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General palaeontology

A new tool for determining degrees of mineralization in fossil amphibian skeletons: The example of the Late Palaeozoic branchiosaurid *Apateon* from the Autun Basin, France

Un nouvel outil pour la détermination du degré de minéralisation du squelette chez les amphibiens fossiles : l'exemple du branchiosauride Apateon du Paléozoïque supérieur du Bassin d'Autun, France

Sophie Sanchez[a,*,b], Gaël de Ploëg[c], Gaël Clément[d], Per Erik Ahlberg[a]

[a] *Evolutionary Biology Centre, Subdepartment of Animal Evolution and Development, Department of Physiology and Developmental Biology, Uppsala University, Norbyvägen 18A, 75236 Uppsala, Sweden*
[b] *European Synchrotron Radiation Facility, 6, rue Jules-Horowitz, BP 220, 38043 Grenoble cedex, France*
[c] *Centre permanent d'initiatives pour l'environnement, ferme du Château-d'Aramont, 1, rue St-Pierre, 60410 Verberie, France*
[d] *UMR-CNRS 7207 CR2P, Département Histoire de la Terre, Muséum national d'Histoire naturelle, CP 38, 57, rue Cuvier, 75005 Paris, France*

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ABSTRACT

Studying ontogenetic features of fossil tetrapods is of major interest for investigating the adaptive strategies of early tetrapods to their palaeoenvironments. To determine the degree of calcification of skeletal elements, biologists have until now relied on X-ray radiographs of organisms or isolated bones, or on thin sections. An X-ray tomographic scan of *Apateon*, a Carboniferous – Permian branchiosaurid from the Autun Basin, France, reveals distinct density properties related to different mineralized tissues (calcified cartilage *versus* bone). The rendering of *Apateon* as a "test individual" provides a 3D map of the degrees of ossification of the axial and cranial elements. The combination of these anatomical observations with histological information from classical thin sections made in limb bones of several other specimens of the same locality allows the detailed determination of their ontogenetic stage. A comparison with the well-known specimens of the Saar-Nahe Basin, Germany, makes it possible to investigate the influence of different palaeoenvironments on ontogenetic features.

RÉSUMÉ

L'étude des caractéristiques développementales des tétrapodes fossiles constitue un intérêt majeur pour mieux comprendre les stratégies adaptatives des premiers tétrapodes au sein de leurs environnements. Pour déterminer le degré de minéralisation des éléments squelettiques, les biologistes fondaient jusqu'à présent leurs analyses sur l'observation de radiographies d'organismes, d'os isolés ou de coupes histologiques. L'imagerie par tomographie à rayons X d'un spécimen d'*Apateon*, branchiosauridé du Carbonifère – Permien du Bassin d'Autun, France, révèle des différences de densité correspondant à différents tissus

* Corresponding author.
E-mail address: sophie.sanchez@ebc.uu.se (S. Sanchez).

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minéralisés (cartilage calcifié *versus* os). La modélisation d'*Apateon* en tant qu'« individu test » fournit une carte en trois dimensions des degrés d'ossification des éléments vertébraux et crâniens. La combinaison de ces observations anatomiques par imagerie 3D avec l'information histologique, obtenue à partir de coupes histologiques réalisées dans les os longs d'autres spécimens du même site, permet une détermination détaillée des stades ontogénétiques. Une comparaison avec les spécimens du Bassin de Saar-Nahe, Allemagne, permet d'étudier l'influence de différents paléoenvironnements sur les caractéristiques ontogénétiques.

1. Introduction

The study of ossification sequences has been of primal importance in palaeontology for understanding the evolution of different types of development from fossil to extant species. Because development is readily influenced by the living environment of the animal, the record of perturbations in bone growth can reflect palaeoenvironmental factors such as palaeoclimate, (Steyer et al., 2004), palaeoaltitude (Sanchez et al., 2010b), demographic distributions, and conditions within palaeoecological niches (Sanchez et al., 2010a; Witzmann, 2009). Until now, such studies have focused mainly on non-amniotic tetrapods (including Late Palaeozoic forms), because they mostly occur in aquatic environments, which present good conditions for the fossilization process. In such taphonomical conditions, complete growth series comprising large numbers of complete specimens have been preserved (Fröbisch and Schoch, 2009).

