General Palaeontology, Systematics and Evolution (Vertebrate Palaeontology)

Chondroid bone in dinosaur embryos and nestlings (Ornithischia: Hadrosauridae): Insights into the growth of the skull and the evolution of skeletal tissues

Présence de tissu chondroïde chez des embryons et des jeunes de dinosaures (Ornithischia : Hadrosauridae) : implications sur la croissance du crâne et l'évolution des tissus squelettiques

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ABSTRACT

In histology textbooks, the vertebrate skeleton is represented as almost entirely made of bone and cartilage. This is a false dichotomy and in fact, a continuum of intermediate tissues between bone and cartilage exists. Chondroid bone ([CB] or chondroid tissue), one of the most well-known intermediate tissues, has been reported in mammals, birds and crocodilians. It accommodates (1) rapid growth of the skull and (2) the development of craniofacial sutures. Since CB is present in the extant phylogenetic bracket of the Dinosauria, we hypothesized that it was also present in non-avian dinosaurs. By means of paleohistological examination and microradiography, we report for the first time the presence of CB in non-avian dinosaur embryos and nestlings (Ornithischia: Hadrosauridae). It was found in five locations: (1) scattered within the bone trabeculae of an embryonic surangular; (2) and (3) in the coronoid process and in the alveolar processes of an embryonic dentary; (4) in the mandibular symphyses of an embryonic and a post-hatching dentary; (5) at the fronto-postorbital suture of an embryo. In these areas, CB was present in large amounts, suggesting that it played an important role in the rapid growth of the hadrosaurian skull during embryonic development. Moreover, the CB present in the sutural borders of a Hypacrosaurus frontal suggests that it was also involved in sutural growth, as it has been reported to be in mammalian and avian sutures. This is the first step taken to document and understand dinosaurian sutures from a histological perspective and it sheds light on an old problem by reporting the presence of CB in an additional clade within the Vertebrata. It is parsimonious to propose that CB in the chick embryo, Gallus gallus, the American alligator, Alligator mississippiensis and the hadrosaurs of the present study are homologous and that CB arose once and was inherited from their common ancestor.

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RÉSUMÉ

Selon les précis d’histologie, le squelette des vertébrés est formé exclusivement d’os et de cartilage. Ceci est une dichotomie erronée, car il existe en fait un continuum de tissus intermédiaires entre l’os et le cartilage. Le tissu chondroïde (TC, ou os chondroïde) fait partie des tissus intermédiaires les plus connus et a été rapporté chez les mammifères, les oiseaux et les crocodiliens. Il autorise (1) la croissance rapide du crâne et (2) le développement des sutures crano-faciales. Puisque le TC est présent dans le clade d’inférence justifiée (extant phylogenetic bracket) de Dinosauria, nous avons émis l’hypothèse qu’il était également présent chez les dinosaures non aviens. Grâce à une analyse paléohistologique et microradiographique, nous documentons pour la première fois la présence de TC chez des embryons et de jeunes dinosaures non aviens (Ornithischia : Hadrosauridae). Ce tissu a été observé dans cinq localisations : (1) au milieu de travées osseuses d’un suranulaire embryonnaire ; (2) et (3) dans les processus coronoidé et alvéolaires d’un dentaire embryonnaire ; (4) au niveau des symphyses mandibulaires d’un embryon et d’un jeune hadrosaure ; (5) au niveau de la suture fronto-postorbitaire d’un embryon. Dans ces cinq sites, le TC était présent en grande quantité, ce qui suggère un rôle important dans la croissance rapide du crâne des hadrosaures durant leur développement embryonnaire. De plus, la présence de TC dans les aires suturales du frontal d’Hypacrosaurus suggère que ce tissu jouait aussi un rôle dans la croissance suturale, comme déjà rapporté chez les mammifères et les oiseaux. Ceci est un premier pas pour documenter et mieux comprendre les sutures des dinosaures d’un point de vue histologique. Cette étude rapporte la présence de TC dans un clade additionnel de vertébrés. Il est paradoxal de proposer que le TC préexiste chez les embryons de poulet, Gallus gallus, chez les embryons d’alligators américains, Alligator mississippiensis, et chez ces embryons d’hadrosaures est homologue et qu’il leur aurait été transmis par leur ancêtre commun.


1. Introduction

The term ‘palaeohistology’ refers to the study of the bone and tooth microstructure of fossil vertebrates. The vast majority of studies involve analysis of tetrapod limb bone and the identification of types of bony tissues that reflect different growth rates, ontogenetic stages, phylogenetic positions or biomechanical factors (Padian, 2013). It is reasonable to focus exclusively on bone, because it composes the overwhelming majority of the fossilized remnants of tetrapods. Cartilage is the second most abundant supporting connective tissue in tetrapods (extant or fossil) and very few investigators have focused on fossilized cartilage (e.g., Barreto et al., 1993; Horner et al., 2001). In histology textbooks (especially those with medical applications), the skeleton is presented as made entirely of these two tissues: bone and cartilage; but this is a false dichotomy. Indeed, it is known that there is actually a continuum of tissues between bone and cartilage, known as ‘intermediate tissues’ (Hall, 2005; Smith and Hall, 1990; Witten et al., 2010), because they share features of both of these tissues. Studies on intermediate tissues in fossils are almost nonexistent (except perhaps in early vertebrates e.g., Ørvig, 1951) because (1) they are hard to identify, even in extant species, (2) they are much more rare than bone or cartilage, and (3) because the terminology employed is obscure and inconsistent. The most well-known intermediate tissues are secondary cartilage and chondroid bone (Beresford, 1981; and see Hall, 2005, Chapter 5). Secondary cartilage was recently reported in hadrosaur embryos and nestlings (Bailleul et al., 2012, 2013; and see section 4), therefore, here, we will focus on chondroid bone in the same taxa.

