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Ontogenetic changes in the histological features of zonal bone tissue of ruminants: A quantitative approach



Changements ontogéniques dans le tissu osseux zonal de ruminants : une approche quantitative

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ABSTRACT

Bone histology is a powerful tool to explore the growth patterns of vertebrates. There is a broad consensus that a deeper understanding of bone development in living taxa is still lacking. Here, we aim to explore the ontogeny of the fibrolamellar (FLC) zonal bone of mammals by studying histological sections of the femoral growth series of 84 wild ruminants. Our results indicate that large ruminants do not preserve a complete ontogenetic record of primary bone growth, so it is necessary to use methods of age retrocalculation. Our study also stresses the ontogenetic variation in histological features of the FLC-zonal bone (vascular orientation, vascular and osteocyte lacunae density) that may reflect the slowdown in growth associated with the onset of physiological maturity. We conclude that the transition from FLC bone to lamellar bone (EFS) in ruminants records the fundamental life history trade-off between growth and reproduction (reproductive maturity).

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R É S U M É

L'histologie osseuse est un puissant outil d'exploration des patrons de croissance des vertébrés. Il existe un vaste consensus sur le fait qu'une connaissance approfondie du développement des os dans les taxons vivants n'existe pas encore. Et le but du travail présenté ici est d'explorer l'ontogénie de l'os fibro-lamellaire zonal (FLC) de mammifères, grâce à l'étude de coupes histologiques d'une série sur la croissance du fémur de 84 ruminants sauvages. Nos résultats indiquent que les grands ruminants ne conservent pas d'enregistrement ontogénique complet de la croissance osseuse primaire, ce qui oblige à utiliser des méthodes de rétrocalcul de l'âge. Notre étude est focalisée sur la

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variation ontogénique des traits histologiques de l'os fibro-lamellaire zonal FLC (orientation vasculaire, densité des vaisseaux et des lacunes d'ostéocytes), qui peuvent refléter le ralentissement de la croissance, associé à l'apparition de la maturité physiologique. Nous en concluons que la transition entre os fibro-lamellaire (FLC) et os lamellaire (EFS) chez les ruminants enregistre le compromis fondamental, dans l'histoire de la vie, entre croissance et reproduction (maturité reproductive).

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1. Introduction

Amprino (1947) and later Enlow and Brown (1956–1958) were the first to relate the histological organization of bone tissue (spatial arrangement of collagen fibers and vascular network) to the rate of periosteal bone deposition. Accordingly, a poorly organized (loosely-packed collagen fibrils) and densely vascularised bone tissue grows relatively faster than highly-organized (closely-packed collagen fibrils) and poorly vascularised bone (Francillon-Vieillot et al., 1990). Several recent studies have focused on the relationship between bone tissue traits and bone deposition rate, termed Amprino's rule, through experimental analyses using *in vivo* bone labelling (Castanet et al., 2000; de Margerie et al., 2002, 2004; Montes et al., 2010).

The presence of regular growth marks (i.e., lines of arrested growth 'LAGs') in the transverse sections of bones of many vertebrates has traditionally been interpreted as a result of seasonal cycles of bone growth following natural environmental cycles, similar to the growth rings of trees (Castanet et al., 1993; Chinsamy-Turan, 2005; Klevezal, 1996). Today, there is strong evidence of annual cyclicity of LAG formation from most groups of living vertebrates (Castanet, 2006; Huttenlocker et al., 2013; Köhler et al., 2012; Woodward et al., 2013).

All the above is the basis for many investigations of physiological and life history aspects of extinct and extant vertebrates using bone histology. The study of the microstructure of fossil bones is widely considered the most reliable analytical tool to reconstruct growth strategies and physiology of extinct vertebrates (Chinsamy-Turan, 2005; Horner and Padian, 2004; Köhler and Moyà-Solà, 2009; Padian and Lamm, 2013). On the other hand, bone histology is a powerful tool for determining life history traits (e.g. longevity) of extant wild populations, key data for demographic studies of survival rate, and life expectancy and generation time. These studies also have major implications for the field of conservation biology (García-Martínez et al., 2011; Marín-Moratalla et al., 2013).

Despite its potential, however, there is broad consensus that a better empirical knowledge of living taxa is necessary for the correct interpretation of bone histology (Marín-Moratalla et al., 2014; Woodward et al., 2014). Experimental studies based on vital labelling are undoubtedly the best way to correlate bone tissue patterns with life history events and growth rates (Castanet et al., 2000; de Margerie et al., 2002, 2004). However, these kinds of studies are practically inviable in wild populations without interfering with their development, and because of the

difficulties involved in recapture and newly marking of wild individuals. Hence, non-experimental studies linking bone microstructure with life history traits and physiology of wild populations are also essential (Köhler et al., 2012; Marín-Moratalla et al., 2013, 2014).

Recently, Köhler et al. (2012), using a comprehensive global study of wild ruminants, provided the strongest evidence to date that the presence of regular LAGs in bone tissues (zonal bone) is a plesiomorphic condition shared by all vertebrate groups (Padian, 2012; Padian et al., 2013) and not a feature restricted to vertebrate ectotherms (e.g., amphibians and reptiles) as traditionally thought (Chinsamy-Turan, 2005; Klevezal, 1996). This study provided a model for the correlation between cyclical bone growth and seasonal physiology in endotherms. According to this model, growth is arrested during the unfavourable season concurrently with decreases in physiological and endocrine parameters forming part of a thermometabolic strategy for energy conservation. On the other hand, intense tissue growth at the beginning of the favourable season reflects the capability of efficiently exploiting and allocating abundant resources to growth when food is plentiful (Köhler et al., 2012).

The study of Köhler et al. (2012) has allowed a better understanding of the formation of zonal bone in mammals. However, other aspects of the ontogenetic variation in histological features of bone tissue as well as in bone remodelling are less well-known. So far, these traits have not been quantitatively assessed in studies of growth in extant mammals. Generally, periosteal growth rate slows as the bone develops (zone thickness between LAGs typically decreases), along with the vascular and cellular density. Moreover, secondary bone and medullary cavity expansion remodel the primary cortex, obscuring much of the record of growth (Woodward et al., 2013). These general ontogenetic changes in bone tissue are important in mammals because the growth and development in endotherms, in an allometric context, is faster than in ectothermic tetrapods (Castanet, 2006; Castanet et al., 2004). This, together with the misleading assumption that LAGs are uncommon in mammals, explains why there are so few studies of bone histology in this group in comparison to, for instance, the great number of studies of reptiles.