To study the ossification patterns in growth series of early tetrapods, authors first observed and described the development of anatomical features during ontogeny (e.g., Bolt, 1977; Boy, 1971). Nowadays, this traditional method is still used and constitutes our main basis for understanding the ontogeny of early tetrapods (e.g., Klembara, 1995; Lohmann and Sachs, 2001; Schoch, 1992, Witzmann, 2006). However, more recently, different methods have been developed and added to this traditional description. Steyer (2000) integrated ontogenetic features into the phylogeny of temnospondyls; Fröbisch et al. (2007) compared developmental sequences traced by staining among extant salamanders to sequences of ossification observed in the fossil branchiosaurid *Apateon* von Meyer, 1844; Germain and Laurin (2009) tested statistical methods on ontogenetic sequences within a phylogenetic framework. Developmental features have also been observed at different scales and bone histology has become an interesting component for understanding details of the ontogeny of early tetrapods (e.g., interpretation of the histological organization, de Ricqlès, 1979; skeletochronology, Sanchez et al., 2008).

Histological patterns of ossification have been rendered visible for microscopic observations by making histological paraffin thin sections (Castanet et al., 2003). In extant non-amniotic tetrapods, radiographs of resin thin sections also clearly reveal the distinction between areas made of bone, calcified cartilage and cartilage (Francillon, 1981). However, this technique has never been applied on extinct animals. Here we perform the equivalent of such a radiographic investigation at the anatomical level in three dimensions on the skeleton of a fossil non-amniotic tetrapod still embedded in the rock. This new CT-scan approach yields a 3D map of the degrees of ossification of a test specimen, *Apateon pedestris* von Meyer, 1844, a branchiosaurid from the Autun Basin, France. Many exceptionally preserved growth series of species of *Apateon* are well known from several localities in Germany and have been the subject of intensive anatomical and histological detailed studies (Fröbisch et al., 2007; Fröbisch and Schoch, 2009; Sanchez et al., 2010a, 2010b; Schoch, 1992, 2004; Schoch and Fröbisch, 2006). As a complement to this work, the present study provides a comparative description of the ossification patterns of a French branchiosaurid specimen at the anatomical and histological levels, combining CT-scan and histological data.

Apateon is a branchiosaurid (tetrapod, temnospondyl) from the Carboniferous – Permian of Europe (Schoch and Milner, 2008). It is of relatively small size. The skull length of the largest specimen known from this genus measures 38 mm (Fröbisch and Schoch, 2009). The branchiosaurids are a group of considerable interest, closely related to extant salamanders according to numerous authors (Ruta and Coates, 2007; Schoch and Milner, 2008) and showing great developmental plasticity (Fröbisch and Schoch, 2009; Sanchez et al., 2010a). Studying the ontogenetic features of species of *Apateon* allows us better to understand the origin and evolution of lissamphibian characters, and the plasticity and adaptation of branchiosaurids and lissamphibians within their ecological niches. Specifically, the specimens of *Apateon* from the Autun Basin present a remarkably homogeneous sample of small individuals, raising the question of whether they represent juveniles or a kind of dwarf-adult "population".

2. Material and method

The fossil specimens of *A. pedestris* come from the Carboniferous – Permian locality called "Site du Lauvernay" at Tavernay, in the Autun Basin, France. These specimens (numbered Lal) belong to the collections of the Muséum d'histoire naturelle, Autun, France.