1.1. Generalities about chondroid bone

As mentioned earlier, the terminology of chondroid bone is very inconsistent and it has been designated by various names since the early 1900s (see review by Beresford, 1981). Therefore, it is important to note that it might be difficult to accurately review where chondroid bone has been found in some instances (but see next paragraph, and Chapter 5 in Hall, 2005, 2014). The term “chondroid tissue” has been proposed to replace the older term “chondroid bone” by Goret-Nicaise and Dhem (1982). We think that both terms are legitimate and we will consider them synonymous in this study.

Chondroid bone (CB) is intermediate between bone and cartilage because it has cartilage-like rounded cells that are closely packed together and that are embedded in a bone-like matrix (Beresford, 1981; Gillis et al., 2006; Hall, 1971, 1972; Lengélé et al., 1990; Murray, 1963). This extracellular matrix (ECM) possesses collagen type I which is typical of bone, and collagen type II which is typical of cartilage (Goret-Nicaise, 1984). It has been found in all the craniofacial bones and the mandible (in both endochondral and membrane bones) of human fetuses and infants (Goret-Nicaise, 1984, 1986; Goret-Nicaise and Dhem, 1982, 1985; Goret-Nicaise et al., 1988; Manzanoares et al., 1988; and see Fig. 1), cat fetuses (Goret-Nicaise et al., 1984) and chick embryos (Lengélé, 1997; Lengélé et al., 1990, 1996a, 1996b). It plays two major roles: it facilitates:

- rapid growth (Gillis et al., 2006; Goret-Nicaise, 1986; Huysseune and Verraes, 1986; Taylor et al., 1994);
- sutural growth (and later sutural fusion).
Indeed, its growth rate was estimated by fluorescence labeling in the cat mandible at 44 to 67 microns/day, while the rate of lamellar bone formation was only 5.3 to 8.9 microns/day (Goret-Nicaise, 1986). It was reported in all the cranial sutural edges in humans (from 20 weeks-old fetuses until at least 9 months old babies; Goret-Nicaise et al., 1988), and in all the cranio-facial sutural edges in chick embryos (at the 9th, 12th and 14th day of incubation; Lengelé et al., 1990, 1996a, 1996b). More recently, Rafferty and Herring (1999) found CB in the nasofrontal suture of 4 to 6 month-old miniature pigs. These studies have shown that during early ontogenesis, it is CB that forms the sutural borders, not bone (contra Kokich, 1976; Pritchard et al., 1956).

CB is thought to arise directly from mesenchymal cells, i.e., these latter can give rise to chondroblasts, osteoblasts or chondroid bone cells depending on the stimuli (Lengelé, 1997; Lengelé et al., 1996b; but see section 4 for alternative hypotheses). The stimulus of CB is thought to be tension (and not compression, like the primary or secondary cartilage; Hall, 1967; Lengelé, 1997). While the extent of calcification of CB and woven bone are similar, CB is more mineralized than lamellar bone but less mineralized than calcified cartilage (Goret-Nicaise and Dhem, 1985). Curiously, it has a unique mode of calcification, different from that of bone and cartilage: it calcifies centripetally around the cell lacunae and irregularly (Goret-Nicaise and Dhem, 1982). Note that this unique calcification has been reported in humans and chick embryos, but it might not be true for all vertebrates.

Ontogenetically, this tissue is resorbed quickly in embryonic bones but it may persist at later stages of development as residual blocks scattered in bone trabeculae, and in articular and sutural areas (Lengelé et al., 1996a, 1996b). It can also arise after hatching (or after birth) in zones that are still under tensional stress. CB has not been investigated in many taxa, but beside from mammals and birds (the preferred laboratory animals in skeletal biology and histology), it has been found in agnathans (Ørvig, 1951), teleostean (Gillis et al., 2006, Huyseune and Verraes, 1986, Taylor et al., 1994), and more recently in the skull of Alligator mississippiensis embryos (Vickaryous and Hall, 2008; see their see their figures 6G and 6H p 411). Therefore, from a phylogenetic perspective, CB seems to be widely distributed among Vertebrata.

1.2. Chondroid bone and dinosaurs

One important fact to highlight from our summary above is that two species that belong to the extant
phylogenetic bracket of the Dinosauria, the chick, Gallus gallus, and the American alligator, Alligator mississippiensis, possess CB (Hall, 1971, 1972; Lengelé et al., 1990; Vickaryous and Hall, 2008). This strongly suggests that CB could have been involved in the craniofacial development of non-avian dinosaurs (Witmer, 1995). Note that there is always a possibility that CB arose in parallel in crocodilians and birds. Whether avian and crocodilian CB is homologous or analogous, it is important to investigate whether or not non-avian dinosaurs had the ability to form CB and how ancient this tissue was within the Archosauria. Therefore, the skull of some Lambeosaurinae and Hadrosaurinae dinosaur embryos and juveniles were analyzed by means of paleohistological techniques and microradiography (Table 1). Paleontological crews of the Museum of the Rockies (MOR, Bozeman, Montana) have unearthed hundreds of remains of young hadrosaurs from nesting grounds (Horner, 1982; Horner and Currie, 1994; Horner and Makela, 1979; Horner et al., 2000), and this abundant material was therefore selected for this investigation. Reporting CB in hadrosaurs would bring two important insights into the growth of their skull:

- first, even though it is commonly accepted that most hadrosaurs grew fast, fast growth has never been reported in the cranium, and most studies concern the post-cranium (e.g., Cooper et al., 2008; Horner et al., 2000; Padian et al., 2001). Moreover, the presence, absence or relative abundance of this tissue in hadrosaurs would give qualitative insights into the growth rate of their skull;
- second, this study could shed light on the mode of sutural growth of hadrosaur skulls, i.e., indicate whether or not they used CB as a vector of growth in the sutures.