A deeper understanding of the ontogeny of bone tissue in wild mammals is crucial to determine aspects of the life history and physiology of this group of vertebrates, both extant and fossil (Köhler and Moyà-Solà, 2009; Marín-Moratalla et al., 2011). Today, it is not clear to what extent the study of growth marks in the bone tissue of mammals (skeletochronology) reliably records the chronological age of the animal, because bone remodelling is so important in

Table 1

Sample of study. Femurs of 84 wild individuals from 30 ruminant species.

Tableau 1

Échantillonnage de l'étude. Fémurs de 84 individus sauvages appartenant à 30 espèces de ruminants.

Family	Subfamily	Species	Juveniles		Adults		Total	
			F	M	F	M		
Bovidae	Bovinae	<i>Tragelaphus imberbis</i>	–	–	–	1	1	
		<i>Tragelaphus scriptus</i>	1	–	–	3	4	
		<i>Tragelaphus speikii</i>	–	1	–	1	2	
		<i>Tragelaphus strepsiceros</i>	–	1	–	1	2	
		<i>Tragelaphus derbianus</i>	–	–	–	1	1	
		<i>Aepyceros melampus</i>	–	1	–	2	3	
	Antilopinae	<i>Oreotragus oreotragus</i>	–	–	1	1	2	
		<i>Raphicerus campestris</i>	–	–	1	2	3	
		<i>Antidorcas marsupialis</i>	1	–	1	2	4	
		<i>Eudorcas rufifrons</i>	–	–	–	2	2	
		<i>Eudorcas thomsonii</i>	–	–	–	1	1	
		<i>Nanger granti</i>	–	–	–	1	1	
		<i>Gazella dorcas</i>	1	–	–	2	3	
		Cephalophinae	<i>Sylvicapra grimmia</i>	1	–	–	2	3
			<i>Philatomba monticola</i>	–	–	1	–	1
			<i>Cephalophus natalensis</i>	–	–	1	1	2
			<i>Cephalophus callipygus</i>	–	–	1	1	2
	Reduncinae	<i>Redunca fulvorufula</i>	–	–	–	1	1	
		<i>Redunca redunca</i>	–	–	–	2	2	
		<i>Kobus kob</i>	–	–	–	2	2	
		<i>Kobus ellipsiprymnus</i>	–	–	–	2	2	
		<i>Kobus megaceros</i>	–	–	–	1	1	
	Alcelaphinae	<i>Connochaetes gnou</i>	–	–	–	2	2	
		<i>Damaliscus lunatus</i>	1	–	1	1	3	
	Hippotraginae	<i>Addax nasomaculatus</i>	–	–	1	1	2	
		<i>Oryx dammah</i>	–	–	–	2	2	
		<i>Oryx gazella</i>	–	–	2	1	3	
Cervidae	Cervinae	<i>Cervus elaphus hispanicus</i>	1	4	3	3	11	
		<i>Rangifer tarandus platyrhynchus</i>	2	4	3	4	13	
	Capreolinae	<i>Capreolus capreolus</i>	–	–	–	3	3	
Total			8	11	16	49	84	

F: females; M: males. Ontogenetic stage determined by the state of epiphysis fusion.

this clade (Castanet, 2006; although it is also clear in many reptiles: Padian et al., 2015). Moreover, there is an important controversy over the biological meaning (sexual or skeletal maturity) of the onset of deposition of the external fundamental system (EFS: Cormack, 1987), the avascular lamellar tissue present in the outer cortex of adults. On the other hand, it is necessary to know to what extent it is important to take into account the ontogenetic variation in comparative studies on bone histology (Marín-Moratalla et al., 2013, 2014).

To shed light on all the above issues, here we explore the ontogenetic changes throughout the zonal bone of extant wild ruminants. We studied growth series of histological sections of the midshaft femur of 30 species of almost all ruminant tribes from a wide range of habitats. Some histological features such as the number of LAGs, growth cycles (GC), the cellular (osteocyte lacunae) and vascular density, and the vascular orientation in primary bone were quantified. Ruminants are an excellent group on which to conduct such a study, because most species have a relatively long juvenile period, with osteogenesis continuing beyond at least 1 year, a basic condition for the formation of zonal bone. The primary bone tissue deposited during the juvenile period reflects the fibrolamellar complex (FLC: de Ricqlès, 1974), which may be interrupted

by LAGs (FLC-zonal bone). Interspecific variation in vascular arrangement and osteocyte density in FLC bone has been related mainly to body mass: larger bovids display more circular canals and lower cell densities than smaller bovids (Marín-Moratalla et al., 2014). After maturity, adults deposit lamellar bone with rest lines in the outermost cortical layer, called EFS (Köhler et al., 2012).

2. Material and methods

We analyzed thin sections from right femora of 84 wild individuals from 3 species of cervids and 27 species of bovids (Table 1, see supplementary material for more information).

Some species include growth series, and others include only adult individuals (epiphyses fused). The sample of red deer (*Cervus elaphus hispanicus*) and roe deer (*Capreolus capreolus*) come from hunting, as well as from carcasses collected in the wild in Spain. Fresh carcasses of Svalbard reindeer (*Rangifer tarandus platyrhynchus*) were collected and sexed by researchers from Norwegian Polar Institute (NPI) during a field campaign in Svalbard archipelago (Norway) in July 2010. All skeletal remains of deer species are housed at the Institut Català de Paleontologia Miquel Crusafont (ICP). Bovid species were hunted from the 1950s