One fossil individual (Lal 17, MHNA) has been scanned with a conventional CTscan (X8050-16 Viscom model) at the University of Poitiers, France (Fig. 1). The scan has been performed according to the following parameters: energy: 100keV; intensity: 170 μA; gain of the camera: 44%; integration number: 16; zoom of the camera: mode 1; projections: 1500/360°, i.e. 1/0.24°. A correction of ring artefacts has been performed, and the original μCT

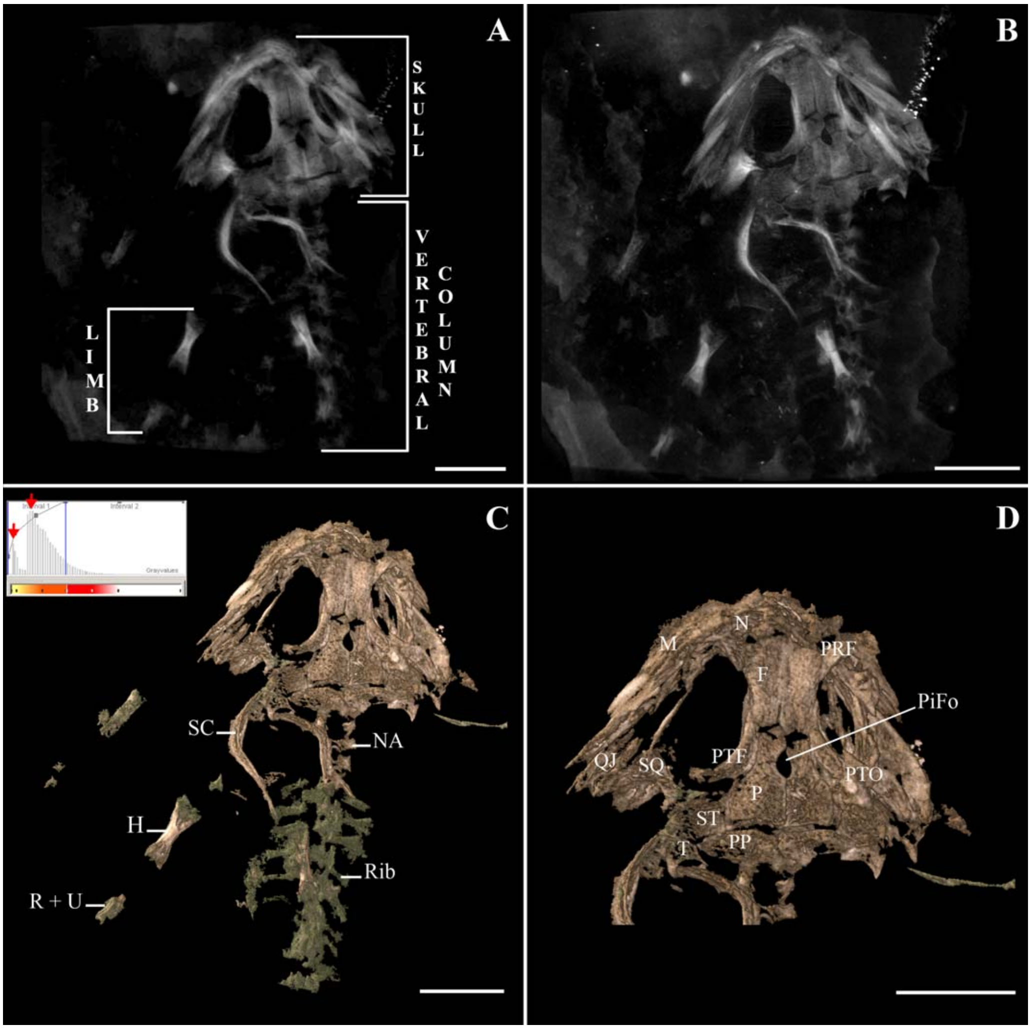

Fig. 1. Illustration of the CT-scan data: **A**. Z-projection of average intensity of 75 images (with ImageJ) simulating the visual aspect of a radiograph of specimen Lal 17, MHNA of *Apateon pedestris* from the Carboniferous – Permian Site du Lauvernay (Autun Basin, France). The distribution of different degrees of ossification is reflected by the distribution of grey levels but the distinction between bone and calcified cartilage remains difficult to interpret. **B**. Z-projection of maximal intensity of 75 images (with ImageJ) (specimen Lal 17, MHNA). The distribution of grey levels reveals a much clearer picture of the degrees of ossification. **C**. 3D rendering of specimen Lal 17, MHNA shown alongside a histogram of its grey levels. After elimination of the noise, the histogram shows two peaks (red arrows) corresponding to the distinct tissues of bone (rendered orange in 3D model) and calcified cartilage (rendered green). **D**. Detail showing the identification of the skull bones. F: frontal; H: humerus; M: maxilla; NA: neural arch; N: nasal; P: parietal; PiFo: pineal foramen; PP: postparietal; PRF: prefrontal; PTF: postfrontal; PTO: postorbital; QJ: quadratojugal; R + U: radius and ulna; SC: scapulocoracoid; SQ: squamosal; ST: supratemporal; T: temporal. Scale bars: 5 mm.

