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Rethinking the nature of fibrolamellar bone: An integrative biological revision of sauropod plexiform bone formation

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ABSTRACT

We present novel findings on sauropod bone histology that cast doubt on general palaeohistological concepts concerning the true nature of woven bone in primary cortical bone and its role in the rapid growth and giant body sizes of sauropod dinosaurs. By preparing and investigating longitudinal thin sections of sauropod long bones, of which transverse thin sections were previously published, we found that the amount of woven bone in the primary complex has been largely overestimated. Using comparative cellular and light extinction characteristics in the two section planes, we revealed that the majority of the bony lamina consists of longitudinally organized primary bone, whereas woven bone is usually represented only by a few cells thin layer in the laminae. Previous arguments on sauropod biology, which have been based on the overestimated amount, misinterpreted formation process and misjudged role of woven bone in the plexiform bone formation of sauropod dinosaurs, are thereby confuted.

To explain the observed pattern in fossil bones, we review the most recent advances in bone biology concerning bone formation processes at cellular and tissue levels. Differentiation between static and dynamic osteogenesis (SO and DO) and the revealed characteristics of SO- vs. DO-derived bone tissues shed light on several questions raised by our palaeohistological results and permit identification of these bone tissues in fossils with high confidence. By presenting the methods generally used for investigating fossil bones, we show that the major cause of overestimation of the amount of woven bone in previous palaeohistological studies is the almost exclusive usage of transverse sections. In these sections, cells and crystallites of the longitudinally organized primary bone are cut transversally, thus cells appear rounded and crystallites remain dark under crossed plane polarizers, thereby giving the false impression of woven bone. In order to avoid further confusions in palaeohistological studies, we introduce new osteohistological terms as well as revise widely used but incorrect terminology.

To infer the role of woven bone in the bone formation of fast growing tetrapods, we review some aspects of the interrelationships between vascularity of bone tissues, basal metabolic rate, body size and growth rate. By putting our findings into the context of osteogenesis, we provide a new model for the diametrical limb bone growth of sauropods and present new implications for the evolution of fast growth in vertebrates. Since biomechanical studies of bone tissues suggest that predominant collagen fibre orientation (CFO) is controlled by endogenous, functional and maybe phylogenetic factors, the relationship between CFO and bone growth rate as defined by Amprino's rule, which has been the basis for the biological interpretation of several osteohistological features, must be revised.

Our findings draw attention to the urgent need for revising widely accepted basic concepts of palaeohistological studies, and for a more integrative look at bone formation, biomechanics and bone microstructural features of extant and extinct vertebrates to infer life history traits of long extinct, iconic animals like dinosaurs.

Key words

sauropod dinosaur; fibrolamellar bone; woven bone; plexiform bone, palaeohistology; osteocyte lacuna; section planes, growth rate; collagen fibre orientation

CONTENTS

I. INTRODUCTION

(1) General introduction

Dinosaurs grew at rates rivaling those of extant birds and mammals. High growth and metabolic rates have been assessed by modern paleohistological studies using fossilized bone tissues of extinct dinosaur and other archosaurian taxa. The main aim of paleohistology is to understand life histories of these extinct groups, and also to extrapolate these results into a phylogenetic context to infer evolutionary patterns of growth strategies (e.g. Chinsamy-Turan, 2005; Cubo et al. 2005, 2008, 2012; Erickson, Rogers & Yerby, 2001; Erickson, 2005; Horner, de Ricqlès & Padian, 2000; Horner, Padian & de Ricqlès 2001; Horner & Padian, 2004; Sander et al. 2004; de Ricqlès, Padian & Horner, 2003; de Ricqlès, 2008).

(2) Sauropod dinosaurs as prime example of high growth rates

Sauropod and sauropodomorph bone microstructure has been widely studied over the past decade (e.g. Sander, 2000; Sander & Tückmantel, 2003; Sander et al. 2004, 2006; Lehman & Woodward, 2008; Woodward & Lehman, 2009; Klein & Sander, 2008; Klein, Sander & Suteethorn, 2009; Stein et al. 2010; Company, 2011; Sander et al. 2011). These studies mostly report long bones with a primary cortex consisting of laminar to plexiform fibrolamellar bone. Fibrolamellar bone is defined as a composite tissue comprising a rapidly growing, woven fibred bone matrix in which osteonal lamellar bone later infills the space between woven bone and primary vascular canals (de Ricqlès, 1974). A main characteristic of woven bone (often synonymized with fibrous bone, Francillon-Vieillot et al. 1990) is the random orientation of the collagen fibrils in the matrix, the deposition of which is thought to require less time than that of a highly organized bone matrix (Amprino, 1947; Curry-Rogers & Erickson, 2005). In contrast, in the lamellar bone of the primary osteons, the collagen fibrils have a highly structured parallel spatial organization, and thus a lower depositional rate. In cross section, laminarity refers to the mainly

circumferential orientation of the vascular canals with a few radial anastomoses, which hence divide the bony constituent into "brick-like" structures. Based on its general isotropic appearance under cross polarized light (Francillon-Vieillot et al. 1990; Castanet et al. 1993), the majority of the laminae in transverse long bone sections of sauropod dinosaurs has been identified as woven bone. Rapid osteogenesis of fibrolamellar bone is thus inferred from the presumed random orientation of the crystallites of the bony laminae, which reflects the original haphazard collagen fibril orientation of the living bone. It is widely accepted that this form of fast osteogenesis, probably already present in basal archosaurs (de Ricqlès et al. 2003, 2008; Cubo et al. 2012), was an exaptation that allowed sauropods to rapidly grow to enormous sizes (Curry-Rogers & Erickson, 2005; Sander et al. 2004, 2011).

However, our findings based on longitudinal sections of sauropod long bones question the woven nature of the non-lamellar component of fibrolamellar bone, thereby contesting previous descriptions. These results have led the current study to review as well as revise former ideas about fibrolamellar bone in fossil and extant vertebrates.

II. NEW RESULTS CONTESTING GENERAL CONCEPTS

(1) Acquiring new insight: Materials and methods

Samples of sauropod long bones (listed in Table 1.) were obtained by histological core drilling (Sander 2000; Stein & Sander, 2009). Transverse sections of these samples had been made for previous studies and were described as laminar fibrolamellar bone (Sander & Tückmantel, 2004; Klein & Sander, 2008; Stein et al., 2010; Sander et al., 2011; Klein et al., 2012). Remaining halves of the drill cores were cut along the long axis of the sampled long bone (perpendicular to the original sections), and thin sectioned with standard paleohistological methods (Stein & Sander 2009). The sections were then investigated with linearly polarized light (LPL) microscopy with a

Leica DM 4500 LP microscope (Leica Microsystems, Wetzlar, Germany), and compared with transverse sections of the same drill core. Images were obtained with a Leica firecam (DFC 450) and processed and measured with Imagic ImageAccess software. Additional specimens of two undescribed ornithomimid theropods were sectioned and investigated to provide a broader phylogenetic comparison. Further details of the investigated specimens are provided in Table 1. Basic statistical evaluation of measured histological features was performed in SOFA (Statistics Open For All v.1.1.5, Paton-Simpson & Associates Ltd, Auckland, New Zealand).

(2) Novel findings

All of the longitudinal sections of relatively well known sauropods (*Camarasaurus*, *Apatosaurus*, *Alamosaurus* and an indeterminate diplodocid from the Morrisson Fm.; detailed in Table 1) confirm the laminar to plexiform vascular architecture previously reported for these animals. However, strong anisotropy characterizes almost the entire primary cortical bone (Figs 1, 2). The osteocyte lacunae in this anisotropic matrix show a typical spindle shape, indicating that they were cut along their longitudinal axis (Fig. 3), and the long axis of most of them adheres to the long axis of the bone element. In transverse section, the general appearance of most of the osteocyte lacunae is rounded (sometimes referred to as 'plump') (Fig. 3). This preferential cell orientation suggests a mostly longitudinal structural organization of the primary bone (from now on referred to as highly organized primary bone, HOPB) in all investigated specimens (Figs 2, 3). These features were also observed in the undescribed theropod specimens.

A very thin layer of bone, usually central, but often offset from the centre of the bony strut (the bony constituent of laminae *sensu* Sander [2000]) shows general light isotropical features on tissue scale in longitudinal section (Fig. 2). In the sauropod samples, this layer is one to maximum four osteocyte lacunae wide (thickness of 20 - 40 µm), and has been described as hypermineralized 'bright line' in transverse sections by other authors (Currey 1962; Francillon-Vieillot et al. 1990; Kerschnitzki et al. 2011). In this region, the osteocyte lacunae do not show any kind of preferential

orientation in longitudinal nor in transverse sections. Generally, the lacunae have a random shape, are more densely spaced and mostly appear larger than the osteocyte lacunae in HOPB. Using the definition of woven bone provided by Francillon-Vieillot et al. (1990), this 1-4 cells thin layer is the only true woven bone in the primary cortex. Locally in this layer, two to three cells may have the same spatial orientation. On cellular scale, the matrix surrounding these cells shows anisotropic features under cross polarized light suggesting the presence of small patches of highly organized fibres. The average thickness of the woven bone layer ranges from14 to 22% of the width of the entire bony lamina and both absolute and relative thickness (Table 2) varied across taxa (ANOVA, $p < 0.001$). The average values of absolute thickness of the woven bone layer increase with increasing long bone shaft circumference (linear regression, Pearson $R = 0.783$; $p = 0.007$), but there is no correlation between the average fraction of woven bone and shaft circumference (linear regression, Pearson $R = -0.128$; $p = 0.725$). The correlation between shaft circumference and average thickness of the bony lamina (Pearson $R = 0.642$) was statistically not significant (linear regression, $p = 0.045$). These results suggest that the proportional thickness of the woven bone layer is variable within the bony lamina across different taxa. For further details on statistical output, please see Table 2 and 3.

The highly organized primary bone (HOPB), which is characterized by the long range order of matrix and cell lacuna orientation, may consist of lamellated and non-lamellated structural subunits in the investigated specimens (Fig. 4). Lamellation is easily detectable in transverse and longitudinal sections if the successive lamellae appear alternating dark and bright under cross polarized light. Such a pattern of alternating dark and bright bands adjacent to the vascular space was observed in *Apatosaurus* (two in femur SMA 'Chris'; up to five in humerus CM3378, Fig. 4A). In these specimens, usually the lamellation starts with a dark lamella in longitudinal section, and the same lamella will appear bright in transverse section, hence exhibiting the complementary pattern of interference. Wherever osteocyte lacunae are embedded in the lamellae, the appearance of them in the dark bands is generally rounded, whereas in the bright bands they have an elongated spindle

shape in either section plane. These features imply a plywood structure with changing fibre orientation in successive lamellae in the living bone (Francillon-Viellot et al. 1990 and references therein). Fine lines visible under plane polarized light are mostly associated with these lamellated perivascular regions (Fig. 4B). However, the borders of the individual lamellae defined by their extinction pattern do not always match these lines. Vascular canals surrounded by such lamellated areas have been referred to as primary osteons by other authors (e.g. de Ricqlès 1968; Currey, 2002; Chinsamy-Turan, 2005). If adjoining lamellae have a uniform extinction pattern rather than an alternating dark and bright appearance, distinguishing them may be more difficult. This is the case in *Alamosaurus*, where 7 to 10 thin lamellar bands are detectable under single plane polarizer based on the presence of fine lines, but these appear uniformly dark under cross polarized light in transverse section and uniformly bright in longitudinal section (Fig. 4B). In transverse section, these dark lamellae are sometimes lined with one bright lamella positioned on the internal surface of the vascular space. The dark lamellar bands have longitudinally oriented osteocyte lacunae (rounded in transverse section; strongly elongated in longitudinal section), whereas the long axes of the osteocyte lacunae in the bright lamellar band are oriented in the transverse plane.

In large areas of the highly organized primary bone, no trace of lamellation can be revealed under single or crossed plane polarizers. This non-lamellated HOPB is usually sandwiched between the thin woven bone layer and the lamellae bordering the vascular space (e.g. *Apatosaurus*, *Camarasaurus*), but it may also form the only structural subunit of the non-woven primary bone (e.g. diplodocid indet. NHUB Ki2, Fig. 4C) if no lamellae had been deposited at the time of death. The transition from the woven bone layer to this non-lamellated HOPB is marked by the appearance of osteocyte lacunae with gradually more longitudinal orientation (parallel to the longitudinal axis of bone), but no particular growth marks or any other distinct features indicating an abrupt change in the deposited tissue type are present.

The relative amount of lamellated and non-lamellated components in the HOPB is variable in different specimens, even though the exact border between the two subunits is usually hard to

define (Figs 4, 5). This ratio does not only differ in different areas of the same section and between different ontogenetic stages of the same species but may vary among species representing similar histologic ontogenetic stages (HOS). For example, in most areas of the section of *Apatosaurus* CM3378 (HOS 11), the ratio of lamellated and non-lamellated HOPB is approximately 1/1, whereas in *Alamosaurus* TMM46002 (HOS 7), the lamellae are frequently deposited directly on the woven bone, thus the highly organized primary bone consists solely of lamellae in these regions. In "*Barosaurus"* Ki2 (HOS 10.5), the lamellated tissue is usually completely absent. Figure 5 shows further details on the average proportional distribution of different constituents of the laminae (*sensu* Sander, 2000) in the investigated specimens.