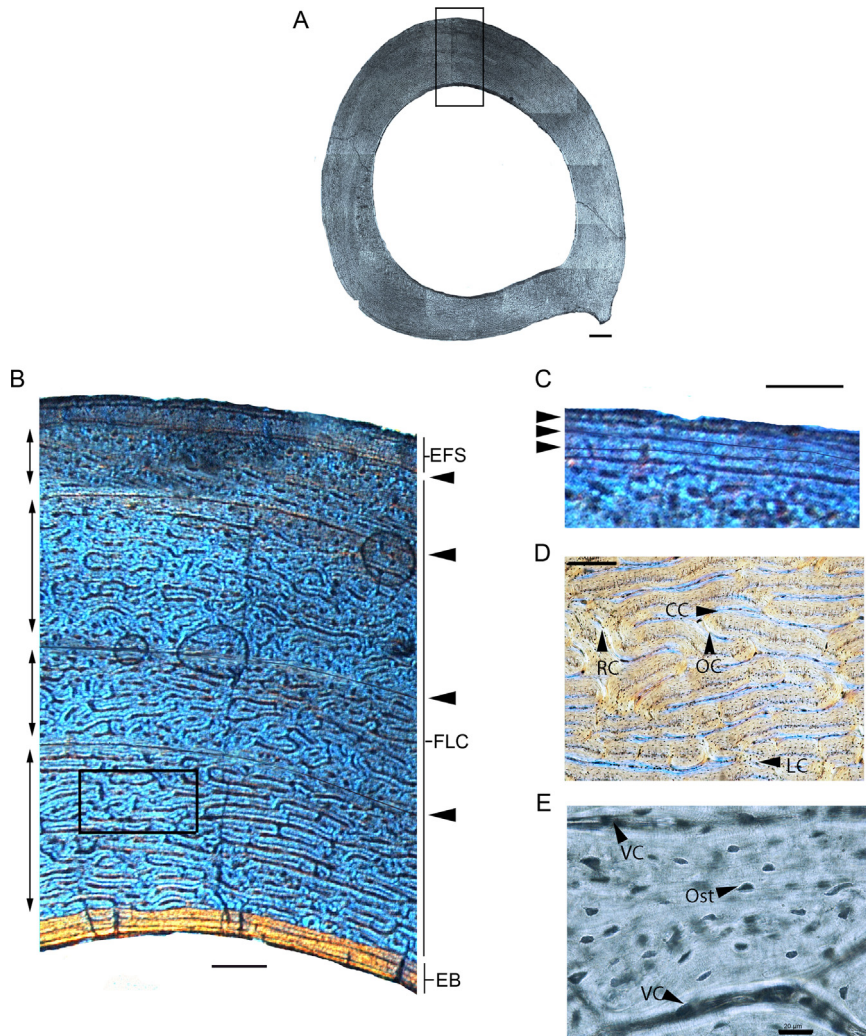


Fig. 1. (Color online.) Histological section of femur of *Cervus elaphus hispanicus* IPS 60873. A. Quantified area of histological data in the anterior region (frame). Scale bar: 2 mm. B. Magnification of the framed area in A. EB: endosteal bone consisting of avascular lamellar tissue; FLC: fibrolamellar complex zonal bone consisting of densely vascularised woven-parallel complex bone interrupted by lines of arrested growth (LAGs); EFS: external fundamental system consisting of avascular lamellar tissue. Zones in FLC (arrows). LAGs (arrowheads). Generally, one zone plus one LAG corresponds to a yearly growth cycle (GC). Scale bar: 500 μm . C. Detail of the EFS showing 3 LAGs (arrowheads). Scale bar: 50 μm . D. Magnification of the framed area in B showing the different types of orientation of vascular canals. CC: circular canal; LC: longitudinal canal; RC: radial canal; OC: oblique canal. Scale bar: 200 μm . E. Quantifying area of osteocyte lacunae density. Ost: osteocyte lacuna; VC: vascular canal. Scale bar: 20 μm .

Fig. 1. (Couleur en ligne). Coupe histologique de fémur de *Cervus elephus hispanicus* IPS 60873. A. Zone quantifiée de données histologiques dans la portion antérieure (cadre). Barre d'échelle = 2 mm. B. Agrandissement de la zone encadrée en A. EB : os endostéique consistant en un tissu lamellaire non vascularisé ; FLC : os fibro-lamellaire zonal complexe consistant en un os complexe très vascularisé à éléments parallèles interrompus par des LAGs ; EFS : système fondamental externe consistant en un tissu lamellaire non vascularisé. Zones en FLC (flèches). LAGs (têtes de flèche). En général, une zone plus une LAG correspond à un cycle de croissance annuel (GC). Barre d'échelle = 500 μm . C. Détail de l'EFS montrant trois LAGs (têtes de flèche). Barre d'échelle = 50 μm . D. Agrandissement de la zone encadrée en B montrant les différents types d'orientations des canaux vasculaires. CC : canal circulaire ; LC : canal longitudinal ; RC : canal radial ; OC : canal oblique. Barre d'échelle = 200 μm . E. Zone quantifiant la densité des lacunes d'ostéocytes. Ost : lacune d'ostéocyte ; VC : canal vasculaire. Barre d'échelle = 20 μm .

until the late 1970s by H. Oboussier, professor at the University of Hamburg (Germany), during several expeditions to Africa. Data (body mass, sex, site and date of death) of most of these individuals are associated (Köhler et al., 2008). The skeletal remains are currently housed at the scientific collections of the Zoological Museum Hamburg University.

Cross-sections of femora were taken at midshaft and thin sections were prepared following standard procedures of our laboratory described in García-Martínez et al. (2011),

Köhler et al. (2012) and Marín-Moratalla et al. (2013). Thin sections were examined under transmitted and polarized light equipped with a digital camera (Leica DM 2500P). The software ImageJ was used to quantify and measure histological traits. Histological data were always quantified in the anterior region of the femora (Fig. 1).

The limb bones such as femur and tibia are commonly preferred in studies of bone development because they are often less subject to secondary remodelling in their

internal cortices than shorter long bones. Moreover, these long bones are generally considered the best skeletal elements for skeletochronology in tetrapods because they preserve the most complete record of growth marks (García-Martínez et al., 2011; Horner et al., 1999; Woodward et al., 2013). In this study, we conducted an analysis for the retrocalculation of missing GC or LAGs in order to explore whether a complete ontogenetic record of primary bone growth is preserved in the femora of ruminants. To carry out this analysis, we measured the antero-posterior diameter of LAG circumferences (APD-LAG) in the successive growth cycles of cortical sections (Marín-Moratalla et al., 2013). The growth curves derived from the sequences of APD-LAG were graphically overlapped among individuals of different ontogenetic stages within-species. In highly dimorphic species (e.g., *C. elaphus hispanicus* and *R. tarandus platyrhynchus*), each sex was analysed separately. We assume an annual periodicity of GC and that cortical cross-sections are similar-sized in similar-aged individuals. The analysis of retrocalculation of age was conducted in those species represented by at least two ontogenetic stages (juvenile and adult), determined by the state of fusion of epiphyses (10 species, Table 1 and supplementary material).