Indeed, sutures are often mentioned in paleontological studies because they are used for maturity assessment (e.g., Bakker and Williams, 1988; Longrich and Field, 2012; Sereno et al., 2009). However very little is known about the sutures of dinosaurs (or even extant archosaurs) from a histological perspective and only a few living mammalian species have been sectioned (i.e., some humans, Kokich, 1976; Koskinen et al., 1976; Latham, 1971; Miroue and Rosenberg, 1975; Opperman, 2000; Persson and Thilander, 1977; Sislen, 1933; and some rats and rabbits, Moss, 1958; Persson, 1973; Persson et al., 1978; Persson and Roy, 1979; Pritchard et al., 1956). For future paleontological studies, it is important to document the osteohistology of sutures in order to understand their morphology and their (potential) relationship to ontogeny.

Finally a third point to note is the possible importance of the study of CB to understand the evolution of skeletal tissues within the Archosauria or the evolution of skeletal tissues in general. Indeed, as mentioned earlier, CB has been observed in birds (e.g., Lengelé et al., 1990), reptiles (Vickaryous and Hall, 2008) and mammals (e.g., Goret-Nicaise, 1984) but neither in anuran nor in urodele amphibians (e.g., Beresford, 1981; Hall, 2003). This study could give more insights into the phylogenetic distribution of this intermediate tissue.

### 2. Material and methods

#### 2.1. Paleohistology

In extant species, CB can be identified histologically with the use of stains on decalcified sections (e.g., with Mallory’s trichrome, Vickaryous and Hall, 2008; or methylene blue, Lengelé et al., 1990). Although it is possible to stain decalcified archaeological bone to observe histological structures (e.g., with Toluidine blue, Garland, 1989), stains used on dinosaur bones cannot show histological structures or different types of tissues per se, as they are usually histochemical (e.g., Sudan black can show

<table>
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<td></td>
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#### Table 1

List of bones sectioned and analyzed in the present study.

Liste des os sectionnés et analysés dans la présente étude.
lipids, Pawlicki, 1977; the Hoechst 33258 dye reveals DNA; Schweitzer et al., 1997). Therefore, we could not use a specific stain that would allow the identification of CB in these hadrosaurs. Instead, CB was identified qualitatively on undecalcified and unstained sections (i.e., standard paleohistological sections). As mentioned earlier, CB cells are round (cartilage-like) and embedded in a bone-like matrix. It is possible to differentiate CB from bone or cartilage under natural light (see section 3 for complete explanation).

For this study, we re-analyzed the paleohistological thin sections that were made for two previous studies on secondary cartilage (Bailleul et al., 2012, 2013) because secondary cartilage and CB are often found in the same areas. About a hundred sections were analyzed, from a total of 21 separate skull bones (see Table 1). All elements were found to be distinct and were collected from nesting horizons of isolated nests in the Two Medicine and Judith River formations (Upper Cretaceous), Montana, USA (see Bailleul et al., 2012, 2013 and Horner and Currie, 1994 for more information about the localities).

Paleohistological methods were employed according to the procedure for small specimens presented in Lamm (2013). The embryonic bones were embedded in epoxy resin and cut with a Norton 5" diamond blade on an Isomet precision saw. Thick sections (between 1.0 and 1.3 mm) were mounted on plastic (Plexiglas) slides with cyanoacrylate glue, then ground and polished by hand on a Buehler Ecomet grinder with silicon carbide paper of decreasing grit sizes. Finished thin sections (with a thickness surrounding 100 microns) were studied by light microscopy under normal and polarized light with a Nikon Optiphoto-Pol polarizing microscope. Photographs were taken with a Nikon DS-Fi1 digital sight camera and the NIS Elements BR 4.13 software.

2.2. Microradiography

In extant species, another efficient method to identify CB is microradiography, an X-ray technique that shows the mineral distribution in calcified tissues at the microscopic scale (e.g., Goret-Nicaise and Dhem, 1982). Under microradiography, CB appears as radioopaque mineralized struts or islets containing irregular patches of confluent cellular lacunae that are radiotransparent (i.e., they appear dark). These struts and islets can be adjacent to woven bone or surrounded by lamellar bone, which have a distinct radiopacity and cell organization (Fig. 1A-D). Microradiography is also used on archaeological bone (e.g., Garland, 1989) and to our knowledge, only one study performed microradiography on Mesozoic fossils (on reptilian and amphibian teeth from the Triassic, Wyckoff et al., 1963). Since paleohistological and histological methods are not standardized, we encountered problems while performing microradiography analysis, and we were only able to microradiograph five slides successfully (Table 2). Even though this is a small sample size, this study introduces a powerful tool that can be used by paleohistologists in the future.

