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Reconstructing life history traits from bone histology in extant and fossil ruminants

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ABSTRACT

The study of life histories is of special importance because it provides valuable insights into ecological conditions, biodiversity, demography, vulnerability and many other aspects of a species' biology. Bone histology is a widely used tool to reconstruct vertebrate life histories, either by analysing primary bone tissue or by counting the number of growth marks (skeletochronology). However, it has long been considered that endotherms, unlike ectotherms, display a continuous or noncyclical bone growth, disabling bone histology for life history inferences in mammals. The general purpose of the research presented in this PhD Thesis is to challenge this statement, contributing to the foundations of mammalian bone histology as a tool for inferences on life history strategies. The specific aims are: i) to analyse the reliability of the skeletochronology in mammalian bone, ii) to explore the association of bone tissue features with environment, physiology, ontogeny and life history, and finally iii) to reconstruct life history traits in fossil and living mammals to get insights on life history evolution and conservation biology.

A sample of 274 bone cross-sections (generally femur) from 225 individuals belonging to extant dormice (Gliridae) and extant and fossil ruminants (Bovidae, Cervidae, Moschidae and Tragulidae) have been analysed under polarized and transmitted light microscopy. Both, qualitative (bone tissue type) and quantitative (number of growth marks, vascular density, vascular orientation and cellular density) analyses have been carried out. This large sample allows the exploration of bone histology in wild mammals of a wide range of body sizes and dwelling in very different environments.

Qualitative analyses of primary bone tissue show that, in early stages of ontogeny, ruminants deposit fibrolamellar complex (FLC) bone mainly laminar or plexiform, while dormice deposit mainly parallel fibered bone (PFB) with longitudinal primary osteons. When adults, both mammalian groups deposit a dense lamellar bone, generally known as external fundamental system (EFS). The results also show that Lines of Arrested Growth (LAGs) are universally present in both mammalian groups analysed in this work. These growth marks are present throughout both, the fast-growing bone tissue deposited during growing period (FLC or PFB) as well as the slow-growing dense lamellar tissue deposited during the adulthood (EFS). The number of rest lines in cortical bones fits well with chronological age of the animals, providing evidence of the annual periodicity of bone growth marks in these mammals. The femur is clearly the most reliable bone for skeletochronology analyses because it records the greatest number of LAGs. Despite this, bone remodelling and resorption can potentially delete or obscure the earliest ontogenetic record, especially in large ruminants. This research further

indicates that bone growth is arrested during the energetically challenging period (low resource supply), coupled with seasonal physiological variation. These findings provide support that growth arrest forms part of a thermometabolic strategy for energy conservation.

Moreover, this work shows that vascular and cellular features of primary bone tissue undergo strong ontogenetic variation associated with a decrease on growth rate as maturity approaches in mammals. Specifically, vascular and cellular densities decrease whereas the proportion of longitudinal canals in relation to circular ones increases throughout ontogeny until reach maturity. However, the most significant change along ontogeny occurs during the transition between the main primary tissues, from FLC/PFB to EFS. This work provides evidences that this transition reliable records the trade-off between growth and reproduction in ruminants. According to these findings, the age at reproductive maturity can be determined by counting the number of growth cycles within the fast growing tissue before the EFS.

The result of comparing histological quantitative features between bovids suggests that vascular and cellular parameters are related to body mass and metabolism rather than to exogenous factors, such as climate. Accordingly, the FLC bone of larger bovids tends to show more circular canals and lower cellular densities than the smaller ones. A phylogenetic signal is found in some histological parameters, as the proportion of longitudinal and circular canals as well as the cellular density.

Finally, the findings on fossil species provide evidence that bone histology is a valuable tool to explore evolutionary trends in mammalian life histories. Moreover, the results of bone histology to get some life history traits in endangered mammals highlight its usefulness on the field of conservation biology.

To conclude, the findings of this work provide evidences that, in mammals, bone growth is mainly regulated by endogenous rates and synchronized with seasonal resource availability. The evidence of cyclical bone growth debunks the classical assumption that homeothermic endotherms grow continuously until they attain maturity, providing a clear support to the usefulness of bone histology to reconstruct life history traits in extinct and extant mammals.

Contents

PROLOGUE

Skeletochronology (the count of growth marks in bone cross-sections for age estimations) emerged during the first half of the 20th century, when many studies linked the layered structure of cortical bone cross-section in reptiles to annual growth marks. In 1947 Rodolfo Amprino established for the first time the relationship between bone tissue types and rates of bone deposition. This relationship, coined *Amprino's rule,* is nowadays the foundation for many investigations on growth strategies in vertebrates. In the fifties Donald H. Enlow explained bone histodiversity taking into account the ontogenetic changes and remodelling dynamics. In accordance with the terminology introduced by R. Amprino and D. H. Enlow, more recently Armand de Ricqlès proposed a very detailed classification of bone tissues and a standardized nomenclature in the field of bone histology and paleohistology.

These studies established the foundations of bone histology, allowing inferences of growth patterns in extinct and extant vertebrates. During the last decades many studies have expanded *Amprino's rule* through quantitative experimental analyses, whereas other studies have improved the knowledge on growth strategies of many fossil vertebrates. Currently, bone histology is a tool widely used to reconstruct life history traits such as age at maturity, longevity and growth rates, mainly in amphibians and reptiles. Specifically, skeletochronology is an approach commonly used by herpetologists to obtain demographic data from wild populations, as well as by palaeontologists to reconstruct growth strategies of extinct taxa, mainly dinosaurs.