Fig. 1. Illustration des données obtenues par microtomographe conventionnelle. **A**. Projection sur l'axe *z* de 75 images avec une moyenne d'intensité (traitée sous ImageJ) simulant l'aspect visuel d'une radiographie du spécimen d'*Apateon pedestris* Lal 17, MHNA provenant du site du Lauvernay (Bassin d'Autun, France) datant du Carbonifère – Permien. La distribution des différents niveaux de gris permet de refléter une cartographie du degré d'ossification, mais la distinction entre l'os et le cartilage calcifié reste difficile à interpréter. **B**. Projection sur l'axe *z* de 75 images avec une valeur maximale d'intensité (traitée sous ImageJ) (spécimen Lal 17, MHNA). La distribution des niveaux de gris fournit ici une cartographie des degrés d'ossification du squelette nettement plus évidente. **C**. Modélisation en trois dimensions du spécimen Lal 17, MHNA et l'histogramme de niveaux de gris associé. Après élimination du bruit, l'histogramme présente deux pics (flèches rouges) qui correspondent aux deux tissus distincts de l'os et du cartilage calcifié, respectivement représentés en orange et vert sur les modèles 3D. **D**. Détail de la région crânienne présentant les os identifiés. F : os frontal ; H : humérus ; M : maxillaire ; NA : arcs neuraux ; N : os nasal ; P : os pariétal ; PiFo : foramen pinéal ; PP : os postpariétal ; PRF : os préfrontal ; PTF : os postfrontal ; PTO : os postorbitaire ; QJ : os quadratojugal ; R + U : radius et ulna ; SC : scapulocoracoïde ; SQ : os squamosal ; ST : os supratemporal ; T : os temporal. Barre d'échelle : 5 mm.

slices have been reduced. The final sections (1004) of 1004×211 pixels (8 bits, tif format) have a resolution of 30.5272 μm. The images have been studied in 2D with ImageJ 1.38×. The 3D model has been rendered using the software VGStudioMax 2.0 (Volume Graphics, Germany).

Traditional thin sections have been made through the limb bones of six specimens of *A. pedestris* from the Site du Lauvernay (Lal 1; Lal 2; Lal 4; Lal 5; Lal 8; Lal 11, MHNA, Fig. 2; Table 1). Transverse sections have been made at mid-shaft, where bone growth is supposed to be fully recorded: one section has been made in each bone. The