All microradiography experiments were performed at the ‘Université catholique de Louvain’ (UCL, Brussels, Belgium). The pre-made paleohistological thin sections (70 to 90 microns thick, except for slide DE1-12 that was as thick as 175 microns in some places) were microradiographed in contact with a fine grain emulsion (VRP-M, Slavich-Geola, Lithuania), exposed to long-wavelength X radiations produced by a Machlett tube (Baltograph BF-50/20, Balteau, Liège, Belgium) at 14 kV and 15 mA. In standardized sections (i.e., on extant material), the exposure usually lasts around 50 min, but because the X-rays did not go through the Plexiglas used at the MOR (i.e., it was too radio-opaque), the exposure lasted 4 hours for a film-focus distance of 106 mm. At UCL, the sections were previously flipped and the Plexiglas (not the bone) was ground down to about 100 microns in order to make it less radio-opaque. The microradiographs were observed and photographed with a Nikon Digital Sight DS-5MC camera (NIS Elements BR 3 software) attached to a microscope Leitz DMRB (Leica).

3. Results

In the present study, we report for the first time the presence of CB in some non-avian dinosaurs. CB was found in five different locations:

1) scattered within the bone trabeculae of an embryonic surangular (Fig. 2);
2) in the coronoid process of an embryonic dentary (Fig. 3);
3) in the alveolar processes of an embryonic dentary (Fig. 3);
4) in the mandibular symphyses of an embryonic and a post-hatching dentary (Figs. 4 and 5);
5) in the sutural borders of an embryonic frontal (at the fronto- postorbital suture, Fig. 6).

Note that locations 2), 3) and 4) are all from the same bone (a dentary of a Hypacrosaurus embryo, MOR 559; Figs. 3 and 4). Even though CB was most evident in these five locations, it was also present deep within the bone trabeculae and at the periosteal borders of the other embryonic bones (Table 1), but it was much more scattered and scarce in the post-hatching bones of this sample (data not shown).

As mentioned earlier, under natural light, this tissue possesses round cell lacunae that are closely packed in clusters, and are embedded in a bone-like matrix. Fig. 2 shows transverse sections of an embryonic hadrosaurid surangular (MOR 1038) with a central string of tissue that runs along the whole length of the bone (Fig. 2A-D). Indeed, this bone was serially sectioned (giving a total of 10 thin sections) and this central tissue can be observed on each slide (data not shown, Fig. 2 only presents images from slide SU1-4). At higher magnification, numerous round cell lacunae can be observed (white arrows, Fig. 2E) and such roundness cannot be attributed to standard bone cell lacunae. Adjacent osteocyte lacunae can be seen (e.g., green arrow, Fig. 2E) and they are much more elongated than the CB cell lacunae in the center. The CB matrix is brighter and more translucent (light brown) than the bone matrix (darker brown, Fig. 2B, D). This difference in light transmission can be seen under polarized light as well (Fig. 2C). Finally, Fig. 1F and 1G show this same tissue in an adjacent section. Alas, we were not able to obtain a microradiograph
of this embryonic surangular because the center of the slide peeled off during experimentation.

Figures 3 through 6 show microradiographs and their corresponding natural light photographs (in color). Note that the corresponding natural light pictures represent the exact same locations, with the same scale, as the microradiographs. However, since it was not possible to re-take pictures after microradiography had been performed, the corresponding natural light pictures are sometimes taken from an adjacent section (about 1.0 to 1.3 mm away). It is the case for Fig. 3C, J, 4F–G, I, 6C and E. Fig. 3 represents longitudinal sections of an embryonic dentary (MOR 559, previously published in Bailleul et al., 2013). As mentioned earlier, CB can be identified under microradiography because it appears as patches of radiopaque matrix (appearing light) that contain radiotransparent (i.e., that appear dark), irregular and confluent cellular lacunae (indicated by white arrows) surrounded by bone layers with a more homogenous radiopacity and containing fewer cell lacunae (indicated by black arrows). Such clusters can be observed in the coronoid process (Fig. 3D, F) and in the alveolar processes (Fig. 3H–I). It is absolutely comparable to the CB observed in the microradiographs of extant human fetuses (Fig. 1) and extant bird embryos (see figures of Lengelé et al., 1990 and Lengelé et al., 1996a). In the coronoid process, CB is present deep within the bone trabeculae and is organized as little islets surrounded by lamellar bone (Fig. 3D and F) but in the alveolar processes, it is organized into struts that radiate out in the alveoli (Fig. 3H–I). The white arrows in these microradiographs show clear-cut, typical characteristics of CB and not those of other tissues (such as woven bone, or osteoid that is radio-transparent in microradiographs). The corresponding natural light pictures show two types of morphologies: round cell lacunae closely packed together in clusters (with a very high cellular density, Fig. 3C, 3E); and a more ‘normal’ appearance, where the cells are not organized in clusters but are distributed evenly throughout the alveoli (Fig. 3J). Had we not performed microradiography, the latter tissue would have probably been identified as ‘normal’ woven bone rather than CB (Fig. 3J). These CB struts in the alveoli ensure rapid growth and they are most likely more recent than the deeper islets of CB surrounded by bone in the coronoid process.