III. REVIEW AND REVISION PROMOTED BY THE NEW RESULTS

(1) Observed structural composition of sauropod primary bone

Strong polarized light anisotropy in longitudinal sections of sauropod long bone cortices indicates a mostly longitudinal crystallite orientation in the non-lamellar primary bone as well (Fig. 1). These crystallite orientations correspond to the original crystallite and matrix orientation in the bone tissues of the living animals (Kolodny et al. 1996; Hubert et al. 1996; Trueman & Tuross, 2002). Truly woven bone, that appears generally darker in longitudinal sections too, is represented only by a thin layer centrally positioned in the bony laminae (Fig. 2). This thin layer corresponds to the highly mineralized 'bright line' (Francillon-Viellot et al. 1990), and is the oldest part of the bony lamina. The remainder of the non-lamellar primary bone is highly organized with a longitudinal preferential organization. This extensive longitudinal structural arrangement in the non-lamellar HOPB is also reflected by the long range spatial order of the osteocyte lacunae being uniformly cut along their long axis in longitudinal sections. These results are inconsistent with widely reported claims that the bulk of the non-lamellar primary bone in sauropod plexiform bone

(often synonymized with laminar fibrolamellar bone, see also de Ricqlès 1968a; 1974) consists entirely of woven bone with originally randomly oriented collagen fibrils and apatite crystallites. In contrast, our results indicate that the microstructural arrangement of sauropod long bones corresponds to the strong longitudinal organization also observed in ovine and bovine plexiform (fibrolamellar) primary bone (Kerschnitzki et al. 2011). Kerschnitzki and coauthors (2011) have shown that the amount of woven bone in ovine and bovine plexiform bone is limited to a layer only a few cells thin, similar to our findings in sauropod primary bone.

In the sauropod samples presented here, highly organized primary bone may appear in the form of lamellated and/or non-lamellated subunits, both of which show a long range uniform extinction pattern and parallel orientation of the osteocyte lacunae. The histological distinction of these two HOPB subunits is often ambiguous for several reasons. First of all, the true nature of lamellation is still under debate. Lamellated appearance has been interpreted as the result of changing fibre orientations (Gebhardt, 1906; Giraud-Guille, 1988) or changing fibre and mineral densities (Boyde & Hordell, 1969; Marotti, 1993; Marotti, Palazzini & Palumbo, 1993) in subsequent lamellae both of which eventuate in an alternating extinction pattern under cross polarized light. Lamellation has also been defined by the presence of interlamellar zones that separate the successive lamellae, consist only of less organized collagen fibres and appear as darker stripes between the individual lamellae under crossed plane polarizers (Ascenzi & Bonucci, 1968; Bromage et al, 2009). We do not support the exclusive validity of the first definition of lamellation, because we could still detect lamellation based on the perivascular fine lines visible under plane polarized light in specimens with uniform fibre orientation in successive lamellae (e.g. *Alamosaurus*, Fig. 4B). However, the structure of these fine lines is unclear, because they do not always correspond to the border of lamellae with alternating extinction pattern or to the dark stripes that might represent interlamellar zones either. We hypothesize that the fine lines represent temporal interruptions in matrix deposition that might be followed by synchronized spatial reorganization of the osteoblasts and thus uniform change in fibre orientation. In this concept, all

previously proposed definitions of lamellation may be valid representing a considerable variability in the formation, structure and composition of lamellated bone. Furthermore, the thickness of the lamellae can vary considerably (Bromage et al, 2009; 2011), thus local changes in fibre orientation in the non-lamellated HOPB may appear as lamellae. Finally, the border between the first lamella and the non-lamellated HOPB is not always distinct which is probably due to the same principles of formation of the two subunits on a cellular level. Hence, we prefer to rely on the combination of the presence of fine lines and extinction pattern when defining HOPB lamellar patterns. Even though their image of plexiform bovine bone shows lamellated and non-lamellated HOPB, Kerschnitzki et al. (2011) did not differentiate these two structural appearances but rather referred to all HOPB deposited on the woven bone layer as lamellar bone. Similarly, other authors consider any type of primary bone as lamellar if it reveals high degree of parallel fibre and crystallite orientation. We propose a consistent usage of lamellar bone for HOPB tissues which show clear characteristics of lamellation. Since HOPB, be it lamellated or non-lamellated, always exhibits high degree of parallel organization, the term 'parallel-fibred bone' is more adequate to describe both types of HOPB rather than an intermediate degree of structural order as it was suggested by other authors (e.g. Francillon-Viellot et al. 1990; Currey, 2002). Thus, HOPB should be synonymized with primary parallel-fibred bone. However, with the unification of the two forms of appearance (lamellar and non-lamellar) of parallel-fibered bone by introducing the new term HOPB, we can avoid confusion originating from the inconsistent or even incorrect usage of the term parallel-fibered bone. Furthermore, based on the common formation principles (see below) and because of the difficulties related to the distinction of lamellated and non-lamellated HOPB, we suggest that any form of HOPB deposited on the initial woven bone layer be considered as the bony constituent of primary osteons (cf. Ferretti et al. 2002).

(2) Woven bone and HOPB in extant and fossil vertebrates

Our results clearly indicate that the amount of woven bone in the long bones of sauropods and probably a number of other dinosaurs has been largely overestimated. In a classical transverse section under crossed plane polarizers, the dark part of the lamina that was previously referred to as woven bone is up to three to four times thicker than the actual layer of woven bone (Fig. 2). This overestimation has led to a number of conclusions and generally accepted interpretations that, in the light of the current observations, call for urgent revision and modification. To overcome confusion and avoid further misconceptions when working with fossil bones, the true nature of woven bone and HOPB needs to be considered on cellular level. Understanding the formation process of these bone tissues is the key to understanding their microstructure, cellular composition, spatial arrangement, growth, and developmental role. This background knowledge is essential to draw any biological inferences based on the presence of woven bone and HOPB in extinct animals.

(a) *Primary bone formation: SO vs. DO*

Recent histological studies of extant vertebrates revealed two major types of primary bone formation: stationary and dynamic osteogenesis (Marotti et al. 1999; Ferretti et al. 2002; Palumbo, Ferretti & Marotti, 2004; Marotti, 2010).

In stationary or static osteogenesis (SO), the ossification center is composed of highly vascularized mesenchymal tissue in which plump cells of diverse shapes start to differentiate into osteoblasts half way between two adjacent primary vascular spaces. In this layer, the thickness of which does not exceed 2-3 cells, the osteoblasts are randomly arranged with respect to each other; however, each of them is functionally polarized with definite secretory territory (Ferretti et al. 2002; Palumbo et al. 2004; Marotti, 2010). The secretory territory is a membrane surface area characterized by finger-like processes at the base of which collagen fibrils secreted in exocytotic vesicles assemble to form extracellular fibril bundles (Palumbo et al. 1990; Pazzaglia et al. 2010). As these osteoblasts start to secrete the preosseus matrix, they do not form a movable sheet of cells such as a monostratified osteogenic lamina, but stay at the same place in this spatially unorganized

manner “trapped” in the matrix they produce. Because their orientation is random with respect to the adjacent osteoblasts, their secretory territories are differently directed resulting in a highly porous matrix. Nevertheless, fibres still form highly organized bundles around the individual cells that secrete them. This short range (cellular scale) order of fibres has been referred to as ‘microlamellar bone’ by Kerschnitzki et al. (2011). The osteoblasts soon begin to differentiate into large, globous osteocytes *in situ* forming the initial bony trabeculae that are positioned at constant distance from the adjacent primary vascular spaces. Thus, the term stationary or static osteogenesis refers to the immobile nature of these osteoblasts and consequently to their transformation into osteocytes at the same place where they differentiated from mesenchymal stem cells. Shortly after osteocyte transformation, the preosseus matrix undergoes considerable mineralization. Some of the osteocytes are enclosed in groups within the same large, confluent lacunae (Ferretti et al. 2002; Palumbo et al. 2004; Marotti, 2010). This porous, irregular spatial construction is probably responsible for the fast volume increase i.e. high formation rate of this *de novo* bone tissue. As SO proceeds, it encloses the capillary network, thereby significantly expanding the volume of the forming bone in a relatively short amount of time. Thus, SO results in a connective tissue type that clearly corresponds on each hierarchical level to what has been defined as woven bone. This equivalence has also been pointed out by Palumbo et al. (2004) and Marotti (2010).

In contrast to static osteogenesis, dynamic osteogenesis (DO) involves a movable set of osteoblasts organized in a monolayer osteogenic lamina (Ferretti et al. 2002; Palumbo et al. 2004; Marotti, 2010). Within this lamina, the osteoblasts are all polarized in the same direction and produce matrix in a highly organized manner (Marotti, 2010; Pazzaglia et al. 2010). The designation ‘moveable’ implies that the lamina adheres to the preosseous matrix it secretes, continuously thickening the deposited layer from which the lamina seems to “move away”. In a regular pattern, those osteoblasts that are destined for differentiating into osteocytes, stop producing matrix, whereby the neighboring, matrix-secreting osteoblasts bury them one by one into the deposited layers of bone (Marotti et al. 1992; Franz-Odendaal, Hall & Eckhard Witten, 2006).

However, the formation of an osteogenic lamina with synchronized secretion activity requires the presence of an initial substrate, on the surface of which the lamina can differentiate (Marotti, 2010; Kerschnitzki et al. 2011). This substrate can be provided by the cord of woven bone trabeculae formed *de novo* by SO or on the surface of cartilage precursors in endochondral ossification (Marotti, 2010). Thus, DO corresponds to accretional or appositional bone growth and always results in the formation of structurally highly organized bone tissue with preferential fibre and cell orientation. DO is responsible for the thickening of the 2-3 cells thin SO-trabeculae, and thereby for the compaction of bone through infilling of the primary vascular spaces using SO-trabeculae as the primer substrate.

On the basis of the above review on primary bone formation, it becomes evident that *de novo* i.e. woven bone is formed by static osteogenesis, whereas HOPB of parallel structural arrangement is the product of dynamic osteogenesis. Although the well-known classification of intramembranous ossification (e.g. formation of most cranial bones) *versus* endochondral ossification (e.g. formation of long bones) is also defined on tissue level, it is clear that bone formation in both involves the combination of static and dynamic osteogenesis. This has significant bearings on the evolutionary considerations of different bone tissue types (see below).

(b) *SO- vs. DO-derived osteocytes and lacunae*

The morphology of the osteocytes also helps identifying whether the investigated primary bone tissue is derived from SO or DO, even though there is no structural or ultrastructural difference between immobile and mobile osteoblasts (Marotti, 2010).

Osteocytes originating from SO mostly retain features of their incipient osteoblast-morphology. They are relatively large, globous (plump), and have short dendritic processes which radiate symmetrically all around the cell body and run in the canaliculi to connect to the adjacent osteocytes by means of gap junctions (Palumbo et al. 2004). Since some of these osteocytes share

the same confluent lacunae, generally the lacunae are also larger in size and exhibit more irregular shapes (Ferretti et al. 2002). No mutual alignment of SO-cells and their lacunae and canalicular system can be observed.

DO-derived osteocytes, on the other hand, are smaller, more elongate and flattened with more and longer cytoplasmic processes which run mainly perpendicular to the long axis of the cell. The long axis of DO-osteocytes is perpendicular to the thickness of the osteoid deposited in their osteoblast-stage (Currey, 2003; Franz-Odendaal et al. 2006). Due to the uniform morphology of these osteocytes, their lacunae and canaliculi follow this pattern exhibiting dense canaliculi that are oriented perpendicularly to the long axis of the mostly spindle-shaped lacunae (Kerschnitzki et al. 2011). The DO-osteocytes show a long-range uniform orientation in the deposited HOPB.

(c) *Occurrence, developmental sequence and role of SO- and DO-derived primary bone*

The major differences in the formation principal of SO and DO-derived primary bone determine their occurrence, role and timing in skeletal development. SO is needed wherever there is no initial substrate surface present upon which osteoblasts could orderly assemble (Sugawara et al. 2005; Mori et al. 2007; Shapiro, 2008; Kerschnitzki et al. 2011). Thus, *de novo* bone formation occurs in the intramembranous ossification centers in which mesenchymal cells condensate in a 2-3 cells thick layer to form the woven bony strut between primary vascular spaces. The formation principals of this process do not differ from the woven bone formation during the diametrical growth of the ovine and bovine long bones cortices (Kerschnitzki et al. 2011). Thus, certain regions of the bone collar, which is the densely vascularized perichondrial mesenchymal tissue condensated around the growing long bone shaft (Karaplis, 2008), can be interpreted as potential local intramembranous ossification centers. However, based on the demonstrated differences in their genetic regulatory systems, intramembranous ossification and bone collar formation have been suggested to be developmentally distinct (Karaplis, 2008). Nevertheless, in the perivascular ossification centers of the bone collar, SO most probably provides the fast volume expansion of the

growing bone through the enclosure of extensive primary cavities (Ferretti et al. 2002). SO has also been reported in the first phase of bone fracture repair producing woven bone in the callus (Marotti, 2010). Since SO produces the scaffold for further bone deposition, woven bone along with cartilage (chondrification into the fracture gap, cf. Pritchard & Ruzicka, 1950) is needed in fracture repair probably because the fracture surface is an ill-defined osteogenic surface with no directional information for organized bone deposition. Alveolar bone also forms *de novo* separated from the jaw bones in embryonic stages (Osborn, 1984; Smith & Hall, 1993; Smith & Coates, 2000) and woven bone formation has also been reported adjacent to alveolar endosseous implants (e.g. Berglundh et al. 2003). In some cases, SO can be induced during endochondral ossification where hypertrophied chondrocytes are resorbed or transdifferentiate into woven bone producing osteoblasts (Franz-Odendaal et al. 2006 and references therein). In each case, SO provides the scaffold for the subsequent deposition of DO-derived, hence more organized primary bone tissue (Ferretti et al. 2002; Marotti, 2010; Kerschnitzki et al. 2011; Mori et al. 2007; Shapiro 2008; Sugawara et al. 2005). As bone matures, SO is always followed by DO, consequently the presence of HOPB is always expected around the woven bone framework in later ontogenetic stages.