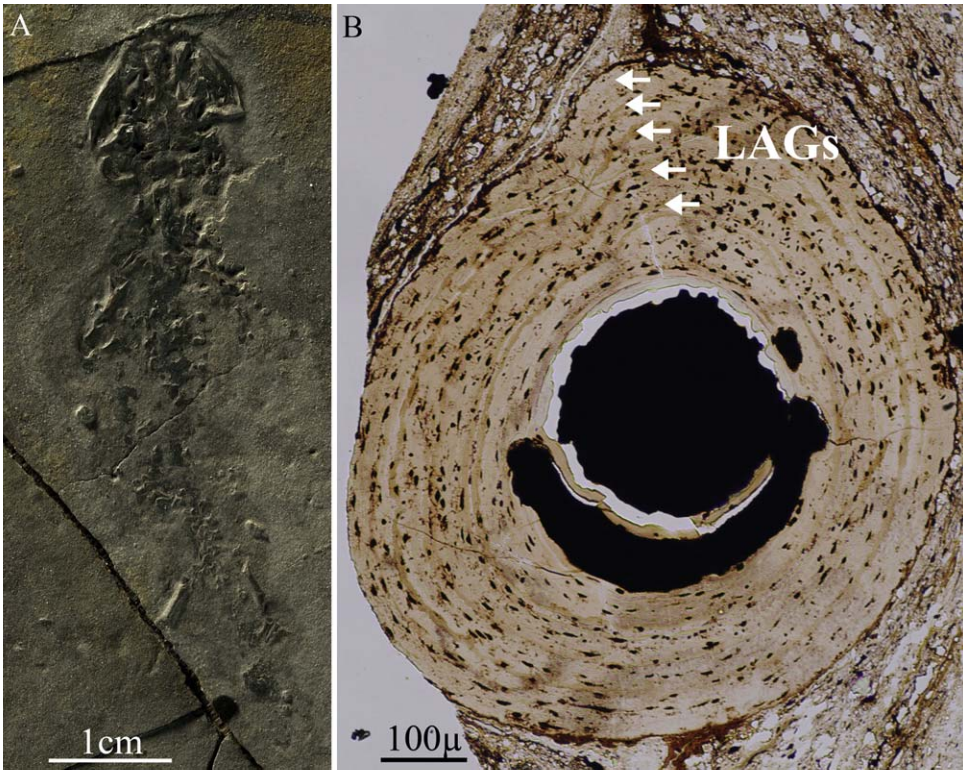

Fig. 2. Analysis of the histological data: **A**. Photo of specimen Lal 5, MHNA of *Apateon pedestris* from the Carboniferous – Permian Site du Lauvernay (Autun Basin, France). **B**. Detail of the femoral mid-shaft bone histology of specimen Lal 5, MHNA showing five lines of arrested growth ([LAGs], indicated by the white arrows).

Fig. 2. Analyse des données histologiques. **A**. Photographie du spécimen d'*Apateon pedestris* Lal 5, MHNA provenant du site du Lauvernay (Bassin d'Autun, France) datant du Carbonifère – Permien. **B**. Détail de l'histologie osseuse en mi-diaphyse du fémur du spécimen Lal 5, MHNA présentant 5 lignes d'arrêt de croissance ([LAGs], indiquées par les flèches blanches).

bones were sectioned by embedding them in polyester resin, sawing them with a diamond powder disk, and polishing the resulting slices to the desired thickness (around 40 μm).

3. Results

3.1. Degrees of mineralization revealed by CT scan

The X-ray data from a CT scan not only provide information on the degree of mineralization of skeletal elements, as do radiographies (Fig. 1A), but also yield results in three dimensions (cf. Movie 1 as a supplementary information online). With this technique, the densities of bone and calcified cartilage are clearly distinguishable (Fig. 1B) and can be independently modelled (two peaks of distinct densities are shown by the histogram of Fig. 1C and result in 3D in two coloured areas: bone in orange and calcified cartilage in green). The anterior part of the skeleton of one specimen of *A. pedestris* (Lal 17, MHNA) has been modelled. Some elements of the cranial skeleton still show incomplete ossification (see details below), and the postcranial skeleton still contains some calcified cartilaginous components. A further detailed description of each element refers to the determination of ontogenetic stages published by Schoch (1992, 2004).

Table 1
Table summarizing the ontogenetic information provided by skeletochronological analysis (based on the account of lines of arrested growth [LAGs]) on the specimens of *Apateon pedestris* from the Carboniferous – Permian Site du Lauvernay (Autun Basin, France).

Tableau 1
Tableau résumant l'ensemble des informations ontogénétiques fournies par analyse squelettochronologique (sur la base du décompte des lignes d'arrêt de croissance [LAG]) sur les spécimens d'*Apateon pedestris* provenant du site permo-carbonifère du Lauvernay (Bassin d'Autun, France).

