès \(1974: 10\)](#) had already concluded that *O. retroversus* exhibited “certain adaptations to an aquatic life”, an interpretation based both on the dense vascularization of bone cortex and on the progressive transition from a compact cortex to an abundant medullary spongiosa. So far, the various clues used to infer the lifestyle of *Ophiacodon*, such as taphonomy ([Romer and Price, 1940](#)), bone microstructure ([de Ricqlès, 1974](#); [Germain and Laurin, 2005](#)), and vertebral anatomy ([Felice and Angielczyk, 2014](#)), proved to be ambiguous or inconclusive. Incomplete resorption of calcified cartilage is a very common and typical feature in tetrapods re-adapting to life in water. However, remnants of this tissue are absent in the medullary trabeculae of our specimen (and were never mentioned by previous authors). This situation raises doubts about the interpretation of some skeletal peculiarities of *Ophiacodon* that were simply considered to result from retardation in endochondral ossification due to aquatic life ([Reisz, 1986](#); [Romer and Price, 1940](#)). The present study infers a terrestrial habitat for *O. uniformis*, but some caveats remain to be discussed. Even if an aquatic (or amphibious) lifestyle for *Ophiacodon* cannot be definitely refuted because of its relatively thick cortex, the

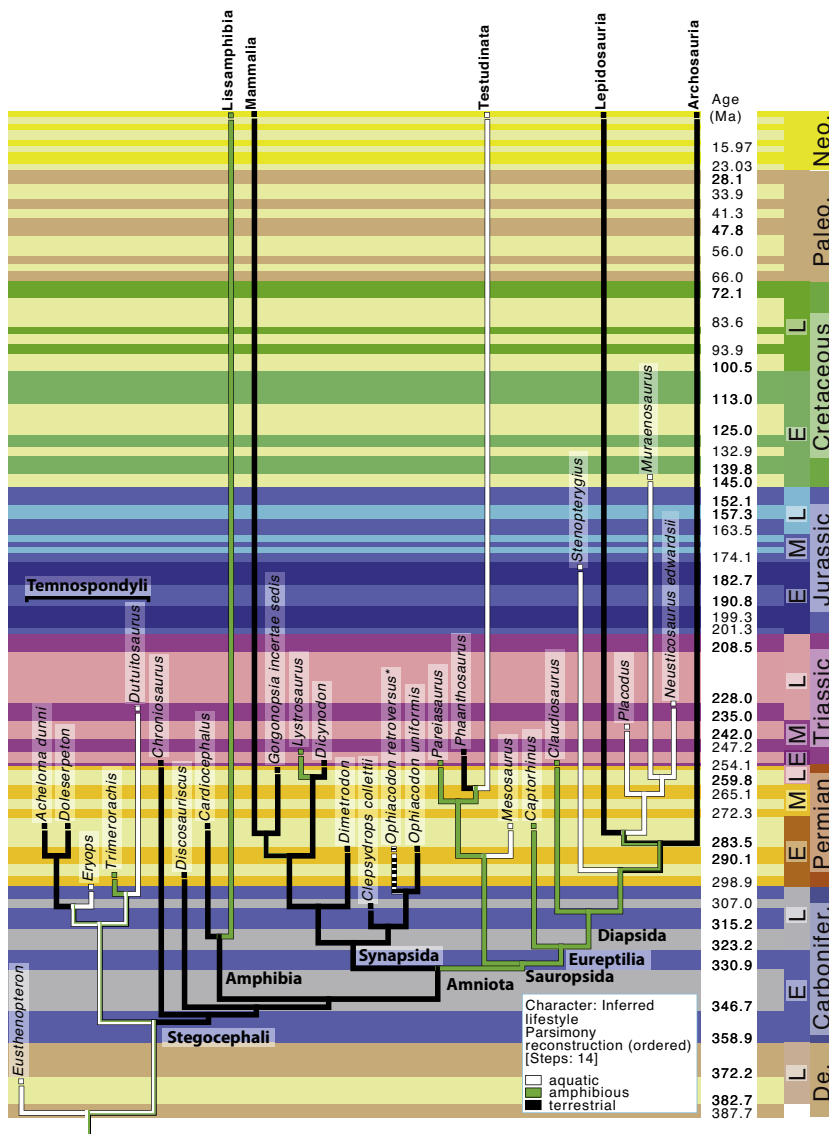


Fig. 6. (Color online.) Optimization of lifestyle of early stegocephalians based on bone microanatomy. Hatching on the branch leading to *Ophiacodon retroversus* indicates substantial uncertainty resulting from the possibly metaphyseal level of the radius cross-section used for that reference. This figure is based on a screen capture in Mesquite 3.0 (Maddison and Maddison, 2014) with the stratigraphic scale superposed using the Stratigraphic Tools (Josse et al., 2006). For higher extant taxa, only the inferred primitive lifestyle (Laurin, 2010; Vallin and Laurin, 2004) is shown; subsequent ecological diversification (such as an aquatic lifestyle for several extant mammals) is not shown because it is not relevant to inferring the history of habitat shifts in the Paleozoic.

Fig. 6. (Couleur en ligne.) Optimisation du mode de vie des stégocéphales anciens, fondée sur la micro-anatomie osseuse. La texture hachurée sur la branche menant à *Ophiacodon retroversus* indique une incertitude substantielle, liée au niveau potentiellement métaphysaire de la section transversale de radius utilisée pour cette inférence. Image fondée sur une capture d'écran de Mesquite 3.0 (Maddison et Maddison, 2014) avec superposition de l'échelle stratigraphique grâce aux « Stratigraphic Tools » (Josse et al., 2006). Pour les taxons actuels de rang supérieur, seul le mode de vie primitif inféré (Laurin, 2010; Vallin et Laurin, 2004) est montré; leur diversification écologique ultérieure (comme le mode de vie aquatique de certains mammifères) n'est pas montrée, car elle n'est pas pertinente pour inférer l'histoire des changements d'habitats pendant le Paléozoïque.