DO is a substrate-dependent process, and the function of the osteogenic lamina is characterized by the accretional deposition of highly structured primary bone (Ferretti et al. 2002; Marotti, 2010). The initial substrate can vary, therefore DO can occur wherever accretional bone growth thickens the cortex or compacts the primary cavities. The diametrical accretional thickening occurs in perichondral, periosteal and endosteal bone growth on the surface of cartilage precursor or on already existing bony surface. Cavity compaction can start on the framework consisting of SO-derived wove bone (Marotti, 2010) or on the surface of the erosion cavities or cutting cones in secondary remodeling resulting in secondary osteons (Pazzaglia et al. 2011). Thus, in contrast to SO, DO deposits primary as well as secondary bone and generally reflects more mature ontogenetic stages.

Since the end product of SO, i.e. woven bone is a poor quality, porous and brittle bone with low resistance against various mechanical loading (Marotti 2010), we suggest that this is very likely a temporarily as well as spatially limited mode of bone formation which is evidenced by the proportionally low amount of woven bone in larger, fast growing animals. In concurrence with this assumption, SO is believed to be induced by inductive stimuli such as different growth factors, whereas DO is triggered by mechanical stimuli transmitted through the osteocytes of the mechanically less resistant SO-trabeculae (Marotti, 2010). This draws attention to the predominantly biomechanical significance of the highly organized fibre arrangement in the bone, as concluded by several previous studies investigating the relation between mechanical loading and long range fibre orientation in various skeletal elements (Ascenzi & Bonucci, 1967, 1968; Boyde & Riggs, 1990; Ascenzi et al. 1987; Martin & Ishida, 1989; Bromage, 2003; Skedros & Hunt, 2004, Skedros et al. 1996, 2006, 2007).

(d) *Distinguishing SO & DO characteristics in fossil bone*

After demonstrating SO and DO in extant animals, identifying SO- and DO-derived tissues in fossil bones becomes possible with much higher confidence than before. This has important bearings on functional and evolutionary interpretations.

Our notion that only a centrally positioned, few cells thin cord represents woven bone in the bony laminae of sauropod plexiform bone (Fig. 2) is supported by its SO-derived characteristics. One of these characteristics is its isotropic nature on tissue level, but its local birefringence on cellular level under cross polarized light (in accordance with the ‘microlamellar’ concept of woven bone, Kerschnitzki et al. 2011, see below). Another SO-derived feature is reflected in the irregular arrangement and morphology of the cell lacunae. In addition, osteocyte lacunae are more densely spaced in the woven bone cord than in HOPB. The absolute thickness of the woven bone trabeculae ranging from 2 to 4 cells in sauropods and other investigated dinosaurs (theropods) also fits the range of that found in the same structure of ovine and bovine bone (Kerschnitzki et al. 2011). These

features of SO-derived trabeculae are in sharp contrast with the rest of the non-lamellar part of the bony laminae that clearly shows anisotropic features and elongate osteocytes with uniform longitudinal alignment in longitudinal sections (Fig. 2). The lamellated part also reveals high structural organization with or without alternating extinction pattern and osteocyte orientation in subsequent lamellae (Fig. 4). This clearly implies that the bulk of the bony lamina in sauropod long bones consists of DO-derived primary parallel-fibred bone (synonymized with HOPB) similarly to the primary cortex of ovine and bovine long bones (Ascenzi et al. 1967; Martin & Ishida, 1989; Kerschnitzki et al. 2011). The majority of HOPB exhibits structural alignment along the longitudinal axis of the bone with some lamellae showing transverse organization.

Fibre orientation in SO- and DO-derived fossil bones cannot be observed directly, however, it can be inferred with high confidence. Until now, the optical behaviour of the thin section under cross polarized light was the most frequently used method in determining original fibre orientation in fossil bones. This method is based on the assumption that the apatite crystallites are aligned along the longitudinal axis of the anisotropic collagen fibres in the living bone, and will faithfully show the same arrangement after fossilization (Kolodny et al. 1996; Hubert et al. 1996; Trueman & Tuross 2002). The anisotropy of fibres and/or crystallites is expressed by a direction-dependent variation in brightness under cross polarized light. Depending on how the plane of section relates to the longitudinal axis of the fibres or apatite crystallite rows, they will show different extinction pattern. If the plane of cut is parallel with the long axis of fibres/crystallites, they will exhibit maximum brightness, whereas if the fibres/crystallites are cut transversally, they will appear completely dark under crossed plane polarizers. Any other direction of the section plane relatively to the long axis of the fibres/crystallites will result in intermediate brightness values (Ascenzi & Bonnuci, 1968; Bromage et al. 2003). The use of full-wave (λ) or half-wave (λ/2) plates may improve the quality of the image by introducing a controlled phase shift between the two polarization components of a light wave, altering its polarization and resulting in different color patterns which can help identifying tissues. Quarter-wave plates (λ/4) convert linearly polarized

light (LPL) to circularly polarized light (CPL) thereby eradicating the obscuring effect of extinction crosses. Guidelines and methodological considerations for the use of CPL are provided by Bomage et al. (2003). If standardized thickness of the thin sections (100 ± 5 μm) is achieved, with the help of a circumpolarized light and grey-level analysis, finer-scale quantification of fibre orientation becomes possible (Bromage et al., 2003, 2009, 2011). However, the efficiency of this method might be interfered by diagenetic extinction-alterations which usually determine how thick the section of fossil bones must be to get the desired resolution of the microstructure. Hence, it is clear that there is always a need for at least two section planes to reconstruct the original three-dimensional structure based on the preserved optical features in a fossil bone. Nevertheless, important aspects of this optical-directional coherence have largely been overlooked by paleohistological investigations which mainly or exclusively relied on transverse sections when drawing inferences on fibre orientation in fossil specimens. This is the most important factor that has led to mistaking longitudinally oriented HOPB for woven bone in sauropods, and most probably in a number of other dinosaurs too (longitudinally structured HOPB will appear completely dark and isotropic in transverse section on the basis of which it has been interpreted as woven bone, see below). However, even if the bone sample is cut in multiple planes, relying only on optical features revealed using either LPL or CPL techniques may be misleading, since diagenetic alterations may after all have modified the original extinction pattern of the fossil bone tissue. Based on the findings of Kerschnitzki et al. (2011), lacunocanalicular features of osteocytes help decrease the uncertainties related to determining original fibre orientation in a fossil bone. Kerschnitzki et al. (2011) visualized the osteocytic network in ovine, bovine and murine primary bone using staining and different microscopic methods. They found a strong organization of the lacunocanalicular network (LCN) in DO-derived, thus parallel-fibred primary bone with the long axis of the lacunae aligned parallel to the predominant orientation of collagen fibres, and the canaliculi running perpendicular to the fibres as well as to the long axis of the lacunae. The same correlation between the LCN and fibre orientation exists in secondary bone, but there is no regular pattern of LCN in the SO-derived

woven bone (Kerschnitzki et al. 2011). This finding is in accordance with the irregular spatial arrangement and thus randomly oriented interconnections of the immobile SO-osteoblasts. Hence, the structural organization of LCN is also highly indicative of large scale collagen fibre arrangement, and thereby SO- vs. DO-derived bone tissues (i.e. woven vs. parallel-fibred bone) can clearly be distinguished in fossil tissues as well.

Because the spatial organization of collagen fibril bundles produced by the individual SO-osteoblasts does not exceed the canalicular radius of a few cells, Kerschnitzki et al. (2011) referred to woven bone as 'microlamellar bone', indicating the high organization of the collagenous matrix on a cellular level. However, since we restrict the term 'lamellar' to structures described in the above sections, we do not adopt this terminology for woven bone. On the other hand, it follows that at cellular level, woven bone may not appear completely dark and isotropic but locally can also reveal light extinction under crossed plane polarizers (also evident in murine woven bone, Kerschnitzki et al. 2011). Indeed, in some cases we found more expressed extinction in the woven trabeculae than in the HOPB which remained all dark in the transverse sections of sauropod long bones. Such a pattern is to be expected if HOPB is longitudinally organized and the fibres/crystallites are cut perpendicular to their longitudinal axis. This strengthens the identification and structural interpretation of woven bone and HOPB in the sauropod long bones investigated in the current study (Fig. 2).

In the light of our results it is evident that future paleohistological studies will have to consider the combination of optical features and lacunocanalicular morphology in multiple section planes to draw inferences on the original micro- and ultrastructure of fossil bones. Figure 6 demonstrates a revised general model of the macro- and microstructure (A-C) and optical behavior (D-F) of the plexiform bone of sauropods representing the new interpretations of this study. The model in Figure 6 was based on the investigated specimen *Apatosaurus* CM 3378 and thereby represents only one of the various possible structural organizations of sauropod primary bone. In order to sum up the effects of the plane of sectioning on the appearance of histological features, this

model indicates the cellular (B,C) and optical appearance (E,F) of the thin section in two section planes.

(3) Causes of misinterpretations and erroneous reasoning in paleohistological studies

The term 'fibrolamellar' was devised to describe the composite nature of this bone tissue type consisting of 'fibrous' and lamellar bone. 'Fibrous bone' is the direct translation of 'Faserknochen', a term that had been applied by Gross (1934) to all types of periosteally deposited 'non-osteonal' primary bone, which he therefore synonymized with 'Periostknochen'. Thus, Gross' category 'Faserknochen' did not only refer to true woven bone with randomly distributed cells in the unorganized matrix (which he termed 'restlicher Faserknochen'), but also to the structurally organized non-lamellar primary bone (which he called 'zonarer Faser- oder Periostknochen)', and even to the acellular periosteal bone (Gross, 1934). With this term, he aimed to express that 'fibrous bone' mostly shows clear signs of 'being fibred' ("Der Faser- oder Periostknochen…ist meist sehr deutlich gefasert."); a feature that is much less apparent in woven bone than in primary bone with highly organized fibre arrangement. In spite of that, subsequent authors have synonymized fibrous bone with woven bone (e.g. de Ricqlès, 1974; Francillon-Viellot et al. 1990), and in the 'fibrolamellar' concept it has been systematically used this way ever since. Possibly Gross' (1934) description of 'Faserknochen' was misinterpreted and mistranslated from German, resulting in a suite of confusing bone histological terminology. Fibrous bone (*sensu* Gross, 1934) and woven bone are thus not the same. Because the formation principals of the SO-derived woven bone and the DO-derived non-lamellar HOPB are so different, we do not support the unification of these structures under the common term 'fibrous bone' as Gross (1934) suggested. Moreover, infilling of the primary vascular spaces does not necessarily involve formation of lamellae (e.g. diplodocid indet. NHUB Ki2). Thus, the term 'fibrolamellar bone' reflects neither the developmental origin of the different bony constituents nor the observed diversity in the microstructures of sauropod primary bone, and therefore should be abandoned. Instead, here we suggest that fast growing, thus

highly vascularized composite bone tissue types that consist of the combination of woven bone and HOPB, be categorized only based on their vascular organization. Hence, in the case of sauropod long bones, primary bone tissue should simply be referred to as laminar to plexiform primary bone. This terminology is also consistent with the new concept of DO-defined primary osteons.

Another misleading conception originates from the superficial observation of the optical behavior of woven bone. Due to the well-known haphazard orientation of the fibres and crystallites in woven bone (Chinsamy-Turan, 2005; Currey, 2002), it has generally been claimed to remain dark under crossed plane polarizers, which is in sharp contrast to the alternating bright and dark patterns exhibited by the surrounding lamellar bone when the stage is rotated (e.g. Francillon-Vieillot et al. 1990). This statement became so dogmatic in paleohistology that almost any kind of primary matrix type that stays dark under crossed plane polarizers has been referred to as woven bone. However, it is clear that those areas of the matrix which steadily appear the darkest in the sections are always the ones that contain fibres/crystallites which are cut exactly transversally (Ascenzi & Bonnuci, 1968; Bromage et al. 2003, 2009). Hence, the darkest matrix areas that are rather extensive (measurable on millimeter scale) certainly have a long range order of collagen and/or crystallite organization, i.e. they show a uniform preferred orientation and run perpendicular to the plane of sectioning. In contrast, woven bone does show extinction due to the presence of locally organized fibre bundles at the cellular level (Kerschnitzki et al. 2011). It follows from this that woven bone will always have higher brightness values than the transversally cut HOPB under crossed plane polarizers, even though this brighter appearance is the result of a random composite of darker and brighter areas. The vulgar error that woven bone is completely dark under crossed plane polarizers combined with the almost exclusive usage of transverse sections in paleohistological studies have likely led to mistaking longitudinally organized (thus transversally cut) primary bone for woven bone in many cases.