Fig. 4 shows cross-sections through the mandibular symphysis (at the most rostral tip of the dentary) of the same Hypacrosaurus embryo (MOR 559). All the microradiographs also show islets of CB on the periosteal borders (white arrows, Fig. 4D–E, H). On the lingual face of this mandibular symphysis, natural light pictures show a light tissue with a high cellular density surrounded by struts of darker lamellar bone (Fig. 4B–C). Cementing lines are present between the internal CB and the more external lamellar bone, attesting the resorption of CB followed by lamellar bone apposition (Fig. 4B–C). Note that this difference in color (light vs. brown) was also observed in the surangular (Fig. 2), but not in the coronoid process and the alveoli (Fig. 3; see discussion for further elaboration). At the very dorsal tip of the mandibular symphysis, the microradiograph indicates the presence of CB (Fig. 4H), but under natural light, it almost looks like a diagnostically altered bone (Fig. 4F–G, I). Indeed, the tissue appears very thin and the shape of the cell lacunae are irregular (Fig. 4I) instead of round like those presented in Fig. 2E. This is curious and it would appear that CB cell lacunae can have multiple morphologies (see section 4). We wish to emphasize here that even though this tissue (at the most rostral tip of the mandibular symphysis) has the appearance of a ‘diagnostically altered’ bone in natural light, it is unlikely that this observed morphology is the product of diagenesis for two reasons:

- the corresponding microradiograph (Fig. 4H) shows a pristine preservation of the mineral distribution, comparable to that of the CB of extant species (see Fig. 1A,C);
- beside from the rostral tip of this bone, all periosteal borders present histological structures that are well preserved (e.g., Fig. 4B).

Only a much localized diagenetic alteration at the mandibular symphysis (and not on the rest of the bone) could explain these differences, but this does not seem like a plausible explanation.

This ‘diagnostically altered’ appearance is in fact present in the mandibular symphysis of a Hypacrosaurus post-hatching specimen as well (MOR 548), at the exact same location (Fig. 5). Even though we could not perform microradiography on this slide (because it is mounted on glass and X-rays cannot go through glass), the fact that:

- this tissue presents a similar appearance as to that of MOR 559 (Fig. 4);
- it is present at the exact same location as that of MOR 559, suggest this tissue is CB (Fig. 5B–D).

There is a clear limit between the light CB located superficially and the darker bone located more internally (Fig. 5C, see blue line). Just like the embryo MOR 559, the periosteal borders of MOR 548 show good histological preservation (data not shown) suggesting that this change in color in the
Fig. 2. (Color online.) Cross sections in the surangular of a hadrosaur embryo (MOR 1038, Hadrosauridae indet.) under natural and polarized light. A. Cross section under natural light. B. Detail of the box in (A) showing a long strut of CB under natural light. C. Same detail of the box in (A) under polarized light. D. Detail of the box in B showing CB at a higher magnification. This tissue appears brighter than the surrounding bone under natural light. E. Detail of the box in (D), showing CB cells that are very round and cartilage-like (white arrows). Compare with the elongated morphology of an osteocyte (green arrow). F. Adjacent section of this same bone showing the central strut of CB. G. Detail of the box in F. Abbreviations: Do: dorsal; La: labial; Li: lingual; Ve: ventral.

Fig. 2. (Couleur en ligne.) Coupes transversales dans le surangulaire d’un embryon d’hadrosaure (MOR 1038, Hadrosauridae indet.), observées en lumière naturelle et polarisée. A. Coupe transversale en lumière naturelle. B. Détail de la zone encadrée dans (A) montrant une travée de TC en lumière naturelle. C. Même détail de la zone encadrée dans (A) en lumière polarisée. D. Détail de la zone encadrée dans B montrant le TC à plus fort grossissement. En lumière naturelle, ce tissu apparaît plus clair que l’os qui l’entoure. E. Détail de la zone encadrée dans (D), montrant que les cellules du TC sont rondes et ressemblent à des chondrocytes (flèches blanches). Ceci n’est pas comparable à la morphologie allongée des ostéocytes (flèche verte). F. Coupe adjacente de ce même os, montrant une travée de TC située dans le centre de l’élément. G. Détail de la zone encadrée dans (F). Abréviations : Do : dorsal ; La : labial ; Li : lingual ; Ve : ventral.
Fig. 3. (Color online.) Longitudinal sections in the dentary of a *Hypacrosaurus* embryo (MOR 559) under natural light with corresponding microradiographic aspect of framed areas. A. Longitudinal section under natural light. B. Detail of the upper box on the coronoid process in (A). C. Detail of the box in (B) under natural light. Clusters of numerous cells can be observed in the center of the bone trabeculae. D. Corresponding microradiograph of (C) showing CB (white arrows) and bone (black arrows). The blue arrow designates an area where microradiography and natural light pictures have contradicting results (see section 4). E. Detail of the lower box on the coronoid process in (A). F. Corresponding microradiograph of (E), CB is indicated by white arrows. G. Detail of the right box in (A). H. Microradiograph of the right box in (G), showing many struts of CB radiating into the alveoli (white arrows), and bone deeper within the element (black arrow). I. Detail of the left box in (G). CB is indicated by white arrows. J. Corresponding natural light photograph of (I). CB has the appearance of “normal” woven bone under natural light. Abbreviations: Ca: caudal; Co: coronoid process; La: labial; Li: lingual; Ro: rostral.

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Fig. 4. (Color online.) Cross sections in the mandibular symphysis of the dentary of a Hypacrosaurus embryo (MOR 559) under natural light with corresponding microradiographs. A. Cross section under natural light. B. Detail of the box B in (A). C. Detail of the box in (B). Struts of CB appear lighter and with a higher cellular density than that of the surrounding bone under natural light. D. Microradiograph of the Box D in (A). The periosteal borders show that CB is very well distributed (white arrows), surrounded by bone struts (black arrows). Bone is also present, deeper within the element. E. Microradiograph of the Box E in (A). The same observations as those of figure (E) can be seen. F. Detail of the box F in (A) under natural light. G. Detail of the box in (F). CB has a “diagenetically altered” appearance under natural light. H. Corresponding microradiograph of (G). CB is extremely abundant at the dorsal tip of this mandibular symphysis. I. Detail of the box in (G), showing irregular CB cell lacunae and their ‘diagenetically altered’ appearance. Abbreviations: Do: dorsal; La: labial; Li: lingual; Ve: ventral.

mandibular symphysis is not due to diagenetic alteration, but instead could be a characteristic of CB in paleohistological ground sections. These results (Figs. 4 and 5) suggest that CB was present during embryogenesis and persisted at least a few months after hatching in the mandibular symphysis of *Hypacrosaurus*. Note that CB has previously been found in the mandibular symphyses of cat embryos and newborns (Goret-Nicaise et al., 1984).