We used phylogenetic generalized least squares 'PGLS' analysis (pglmEstLambda in R package; CAIC library) to correlate the number of GC in the FLC-zonal bone of the species of our sample with their antero-posterior diameter at midshaft femur (within-species average). PGLS analysis computes the least squares regression taking into account the extent of phylogenetic nonindependence (Harvey and Pagel, 1991; Jordana et al., 2014; Orme et al., 2009). We used a synthetic phylogenetic tree with the species of our sample based on a time-calibrated phylogenetic supertree that includes 197 extant and recently extinct species of the suborder Ruminantia (Hernández-Fernández and Vrba, 2005).

To analyse ontogenetic changes in histological features, we quantified vascular orientation, vascular density and cellular (osteocyte lacunae) density in successive growth cycles along the FLC-zonal bone, following the method described in Marín-Moratalla et al. (2014) based on Cubo et al. (2012). Cellular density was quantified as the number of osteocyte lacunae (in one focus plane) divided by the area (mm²) in a given surface of 40× micrographs. Vascular density was quantified as the number of vascular canals divided by the area (mm²) in a given surface of 5× micrographs. Vascular orientation was quantified as the proportion of one kind of vascular canal (longitudinal, circular, oblique and radial) with respect to the total number of canals in a given surface. This analysis was carried out on species composed of at least two males showing at least two GC within the FLC (7 species, see supplementary material). Females were excluded from this analysis due to their small sample size.

3. Results

Fig. 2 shows the growth curves based on the sequence of APD-LAG among individuals of different ontogenetic stages (juveniles and adults). Two males of *C. elaphus hispanicus*

(IPS 60870 and IPS 60875) and one male of *Tragelaphus strepsiceros* (IPS 56211) are the only ones of our sample that have probably lost their first GC (corresponding to the first year of age). Both species are the largest species, and with the longest growth period of our sample. The onset of deposition of the EFS occurs at 5–6 years of age in male red deer and at 6 years in male greater kudu (*T. strepsiceros*). Furthermore, the specimen IPS 60875 is the oldest of the red deer sample, with an estimated age of 10 years based on the number of GC in the whole cortical section including the EFS. However, the 4 years young deer ICP 60870 has also lost its first LAG in the anterior region of the femoral cross-section, though it is still visible in other regions. Therefore, there is some variability within-species. Expansion of the medullar cavity during bone growth and continuous remodeling of the inner cortex during life are responsible for the resorption of the first GC in these specimens. Our results therefore indicate that the largest cervids and bovids may lose their first LAG with increasing age.

We counted the number of GCs in the FLC-zonal bone until the deposition of the EFS in the adult sample (fused epiphyses), which is the same as the length of radial growth of the femoral shaft (Table 2 and Fig. 1). In each species and sex, individuals show equal numbers of GC, except for males of roe deer (*C. capreolus*) and red deer (*C. elaphus hispanicus*) in which the number of GC ranges between 3–4 and 5–6, respectively. Fig. 3 shows the correlation between the number of GC of the species of our sample and their antero-posterior diameter at midshaft femur (within-species average), for males and females separately. As expected, the number of GC increases allometrically which is evidence of the longer growth period of larger ruminants. Moreover, we see that the number of GC is influenced by environmental factors as well: species inhabiting closed environments (deer species), the blue duiker (*Philatomba monticola*) and most species of *Tragelaphini*, as well as those from energy-poor environments (the desert dweller *Addax* and the insular Svalbard reindeer) are above the regression line; while open habitat bovids fall below the regression line. Our results therefore indicate that species of closed habitats and of energy-poor environments show relatively delayed growth periods of the femoral cortex.

We have carried out a compilation of literature data on age of first reproduction of sampled species and we see that the number of GC within the FLC-zonal bone in adults is broadly consistent with data on age at reproductive maturity (Table 2). For instance, in our red deer sample, males show 5–6 GC and females 3 before deposition of the EFS, fitting the age at which red deer normally attains reproductive maturity: 5–7 years in males and 3–4 years in females (Clutton-Brock et al., 1982). It must be clarified, however, that for some species it is not easy to know if the data from literature relate to physiological or to reproductive maturity.

Quantitative changes in vascular orientation, vascular density and osteocyte lacunae density among successive GCs have been explored (Fig. 4). Circular canals are predominant in the FLC-zonal bone of our ruminant sample. However, we observe a general pattern of decreasing frequency of circular canals and an increasing number of