The effect of the plane of sectioning on the appearance of the lacunocanalicular system (Fig. 3) presented further source of misinterpretation. A number of studies have used aspects of shape,

size, orientation and density of the lacunocanalicular system to infer large scale evolutionary patterns without taking bone three-dimensional structure into account. Rensberger & Watabe (2000) claimed that differences in the angle of the canaliculi sprouting from the osteocyte lacuna represented differential rates of osteogenesis. According to these authors, there is a random orientation of canaliculi on the lacunar surface in the long bones of non-avian theropod dinosaurs and birds, which is in contrast with the strong radial orientation found in ornithischian dinosaurs and mammals. Rensberger & Watabe (2000) interpreted the random canalicular orientation indicative of a lower degree of structural organization (i.e. woven bone), implying higher bone growth rates in non-avian theropods and birds than in ornithischians and mammals with a more organized LCN. Here we want to point out that the random and radial canalicular patterns described by Rensberger & Watabe (2000) at the level of the osteon are actually radial and perpendicular to the long axis of the lacuna respectively on a cellular scale. As prime evidence for differences in the degree of structural organization, the authors provided images (Rensberger & Watabe, 2000, figure 2) of the LCN in secondary osteons. However, all secondary bone is DO-derived, and hence characterized by a strongly organized nature. This disputes the irregular structural organization hypothesized by Rensberger & Watabe (2000), and rather points to local differences in cell orientation in the highly organized matrix. Depending on the plane of sectioning of the cell lacuna, transverse or longitudinal, any DO-derived lacuna will exhibit a rounded or flattened shape with radial or perpendicular canalicular pattern respectively. Thus, we hypothesize that Rensberger & Watabe (2000) most likely documented different patterns of loading in the limb bones they studied rather than real differences in the degree of structural organization (see biomechanical considerations below). Morphological distinction between “flattened” and “stellate” osteocyte morphologies based on ground sections of turtle shell bones described by Cadena & Schweitzer (2012) is loaded with the same uncertainties. For instance, the alternating layers of “flattened” and “stellate” osteocytes shown in the internal cortex of the costal bone of *Podocnemis expansa* demonstrates a plywood like arrangement of longitudinally vs. transversally orientated osteocytes

of HOPB rather than osteocytes of genuinely different 3D shapes. Flattened and stellate morphotypes identified among isolated 3D osteocytes of extant species and the organic lacunocanalicular infilling of the fossil species most probably represent DO- vs. SO-derived osteocytes, respectively (note that 'SO' in Cadena & Schweitzer, [2012] does not refer to static osteogenesis but to stellate osteocytes). For the same reasons, studies that use osteocyte lacuna volume are prone to similar mistakes. Lacuna volume has been used in paleogenomic studies to reconstruct evolutionary changes in the genome size of birds compared to other large tetrapod clades (Organ et al., 2007; Organ, Brusatte & Stein, 2010). Lacuna volumes in these studies were calculated with measurements of lacuna long and short axis taken in classical transverse sections. Because of uncertainties related to the plane of sectioning at the cellular level, at least in some species with longitudinally organized bone, the longitudinal axes of the lacunae must have been significantly underestimated. Montanari et al. (2011) provided further criticism by documenting strong differences in estimated genome size of a single individual, depending on which skeletal element was studied. Pending further investigation, we hypothesize that differences in estimated genome size may lie in differences in the plane of sectioning of the lacunae.

Studies focusing on sauropod bone histology suffer from the same drawbacks (cf. Organ et al., 2009). Transversally cut osteocyte lacunae of the longitudinal HOPB appear round or oval in cross section, thus they have often been identified as the typical rounded, "plump" lacunae of woven bone. Since a considerable proportion of the primary plexiform bone of sauropods is composed of longitudinally organized bone, most of the non-lamellated bony laminae show up dark in transverse sections and as such have been referred to as woven bone. These observations and the erroneous conclusions have resulted in a considerable overestimation of the actual amount of woven bone. De Ricqlès (1968a,b, 1974, 1975) provided detailed three dimensional illustrations of sauropod bone, and he drew the fibres of the non-lamellated primary bone with a longitudinal orientation. However, he did not specifically describe the longitudinal arrangement, and this notion has largely been ignored in the following decades. The results of the current study unequivocally

support de Ricqlès' original conception, and suggest furthermore that this structural arrangement is probably much more widespread among dinosaurs and large mammals (Ascenzi et al. 1967, Martin & Ishida, 1989, Kerschnitzki et al. 2011, and references therein) than has been demonstrated so far.

Building upon the overestimated amount of woven bone, paleohistological studies concluded that one of the reasons why sauropods could reach their enormous adult sizes very fast is because the bulk of the bony laminae in their long bones is composed of fast forming woven bone (Sander et al. 2011 and references therein). Although several characteristics of sauropods indicate that in general they must have grown very fast, our study demonstrated that the amount of woven bone in itself cannot account for the assumed high growth rates; hence this reasoning does not hold anymore. So, instead of focusing on the amount of woven bone, it seems much more important to understand the meaning of its *presence* in the long bones of these animals.

(4) Biological inferences based on SO- and DO-derived tissues in fossils

(a) Woven bone: evolutionary implications for high growth rate

The presence of non-pathologic and non-embryonic woven bone in the form of "fibrolamellar bone" has been reported for the long bones of several extant and extinct tetrapods (e.g. Enlow & Brown, 1957, 1958; de Ricqlès et al. 2003, 2008; Ray, Botha & Chinsamy-Turan, 2004; Klein, 2010; Mukherjee, Ray & Sengupta, 2010), however, in the light of our results, some of these studies may have to be revised. Nevertheless, woven bone has been observed during the scale formation of polypteriform and lepisosteiform fish; two extant but basal groups of actinopterygians (Sire, Donoghue & Vickaryous, 2009). It is important to note that the woven fibered connective tissues described in the scales of these fish are real bony tissues and not dentin-like structures, i.e. they are derivatives of osteogenic and not odontogenic processes (Sire et al. 2009). The undoubtedly basal position of these animals on the phylogenetic tree of vertebrates clearly indicates that the potential for SO had been acquired by the time of diversification of more derived vertebrates such as tetrapods. Since intramembranous as well as some phases of endochondral

ossification follows the SO bone formation model (Ferretti et al. 2002; Palumbo et al. 2004; Marotti, 2010) and because intramembranous ossification has "older" evolutionary origin than endochondral ossification (Wagner & Aspenberg, 2011), SO must have appeared at latest by the time intramembranous ossification was invented.

The evolutionary order of appearance of SO and DO is much more obscure. It has been demonstrated in the development of dermal plates and scutes of teleost fish that the formation of woven bone precedes the deposition of parallel-fibered bone (Sire et al. 2009). Furthermore, it was suggested that DO can only take place in the presence of preexisting osteocytes because the orderly recruitment of DO-osteocytes is controlled by the signal-transduction of the preexisting osteocyte syncytium (Marotti, 1996; Palumbo et al. 2004). Both of these indicate certain developmental constraints on the sequence of SO and DO and suggest that the occurrence of SO might have preceded that of DO in the evolutionary scenario, as well. However, their concurrent appearance is the most plausible hypothesis given the adaptive nature of DO-derived highly organized bone which lies in its superior biomechanical characteristics compared to woven bone (Currey, 2002, see also below). This unequivocally implies that woven bone must be widespread among all kind of vertebrates, and that its presence cannot be considered as an evolutionary innovation in achieving high growth rates. The question which should rather be posed is when, why and how SO-derived woven bone got involved into the periosteal growth of long bones of tetrapods and how do these issues relate to growth rates.

(b) Interrelationships between vascularity, BMR, body size and growth rate

Vascular canals play a crucial role in the formation of woven bone. The mostly avascular long bone cortices of small extant amphibians and lepidosaurs (Foote, 1916; Enlow & Brown, 1956, 1957, 1958) exhibit only DO-derived lamellar or non-lamellar highly organized primary bone. This exclusively accretional, slow bone growth limits the growth rate of these animals. However, from a certain size, temnospondyl amphibians and large extant varanids also show various degrees of

vascularization in the bone cortex (Enlow & Brown, 1958; de Ricqlès et al. 2004; Steyer et al. 2004; Buffrénil, Houssaye & Böhme, 2008). In varanids, this size-specific appearance of vascular canals is independent of phylogeny and seems to reflect the absolute growth rate of the cortex (Buffrénil et al. 2008). Cubo et al. (2005) also demonstrated the positive correlation between bone size and vascular density in sauropsids. Nevertheless, it is still to be explored whether there is a critical shaft diameter (which correlates with body mass in graviportal and mediportal tetrapods, [Anderson, Hall-Martin & Russel, 1985; Alexander, 1989]) at which vascular canals start to be incorporated into the long bone cortex of tetrapods. Besides this size factor, the relatively highly vascularized cortices of extant crocodylians could be considered a reversal from a more advanced thermal and metabolic biology that characterized their archosaurian ancestors (de Ricqlès et al. 2003, 2008; Seymour et al. 2004; Cubo et al. 2012). In fact, some studies concluded that extant crocodyles still possess the capability of growing fast under certain circumstances (Tumarkin-Deratzian, 2007; Owerkowicz, Elsey & Hicks, 2009; Cubo et al. 2012).

Size-dependent vascularity, i.e. the trend that larger-bodied forms show more densely vascularized long bone cortices, also applies to extant homeothermic amniotes with generally high basal metabolic rate (BMR) and growth rate, namely birds and mammals (Klevezal, 1996 pp. 22; Padian, de Ricqlès & Horner, 2001; Erickson et al. 2009). Nevertheless, they still possess a higher number of cortical vascular canals than a poikilotherm animal of similar size but of much lower BMR. The presence of this trend in vascularity vs. body size in both groups implies a common cause which must be irrespective of the physiological differences between them. This common cause most probably originates in biomechanics, fluid mechanics and scaling laws related to nutrient transport within the bone tissue (Mishra, 2009). Above a certain distance between the blood supply and cells (~150 μm), further fluid and solute transportation requires additional energy input because of growing hydraulic resistance. The relatively constant distance found in large bodied dinosaurs (*Iguanodon*) and mammals (cow) implies a functional and metabolic optimization of the transportation path distance in the vascular-lacunocanalicular system of bones (Mishra, 2009). On

the other hand, the apparent deviations in the degree of vascularity of equally sized members of the considered clades might be explained by the differences in BMR. High BMR is related to enhanced aerobic capacity associated with elevated oxygen consumption of all cellular tissues (Bennett & Ruben, 1979; Biewener, 2003) including bone, therefore the osteocytes in animals with high BMR have higher metabolic demands than the osteocytes of similar sized animals with lower BMR. Providing the osteocytes with sufficient supply at high rates requires more extensive vascularity in the bone cortex than that found in similar sized animals with low BMR (Montes, Castanet & Cubo, 2010; Seymour et al. 2011). Furthermore, the complexity of the vascular-lacunocanalicular organization of bones also increases with increasing body mass and BMR (Mishra, 2009), and BMR itself universally scales with respect to body mass (Bishop, 1999; White & Seymour 2005; Speakman, 2005; West & Brown, 2005; but see Kolokotrones et al. 2010). In sum, there is a positive correlation between vascularity (density and/or total porosity percentage) and body size, as well as vascularity and BMR (Montes et al. 2010). Vascularity in the long bone cortices of different tetrapods is most likely determined by the combination of body size and BMR with mostly structural and functional components, even though a phylogenetic influence is also to be expected to some degree (Cubo et al. 2005). The obvious relation between BMR and growth rate is that only high BMR can motorize fast growth, since besides sustaining other essential body functions, a considerable amount of energy must be invested into growth (Cubo et al. 2008; Montes et al. 2007, 2010). Dense vascularization of the fast growing bone characterized by elevated tissue metabolism is also essential above a certain cortical thickness (i.e. body size, see above). The fact that body size of the largest poikilotherm tetrapods never exceeds that of the largest endotherms in a similar environment implies that growth rate limits maximum body size/body mass an animal can achieve in its lifespan. Hence, high vascular densities and enormous body sizes of sauropods all point to high BMR and high growth rates in these animals.

(c) The role of woven bone in fast growth: a model for bone histomorphogenesis in sauropods

Due to the characteristics of woven bone (providing *de novo* formed scaffold for DO, see above), the involvement of SO into the primarily DO-based periosteal growth of the long bone shaft greatly extends the potential upper limits of diametrical growth rates. In the following description, we demonstrate how woven bone can contribute to the acceleration of long bone growth throughout sauropod ontogeny. For the sake of simplicity, only diametrical growth of the long bone shaft will be considered here.