Fig. 6 shows transverse sections in a frontal of a *Hypacrosaurus* embryo (MOR 559). The microradiographs show once again struts and islets of CB (Fig. 6B and D). These struts are located on the periosteal surface (rather than deeper within the bone) of the fronto-postorbital sutural border. Indeed, at this suture, almost all the sutureal “bony” projections are made of CB and not of bone. This is very similar to what has been observed in the metopic suture of human fetuses (Manzanares et al., 1988) and other bird sutures (Lengélé et al., 1990, 1996a). The corresponding natural light pictures (Fig. 6C and E) look like “normal” bone, although cellular density can be very high in some areas (black arrows, Fig. 6C).

Lastly, we analyzed a fossilized endochondral bone (a laterosphenoid, Fig. 7) that showed remnants of calcified cartilage, in order to document how CB and calcified cartilage differ under microradiography. Indeed, one could argue that we are misidentifying CB cells for cartilage cells (or vice-versa) since they can share a similar round shape. Fig. 7 shows sections through the laterosphenoid of a post-hatching Lambeosaurinae (MOR 1015). Remnants of the early embryonic cartilage model can be seen within the bone trabeculae (Fig. 7B and C). Articular cartilage can also be seen on the surface that articulates with the postorbital (Fig. 7E). The corresponding microradiographs show cartilage cell lacunae that are very round (instead of irregular), individualized (instead of confluent) and embedded in an extremely radiopaque matrix (instead of a moderately radiopaque matrix, Fig. 7D and F, blue arrows). This appearance is similar to that of the primary and the secondary
cartilage of extant species seen under microradiography (e.g., see Fig. 3C in Goret-Nicaise and Dhem, 1985; and Fig. 5 p 33 in Lengelé, 1997). Moreover, the density of cartilage cells is much lower than that found in CB clusters. This shows that in these hadrosaurs, calcified cartilage and CB have very different characteristics on microradiographs and that it is difficult to misidentify them for one another.

4. Discussion

As mentioned earlier, CB (or chondroid tissue) is widely distributed among vertebrates. It has been reported in fossil agnathans and placoderms (Ørvig, 1951), in teleosts (e.g., in the kype of the Atlantic salmon, *Salmo salar*, Gillis et al., 2006; in the pharyngeal jaws of some African cichlids, Huysseune, 1985; in bony cysts of the yellow perch, *Perca flavescens*, Taylor et al., 1994), in mammals (in deer antlers, Wislocki et al., 1947; in the growing skull of human fetuses and infants, e.g., Goret-Nicaise, 1986; at muscle, tendon and ligament attachments of rabbit long bones, Hurov, 1986; in the cat skull, Goret-Nicaise et al., 1984; in miniature pig sutures, Rafferty and Herring, 1999), in crocodiles (in the embryonic skull of *Alligator mississippiensis*, Vickaryous and Hall, 2008) and in birds (in the skull of chick embryos, Hall, 1971, 1972; Lengelé, 1997; Lengelé et al., 1990, 1996a, 1996b; Murray, 1963). It is also found as
a transitional tissue in bone sarcomas and tumors of human patients (see Beresford, 1981 and Hall, 2005, 2014 in press for full reviews). It appears that the terminology used for CB and other intermediate tissues is still inconsistent. For example, 'chondroid tissue' was recently described during the formation of the notochord of some geckos (Jonsson et al., 2012). However, its mode of formation (differentiating from the chordoid tissue of the notochord) is very different from that of 'chondroid tissue' sensu Goret-Nicaise and Dhem (1982) and these studies designate two different tissues. This demonstrates one of the difficulties when studying CB. Nevertheless, here, we report for the
first time the presence of CB (synonymous with the “chondroid tissue” sensu Goret-Nicaise and Dhem, 1982; and Lengelé et al., 1990, 1996) in some non-avian dinosaurs (Ornithischia, Hadrosauridae). The identification of this tissue was made possible by means of paleohistological examination under natural light (Fig. 2) and microradiography (Figs. 3–6). As mentioned previously, it was found in five locations: scattered within the bone trabeculae of an embryonic surangular (Fig. 2); in the coronoid process of an embryonic dentary (Fig. 3); in the alveolar processes of an embryonic dentary (Fig. 3); in the mandibular symphysis of an embryonic and a post-hatching dentary (Figs. 4 and 5); and in the sutural borders of an embryonic frontal (at the fronto-postorbital suture, Fig. 6).

Before discussing the physiological and the phylogenetic implications of these findings, we explore morphological criteria identified as characteristic of CB under natural light in standard paleohistological thin sections. This will be useful for future paleohistologists interested in this tissue.