Individual	Skull length (mm)	Number of LAGs	Ontogenetic stage based on the skeletochronological analysis
Lal 1	7	2, almost 3	Early juvenile
Lal 8	6.8	3	Early juvenile
Lal 11	8	3, almost 4	Early juvenile
Lal 4	9	3, almost 4	Early juvenile
Lal 2	8.7	5	Late juvenile (suggested by the progressive tightening of the LAGs)
Lal 5	10.5	5	Late juvenile (suggested by the progressive tightening of the LAGs)

The skull length of the specimen studied here is of 10.82 mm (Fig. 1D). The snout is distinctly narrower than the posterior region of the skull. As indicated by the intensity of light grey levels (Fig. 1B), degrees of mineralization are higher in the median part of the skull (e.g., frontals, parietals) than in the lateral part (e.g., quadratojugals, jugals). The anterior elements (e.g., premaxillaries) seem well ossified but are not well preserved. The frontals are clearly the stoutest bones of the skull (Fig. 1B, 1C). The maxilla almost contacts the quadratojugal. The jugal still remains largely unossified. Bones of the skull roof are sculptured, showing numerous pores, except the frontals, which already exhibit a complex network of relatively deep grooves. The pineal foramen is slightly oval. All these features are characteristic of an ontogenetic stage within the interval of the third to fifth stages as defined by Schoch (2004).

The appendicular skeleton is even more mineralized (Fig. 1B). Schoch (1992, 2004) observed that the mineralization of the humerus, radius and ulna occurs very early during the development of *A. pedestris*, only allowing the confirmation that the specimen was more mature than the third ontogenetic stage defined by Schoch (2004). However, the advanced stage of mineralization of the scapulocoracoid more particularly suggests an advanced stage of development (around the 19th stage defined by Schoch, 1992; or the fourth stage defined by Schoch, 2004).

The vertebral elements would favour the hypothesis that the ontogeny of the specimen studied here would have stopped between the third and fourth stages defined by Schoch (2004). The neural arches are relatively well ossified but not very much developed and the ribs remain mostly cartilaginous (Fig. 1B, 1C).

3.2. *Skeletochronological information*

Lines of arrested growth (LAGs) can be observed at mid-shaft in limb bones of specimens of *A. pedestris* from the Site du Lauvernay (Fig. 2). These LAGs, which have already been studied in other specimens of the same genus (and even the same species) from the Saar-Nahe Basin, Germany (Sanchez et al., 2010a, 2010b) refer to biological cycles for which the periodicity is regular. Skull lengths, LAG counts and histological features related to ontogenetic stages are summarised in Table 1.

Individuals whose skull length is about 7 mm show 2–3 LAGs; specimens whose skull length is about 8 mm exhibit 3–4 LAGs; and specimens up to 10.5 mm show 5 LAGs.

The femoral bone histology of the specimens from the Site du Lauvernay is similar to that of the specimens of *A. pedestris* from the locality of Odernheim, Saar-Nahe Basin, Germany. For the description of the diaphyseal bone histology of the specimens from the Site du Lauvernay, we therefore refer to the description of the femoral microstructure of the specimens from Odernheim made by Sanchez et al. (2010a, 2010b).

4. Discussion

4.1. *Ontogenetic stages*

CT scan and 2D histological data both confirm that all the specimens of *A. pedestris* studied here and coming from the Autun Basin are juvenile individuals.

On the basis of Schoch's work (1992, 2004), the degrees of mineralization of the specimen Lal 17, MHNA determined from the study of the CT scan data, suggest that the individual exhibited a juvenile morphology. Some features permit us to infer that it may have been a relatively late juvenile: the scapulocoracoid is well mineralized and a posterior extension of the maxillae to the quadratojugal is typical for later stages of development (e.g., in the two known metamorphosed specimens of *A. gracilis* the cheek elements become tightly integrated, probably to process larger, harder food; Fröbisch and Schoch, 2009). The advanced ossification of the appendicular skeleton (as compared to the skull) observed in the specimen from Tavernay is typical and apomorphic for branchiosaurids (Fröbisch et al., 2010; Schoch, 2004). In other temnospondyls for which complete ontogenies are known (e.g., *Micromelerpeton*, *Sclerocephalus*) the skull is very well ossified in the smallest known larvae (Schoch, 2004; Fröbisch et al., 2010).

The skeletochronological analysis, based on the study of a simple LAG pattern (as observed in extant tetrapods; e.g., Castanet et al., 1993) at mid-shaft, allows the assessment of the specimens' ages ranging from 2 to 5 years. The slight tightening of the distance between the fourth and fifth LAG indicates that the rate of bone deposition slightly decreased between these two biological cycles suggesting that specimens of five years old (with skull length ranging from 8.7 to 10.5 mm) were rather late juveniles whose growth had just begun to slow.