In the sauropod embryo, the cartilage precursor of limb bones is embedded in a thick layer of densely vascularized mesenchyme. The outermost region of the cartilage precursor is an avascular layer of condensated progenitor cells, called the perichondrium (Hall, 2005). Signaling of the hypertrophied chondrocytes in the cartilage precursor stimulates the invasion of capillaries, and induces the differentiation of the perichondrial cells into osteoblasts, ultimately forming the periosteum (Karaplis, 2008). At this stage of development, the periosteum corresponds to the bone collar, which is the precursor of the cortical region of the limb bone (Ferretti et al. 2002; Hall, 2005; Karpalis, 2008). The bone collar is a thick, vascularized tissue layer surrounding the cartilaginous shaft, in which further maturation of pre-osteoblasts is either controlled by SO or DO processes. As SO starts to take place, randomly oriented mesenchymal cells condensate into 1-3 cells thick layers half way between the adjacent capillaries of the bone collar, and start to differentiate into stationary osteoblasts. At the same time, in other regions of the periosteum, DO-controlled osteoblasts are being organized into osteogenic laminae. Whereas the static osteoblasts produce the first thin trabeculae of porous, irregularly fibred woven bone between the capillaries, the DO-derived osteogenic lamina starts to deposit highly organized bone matrix on already existing surfaces like the limb precursor or the forming SO-derived trabeculae. Since the SO-derived woven framework incorporates large vascular spaces into the bony substance within a short period of time, fast diametrical growth can be realized by means of fast volume expansion of the forming limb bone shaft. SO-osteoblasts start to mineralize their secreted matrix very early, filling up the pores with apatite crystallites. As this SO process extends diametrically and more and more vascular spaces get

enclosed into the shaft, the firm substrate of SO-derived trabeculae provides the initial conditions for the formation of more and more osteogenic laminae on their surface. With the deposition of highly organized bone on the surface of the SO-scaffold, the DO-derived laminae uniformly thicken the trabeculae in all directions and fill up the entrapped vascular spaces. The onset of this process launches the formation of primary osteons (redefined in this study, see above). Meantime, the SO-osteoblasts in the woven trabeculae differentiate into osteocytes *in situ* retaining their unorganized osteoblastic appearance. From time to time, those DO-osteoblasts of the osteogenic lamina which are destined to become osteocytes, stop secreting matrix and get buried into the osteoid continuously produced by the neighboring osteoblasts. As a consequence, the osteogenic lamina moves away from the entrapped osteoblasts. The latter soon differentiate into osteocytes in a spatially highly organized manner aligning their long axis to the long range order of the collagen fibres, parallel to the long axis of the limb bone. The osteoblasts of the osteogenic lamina begin to mineralize the deposited extracellular matrix during which process plate-like apatite crystallites develop along and within the collagen bundles with their c-axis (long axis) following the long axis of the fibres.

After the sauropod embryo hatches, the diametrical expansion as well as compaction of the developing limb bone performed by SO and DO processes, respectively, continue the same way in the active growth phases of postembryonic ontogeny. As DO proceeds and compacts the primary vascular spaces, the osteogenic lamina may at some point start to form lamellae around the progressively narrowing vascular space. These lamellae might have alternating fibre orientation in which case the secretion activity of the osteogenic lamina slows down or ceases periodically while all the osteoblasts in the lamina uniformly and synchronously change their orientation, and soon they start to produce a new sheet of highly organized matrix but with differently oriented collagen bundles. In sum, the combination of SO-controlled fast volume expansion and DO-controlled bone compaction characterizes the phases of active growth, and their proportional contribution to the

growth process determines the diametrical growth rate of the limb bone in the different growth phases.

As the animal matures and approaches its maximum size, less and less vascular canals get encased into the growing bone, whereby the high diametrical expansion rate of the limb bone drastically decreases. Since there is a well-defined bony substrate, the surface of which is much less interrupted by vascular canals by this time, DO-derived bone deposition now dominates the growth of the outermost cortical region. As soon as no SO-derived woven bone is needed anymore, the diametrical growth of the limb bone is ensured exclusively by DO processes. At this stage, the relatively thin periosteum does not exhibit any SO ossification centers, but only its innermost cell-layer is organized into a unified osteogenic lamina which still deposits bone on the surface of the bone shaft. This slow accretional growth provided by the periosteum is not perpetual but rather cyclical producing lines of arrested growth in the almost avascular outermost cortex. The onset of this process is the onset of an EFS (*sensu* Ham, 1953). In each and every subsequent cycle, the amount of bone deposited by the periosteum decreases, and as a consequence, LAGs get more densely spaced. This residual growth can go on for a long time, however, the amount of deposited bone is so negligible that by this time the animal is said to be skeletally fully grown. Finally, the osteoblasts of the periosteum differentiate into lining cells (basically inactive osteoblasts), and this membrane of lining cells covers the fully grown bone shaft.

The model clearly demonstrates that woven bone has a crucial role in fast diametrical bone growth. However, the significance of woven bone does not lie in its absolute amount in the growing bone, since woven bone trabeculae make up only a low fraction (~14-25%) of the entire bony lamina. Its high formation rate is clearly of importance but not in the same sense as it would be if the majority of the primary bony substance was composed of woven bone as it has always been assumed. There is not one particular feature but rather a combination of key features of woven bone (*de novo* formation without substrate, high formation rate and fast mineralization) which allows it to enclose extensive vascular spaces with its forming network of trabeculae, thereby creating a highly

porous, fast expanding composite of vascular cavities and bony substance. Subsequent deposition of DO-derived highly organized bone is responsible for the infilling of the extensive vascular spaces and providing the skeleton with higher resistance against mechanical loading.

Although the SO-controlled volume expansion is much faster than the DO-controlled compaction of the growing bone, it is important to note, that the comparison of the formation rate of SO-derived woven bone with that of DO-derived highly organized bone can be very misleading because they have such different formation principles. First of all, only the actual amount of bony substance should be reckoned with when formation rate of SO- vs. DO-derived bone is considered, since woven bone cannot take credit for the huge enclosed vascular spaces. Second, whereas the irregular orientation of the secretory territories of the static osteoblasts results in a highly porous matrix, the matrix deposited by the osteogenic lamina is much more compact. This means that the bulk of the woven bone matrix consists of holes which only provide extra volume but do not contribute to the dry organic matter of the bone tissue. If osteoid (mainly collagen) secretion rate is considered to be the true measure of bone formation rate, then the secretional activity of the SO- and DO-osteoblasts must be compared. The osteoid secretion rate of each osteoblast depends on the amount of its protoplasm engaged on a given bone surface (Marotti, 1976; Ferretti et al. 2002). Since no structural or ultrastructural differences have been observed between SO- and DO-osteoblasts so far (Marotti, 2010), we hypothesize that secretion activity can be highly variable depending on several exogenous and endogenous factors (e.g. actual physiological state, ontogenetic stage, environmental conditions etc.) but might be irrespective of the SO or DO origin of osteoblasts. This suggests that on the cellular level, the secretion activity i.e. matrix production rate of SO-derived osteoblasts is not necessarily higher than that of DO-osteoblasts.

Osteocyte density clearly depends on the incorporation rate of the osteoblasts into the matrix, and it has been suggested by some authors that it represents overall growth rate (Bromage et al. 2009; Skedros, Hunt & Bloebaum, 2004). SO-osteoblasts differentiate into osteocytes *in situ*, thus the term incorporation rate has no meaning in the context of woven bone. The primary density

of SO-osteoblasts, on the other hand, could be related to the growth rate of the animal; however, the assumed correlation is still to be explored. The density of osteocytes in DO-derived bone tissue depends on how many osteoblasts stop producing matrix in a given area within a given time period (i.e. on the incorporation rate) which, similarly to the secretion activity of the cells, might also be influenced by a variety of exogenous and endogenous factors. Thus, incorporation rate and thereby osteocyte density of DO-derived bone tissues may indeed correlate with growth rate but this relation is still largely unexplored.

It must be emphasized that woven bone is always present in fast growing bones even if its significance does not lie in its absolute volume. This means that its presence in long bones is indeed indicative of fast growth. However, in large bodied animals, woven bone can only initiate fast volume expansion if sufficient amount of blood vessels are also provided. Thus, allowing for the bone growth related issues discussed above, the most reliable histological indicator of bone growth rate of all candidates suggested so far (e.g. vascular architecture, fibre orientation, woven bone amount, osteocyte density etc. see above) is probably the vascular density and/or total porosity percentage (de Margerie, Cubo & Castanet, 2002). Nevertheless, this assumption also needs further testing without neglecting possible phylogenetic, biomechanical and/or developmental constraints.

(d) Evolutionary implications on fast growth

The evolutionary innovation which gave way to fast skeletal growth (with special emphasis on the appendicular skeleton) was the involvement of static osteogenesis into primarily dynamic osteogenic processes, i.e. *de novo* bone formation into accretional bone formation in the diametrical growth of limb bones. Combined with the enhanced blood supply of the developing bone, SO-derived woven bone permits fast volume expansion, whereas DO-derived highly organized bone accounts for the mechanical stability of the growing skeleton. In many vertebrates that exhibit this combination in their limb bone development, fast skeletal growth has been experimentally proven (Castanet et al. 2000; de Margerie et al. 2002; 2004; Starck and Chinsamy 2002).

The incorporation of SO into predominantly DO-controlled skeletal growth processes evolved independently in several tetrapod lineages (see "fibrolamellar bone" in de Ricqlès et al. 2003, 2008; Botha-Brink, Abdala & Chinsamy-Turan, 2012; Ray, Bandyopadhyay & Bhawal, 2009). The independent appearance of fast skeletal growth by means of fast volume expansion through simultaneous encasement of extensive vascular spaces is further supported by the apparent independent evolution of high BMR in birds and mammals. In an integrative biological view, all fundamental resources (mitochondria, advanced circulatory system and lungs for acquiring high BMR, SO- and DO-derived bone) had already evolved in the earliest tetrapods. Evolving a more active metabolism, enabling fast growth, therefore became possible on several lineages. A considerable evolutionary flexibility of physiological traits is exemplified by the reverse thermobiology and growth strategy of modern crocodiles (de Ricqlès et al. 2003, 2008; Cubo et al, 2012; Seymour et al. 2004; Tumarkin-Deratzian et al. 2007; Woodward, Horner & Farlow 2011). Thus, the evolution of high BMR as well as fast growth is most likely motorized by long-term environmental and ecological factors and less constrained by constructional aptitude of vertebrates.

Our new interpretation on the histomorphogenesis of sauropod laminar bone has significant impact on the reconstruction of the evolution of sauropod giantism. However, our results also support the long held view that the growth rate of sauropods was very high, even if this assumption is mostly based on the extensive bone vascularity and enormous body sizes. Once this capacity was there, almost everything became possible: from reaching body masses up to 80-100 tons (e.g. *Argentinosaurus*, [Hokkanen, 1986], *Amphicoelias* [Paul, 1998]) to the complete loss of gigantic dimensions probably along with physiological reversals and becoming island dwarfs (*Europasaurus* [Sander et al. 2006] and *Magyarosaurus* [Stein et al. 2010]).

(e) *Implications for biomechanics*

The main function of the skeleton is to provide the body with mechanical support, thus bone is required to resist mechanical forces. Bone strength depends on the microarchitectural features,

turnover rate and characteristics of the mineral and collagen phases (density, orientation) of the bone tissue. In this complex system, the mineral constituent is responsible for the stiffness of the bone, whereas collagen with its high tensile strength provides the bone with toughness by assuring flexibility and absorbing the energy of forces acting on the bone (Viguet-Carrin, Garnero & Delmas, 2006 and references therein). The mineral component is mostly integrated into the collagen fibrils (Ottani et al. 2001), hence the crystallite orientation is determined by the orientation of the fibres. Since the amount and orientation of these two components are clearly decisive in the biomechanical properties of bones (Acsenzi & Bonucci 1967, 1968; Beniash, 2011; Currey, 1987; Frost, 1994), differences in their spatial arrangement as well as relative proportions reported in woven bone vs. highly organized primary bone must reflect the different mechanical behaviour of SO- and DO-derived bone tissues. The SO-derived woven bone has no long range preferred fibre orientation, it is initially highly porous, and later becomes highly mineralized. Since apatite crystal orientation is parallel to the long axis direction of adjacent collagen fibrils in both intra- and extrafibrillar spaces, the long range random arrangement of fibrils and the extensive extrafibrillar spaces in woven bone result in a more irregular spatial alignment of crystals (Su et al. 2003). As a result, woven bone is very brittle and cannot withstand considerable mechanical impacts. In contrast, in DO-derived highly organized bone, the closely packed fibril bundles with integrated crystallites have a long range preferential orientation. Although it is less mineralized than woven bone, it has improved mechanical properties, which indicates that collagen fibre orientation is much more important than the degree of mineralization in determining the mechanical properties of bone (Martin & Ishida, 1989; Marotti, 2010). Thus, predominance of woven bone in the weight bearing limb elements characterizes mostly embryonic stages, in which mechanical constraints on the skeleton are certainly much less than in any post-hatching stage. In extant animals, a proportionally large amount of woven bone in non-embryonic developmental stages can only be found in the limb bone cortex of very small species with high BMR such as mice (Kerschnitzki et al. 2011). As body mass increases, the proportional amount of woven bone decreases, and DO-derived highly

organized bone tissue will dominate the cortex of the limb elements. The low percentage of woven bone in favour of HOPB in the primary cortex of the sauropod limb bones is therefore to be expected.