In mammals, however, bone histology has not been systematically studied so far. The reason is not that they might be less interesting than, for instance, reptiles, but it is widely believed that long bone tissues of endotherms do not provide such a wealth of information, as do the bones of ectotherms. The metabolism of ectotherms is highly dependent on the seasonal environment, which has been related to the appearance of annual growth marks. This layered pattern constitutes what is classically known as zonal bone (cyclical bone growth), which is the basis for the skeletochronology. In endotherms, however, in which body temperature is regulated within a narrow range, growth rates are generally high and constant. This metabolic strategy has led to the belief that endotherms form azonal bone (uninterrupted bone growth) throughout the cortex until maturity. This is why skeletochronology is considered to not be a reliable method for aging mammals.

The research presented in this PhD Thesis challenges the widespread assumption above mentioned. It arises from the research being carried out at the Evolutionary Paleobiology

department of the *Institut Català de Paleontologia Miquel Crusafont*. One of the most relevant studies presented in this work highlights the universality of annual growth marks among vertebrates, including mammals, which debunked the dichotomy between zonal and azonal bone associated to ectothermy and endothermy, respectively. The investigations of this team provide evidence that some life history traits, such as age at reproductive maturity, age at death (i.e. longevity) and growth rate can be reconstructed in living and fossil mammals using bone histology. Much of this research is part of the work presented here.

The present PhD Thesis is structured according to the standards of the *Universitat Autònoma de Barcelona* for theses as compendium of publications. The Introduction (Chapter 1) comprises an overview that attempts to provide the essential bases for understanding Life History Theory, which is a central matter of this work. Moreover, the Introduction provides a state of the art of the usefulness of bone histology to reconstruct life history traits prior to the knowledge provided by the current work. Therefore, this section includes the classical statements about mammalian bone histology, some of which have been debunked in the studies compiled in this Thesis. Subsequently, the justification of the investigation carried out and the hypotheses and aims raised in this Thesis are presented in Chapter 2. The Material and Methods section (Chapter 3) summarizes the whole material used in the present work and also provides the basic information on thin section procedures and bone histology analyses. The original works (Chapters 4 to 9) correspond to published articles in SCI journals as well as non-published results, which will be the basis for future publications. Because these chapters follow the organization of scientific articles, each of them contains a specific introduction, material and methods and discussion sections. The integrated results and a general discussion are presented in Chapter 10. This section summarizes the main results of the original works and sets a general discussion following the raised aims. Finally, the conclusions of this PhD Thesis are presented (Chapter 11).

CHAPTER 1. INTRODUCTION

1. INTRODUCTION

1.1. LIFE HISTORY THEORY

The study of life histories is one of the most integrative disciplines in the field of biology, including physiology, ecology and evolution (Stearns, 1992). The life history of an organism explains the main features of its life cycle, named life history traits (Stearns, 1992; Roff, 2002). Life history traits are those related with the timing and rates of the life cycle events, such as growth rate, age at first reproduction, longevity and so on. Additionally, those features that co-vary with above traits, such as adult body mass, body mass at birth, litter size, litter per year, among others, are usually considered as life history traits as well (Stearns, 1992).

Life History Theory seeks to explain how selective forces act upon life history traits in order to optimize organism's survival and reproduction (fitness) to face the ecological challenges (Stearns, 1992; Roff, 2002). This theory is rooted in the *r*/K theory of McArthur and Wilson (1967), which proposed resource availability as the main selection pressure acting upon population growth and regulation. Both population parameters, *r* (intrinsic rate of natural increase) and K (environmental carrying capacity), are used to designate two strategies of regulation of natural populations (MacArthur and Wilson, 1967; Pianka, 1970; 2000). On the one hand, *r*-selected populations inhabit hazardous environments with strong environmental fluctuations (Pianka, 2000). These populations alternate favourable periods of fast population growth with periods of high mortality (ecological perturbations). Because these populations do not reach the environmental carrying capacity, they evolve under density-independent factors. In this context, those traits that maximize the productivity, i.e. mature early with a large litter size, small offspring and short lifespan, are selected (Pianka, 2000). On the other hand, K-selected populations inhabit stable ecosystems with few environmental fluctuations (Pianka, 2000). In these ecosystems, natural populations reach the environmental carrying capacity with a population size approximately constant in time (birth and death rates are in equilibrium). Under this context, density-dependent factors (i.e., intra-specific competition) largely determine the survival and fecundity of individuals. Therefore, those traits that maximize the efficiency, i.e., later maturation with a small litter size, large offspring and long lifespan, are those that increase the fitness of individuals (Pianka, 2000).

Life History Theory can be considered as an extension of the *r*/K theory incorporating extrinsic mortality as the main selection pressure. This theory focuses on the evolution of the life history traits that are directly related to reproduction and survival and how they interact through trade-offs (Stearns, 1992; Reznick et al., 2002; Roff, 2002). Organisms have limited

time, energy, and nutrients at their disposal and each organism is faced with the problem of allocation (Ricklefs, 2007). The trade-offs are caused precisely by allocation decisions between two or more processes that compete directly with each other for limited resources in an individual. A trade-off exists when a benefit achieved by the change in one trait is related to a cost paid by the change in other traits. The benefits and costs of a trade-off are not related to energy, nutrients or time, but to the currency of fitness (Stearns, 1992). One of the best-studied trade-offs is between growth and reproduction, which exemplifies this concept: as energy and time are limited, individuals should allocate resources optimally between growth and reproduction. Hence, when the minimum size for successful reproduction is attained, resources are channelled away from growth towards reproduction (Stearns, 1992; Roff, 2002; Ricklefs, 2007).