results of this study confirm that synapsids represent a primitively terrestrial clade (Fig. 6), and that the eventual aquatic or semi-aquatic lifestyle of *Ophiacodon* would be secondary, as already proposed by Olson (1941) for the latest representatives of that taxon. Finally, the proposal by Felice and Angielczyk (2014) to consider a terrestrial ecology for *Ophiacodon* as a “null hypothesis” in future studies is supported by our results. However, the lifestyle of *Ophiacodon* deserves closer scrutiny.

The optimization of lifestyle in stegocephalians resulting from the use of all inference models based on bone microanatomy (but no other types of data) suggests that a terrestrial lifestyle may have appeared fairly early, by the last common ancestor of *Chroniosaurus*, *Discosauriscus*, and the tetrapod crown-group (Fig. 6). However, if *Chroniosaurus* is nested within embolomeres, as previously suggested (Buchwitz et al., 2012; Laurin, 2000), its terrestrial lifestyle is probably convergent with that of

seymouriamorphs and crown-tetrapods (Laurin, 2010: fig. 7.1), given that most embolomers appear to have been fairly aquatic. Unfortunately, we have not yet had the opportunity to apply our inference models to geologically older, more basal embolomers to test this hypothesis. Similarly, the early return to an amphibious lifestyle at the base of Sauropsida may reflect that low number of sampled taxa, along with the fairly basal position of the aquatic mesosaurs. A denser sampling of all these taxa will be necessary to get a more robust, better-documented scenario of the move onto land by vertebrates. This will be the topic of future contributions.

The synthesis presented here (Fig. 6) is an attempt at isolating what bone microanatomy suggests, as compared with the indications provided by other kinds of data. Other relevant types of data, such as the presence of lateral-line canals on the skull, the degree of ossification of the endoskeleton, and other criteria, have been detailed elsewhere (Laurin, 2010; Vallin and Laurin, 2004); these allow the lifestyle of far more taxa to be inferred, but with caveats that differ from those applying to bone microanatomy. Comparison of the signals provided by these various data types should prove useful to raise questions about established ideas or suspicious results provided by our models, and hence, contribute to progress in this field.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.crpv.2015.01.001>.

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