The origin of the predominantly longitudinal organization of DO-derived highly organized bone in sauropods remains an important question. The significance of the orientation of mineralized collagen fibres in bone strength has been shown by several studies (e.g. Ascenzi & Bonucci, 1967, 1968; Martin, 1993; Martin et al. 1996; Martin, Burr & Sharkey,1998; Boyde & Riggs, 1990; Riggs, Lanyon & Boyde, 1993; Bromage et al. 2003; Skedros et al., 2003; Skedros & Hunt 2004; Skedros et al. 2007) all concluding that in a limb bone, longitudinal fibre orientation is found in the cortical regions under tension, whereas transversally running fibres characterize the areas under compressive loading. These results suggest that collagen fibre orientation can be used to infer main loading regimes on the bone. Nevertheless, limitations of this method originate from the diverse endogenous and exogenous factors which determine predominant collagen fibre orientation (CFO) in the limb bones and whose proportional influence can change throughout ontogeny. In earlier developmental stages, CFO seems to be controlled mainly by genetic and epigenetic factors (endogenous), whereas extragenetic stimuli, such as mechanical loading or microcracks only later become the prevailing inducers of fibre arrangement and/or rearrangement (Skedros et al. 2007). Findings of Riggs et al. (1993) support this hypothesis, demonstrating that the consistent CFO pattern found in the radius of adult horses, which unequivocally reflected the consistent pattern of stress-strain distribution in the bone, did not correspond to the predominantly longitudinal CFO revealed in the foals. Longitudinal CFO characterized the entire primary cortical bone, seemingly irrespective of strain directions, however, remodeled areas showed tensile (longitudinal CFO) as well as compressive (transverse CFO) secondary osteons (Ascenzi & Bonucci, 1967, 1968) following the region-specific pattern of mechanical loading (Riggs et al. 1993). Thus, predominantly longitudinal CFO in the primary bone of horses, the histology of which is strikingly similar to that found in the humerus of *Alamosaurus* TMM 46300-2 (Fig. 4B), seems to be

controlled by genetic and epigenetic regulatory systems rather than by mechanical stimuli. This is in accordance with the conclusions of the study of Skedros et al. (2007) which were based on the pattern found in ovine calcanei. Primary plexiform bone exhibits the same preferred longitudinal CFO in ovine, bovine and sauropod limb bones (Ascenzi et al. 1967; Martin & Ishida, 1989; Kerschnitzki et al. 2011; this study). This conformity in the histological composition of the limb bones of very distantly related groups (mammals and dinosaurs) suggests functional (analogous) rather than identical genetic and epigenetic (homologous) fibre organizing principles. On the other hand, the apparent variability in predominant CFO in a monophyletic clade like dinosaurs (e.g. *Scutellosaurus* [Padian, Horner & de Ricqlès, 2004], *Jeholornis* [Erickson et al. 2009] or even *Ampelosaurus* with its "modified laminar bone" [Klein et al. 2012]) further implies that functionality may obscure the phylogenetic signal in this histological feature. The functional aspects of vascular architecture have also been demonstrated (de Margerie, 2002; Skedros & Hunt, 2004, de Margerie et al. 2005). Thus, CFO along with vascular architecture probably reflects the functional constraints present throughout ontogeny (Skedros et al. 2003). However, predominantly longitudinal CFO in the HOPB may also reflect the ancestral condition of tetrapods. Finally, it is important to note that, contrary to Amprino's rule (Amprino, 1947), which is generally applied in paleohistological studies, the concept that CFO in primary bone is in itself suggestive of growth rate is highly unlikely.

IV. CONCLUSIONS

(1) Longitudinal thin sections of sauropod long bones revealed that in contrast to the general reconstruction, the amount of woven bone in sauropod long bones is only a few cells thin layer in the laminae and the majority of the primary bone is longitudinally organized. This structural organization also characterizes the plexiform primary bone of large bodied

mammals, and is likely to be more widespread among large tetrapods than previously thought.

(2) The overestimation of the amount of woven bone in sauropod primary bone originates mostly from the almost exclusive usage of transverse sections examined under crossed plane polarizers, whereby the optical behaviour as well as the LCN characteristics of the different bony constituents have been misinterpreted.

(3) Current biological studies, which revealed two major bone formation types, static and dynamic osteogenesis (SO and DO) resulting in woven and highly organized bone, respectively, gave detailed explanation for the detectable differences between bone tissues of different developmental origin. Our better understanding of the formation process and role of SO- and DO-derived bone tissues demonstrates the need for investigating optical behavior combined with LCN characteristics of thin sections of fossil bones in at least two section planes to make sure that the differentiation of the tissue types is justified.

(4) In the revised palaeohistological terminology presented here, HOPB is introduced to describe all (lamellar and non-lamellar) DO-derived primary bone tissues and differentiate them from SO-derived woven bone. In accordance with the long range parallel fibre orientation in HOPB, the term primary parallel-fibred bone should be synonymized with HOPB rather than used to describe the suggested intermediate state of spatial organization of fibres which, based on the presented bone formation principals, may not even exist. Lamellation should be identified using both the characteristics of extinction pattern and the presence of fine lines. Based on the common formation principals but diverse structural organization of HOPB, we suggest that the term primary osteon include the entire DO-derived primary tissue (lamellated and non-lamellated) which has been deposited on the SO-derived scaffold of woven bone around the vascular canals. The widely used term "fibrolamellar" should be discarded as it demonstrates neither formation principals nor structural diversity of primary bone tissues. Instead, we recommend the usage of vascular

architecture for structural classifications of fast growing primary composite tissue of woven bone and HOPB.

(5) The most important evolutionary innovation, which made fast growth possible, and which seems to have happened independently on several lineages, is most probably the incorporation of woven bone into the primarily DO-derived periosteal growth of the long bone shaft.

(6) The significance of woven bone in long bone growth rates does not lie in its amount but rather in its capacity to rapidly enclose large vascular spaces thereby ensuring fast diametrical growth. SO accounts for the fast volume expansion, whereas DO guarantees compaction and improvement of the mechanical properties of primary bone tissues.

(7) The primary indicator of high bone growth rates is most likely vascular density and/or porosity, whereas woven bone is probably only a secondary requirement needed to encase the extensive vascular network into the growing bone. Since sauropod long bones all exhibit high vascular densities in their plexiform primary tissues, we support the long held view that sauropods had similar growth rates to those of extant endotherms.

(8) The brittle, hypermineralized SO-derived woven bone is mechanically reinforced by the deposition of DO-derived HOPB, in which long range fibre orientation (CFO) is most likely controlled by genetic and epigenetic factors in earlier ontogenetic stages, and by mechanical requirements in more advanced ontogenetic stages. Since CFO in secondary osteons of long bones is a more reliable indicator of loading regimes than the predominant CFO is in primary osteons, functional aspects may be more reflected in secondary than in primary bone tissues.

(9) The mostly longitudinally organized HOPB found in the plexiform bone of sauropods and large bodied mammals might imply functional convergence or alternatively can indicate the ancestral tetrapod condition.

(10) Preferred CFO in the primary bone of dinosaurs or any other tetrapods is not indicative of growth rate disputing this aspect of Amprino's rule which has been the basis of inferences drawn in several palaeohistological studies.

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VI. APPENDIX 1. INSTITUTIONAL ABBREVIATIONS

CM Carnegie Museum of Natural History, Pennsylvania, USA; NHUB Naturkundemuseum of the Humboldt-Universität Berlin, Germany; OMNH Oklahoma Museum of Natural History, Norman, Oklahoma, U.S.A.; SMA Sauriermuseum Aathal, Aathal, Canton Zürich, Switzerland; TMM Texas Memorial Museum, Austin, Texas, U.S.A.

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VIII. FIGURE CAPTIONS

Figure 1. Comparative overview of transverse vs. longitudinal thin sections of the plexiform primary bone of long bones of various sauropods under crossed plane polarizers. Comparison of the paired images of transverse (above) and longitudinal (below) sections brought to the same scale clearly shows that the majority of the dark areas in the transverse section, which has been identified as woven bone in previous studies, light up brightly in the longitudinal section disputing its presumed unorganized nature. True woven bone is represented only by a thin, centrally positioned dark stripe distinct on the longitudinal slides, whereas the rest of the bony lamina is evidently composed of highly organized primary bone (HOPB). The sections presented as paired images were prepared using the same bone drill core of the considered specimen. (A) *Alamosaurus* TMM 43600-2, humerus; (B) *Apatosaurus* CM 3378, humerus; (C) diplodocid indet. NHUB Ki2 ("*Barosaurus*" sensu Janensch, 1961; *Australodocus* sensu Remes, 2007), femur; (D) *Apatosaurus* "Chris" SMA M4/10-1, femur; (E) diplodocid indet. SMA 647/87-1, femur; (F) *Camarasaurus* "ET" SMA 0002, femur. Note that the dark patches in the longitudinal section of *Camarasaurus* do not reflect original fiber orientation but represent preservational artifacts.

Figure 2. Large-scale and projected close-up images of thin sections of the bony lamina. Both, transverse and longitudinal thin sections of (A-F) *Alamosaurus* TMM 43600-2 humerus and (G-L) *Apatosaurus* CM 3378 humerus are presented under single as well as crossed plane polarizers. Large-scale images (A,D,G,J) show the overall cortical microstructure in transverse (A,G) and longitudinal (D,J) sections under plane polarized light with the white square indicating the projected and magnified area of the cortex. 40x magnified transverse sections under plane (B,H) and cross polarized light (C,I), and corresponding longitudinal sections under plane polarized light (E,K) and crossed plane polarizers (F,L). Based on the observed lacunocanalicular network (LCN) features and extinction patterns in transverse and longitudinal sections of the specimens, only a few cells

thin layer of woven bone is present, and the non-lamellar part of the highly organized primary bone (HOPB) has a long range longitudinal structural arrangement. Due to the prevailing longitudinal alignment of HOPB, the LCN is characterized by transversally cut, thus rounded lacunae (tol) with radial canalicular system (rc) in the transverse sections, and by longitudinally cut, thus elongate, spindle-shaped lacunae (lol) with perpendicular canalicular system (pc) in the longitudinal sections. Osteocyte lacunae of the woven bone (olw) are larger and have genuinely irregular shapes and canalicular network irrespective of the cutting plane. The extinction of transverse and corresponding longitudinal sections under crossed plane polarizers is complementary in HOPB, whereas woven bone retains the same, darker appearance in both section planes. Further abbreviations: colw, confluent osteocyte lacuna of woven bone; HOPB, highly organized primary bone; vc, vascular canal (primary cavity). Bony lamina is defined *sensu* Sander (2000).

Figure 3. Interpretative drawing representing a schematic model of the effect of the different cutting planes on the general 2D appearance of the lacunocanalicular network (LCN). 3D osteocyte lacuna model cut in three different planes (transverse, longitudinal and oblique) through (A) and offset from (B) its center of symmetry results in different projected 2D shapes, sizes and canalicular arrangements. Relatively to the long axis of the osteocyte lacuna, a transversally cut lacuna (tol) appears rounded with radial canalicular system, whereas a longitudinally cut lacuna (lol) exhibits elongate, flattened shape with perpendicular canalicular system. Other sectioning planes will result in obliquely cut osteocyte lacunae (ool) with variable transitional lacunocanalicular arrangements. The lacunae will exhibit the largest 2D area when they are cut through their center of symmetry (A). Depending on the level of the offset of the cutting planes relatively to the center of symmetry, the lacunae will grossly retain their shape but will appear smaller (B) compared to any situation represented in (A).

Figure 4. Different degrees of lamellation in the plexiform primary bone of three different sauropod specimens under single and crossed plane polarizers. Paired images are taken of the same section under plane polarized light (above) and crossed plane polarizers (below). The bony lamina consists of woven bone (wb), and lamellated (l) and non-lamellated (nl) HOPB. (A) Longitudinal section of *Apatosaurus* CM 3378 humerus with three to four distinct lamellae which can be distinguished around the primary vascular space (vc) based on the fine lines (fl) under plane polarized light, and on the alternating fiber orientation (al) under cross polarized light. (B) Lamellation in the transverse section of *Alamosaurus* TMM 43600-2 humerus is very extensive, being deposited right on the surface of the woven bone, hence non-lamellar HOPB is absent. Lamellae can be traced in form of densely packed and well-defined fine lines under plane polarized light, but are hard to observe under crossed plane polarizers because originally the fiber orientation did not change in the subsequent lamellae. Due to this preferred longitudinal orientation in each lamella, the entire bony lamina (woven bone + HOPB) appears dark in the transverse section under crossed plane polarizers. (C) At the other extreme, the magnified area of the transverse section of diplodocid indet. NHUB Ki2 femur shows no lamellation, but exclusively non-lamellar HOPB. Woven bone is hardly discernable (wb?) and seems to occur only in scarce patches in this specimen.

Figure 5. Relative amount of lamina constituents in percentage of total lamina thickness in different sauropod long bone samples based on values given in Table 2. Constituents are measured following the definition of lamina *sensu* Sander 2000, from the center of one vascular canal to the center of the subsequent one. This visualisation demonstrates the low amount (max 25% of the total lamina thickness in diplodocid NHUBKi2) of woven bone (wb) compared to the HOPB in all sauropod samples, but also the general pattern in proportional distribution of the different lamina constituents measured in the investigated specimens. The only specimen that shows considerable deviation from this pattern is *Alamosaurus* TMM 43600-2, humerus exhibiting proportionally high amount of lamellar subunit in the total thickness of HOPB.