The main ecological factor determining these allocation decisions (trade-offs) is the level of extrinsic mortality (Stearns, 1992; Roff, 2002; Ricklefs, 2007). In contrast to the dichotomy of the *r*/K theory, life history traits are organized along a single continuum of values. At one extreme, the 'slow' end of the spectrum, organisms exhibit long life span, slow development, delayed maturity, high parental investment, and low reproductive rates. At the 'fast' end of the spectrum, organisms exhibit the opposite life history traits (Ricklefs, 2007). Under conditions of high mortality rates (e.g. high predation risk), species invest in early reproduction and rapid growth (fast life history) due to their demographic benefit: early maturing leads to a higher probability of surviving to reach the adulthood (Stearns, 1992). Hence, early maturation is advantageous for species highly predated. Conversely, a decrease in extrinsic mortality is expected to lead a slow life history, characterized by the opposed traits (Wilbur et al., 1974; Stearns 1992).

Studies of life histories are of special importance because they concern directly on fitness components. Successful conservation and management of endangered species depend on the knowledge of the life history traits that determine the species' demography (Stearns, 1992; Tuljapurkar and Caswell, 1996). That is, species displaying certain life history traits are more vulnerable to extinction than others (McKinney, 1997; Isaac, 2009). Moreover, the evolution of species' life histories from fossil ecosystems is of special importance because it provides a long time perspective that enables to explore the general principles of large-scale population trends. It is useful for understanding how ecological processes act in the absence of anthropogenic factors as well as for understanding extinction processes; e.g. by analyzing the effect of past climatic changes on species' life histories. Therefore, the studies on life history from fossils can contribute to the fields of evolutionary biology and evolutionary ecology (Köhler and Moyà-Solà, 2009; Köhler, 2010; Jordana and Köhler 2011; Jordana et al., 2013; Padian et al., 2013).

1.2. BONE HISTOLOGY AS A TOOL TO RECONSTRUCT LIFE HISTORY STRATEGIES

1.2.1. Skeletochronology in cortical bone

Bone histology has been widely used for life history inferences because bone is a recording structure. Recording structures (Mina and Klevezal, 1970) are those that respond to physiological changes of an organism by changing their morphological characteristics as they grow (Klevezal, 1996). It is widely accepted that bone tissues of vertebrates lay down growth marks, either Lines of Arrested Growth (LAGs) or rings of lamellar bone (annuli), following circannual rhythms (Wallis, 1928; Petter-Rousseaux, 1953; Peabody, 1958, 1961; Castanet and Smirina, 1990; Castanet et al., 1993; Castanet et al., 2004, 2006; Chinsamy-Turan, 2005). However, these annual rhythms are not life history traits *per se.* Life history traits are recorded in bone as single events (Köhler et al., 2013). Then, the timing and duration of life history traits can be determined by counting the number of growth marks in bone tissue (Castanet and Smirina, 1990; Chinsamy-Turan, 2005), an approach widely known as skeletochronology (Castanet and Smirina, 1990; Woodward et al., 2013).

Generally, skeletochronology by means of bone histology may provide information upon three key life history traits: age at maturity, age at death and growth rate. During the juvenile period, growth rate is elevated, and so large quantities of bone matrix are deposited between the growth marks (e.g. LAGs) (Horner et al., 2000). When maturity is reached, bone deposition rate slows down (in ectotherms) or becomes residual (in endotherms) (Sander, 2000; Chinsamy-Turan, 2005). This biological event is reflected in bone tissue by the reduction of the space between rest lines forming an outer cortical layer of dense lamellar tissue, known as External Fundamental System (EFS) (Cormack, 1987). The age at maturity, hence, can be theoretically determined by counting the number of growth marks before the beginning of the EFS (Sander, 2000; Chinsamy-Turan, 2005; Chinsamy and Valenzuela, 2008; Woodward et al., 2013). Furthermore, the number of growth marks along the complete radius of a section including the EFS may provide information on the age at death of the individual (Castanet and Smirina, 1990). Finally, insights on linear growth rate can be provided by measuring the distance between growth layers (Bybee et al., 2006; Cooper et al., 2008; Lee et al., 2013).