4.1. Identification of chondroid bone with microradiography versus natural light microscopy

The microradiographs obtained from these hadrosaurs look exactly like those of humans (Fig. 1) and chicks (see Lengelé et al., 1990, Lengelé et al., 1996a) that possess CB (compare Fig. 1 with Figs. 3–6). Both the extant species and the hadrosaurs show islets of mineralized matrix with clusters of confluent cellular lacunae, typical of CB. This suggests that our identification in the fossil bone is correct. When comparing microradiographs with their corresponding natural light pictures, we found two noteworthy results. Firstly, while CB always has the same appearance under microradiography, different corresponding morphologies can be observed under natural light: the ECM of CB can be much brighter than the ECM of the surrounding bone (Figs. 2B, D, F–G and 4B–C), it can have the appearance of normal woven bone (Figs. 3J, 6C, E), or of a genetically altered bone (Figs. 4F, G, I). Moreover, the cell lacunae can appear round, i.e., almost like hypertrophied cartilage cells (Fig. 2E), or more irregular (Fig. 4G, I). Note that CB cells are sometimes known to present features of hypertrophied chondrocytes (e.g., Tuominen et al., 1996). In all the cases, the cellular density was always higher than that of the surrounding bone, or that of calcified cartilage (Fig. 7).

Second, some areas that look like CB under natural light (with clusters of round cells closely packed together) look similar to bone under microradiography (see Fig. 3C-D, blue arrows). These conflicting results did not enable us to find a clear-cut relationship between microradiography and natural light pictures of CB. Nevertheless, we propose four characteristics that CB presents under natural light in paleohistological ground sections. Note, however, that these characteristics are not necessarily accurate for CB in ground- or decalcified sections of extant animals. Also note that those are preliminary results since further examination and discrimination of this tissue need to be made with paleohistological sections. The characteristics of CB are as follows:

- it presents large ‘cartilage-like’ cell lacunae (similar in morphology to those of hypertrophic cells of extant vertebrates), or irregular cell lacunae;
- its cell density is always higher than that of the surrounding woven or lamellar bone;
- its ECM is more translucent than that of bone (for a section thickness of approximately 80 to 120 μm);
- it may have the appearance of a diagnostically altered bone.

This diversity of morphologies could directly reflect skeletal diversity (i.e., there would be multiple types of CB). Indeed, Beresford (1981) classified two types of CB (type I and type II, see Chapter 1) and noted that it is difficult to draw limits between intermediate tissues, as they can form a “jungle of overlapping categories”. Another example of reported skeletal diversity is that in Benjamin, 1990, where six different types of cranial cartilage where reported in the black molly, Poecilia sphenops (also see Witten et al., 2010). To demonstrate that hadrosaurs possess different types of CB is beyond the scope of this paper, but we would like to note that this is a plausible hypothesis.

It cannot be ruled out that this morphological diversity does not reflect reality but instead is due to diagenesis (e.g., the latter could have altered the overall appearance of the tissues but not the orientation of their crystals). However, as mentioned in the results sections, both the microradiographs and the natural light images of the periosteal borders of the sampled elements showed good preservation (e.g., compare Fig. 4H with Fig. 1A,C). According to our results, diagenetic alterations seem to have been minor. Perhaps a bigger sample size, or a comparison between microradiographs and serial sections of some extant species stained with Masson’s trichrome and/or Mallory’s trichrome (i.e., what was used in Gillis et al., 2006 and Vickaryous and Hall, 2008) could help answer our questions about this morphological diversity.

4.2. Chondroid bone in hadrosaurs: implications for skull growth

In analogy to the five locations presented in this study, CB has been found:

- on the surangular of chick embryos (Lengelé et al., 1996a);
- in the coronoid process of human fetuses and infants (Goret-Nicaise, 1981);
- in the alveolar processes during the growth of the tooth buds in human fetuses and infants (Goret-Nicaise et al., 1984);
- in the mandibular symphysis of the cat and the human fetus (Goret-Nicaise, 1986; Goret-Nicaise et al., 1984);
- at all the sutural borders of the skull of human fetuses and infants (Goret-Nicaise et al., 1988, Manzanares et al., 1988) and chick embryos (Lengelé et al., 1990, 1996a).

Finding this tissue in these duck-billed dinosaurs has two important implications for their skull growth:
• as mentioned earlier, CB is an adaptation to rapid growth (Gillis et al., 2006; Goret-Nicaisé, 1986; Huysseune and Verraes, 1986; Taylor et al., 1994). The fact that CB was found in large amounts in these dinosaur embryos suggests a rapid embryonic skull growth. Alas for now, we can only make qualitative statements since a comparison between microradiographs of slow and fast-growing animals (with quantitative information) has never been undertaken. This rapid development is not surprising because it is already known that the post-cranium of hadrosaurs grew fast and that they were more endotherms than ectotherms (Cooper et al., 2008; Horner et al., 2000; Padian et al., 2001).
• we also report the presence of CB at the fronto-postorbital suture of Hypacrosaurus. The vast majority of the “bony” struts at these sutural borders are made of CB (Fig. 6B, D), in a similar manner as to what has been observed in mammalian and avian sutures (Lengelé et al., 1990, 1996a; Manzanares et al., 1988). Goret-Nicaisé et al. (1988) found that CB was the major driving force of “bone” lengthening at the sutural borders, and that it was not comparable to periosteal growth. Manzanares et al. (1988) concluded that CB also had a role in sutural fusion (in the metopic suture of 6-months-old infants).

Our results suggest that CB might also have been involved in the sutural growth of hadrosaurs. Many dinosaur studies use the degree of closure of sutures to assess the maturity of their specimens and in turn, if they are identified as ‘adults’, this sometimes is considered grounds for naming a new species (e.g., Bakker and Williams, 1988; Sereno et al., 2009). However, such conclusions should be reconsidered since there is a lack of understanding of sutures in non-mammalian species (including the phylogenetic bracket of the Dinosauria) in terms of:

• pattern (i.e., the order in which sutures fuse through ontogeny);
• morphology;
• histology (see Herring, 2000 for an excellent review).