Figure 6. Summarizing model of the macro- and microstructure of sauropod plexiform primary bone. Three-dimensional visualization of the vascular architecture and long range fibre arrangement in a slice of the primary cortex of a sauropod long bone (A), and the theoretical overall extinction pattern of the transversally and longitudinally cut surfaces of the same slice under crossed plane polarizers (D). Black strips are the primary vascular spaces with surrounding lines representing lamellae. Between the vascular canals, the centrally positioned, irregularly shaped grey stripes represent the woven bone layer; the end product of static osteogenesis (SO). Parallel lineation on the longitudinally cut surface and dots on the transversal surfaces (A) indicate the preferred longitudinal fiber orientation of the non-lamellar HOPB formed by dynamic osteogenesis (DO). This longitudinal structural arrangement with the very thin woven layers in the bony laminae results in a predominantly dark appearance of the transversal side, and a mostly bright appearance of the longitudinal side of the 3D slice under crossed plane polarizers (D). White squares on the transversal and longitudinal surfaces indicate the magnified and projected areas (B,C,E,F). (B,C) Schematic drawing of the lacunocanalicular network (LCN) arrangement in the bony laminae in transverse (B) and longitudinal (C) sections with the indication of the cutting planes and the resulted 2D appearance of a 3D osteocyte lacuna model (see also Fig. 2). (E,F) Optical features of (B) and (C), respectively, under cross polarized light. Note that the modeled changes in the LCN and extinction in the subsequent lamellae with alternating fiber orientation and the LCN and extinction in the non-lamellar HOPB all reflect that the long range fiber orientation in DO-derived HOPB corresponds to the long axis of the aligned lacunae, and is perpendicular to the canalicular network (in accordance with Kerschnitzki et al. (2011)). The SO-derived woven bone does not show any regular pattern in LCN, and has a uniformly dark appearance under crossed plane polarizers. On the basis of formation principles, primary osteon is redefined here as the primary vascular space surrounded by lamellated and/or non-lamellated DO-derived HOPB. The illustration is largely based on *Apatosaurus* CM 3378, and therefore represents only one of the several possible

structural organizations of primary tissue in sauropod long bones (for further details see main text). Abbreviations: es, endosteal surface; lp, longitudinal plane; ps, periosteal surface; tp, transversal plane; vc, vascular canal.

IX. TABLES

Table 1. Details of the studied materials providing novel results. First description refers to the source publication of HOS and first description of transverse sections of the specimen. Abbreviations: el, element type (h, humerus; f, femur); HOS, histological ontogenetic stage (*sensu* Klein and Sander, 2008); l, element length; sc, element smallest diaphysis circumference.

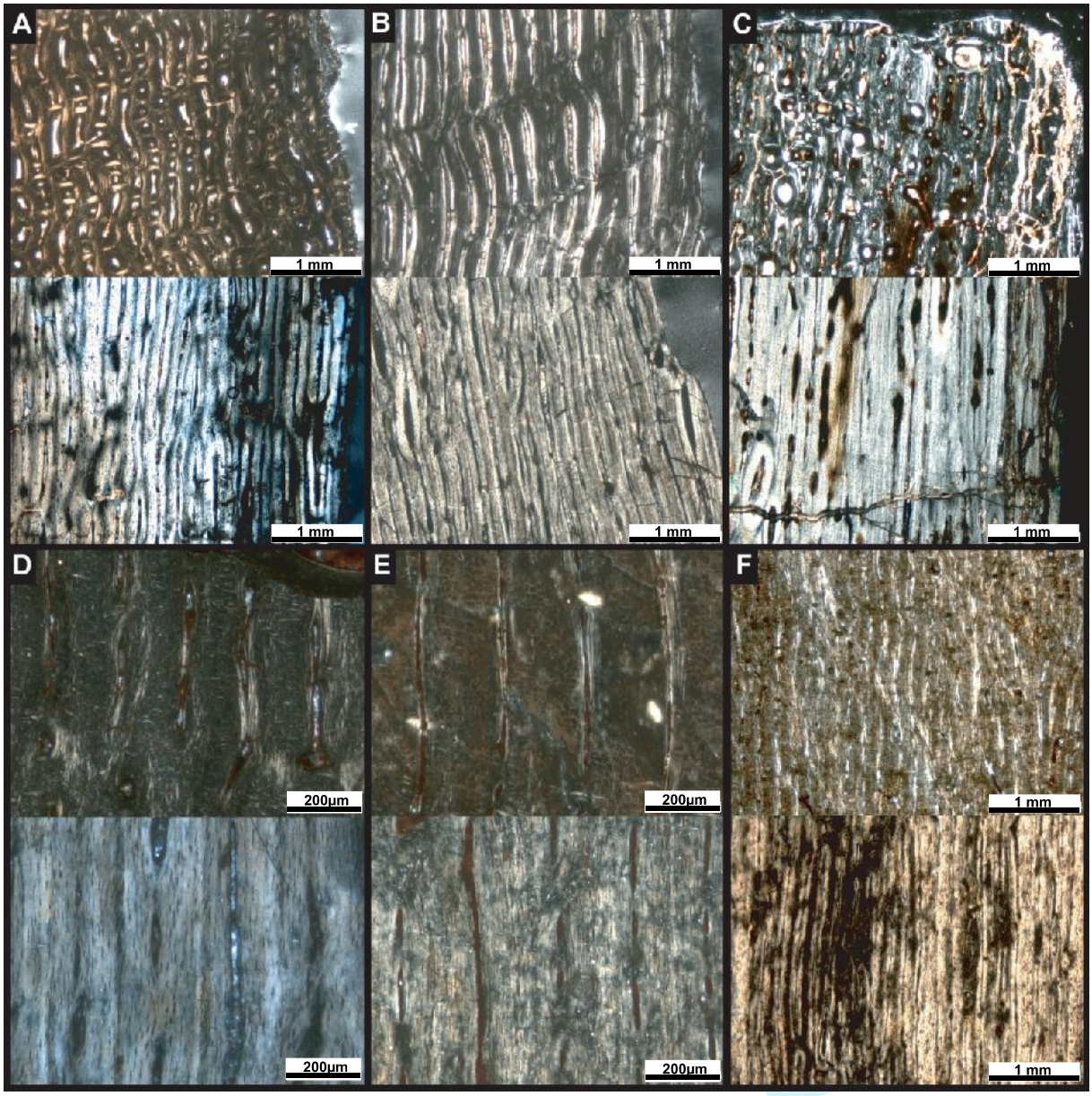
Taxon	Specimen id.	el	l (mm)	sc (mm)	HOS	first description
Alamosaurus	TMM 46300-2	h	915	375	7	Stein et al., 2010; Klein et al. 2012
Apatosaurus	OMN H1279	f	340	190	4	Klein and Sander, 2008
Apatosaurus	OMNH1278	h	258	144	4	Klein and Sander, 2008
Apatosaurus	CM 3378	h	980	492	11	Klein and Sander, 2008
Apatosaurus	SMA "Jaques"	f	1640	725	10	Klein and Sander, 2008
Apatosaurus	SMA "Chris" M4/10-1	f	1440	525	12	Klein and Sander, 2008
Camarasaurus	SMA "ET" 0002	f	935	422	12	Klein and Sander, 2008
diplodocid indet.	NHUB Ki2	f	1190	420	10.5	Sander, 2000; Klein and Sander, 2008
diplodocid indet.	SMA 647/87-1	f	1200	433	8	Klein and Sander, 2008

Table 2. Means and standard deviations of the thickness of different lamina constituents in the measured specimens. Note that identical bony constituents on the left and right side of the woven bone (wb) layer were individually measured and the values are indicated separately. Due to its bad preservation, *Apatosaurus* SMA "Chris" M4/10-1 was excluded from the analysis. Abbreviations: lHOPB, lamellated highly organized primary bone thickness; Mn, mean; N, number of measurements per specimen; nlHOPB, non-lamellated highly organized primary bone thickness; SD, standard deviation; pv, primary vascular canal width; tl, total lamina thickness; wb, woven bone layer thickness; wbf, thickness fraction of woven bone in one lamina. *For some values of lamellar bone, standard deviations are higher than the actual means. This is the consequence of many null-measurements, i.e. complete absence of lamellar components in many laminae.

Specimen	N	pv (µm)		lHOPB (µm)		nlHOPB (µm)		wb (µm)		nlHOPB (µm)		lHOPB (µm)		tl (µm)		wbf (%)	
		Mn	SD	Mn	SD	Mn	SD	Mn	SD	Mn	SD	Mn	SD	Mn	SD	Mn	SD
Alamosaurus TMM46300-2	20	34.66	10.57	55.54	13.60	56.99	10.42	38.73	11.64	38.30	11.22	44.33	14.81	260.76	41.10	15	4
Apatosaurus OMNH1278	22	71.01	18.56	1.80	3.99*	31.11	8.43	37.60	7.63	52.29	15.06	2.07	5.55*	195.89	17.18	19	4
Apatosaurus OMNH1279	19	42.79	22.29	5.28	4.96	39.76	13.49	29.50	5.07	57.72	13.17	3.36	5.13*	178.41	29.53	17	3
Apatosaurus CM3378	21	26.54	13.20	36.20	16.23	61.36	20.69	34.50	5.78	62.21	27.79	32.50	15.11	253.32	29.40	14	2
Apatosaurus SMA Jaques	19	17.24	14.35	16.27	7.32	60.32	19.12	43.61	7.78	68.05	13.73	19.94	8.01	225.42	30.67	19	3
Camarasaurus SMA ET	20	11.58	4.86	5.74	7.22*	52.03	22.58	41.04	10.18	51.87	17.38	4.79	6.94*	167.05	23.14	25	7
diplodocid NHUB Ki2	20	17.57	8.50	0.64	2.84*	54.64	13.82	35.81	9.80	51.22	15.26	0.73	3.28*	160.61	23.97	22	5
diplodocid SMA 647/87-1	19	18.98	11.95	14.52	12.42	63.24	15.02	39.64	9.76	74.15	13.16	14.82	7.30	225.35	18.33	18	4

Table 3. Linear regression parameters for element shaft circumference vs. absolute woven bone thickness, woven bone fraction and total lamina thickness in sauropod plexiform bone based on the mean values given in Table 2.