Until recently, skeletochronology from bone histology has been applied almost exclusively in extinct and extant amphibians and reptiles (Horner et al., 2001; Jakob, 2002; de Ricqlès et al., 2003, 2008; Erickson, 2000; Tumarkin-Deratzian, 2007; Chinsamy and Valenzuela, 2008). The reason is the widespread belief that annual growth marks (LAGs or annuli) are laid

down exclusively in ectotherms during the coldest season[1,2] because their growth is highly dependent on the seasonal environment (Castanet and Smirina, 1990; Klevezal, 1996; Chinsamy-Turan, 2005). Endotherms have been considered to grow continuously or noncyclically (Chinasmy-Turan, 2005). In endotherms, therefore, the 'recording' region of bone (where LAGs are deposited) has been classically considered to be restricted to the outermost part of the bone, the EFS. Growth marks in the middle of the cortex were considered to be fortuitous and related to environmental stresses (Klevezal, 1996; Chinsamy-Turan, 2005). It has been upheld that the number of rest lines in endotherms does not reflect the true age of the animal[2] (Klevezal, 1996). Hence, the usefulness of bone histology and specifically skeletochronology in mammals has been underestimated hitherto. Despite this general belief among the scientific community, some researchers have challenged this presumption (Horner et al., 1999; Castanet et al., 2004, 2006; Sander and Andrássy, 2006; Köhler and Moyà-Solà, 2009; Köhler, 2010).

Castanet et al. (2004) studied a large sample of the small primate *Microcebus murinus* using fluorescent labelling. He found that annual photoperiodicity is the key factor triggering the formation of LAGs and hence, he proposes that skeletochronology can be a good tool to determine age at death in these animals. The authors concluded that the occurrence of growth marks is not limited to ectothermic animals. They suggested that the absence of growth marks in some endothermic species may be due to a short time of bone growth in thickness (less than one year) or as a result of bone remodelling (Castanet, 2006). Horner et al. (1999) documented LAGs embedded in the fast-growing bone matrix of northern-latitude elk (*Cervus canadiensis*). Likewise, Sander and Andrássy (2006) documented the presence of LAGs within the fast-growing bone matrix in some Pleistocene ungulates, suggesting that the glacial conditions during Pleistocene induced the LAG deposition. Köhler and Moyà-Solà (2009), however, provided evidence of LAGs in long bones of *Myotragus*, a bovid that dwelled on the Balearic Islands over more than 5 million years (Plio-Pleistocene) under different climatic regimes. Further work on this taxon focused on the usefulness of bone histology for life history inferences in mammals, especially for reconstructions of longevity and age at maturity (Köhler, 2010). The above-mentioned studies challenged the idea that only the outermost part of the

[1] The thermophysiology of non-avian dinosaurs has been hotly debated for many years within scientific community. The term 'ectotherms' used here includes non-avian dinosaurs following the general idea that many researchers defended until recently. Whereas the presence of growth marks in this group have been considered as a strong evidence of ectothermy (Chinamy-Turan, 2005 and references therein), some researchers challenged this idea because of the presence of fibrolamellar bone, suggesting non-avian dinosaur' endothermy (Padian et al., 2001 and references therein). An original work that conforms this Thesis (see Chapter 5) supports the non-avian dinosaurs' endothermy.

[2] This statement has been debunked by the results presented throughout this Thesis (see Chapters 4 to 10).

bone is a recording structure in mammals (Klevezal, 1996), and opened the door to further studies on life history by using skeletochronology from bone histology.

1.2.2. Histological features of primary bone

The analysis of bone matrix typology as well as vascular and cellular parameters of primary bone tissue can also provide information on growth rates (Francillot-Vieillot et al., 1990; de Ricqlès, 1991). Primary compact bone tissue is commonly categorized in different typologies related to the rate of bone deposition, following *Amprino's rule* (Amprino, 1947; Francillot-Vieillot et al., 1990; Castanet et al., 1996; de Margerie et al., 2002; Montes et al., 2010). In broad terms, a bone tissue can be highly organized (closely-packed collagen fibrils) and poorly vascularised with few osteocytes (e.g. lamellar bone) indicating slow rates of bone deposition. Conversely, a bone tissue highly disorganized (loosely-packed collagen fibrils) and vascularised with high number of osteocytes (i.e. fibrolamellar bone) indicates a fast rate of bone deposition (see Chapter 3 for further explanations).

Recently, Castanet et al. (2000), de Margerie et al. (2002, 2004) and Montes et al. (2010) tested experimentally the *Amprino's rule* essentially in archosaurs. These works expanded on *Amprino's rule*, providing new features related to growth rates. One of these features is the relative cortical bone porosity in primary bone tissue (expressed as total percent of vascularity or as vascular density). These studies have shown that decreases in relative cortical porosity through ontogeny coincide with decreases in periosteal growth rate (Castanet et al., 1996; Horner et al., 2001; Williams et al., 2004; Montes et al., 2010; Cubo et al., 2012). Other features refer to vascular orientation, specifically the radial and circular canals are associated with faster deposition rates than longitudinal canals (Castanet et al., 1996; de Margerie et al., 2004).

Although these studies allow inferences on absolute bone deposition rates only in extinct archosaurs, the discrete spectrum of bone deposition rate may also be used in other taxa such as mammals (Castanet et al., 1996; Montes et al., 2010; Lee et al., 2013; Padian, 2013). However, much remains to be explored in mammals because the scarcity of experimental studies in this group (Montes et al., 2010; Cubo et al., 2012).