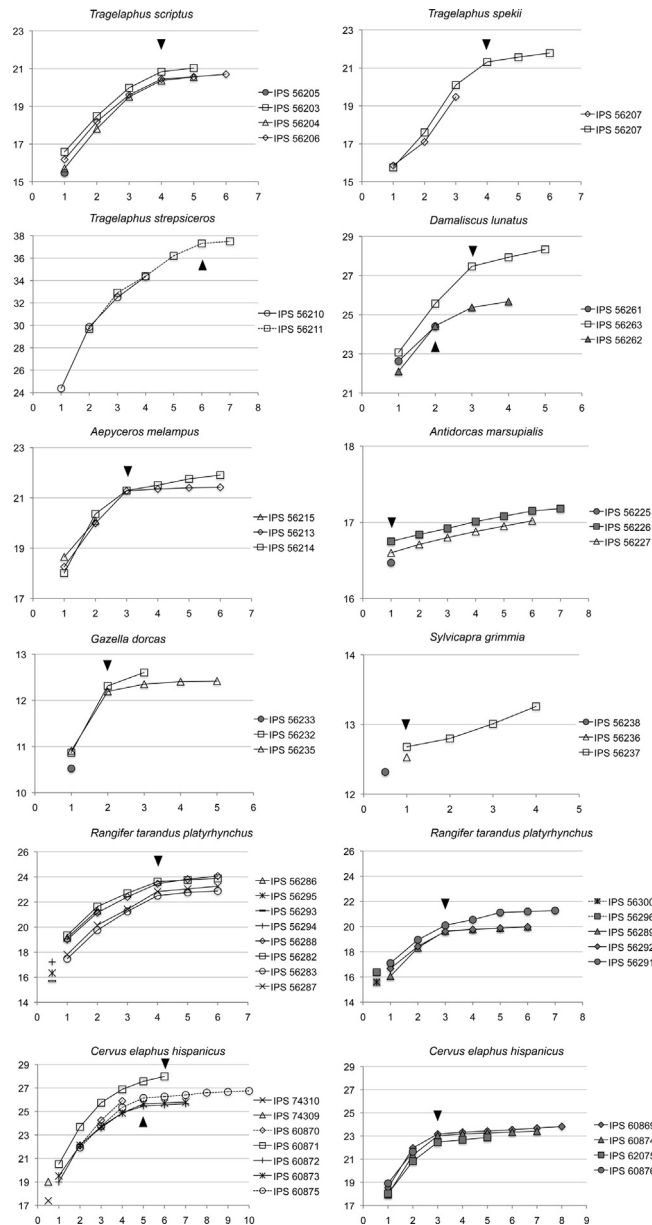


Fig. 2. Plots for the retrocalculation of age. Growth curves of juveniles and adults are overlapped based on the sequence of antero-posterior diameter of line of arrested growth (LAG) circumferences (APD-LAG) in the sample histological sections. Empty points: growth curves of males. Filled points growth curves of females. Arrowhead (onset of deposition of the external fundamental system). Individuals with missing growth cycles are in dotted lines. APD-LAG (mm) in ordinate axis and estimated age (years) in abscissa axis.

Fig. 2. Diagrammes de rétrocalcul d'âge. Les courbes de croissance des juvéniles et des adultes se recouvrent, basées sur la séquence de diamètres antéro-postérieurs des circonférences de LAG (APD-LAG) dans les coupes histologiques de l'échantillonnage. Points représentatifs blancs: courbes de croissance des mâles. Points représentatifs noirs: courbes de croissance des femelles. Tête de flèche (apparition de dépôts du système fondamental externe). Les individus à cycles de croissance manquants sont en lignes pointillées. APD-LAG (mm) en ordonnée, âge estimé (en années) en abscisse.

longitudinal canals along the successive growth cycles from inner to outer cortex. This pattern is more evident in *T. strepsiceros*, *Aepyceros melampus* and *Gazella dorcas*, where the proportion is reversed in the latest growth cycles, becoming longitudinal canals more abundant than circular ones. Radial and oblique canals were less frequent in our sample. As regards vascular and osteocyte lacunae density, our results show that both features tend to decrease in the latest growth cycles. For instance, in our

male red deer sample (Fig. 4F), vascular orientation changes during the third GC, cellular density drops significantly after the fourth GC and vascular density decreases in the fifth and sixth GCs. In general, these histological features tend to change significantly in the last growth cycles of the FLC-zonal bone in analysed species. Exceptionally, this trend does not occur in the Svalbard reindeer, in which the histological features remain comparatively constant (Fig. 4G).

Table 2

Number of growth cycles (GCs) in the FLC - zonal bone of adults in the sampled species and literature data on the age of onset of reproductive maturity (ARM) in the same species.

Tableau 2

Nombre de cycles de croissance (GCs) dans l'os zonal - à FLC d'adultes dans les espèces échantillonnées et données de la littérature sur l'âge d'apparition de la maturité reproductive (ARM) dans les mêmes espèces.

Species	GCs			ARM		References
	N	F	M	F	M	
<i>Tragelaphus imberbis</i>	1	–	5	–	4	Kingdon, 1997
<i>Tragelaphus scriptus</i>	3	–	4	–	3–4	Kingdon, 1997; Wronski et al., 2009
<i>Tragelaphus spekii</i>	1	–	4	–	4	Kingdon, 1997
<i>Tragelaphus strepsiceros</i>	1	–	6	–	6	Kingdon, 1997
<i>Tragelaphus derbianus</i>	1	–	5	–	4	Pappas, 2002; Nowak, 1999
<i>Aepyceros melampus</i>	2	–	3	–	4	Estes, 1991
<i>Oreotragus oreotragus</i>	2	2	2	?	1–2	Kingdon, 1997
<i>Raphicerus campestris</i>	3	1	1	?	?	Kingdon, 1997
<i>Antidorcas marsupialis</i>	3	1	1	1	2	Cain et al., 2004
<i>Eudorcas ruffrons</i>	2	–	2	–	1–2	Nowak, 1999
<i>Eudorcas thomsonii</i>	1	–	1	–	1	Estes, 1991
<i>Nanger granti</i>	1	–	1	–	3	Estes, 1991
<i>Gazella dorcas</i>	2	–	2	–	2	Dittrich, 1972
<i>Sylvica pragrimmia</i>	2	–	1	–	1	Kingdon, 1997
<i>Philatomba monticola</i>	1	2	–	2	–	Kingdon, 1997
<i>Cephalophus natalensis</i>	2	1	1	?	?	
<i>Cephalophus callipygus</i>	2	1	1	?	?	
<i>Redunca fulvorufula</i>	1	–	2	–	3	Estes, 1991
<i>Redunca redunca</i>	2	–	2	–	3	Estes, 1991
<i>Kobus kob</i>	2	–	3	–	3	Estes, 1991; Kingdon, 1997
<i>Kobus ellipsiprymnus</i>	2	–	3	–	5	Owen-Smith, 1993
<i>Kobus megaceros</i>	1	–	3	–	4	Estes, 1991
<i>Connochaetes gnou</i>	2	–	3	–	≥ 4	Von Richter, 1974
<i>Damaliscus lunatus</i>	2	2	3	2–3	>	Kingdon, 1997
<i>Addax nasomaculatus</i>	2	3	4	1–2	> 3	Kingdon, 1997
<i>Oryx dammah</i>	2	–	3	–	?	
<i>Oryx gazella</i>	3	3	3	2	2	Kingdon, 1997
<i>Cervus elaphus hispanicus</i>	6	3	5–6	3–4	5–7	Clutton-Brock et al., 1982
<i>Rangifer tarandus platyrhynchus</i>	7	3	4	2–3	?	Skogland, 1985
<i>Capreolus capreolus</i>	3	–	3–4	–	> 3	Vanpé et al., 2009

N: number of individuals; F: number of GCs in females; M: number of GCs in males.