Linear regression		Pearson's R	df	Two tailed p-value	Intercept	Slope
independent	dependent					
shaft circumference	mean woven bone thickness	0.783	8	0.007	26.41	0.025
	mean lamina thickness	0.642	8	0.045	136.391	0.16
	mean woven bone fraction	-0.128	8	0.725	0.198	-0.0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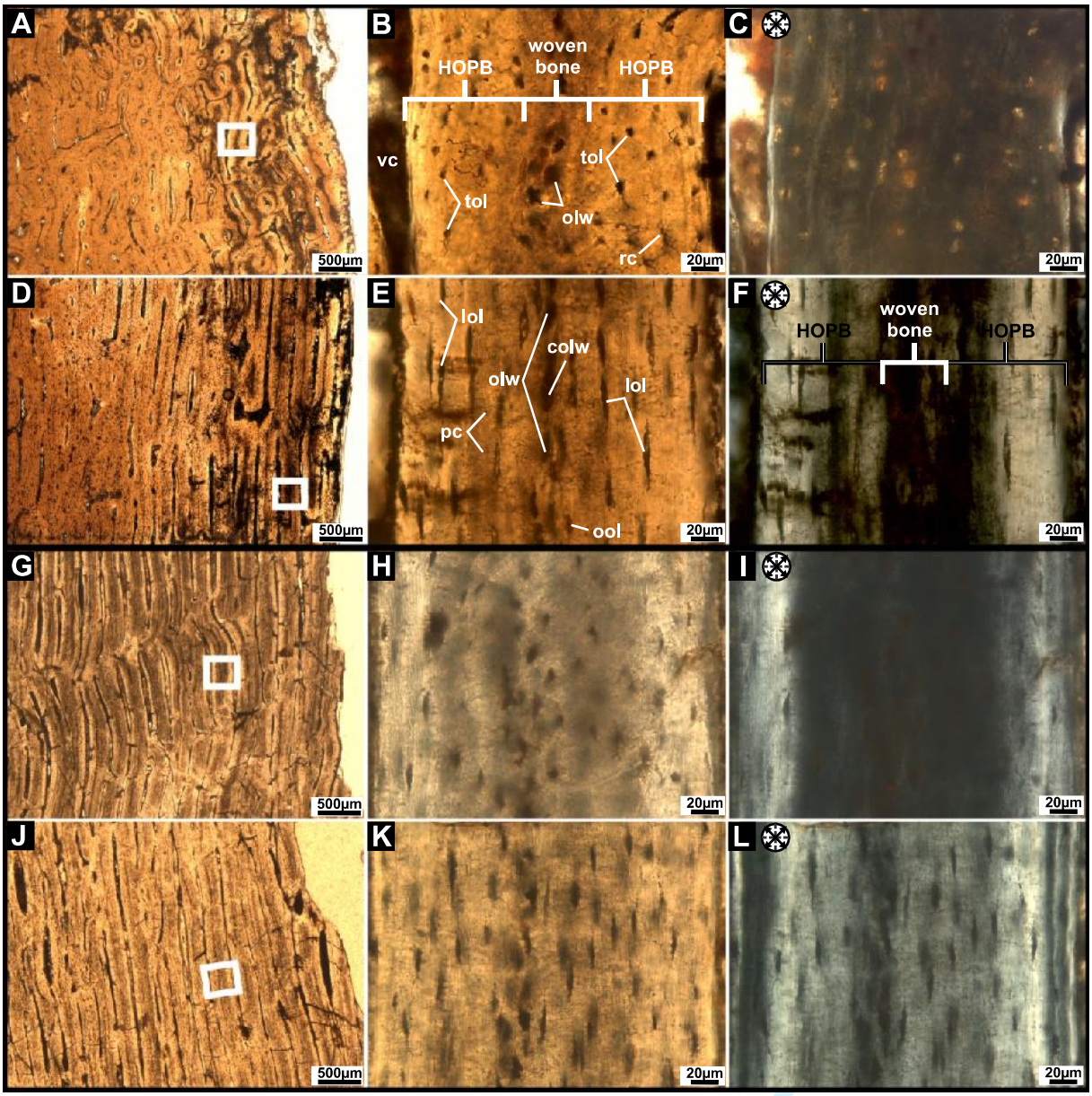
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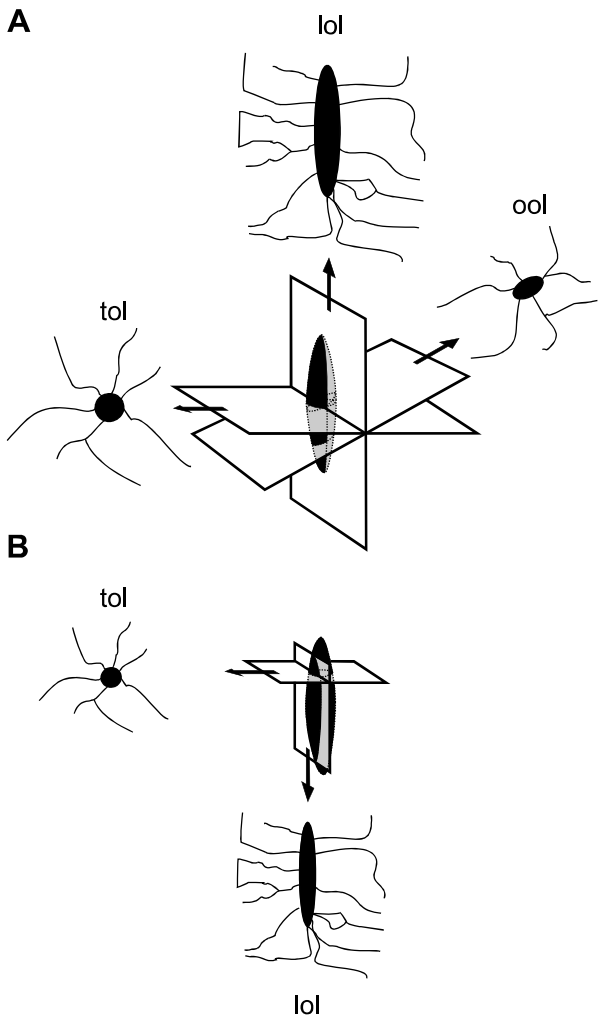
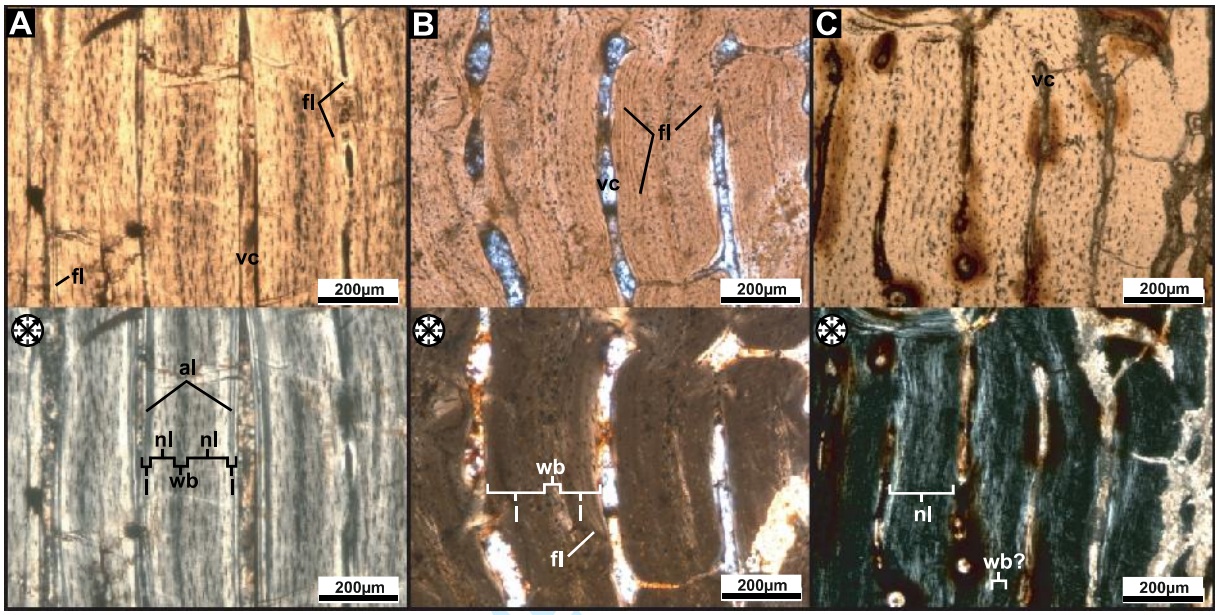
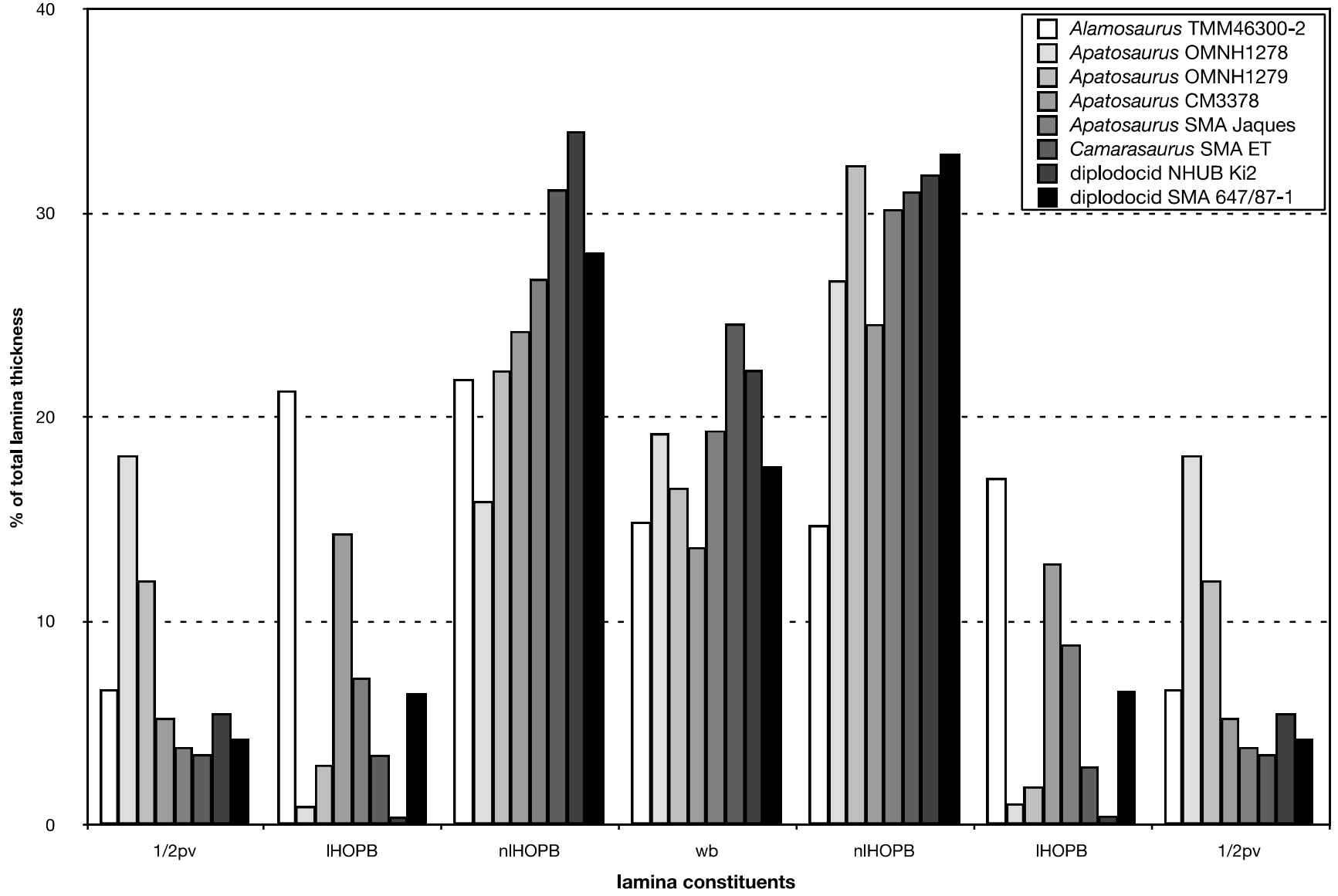
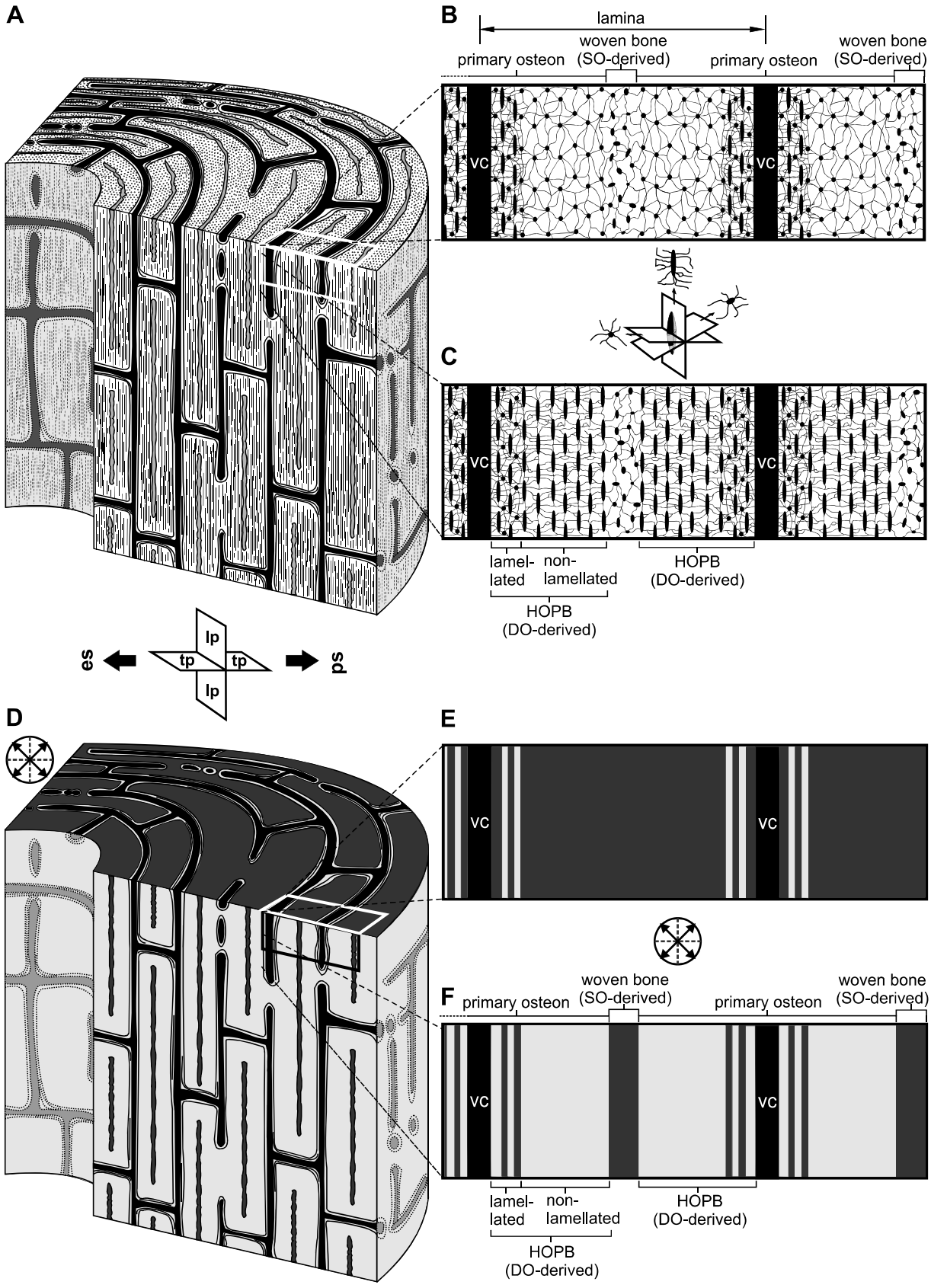
B
HOPB
woven bone
HOPB
vc
tol
tol
olw
rc
20µm
C
20µm
D
500µm
E
lol
olw
colw
lol
pc
ool
20µm
F
HOPB
woven bone
HOPB
20µm
G
500µm
H
20µm
I
20µm
J
500µm
K
20µm
L
20µm






40
30
20
10
0
% of total lamina thickness
Alamosaurus TMM46300-2
Apatosaurus OMNH1278
Apatosaurus OMNH1279
Apatosaurus CM3378
Apatosaurus SMA Jaques
Camarasaurus SMA ET
diplodocid NHUB Ki2
diplodocid SMA 647/87-1
1/2pv
lHOPB
nlHOPB
wb
nlHOPB
lHOPB
1/2pv
lamina constituents


A
B
lamina
woven bone
(SO-derived)
primary osteon
primary osteon
woven bone
(SO-derived)
VC
VC
C
VC
VC
lamel-
lated
non-
lamellated
HOPB
(DO-derived)
HOPB
(DO-derived)
es
ps
lp
tp
tp
lp
D
E
VC
VC
F
woven bone
(SO-derived)
primary osteon
primary osteon
woven bone
(SO-derived)
VC
VC
lamel-
lated
non-
lamellated
HOPB
(DO-derived)
HOPB
(DO-derived)