CHAPTER 2. OBJECTIVES

2. OBJECTIVES

Bone histology has been widely used in extinct taxa, essentially in fossil archosaurs, to reconstruct growth strategies (Horner et al., 2001; Horner and Padian, 2004; Lee and Werning, 2008; Cooper et al., 2008; Cubo et al., 2012; Legendre et al., 2013). In fact, bone development in some extinct dinosaurs is better understood than in most groups of extant taxa. Yet, a specific group of extant vertebrates is well known: those animals used in laboratory studies, such as mouse or chicken, which are considered to be 'model' animals. However, the drawback of this focus is that skeletal developmental processes and patterns tend to be generalized to all vertebrates. These animals are generally medicated and they have altered reproductive rhythms. To make inferences by bone histology from laboratory animals to wild populations may be tempting but incorrect. Therefore, a widespread perception is that once bone histology is understood in wild extant vertebrates, we will be able to make more accurate inferences in fossil taxa (Padian et al., 2013).

Experimental studies based on vital labelling are undoubtedly the best way to correlate bone tissue patterns with life history events. However, this kind of study is practically unviable in wild populations without interfering with their development. Hence, non-experimental studies relating bone microstructure with life history traits on wild populations are also necessary. Only the study of bone on wild extant mammals can provide the basis for its application in fossils.

The studies of life histories on fossil communities are of special significance because they provide a time perspective long enough to discover general principles of large-scale population trends. In this sense, paleohistology becomes an essential tool able to reconstruct life history strategies. The study of extinct mammals using bone histology can provide further knowledge for the study of the evolution of life history.

Moreover, the knowledge of life history is crucial to design conservation policies (Isaac, 2009). In this sense, bone histology applied to endangered species can provide a significant contribution to the field of conservation biology, offering a new approach to gather demographic data on wild endangered populations.

The hypothesis proposed in this PhD Thesis is that bone histology is a powerful tool to reconstruct life history traits of extinct and extant mammals. Accordingly, the general aim of this work is to contribute to the foundations of mammalian bone histology as a tool for inferences on life history strategies. It addresses the following specific aims:

I. To analyze the reliability of the skeletochronology in mammalian bone, as well as the variability of bone elements as recording structures.

II. To explore the association of bone growth cycles, including LAG formation, with environmental and physiological traits in mammals.

III. To explore how ontogenetic and physiological changes influence primary bone tissue in mammals.

IV. To find out the correlates of histological features of mammalian primary bone tissue with environment and life history in a phylogenetic context.

V. To reconstruct life history traits in fossil to get insights on life history evolution and in extant mammals to provide data for conservation biology.

RESEARCH PLANNING OF THE ORIGINAL WORKS

Original works	Objective achieved	Publication
Chapter 4. The ontogeny of bone growth in two species of dormice: Reconstructing life history traits.	I and V	García-Martínez, R., Marín-Moratalla, N., Jordana, X., Köhler, M. 2011. *CR Palevol,* 10: 489-498.
Chapter 5. Seasonal bone growth and physiology in endotherms shed light on dinosaur physiology.	II	Köhler, M., Marín-Moratalla, N., Jordana, X., Aanes, R. 2012. *Nature*, 487: 358-361.
Chapter 6. Exploring ontogenetic signals in the fibrolamellar bone of mammals with implications for life history inferences.	III	Unpublished results.
Chapter 7. Correlations of vascular and cellular parameters of primary bone tissue with life history traits and climate: a phylogenetical approach.	IV	Marín-Moratalla, N., Cubo, J., Jordana, X., Moncunill-Solé, B., Köhler, M. 2014. *Biol. J. Linn. Soc.,* 112: 678-687.
Chapter 8. Tracing the evolution of fitness components in fossil bovids under different selective regimes.	V	Marín-Moratalla, N., García-Martínez, R., Jordana, X., Köhler, M. 2011. *CR. Palevol*, 10: 469-478.
Chapter 9. Bone histology as an approach to provide data on certain key life history traits in mammals: implications for conservation biology.	I, III and V	Marín-Moratalla, N., Jordana, X., Köhler, M. 2013. *Mam. Biol.*, 78: 422-429.

CHAPTER 3. MATERIAL and METHODS

3. MATERIAL AND METHODS

3.1. MATERIAL

The material analysed in this PhD Thesis comprises a sample of 274 bone cross-sections (generally right femur) from 225 individuals belonging to extant dormice (Gliridae) and extant and fossil ruminants (Bovidae, Cervidae, Moschidae and Tragulidae) (Table 3.1). The permission to cut the bones from museum collections was obtained for all cases.

Table 3.1: *Summarize of the material used in this PhD Thesis. For further detail of the material analysed see the specific Chapter.*

Order	**Family**	**Extant species**			**Fossil species**			**Chapter**
		Species	Individuals	Thin section	Species	Individuals	Thin section	
Rodentia	Gliridae	2	16	65	-	-	-	4
Artiodactyla	Bovidae	35	83	83	2	78	78	5 to 9
	Cervidae	3	45	45	-	-	-	5 to 6
	Moschidae	1	2	2	-	-	-	5
	Tragulidae	1	1	1	-	-	-	5 and 7
Total sample		42	147	196	2	78	78	

The sample of dormice comes entirely from wild populations and most of them come from the collection of the Museu de Ciències Naturals de Granollers (Spain).