4.2. *Comparison with some specimens of* Apateon pedestris *from the Saar-Nahe Basin, Germany*

The degree of mineralization of the different bones scanned on specimen Lal 17, MHNA allows the sequence of ossification of this individual to be determined: skull bones (except the jugal) begin to ossify at a relative early stage of development, long bones of the forelimb and scapulocoracoid even earlier, while the ossification of vertebrae, which begins at the anterior end of the column, takes more time and the ribs appear as the last ossified elements. This sequence of ossification shows several differences from the sequence published by Schoch (2004) on specimens of *A. pedestris* from the Saar-Nahe Basin, Germany (Erdesbach and Odernheim). The ossification of the ribs is delayed in the specimen from the Autun Basin. The degree of ossification of some of its median cranial bones (e.g., prefrontal, postfrontal) is relatively advanced while Schoch (2004) observed that it occurred later in the specimens from Germany. Moreover, specimens from Germany of similar skull length are almost fully ossified (showing at least a jugal; Schoch, 2004) while the specimen from France still exhibits weakly ossified ribs and jugal. These observations, mostly suggesting a delay in the development of the French specimen, may be interesting but in order to hypothesize about the significance of these differences, it would first be necessary to apply a similar analysis on a complete ontogenetic series from the Autun Basin.

Histological data provide relatively similar ontogenetic results for the French and German specimens. The oldest French specimens, aged 5 years, are still juveniles as are the German specimens from the localities of Niederkirchen and Odernheim (Sanchez et al., 2010a). However, older specimens have been found in Germany and determined as adults by skeletochronology (Sanchez et al., 2010a).

4.3. Palaeoecological implications

The LAG patterns observed at mid-shaft in long bones of the specimens from the Site du Lauvernay, France are simple LAG patterns as observed in the sample of *A. pedestris* from Odernheim, Germany (Sanchez et al., 2010b) and extant tetrapods which live up to the elevation of 550 m (Caetano et al., 1985; Caetano and Castanet, 1993). From this observation, we can deduce that the Site du Lauvernay in the Autun Basin, France, may have been an aquatic environment at a relatively low palaeoaltitude. The specimens were exposed to only one major palaeoclimatic event per year, causing their growth to stop during a single period each year.

The observation that all the examined specimens are juveniles, ranging in age from 2 to 5 years and not showing any signs of perturbed growth patterns (other than the annual hiatus), suggests that the Site du Lauvernay represents an environment favourable to, and predominantly inhabited by, immature individuals. Their deaths appear to have been scattered through the year rather than occurring in a specific season, given that Lal 1, Lal 4 and Lal 11 died just as they were about to deposit a new LAG whereas Lal 2, Lal 5 and Lal 8 died during the growth period between LAGs (Table 1). One possible interpretation is that the site represents a breeding area that was inhabited by juveniles during their first few years of life but which was only infrequently visited by adults. However, in order to critically examine this hypothesis it will be necessary to obtain a much larger population sample, as well as to investigate the taphonomy, palaeoenvironment and associated fauna and flora of the Site du Lauvernay in detail.

5. Conclusion

Here we present a new non-destructive method that clearly discriminates calcified cartilage and bone in a fossil tetrapod still embedded in its rock matrix. It is the first to provide information on fossil ossification in three dimensions. This method is thus complementary to the other methods used until now to determine degrees of mineralization and sequences of ossification in Palaeozoic tetrapods (Fröbisch et al., 2007; Germain and Laurin, 2009; Schoch, 1992). We illustrate the application of this method by studying the state of mineralization of a specimen of *A. pedestris* from the Autun Basin, France. Supplementary and complementary information have been provided by the observation of classical histological thin sections and a skeletochronological analysis. The combination of these data permits us to draw preliminary conclusions on the development of *A. pedestris* and its conditions of life in the Site du Lauvernay, Autun Basin, France.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.crpv.2010.07.004.

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