4. Discussion

There is controversy in the paleohistological literature about the biological meaning of the onset of deposition of the external fundamental system (EFS; Cormack, 1987), the avascular and highly-organized lamellar tissue present in the outermost cortex of the long bones of adults. The EFS is a residual periosteal growth that marks the end of the intense radial growth of the long bones shaft and that is generally linked to the skeletal asymptoty or maturity (epiphyseal fusion in mammals) (Chinsamy-Turan, 2005; Lee et al., 2013). In the paleohistological literature, the EFS is also frequently related to sexual maturity in mammals (Klevezal, 1996; Köhler et al., 2012; Marín-Moratalla et al., 2013; Straehl et al., 2013). However, other studies disagree with this relationship, since they maintain that sexual maturity precedes skeletal maturity in most vertebrates (Lee et al., 2013; Martínez-Maza et al., 2014). Although we agree with the latter statement in general, we should make some considerations especially for mammals. First, skeletal maturity or skeletal asymptoty of an animal is difficult to assess from a single long bone. Epiphyseal fusion of long bones (a proxy for skeletal maturity) occurs at different rates in the different long bones in mammals (Zeder, 2006). Therefore, only the long bones that fuse their epiphyses later could be used to assess if the individual has already

attained skeletal asymptoty. Second, it is not always clear when sexual maturity in the paleohistological literature refers to physiological or reproductive maturity, which are not always synchronized. For instance, the decoupling between the age at physiological and reproductive maturity is widely observed in ungulates (Mitchell and Maher, 2006). Males of ruminants may produce viable sperm at 6–24 months but they typically delay reproduction until they reach maximum body and horn or antler size (skeletal maturity), many years after the initial onset of fertility at puberty (Clutton-Brock et al., 1982; Festa-Bianchet et al., 2004; Geist, 1971; Stewart et al., 2000; Vanpé et al., 2009).

The results of our histological study on a ruminant sample show that the number of GC within the FLC-zonal bone of adult femora (until the deposition of the EFS) is broadly consistent with data on average age at reproductive maturity obtained from literature (see Table 2). In a few cases, obvious discrepancies exist and different reasons could be behind these inconsistencies. First, it is not always clear whether data on age of sexual maturity from literature correspond to physiological or reproductive maturity. This could explain the difference of almost 2 years between our results on addax females and data on age at maturity from Kingdon (1997).

Second, inter-populational variability may explain some of the discrepancies as well. For instance, we counted

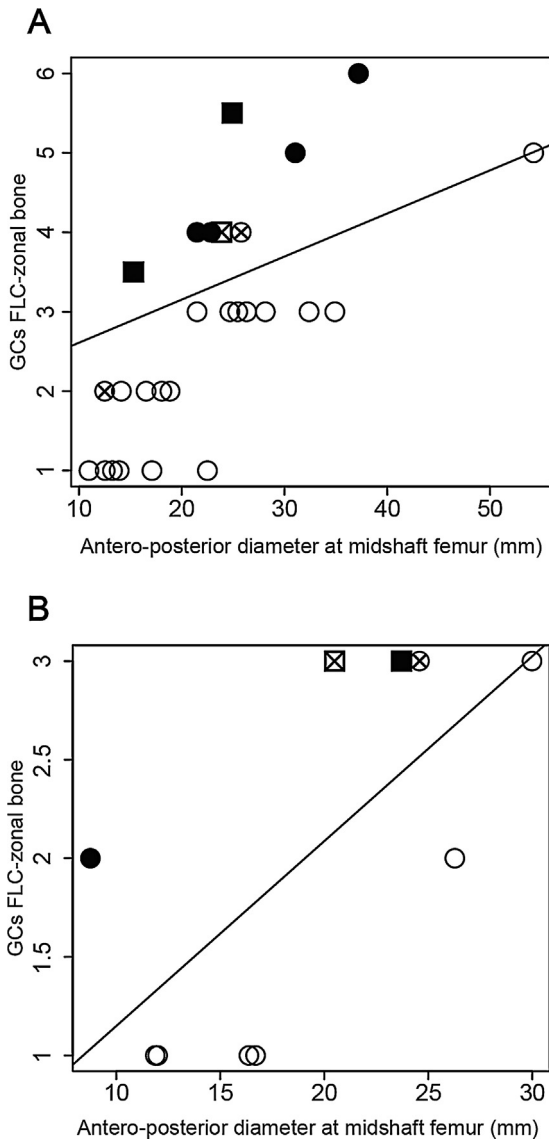


Fig. 3. Scatter plots between the number of growth cycles (GCs) in the fibrolamellar complex-zonal bone and their antero-posterior diameter at midshaft femur (within-species average) of the sampled species. A. Males, $r^2 = 0.24$, $P < 0.01$. B. Females, $r^2 = 0.49$, $P < 0.05$. Empty-circles: bovids from open habitats under more suitable conditions; crossed-circles: bovids from open habitats under resource limitation; filled-circles: bovids from closed habitats; filled-squares: cervids from closed environments; crossed-squares: cervids from open landscapes but under resource limitation.

Fig. 3. Nuage de points représentatifs entre le nombre de cycles de croissance dans l'os zonal à complexe fibro-lamellaire et leur diamètre antéro-postérieur à mi-hauteur du fémur (en moyenne au sein de l'espèce) des espèces échantillonnées. A. Mâles, $r^2 = 0,24$, $p < 0,01$. B. Femelles, $r^2 = 0,49$, $p < 0,05$. Cercles vides : bovidés d'habitats ouverts, dans de bonnes conditions ; cercles avec croix : bovidés d'habitats ouverts à ressources limitées ; cercles pleins : bovidés d'habitats clos ; carrés pleins : cervidés d'environnements clos ; carrés avec croix : cervidés de paysages ouvert, mais à ressources limitées.

a single GC in *Nanger granti*, while Estes (1991) reported an age at reproductive maturity of 3 years for this gazelle. *N. granti* typically dwells in arid to semi-arid habitats (Estes, 1991). In these ecosystems, water restriction affects reproductive patterns (Cain et al., 2006) and gazelles attain maturity according to the water availability. Under favourable conditions, gazelles may attain reproductive maturity earlier than when water is restricted (Baharav, 1983). The *N. granti* individual analysed in this study comes from Lolikisale Conservation Area (Tanzania), characterized by a tropical savannah climate where water is less limited than in the typical habitat of this species.