The sample of ruminants comprises both wild and captive individuals. Most extant bovids were hunted in Africa between the fifties and the seventies by H. Oboussier (Köhler et al., 2008), while few of them come from zoos (Hannover, Berlin and Frankfurt). Moschidae individuals come from the Zoo of Berlin, and the Tragulidae comes from India (unknown locality). All these species are housed at the scientific collections of the Zoological Institute of Hamburg University (Germany). Most of them have associated data (body mass, sex, site and date of death). The deer sample is comprised by individuals of red deer (*Cervus elaphus*) from a flock (conditions of semi-captivity) obtained from the Research Institute of Wildlife Ecology (Vienna, Austria); by specimens of Spanish red deer (*Cervus elaphus hispanicus*) from the wild through hunter activities; and by individuals of

Svalbard reindeer (*Rangifer tarandus platyrhynchus*) from the wild through the collaboration with researchers from the Norwegian Polar Insititute (NPI). All deer skeletons are housed at the Institut Català de Paleontologia Miquel Crusafont (ICP).

The fossil sample is comprised by two bovid fossil species, *Myotragus balearicus* Bate, 1909, and *Gazella borbonica* Depéret, 1884 housed at the ICP.

3.2. METHODS

3.2.1. Histological sections

3.2.1.1. Thin section procedures

Once selected the material to be sectioned, the bones are photographed and measured carefully, and the stage of epiphyseal fusion of long bones is annotated. These data together with available data of the specimens are included in the extensive histological database analysed in this PhD Thesis.

Before sectioning, the material is embedded in epoxy resin (Araldite 2020). Small bones (< 5cm) are embedded completely, whereas larger bones (> 5cm) are previously sectioned at midshaft for obtaining a roughly 2 cm chunk of the shaft proximal to the centre (Figure 3.1A). The section is oriented, which means that the proximal, distal, medial and lateral axes are marked on the bone. This orientation is carefully kept along all the process to finally mark the orientation planes in the thin section. Once the bone is embedded in epoxy resin, the surface of interest is exposed (Figure 3.1B) using a Buehler Isomet for small bones (Figure 3.1H) or a Diamant ND-300 saw for large ones (Figure 3.1I). Previous to gluing the hardened resin block to a glass slide, both surfaces (the block and the glass) are polished with carborundum powder of decreasing particle size (350, 800 and 1000 grit, 800 and 1000 grit, respectively) (Figure 3.1C). Between each step, the block is cleaned in an ultrasonic bath during 30 seconds, whereas the slide is cleaned with a degreasing agent. Both the block and the slide are dried in an oven (30ºC during 2-3 hours and 30ºC during 1 hour, respectively). Once the surfaces are totally polished and dry, the resin block is fixed on the slide using ultraviolet curing glue (Loctite 358) during 30 minutes (Figure 3.1D-F). The excess of glue is removed with alcohol (96ºC).

The thin section is performed with a diamond saw (Buehler, PetroThin, Figure 3.1H). This saw includes two discs: one for cutting the sample to ≈ 400 µm and the other one for polishing the sample until 130 µm. The final thin section is obtained by hand polishing with a gradient of carborundum (800, 1000 and 1200 grit) until the desired thickness is attained (roughly 100 µm), always controlling the thickness with a polarized microscope. The excess of carborundum is removed with an ultrasonic bath during no more than 10 seconds, as the vibration may detach the sample from the slide. In fossils, the time should not be longer than 5 seconds because they are frailer than extant bones. A micrometer is required to approach the final thickness of the thin section (≈100 µm). Once the thin section is totally polished, it is dried overnight at room temperature.

The thin section is mounted with a DPX medium (Scharlau) because it preserves and enhances the visualization of bone tissue by modifying the refractive index of the sample. DPX is not soluble in water but in xylene. Therefore, the thin section is first dehydrated using a graded series of alcohol baths with increasing concentration: 70-96-100º during 20-30 seconds in each bath. To provide a soluble medium for the DPX, the thin section is immersed (2 baths of 2.5 minutes) in a clearing agent (Histoclear), which is a non-toxic substitute of xylene (Figure 3.1G). Finally, the sample is covered with DPX, and is left for drying during 24 hours at room temperature.