Therefore, it is important to start documenting and understanding the osteohistology of sutures in extant and extinct archosaurs. Our results suggest that sutural growth was very similar in birds, mammals and hadrosaurs (at least during embryonic development), but other preliminary results suggest that this similarity fades through ontogeny and leads to more unique modes of fusion (Bailleul and Horner, 2013). Even though CB was only found in one suture of Hypacrosaurus (i.e., the only suture that was microradiographed), this is the first step in understanding more about sutural growth in non-avian dinosaurs, a process that we need to comprehend to accurately assess their maturity.

4.3. Chondroid bone: phylogenetic implications

CB has not been studied in many clades of vertebrates, therefore hasty and preliminary conclusions should not be made. However, it is not surprising to find CB in non-avian dinosaurs, because members of their extant phylogenetic bracket also form this tissue (Hall, 1971, 1972; Lengelé et al., 1990; Vickaryous and Hall, 2008). It is parsimonious to say that the CB in Gallus gallus, Alligator mississippiensis, and hadrosaurs are homologous and that it was present in their most recent common ancestor. If our hypothesis of homology holds true, CB would be present in the other clades of dinosaurs as well, but only more paleohistological and microradiography examinations could verify this.

It is not clear exactly when CB arose within the Archosauromorpha, and if it is homologous to the CB found in less derived vertebrates (e.g., in Gallus gallus, Alligator mississippiensis, and hadrosaurs are homologous, and that it was present in their most recent common ancestor. If our hypothesis of homology holds true, CB would be present in the other clades of dinosaurs as well, but only more paleohistological and microradiography examinations could verify this.

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1.4. Dinosaursian chondroid bone and secondary cartilage

Secondary cartilage and chondroid bone are often found together throughout the skull of mammals and birds (Goret-Nicaisé and Dhem, 1982; Hall, 1971, 1972; Lengelé et al., 1996b). In our small sample size, we found CB (present study) where dinosaurian secondary cartilage had previously been reported (Bailleul et al., 2013, in the alveoli of MOR 559). We also discovered a new secondary cartilage location while we were looking for CB, on the coronoid process of a post-hatching Hypacrosaurus (slides DE10 and DE13 from project 1988–13, MOR 548, data not shown).

The possibility of the existence of multiple types of CB, as presented in the first part of the discussion, was partly suggested by the fact that CB cell lacunae present two different morphologies: they can have the appearance of hypertrophied cartilage (Fig. 2E) or can appear irregular (e.g., Fig. 4G, 1). Even though it has been reported that CB cells sometimes have the appearance of hypertrophied chondrocytes (Mizoguchi et al., 1997; Tuominen et al., 1996), since we could not perform microradiography on the element present in Fig. 2 (MOR 1038), the possibility that those cells are actually secondary cartilage cells, and not CB cells, cannot be ruled out. Secondary cartilage is usually organized as nodules on the periosteal surface of membrane bones (Hall, 1967, 1968), while here, it is present as a long central strut. The absence of resorption suggests that it is not an external secondary cartilage nodule that has been relocated from the periosteal surface to the interior of the bone. Rather, these CB cells could be transforming into cartilage cells via metaplasia (Beresford, 1981). Lengelé et al. (1996b) showed that CB and secondary cartilage were two autonomous tissues, arising independently from the cephalic mesenchymal cells under different biomechanical inductions. However, if indeed there is not one, but many types of CB, perhaps metaplasia between CB and
secondary cartilage cells is possible in some cases. Metaplasia, the permanent transformation of cell identity from one cell type to another (Beresford, 1981; Hall, 2005), is a common mechanism in vertebrate lineages in skeletal and non-skeletal tissues; for e.g., hypertrophied chondrocytes can transform directly into osteoblasts in turtle and lizard long bones (Haines, 1969); fibroblasts can transform into fibrocartilage (e.g., at mammalian tendon or ligament insertions, Gao et al., 1996; Hurov, 1986; McLean and Bloom, 1940); during antlerogenesis; and at the anterior tip of the rat penile bone and during the fracture repair of bones in some frogs and lizards; see Beresford, 1981 and Hall, 2005, 2014 in press for full review). Metaplasia has also been reported in the extant phylogenetic bracket of non-avian dinosaurs: the osteoderms of Alligator mississippiensis arise by in situ transformation of dense irregular connective tissue (perhaps a population of fibroblasts; M. Vickaryous, personal communication) into bone (Vickaryous and Hall, 2008). Tenocytes of avian tendons can transform into osteoblasts (Adams and Organ, 2005; Agabalyan et al., 2013) and most importantly, secondary cartilage can transform into CB in paralyzed embryonic chicks (Hall, 1972). Moreover, even though it is not possible to know the exact mode of formation of fossilized tissues, due to the unusual histology of some dinosaur mineralized tissues, metaplastic transformations have been hypothesized to play a role in the formation of dinosaur skulls (Goodwin and Horner, 2004; Hieronymus and Witmer, 2008; Horner and Lamm, 2011; Horner et al., 2015) and osteoderms (Main et al., 2005, Scheyer and Sander, 2004). It appears also that this unusual histology was much more abundant in the skulls of dinosaur than in those of extant mammals and birds (J. Horner personal observations). Therefore, these numerous examples of metaplasia suggest that it is reasonable to hypothesize the transformation of CB cells into secondary cartilage cells in our sample. Of course, this is beyond the scope of this paper, and we are simply considering additional possibilities. More neontological studies on extant archosaurs are necessary for the current and futures advances in the growing field of dinosaur paleohistology.

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