Last, bone remodelling is another important factor to take into account for understanding the discrepancies between our results from bone histology and data from literature on age at reproductive maturity. Our results show that certain large species (i.e. red deer males and the greater kudu male) have lost their first GC due to expansion of the medullar cavity and endosteal remodelling during ontogeny. This suggests that large ruminants might not preserve a complete ontogenetic record of primary bone growth, becoming necessary the use of different ontogenetic stages to determine the age of the specimen, as is common in other large non-mammalian vertebrates (Woodward et al., 2013). This could also be the case for the large antelope *Kobus ellipsiprymnus* (> 225 kg), in which we counted 3 GC in the FLC-zonal bone of two males; however, this species generally attains reproductive maturity at 5 years (Owen-Smith, 1993). Nevertheless, we could not conduct retrocalculation of age in this species because we only have adult specimens.

The age at reproductive maturity is often the life history trait most sensitive to ecological conditions (Clutton-Brock et al., 1982; Gaillard et al., 2000). Specifically, predation is considered to exert strong selection on age at first reproduction (Stearns, 1992). Under conditions of high mortality rates (i.e. high predation risk), species invest in rapid growth and early maturation that entails higher probabilities of surviving to first reproduction (Festa-Bianchet et al., 2006; Gaillard et al., 2000; Stearns, 1992). Conversely, in populations under low extrinsic mortality environments (low predation pressure), selection favours delayed age of reproductive maturity, allowing an investment in growth and survival (Ricklefs, 2007; Stearns, 1992). Similarly, the length of radial growth of the femoral cortex (or the number of GC in the FLC-zonal bone) of our ruminant species also appears to show this dependence on ecological factors, specifically on predation. Our study shows that among our ruminant sample, deer species and some bovid species such as the blue duiker (*P. monticola*), addax (*Addax nasomaculatus*) and most of tragelaphine antelopes (*Tragelaphus imberbis*, *T. scriptus*, *T. speki*, *T. strepsiceros*), display a relatively long growth period (relative to their size). These species, except for addax and the insular Svalbard reindeer, dwell in wooded and closed habitats, which are environments characterized by lower predation pressure than open habitats (Clutton-Brock et al., 1982; Köhler, 1993; Owen-Smith, 1993; Wronski et al., 2009). Addax dwells in desert, habitat characterized by resource limitation and scarcity of predators (Mallon and Kingswood, 2001). Similar selective pressures