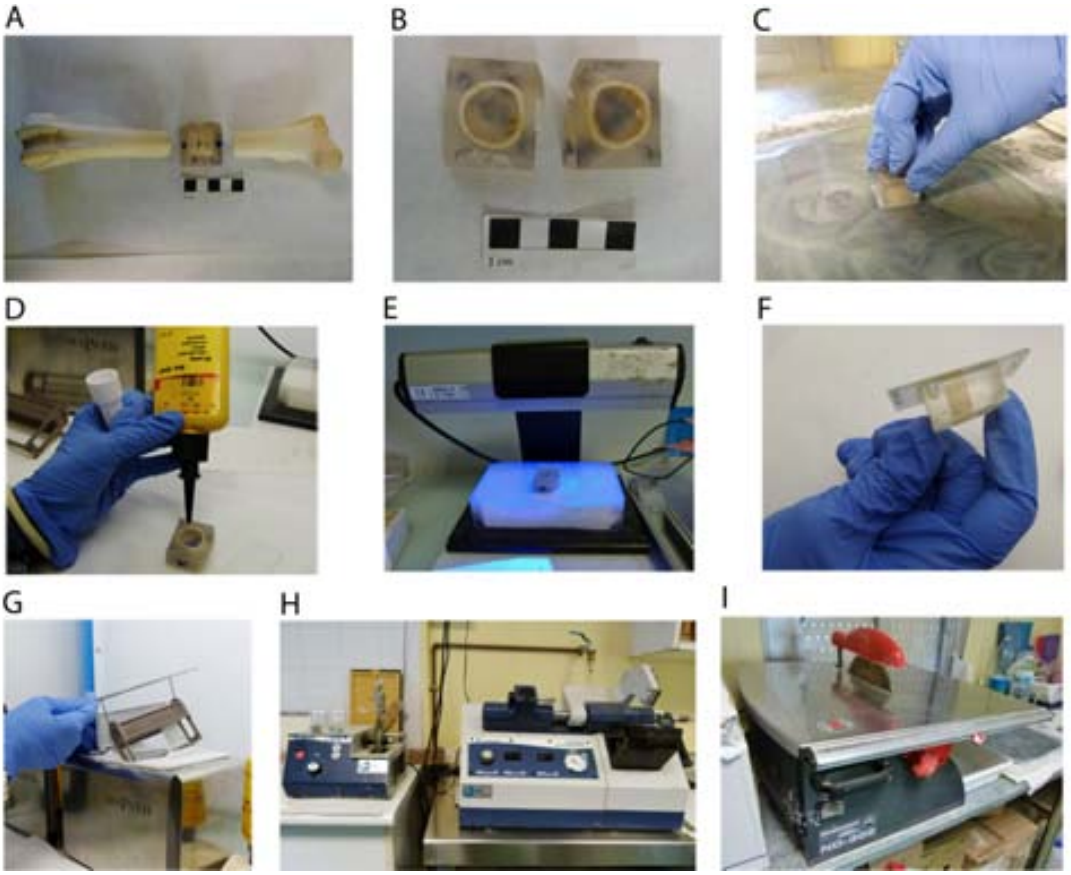

Figure 3.1: *Thin section procedure. A. A chunk of the diaphysis of the femur is cut at midshaft and embedded in epoxy resin. B. The block is cut with either the Buehler Isomet or the Diamant ND-300 saw depending on bone size, thus obtaining the surface of interest. C. The block is polished with carborundum. D-F. The block is glued to the glass slide using Loctite under UV light. G. The thin section is immersed in the histoclear bath. H. left: Buehler Isomet saw, right: Buehler, PetroThin saw. I. Diamant ND-300 saw.*

3.2.1.2. Microscopy

Two kinds of microscopes, transmitted and polarized light, can be used to analyse bone thin sections. On one hand, linear polarized light (LPL) allows to distinguish the collagen fiber orientation of the bone. Collagen is an anisotropic material with two different refractions index, characteristic named birefringence. This birefringence is expressed as alternations between darkness and brightness of the collagen under LPL (Figure 3.2A), allowing the identification and classification of bone tissue (e.g. lamellar *vs.* woven bone). It consists an arrangement of two polarizing filters. The first one, the polarizer, is located between the light source and the sample, whereas the second filter, the analyzer, is situated between the sample plane and the observer, with its vibration direction at 90 degrees to that to the polarizer (Figure 3.3) (Bromage et al., 2003; Ross and

Pawlina, 2011). Apart from these two polarizing filters (the polarizer and the analyzer), other lambda filters can be used in order to delay the light's wave (1λ or ¼λ). They provide essentially colour to contrast the different orientations of collagen fibers (Figure 3.2B). The inconvenience of this kind of microscopy is that, under high magnifications (e.g. 63x), the polarized filters limit the quantity of light that arrives to the observer. Hence, the best option for analysing bone microstructures that require high magnifications, such as osteocytes, is the transmitted light microscopy (Figure 3.2C).

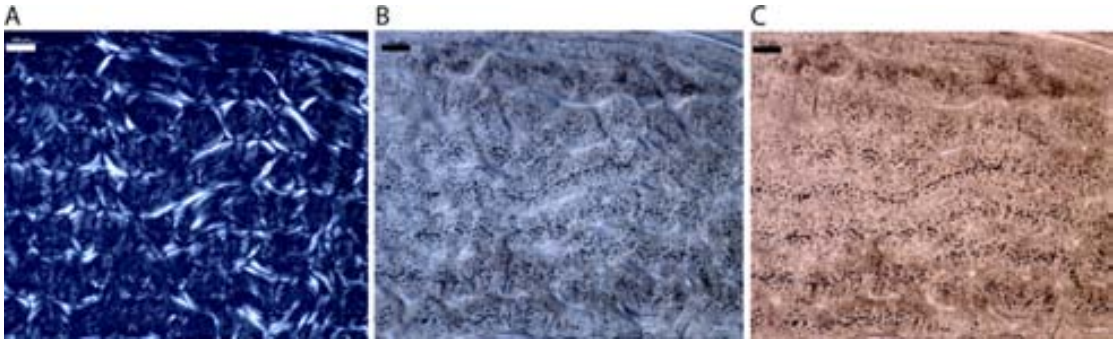

Figure 3.2: *Images of bone tissue by using different kind of microscopy. A. Fibro-lamellar bone under cross-polarized light microscope (linear polarized at 0º; analyzer at 90º), showing the maximum birefringence of the collagen (dark areas correspond to woven bone whereas bright areas correspond to lamellar bone). B. Under cross-polarized light with ¼ lambda filter. C. Under transmitted light microscope. Scale bar: 100 µm. Note that all images correspond to the same zone of the bone tissue of the ruminant* Moschus moschiferus (IPS 56276).