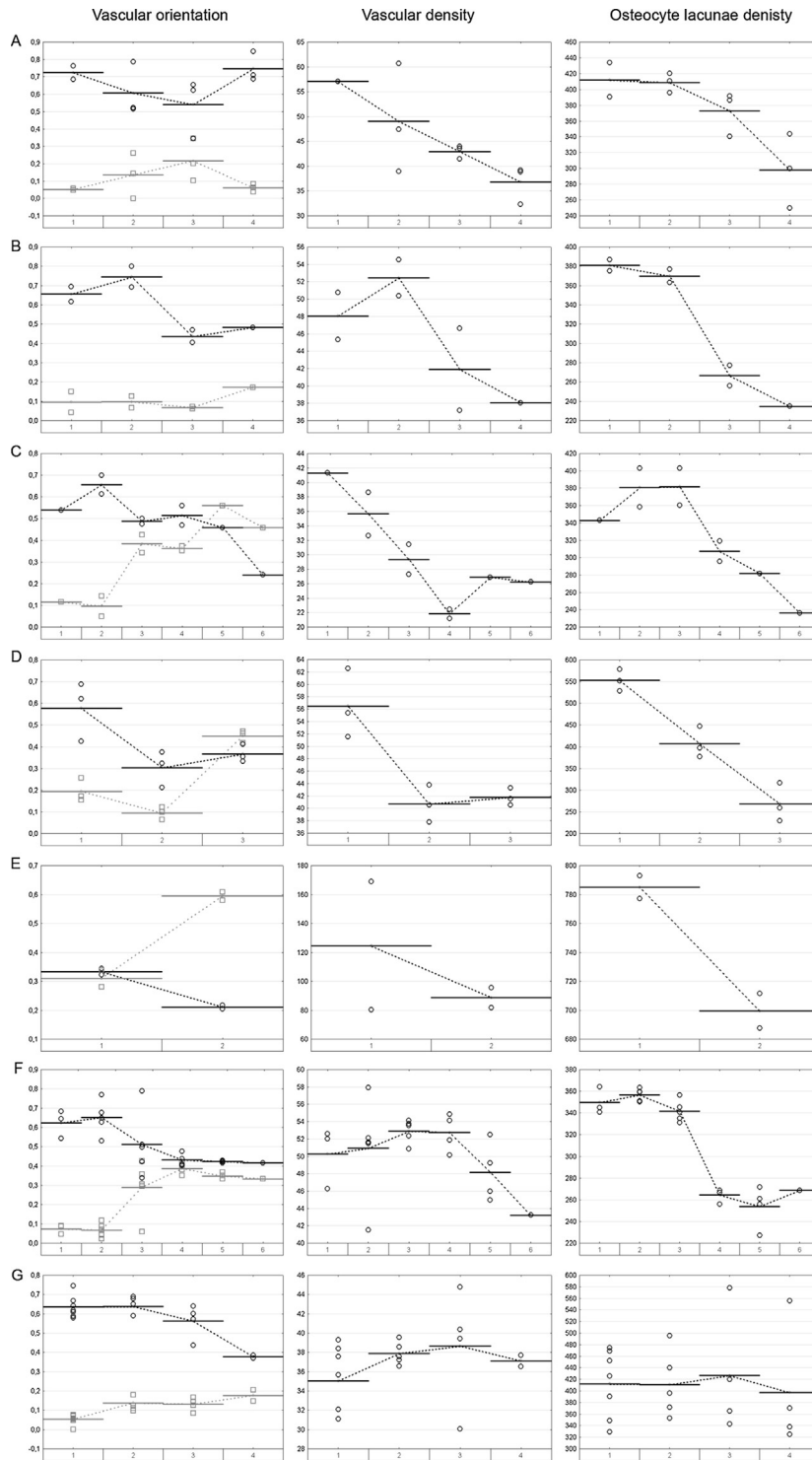


Fig. 4. Quantitative changes in vascular orientation (ratio), vascular density (No. vascular canal/mm²) and osteocyte lacunae density (No. osteocyte lacunae [OL]/mm²) (ordinate axis) among successive growth cycles (abscissa axis). Raw data (dots). Means (horizontal lines) connected by dotted lines. Vascular orientation column: proportion of circular canals (black) and proportion of longitudinal canals (grey). A. *Tragelaphus scriptus*. B. *Tragelaphus speikii*. C. *Tragelaphus strepsiceros*. D. *Aepyceros melampus*. E. *Gazella dorcas*. F. *Cervus elaphus hispanicus*. G. *Rangifer tarandus platyrhynchus*.
Fig. 4. Changements quantitatifs dans l'orientation vasculaire (rapport), la densité vasculaire (n° canal vasculaire/mm²) et la densité de lacunes d'ostéocytes (n° OL/mm²) (en ordonnée) pour les cycles successifs de croissance (en abscisse). Données brutes (points). Moyennes (lignes horizontales) reliées par des lignes pointillées. Colonne d'orientation vasculaire : proportion de canaux circulaires (en noir) et proportion de canaux longitudinaux (en gris). A. *Tragelaphus scriptus*. B. *Tragelaphus speikii*. C. *Tragelaphus strepsiceros*. D. *Aepyceros melampus*. E. *Gazella dorcas*. F. *Cervus elaphus hispanicus*. G. *Rangifer tarandus platyrhynchus*.

also characterize the habitat of the arctic reindeer from Svalbard archipelago (Aanes et al., 2000). Therefore, our study suggests that the transition from fast-growing FLC bone to slow-growing lamellar bone (EFS) in the femoral cortex of ruminants records the fundamental life history trade-off between growth and reproduction (Stearns, 1992). Hence, this transition between main bone tissue types is a good proxy for reproductive maturity, one of the most important life history traits that determine the species' fitness.

Changes in vascular orientation, vascular density and osteocyte lacunae density along the FLC-zonal bone of ruminants have also been explored in this study. Our results show an important ontogenetic variation of these histological features. Circular canals predominate during early bone development, but later the number of circular canals decreases while that of longitudinal ones increases. Moreover, vascular and cellular (osteocyte lacunae) density tend to decrease significantly during bone development. Several studies have revealed that these histological features offer insights into relative periosteal growth rates (Bromage et al., 2009; Castanet et al., 2000; Cubo et al., 2012; de Margerie et al., 2002; Montes et al., 2010; Mullender et al., 1996). According to them, the histological changes that we quantified during the development of the FLC-zonal bone of our ruminant sample reflect the slowdown of growth as maturity approaches (Lee et al., 2013). Actually, the most significant variation in the histological features along the FLC-zonal bone occurs within the last growth cycles before deposition of the EFS, indicating that there is an important slowdown of growth shortly before reproductive maturity. This trend could be related with the onset of physiological maturity (Clutton-Brock et al., 1982; Georgiadis, 1985; Vanpé et al., 2009). For instance, males of red deer (*C. elaphus hispanicus*) usually reach sperm maturity during their third year of age (Garde et al., 1998), coinciding with the age (third GC) when major changes in the histological features of our male deer appear (see Fig. 4F). Likewise, bushbucks (*T. scriptus*) reach physiological maturity at 1.5 years (Apio et al., 2007), and the significant changes in vascular orientation and density during their second year could be related to this physiological change (see Fig. 4A). Oddly, the arctic reindeer from Svalbard archipelago (*R. tarandus platyrhynchus*) does not show this important ontogenetic variation in histological features of the FLC-zonal bone (see Fig. 4G). This could be related to physiological adaptations to their extreme environment and would require further research.

The important ontogenetic variation in the histological features of FLC-zonal bone in ruminants has major implications for comparative studies on bone histology. Our results point out the need to standardize ontogeny for comparisons of bone tissues of different individuals or species, both quantitatively and qualitatively. Some studies quantify histological features in the innermost part of the primary bone cortex because this is the region formed during the highest growth rate phase (Cubo et al., 2012; Legendre et al., 2014; Marín-Moratalla et al., 2014), thus maximizing growth rate differences between individuals or species. Our study shows that large ruminants might not preserve a complete ontogenetic record of primary bone

growth. Hence, in these species we run the risk of comparing features of different chronology. We further show that important changes in histological features occur later in bone development, during the last growth cycles before deposition of the EFS. Therefore, in ruminants with a long growth period, i.e. 4 or more annual cycles of radial growth in the femoral cortex such as red deer, there are no important differences in histological features between the first and the second year. Other studies, however, quantify histological traits in the outer third of the bone cortex before the EFS (Stein and Werner, 2013). However, our results indicate that in ruminants this zone is formed after a significant drop in the rate of periosteal growth; therefore, it represents an ontogenetic period of minimum growth rate, minimizing differences between groups.

5. Conclusions

Our study suggests that the transition between the fast-growing FLC tissue and the slow-growing EFS in femoral cortical bones of ruminants records the fundamental life history trade-off between growth and reproduction. Accordingly, the number of growth cycles in the FLC-zonal bone of femurs of ruminants is a good proxy for the onset of reproductive maturity. Large ruminants such as red deer, however, might not preserve a complete ontogenetic record of primary bone growth, making the use of ontogenetic series necessary for skeletochronological studies. Moreover, our study also provides evidence of an important ontogenetic variation in the FLC-zonal bone of ruminants. Circular canals that are predominant during the early juvenile stage decrease in frequency during bone development along with an increase in longitudinal canals. Additionally, vascular and cellular (osteocyte lacuna) densities tend to decrease during the development of FLC-zonal bone as well. Significant changes in these histological features tend to occur during the last growth cycles and could be related to the important slowdown in growth associated with physiological maturity. Finally, our study suggests that the innermost cortex should be the preferred zone for comparing histological features of primary bone tissue between individuals or groups, because this zone is formed during the highest postnatal growth rate, hence maximizing differences in this parameter between groups.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version available at <http://dx.doi.org/10.1016/j.crpv.2015.03.008>.

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