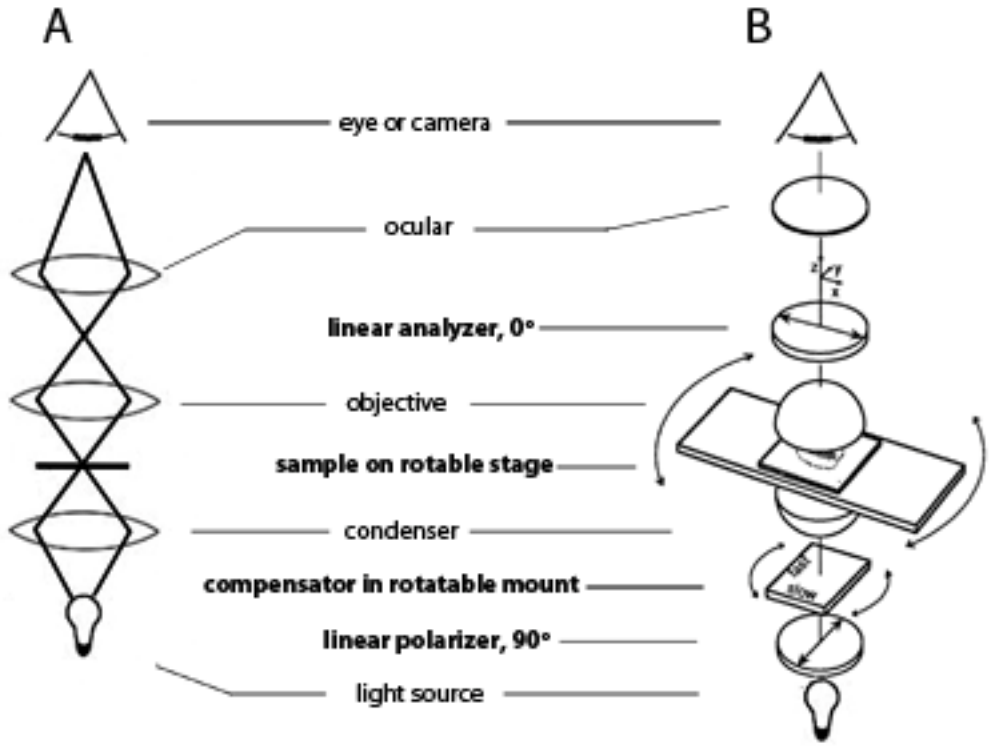

Figure 3.3: *Schematic of a transmitted light (A) and a polarizing microscopy (B). In bold the specific elements of a polarized light microscope (modified from Inoué and Oldenbourg, 1998).*

3.2.2. Bone histological analyses

Bone is a complex organ system that can be studied at many different levels of biological organization (Currey, 1984). The first level (macrostructural) of organization involves the descriptive and comparative anatomy. The second level (meso- or micro-structural) comprises the bone tissue, namely collagen fiber orientation, the cells arranged within the extracellular matrix (ECM) as well as the vascular canals. The third and fourth levels (sub-micro- to nano- structural) refer to the chemical and structural organization of its organic and mineral components (Francillot-Vieillot et al., 1990; Rho et al., 1998; Huttenlocker et al., 2013). The level of organization considered in the present work comprises exclusively the second order, the histological level.

3.2.2.1. Qualitative analyses: bone tissue nomenclature

Because of bone tissue comprises a highly variable structural patterns, it must be recognized and classified taking into account the biological significance. This Thesis follows the bone typology and nomenclature proposed by de Ricqlès (1991).

Typological classification of bone tissue:

Primary periosteal bone tissue, which is deposited during post-natal growth, can be classified taking into account the bone matrix type and vascularisation (de Ricqlès, 1991; de Margerie et al., 2002).

Bone matrix can be classified into lamellar, parallel-fibered or woven bone. Lamellar bone corresponds to a high level of spatial organization, formed by successive layers called lamellae. Each lamella is deposited by alternating the direction of deposition, which is visualized by alternating dark and light pattern when it is observed under cross-polarized light. Within each lamella, some flattened bone cells (osteocytes) can be observed. Parallel-fibered bone (PFB) is formed by a high quantity of closely packed collagen fibrils running parallel to each other. PFB shows an anisotropy and hence it appears homogenously dark or light under polarized light, accordingly to collagen orientation. Cells are flattened and more or less randomly distributed. PFB is deposited slowly, but not as slowly as lamellar bone (Castanet et al., 1996, 2000). Both lamellar and PFB bone are often presented in turtles, crocodilians and other reptiles (Francillot-Vieillot et al., 1990; Huttenlocker et al., 2013). If these bone tissue types (either lamellar or PFB) do not present any vascular canals, the bone tissue is named *lamellar (or parallel-fibered) non-vascular bone (LNV)*. Conversely, these bone tissue types can present vascular canals either simple-vascular canals or primary osteons. The first one consists in canals that are enclosed within the mineralizing matrix, forming the *lamellar (or parallel-fibered) simple-vascular bone (LSV)*. Primary osteons are formed by vascular canals surrounded by concentric bone lamellae, forming the *lamellar (or parallel-fibered) bone with primary osteons (LPO)*. It is worth to note that the vascularisation in lamellar bone or PFB is generally scarce (de Margerie et al., 2002).

The other typology of bone tissue matrix is the woven or fibrous bone, which consists in a loosely packed collagen fibers distributed without spatial arrangement and several spaces within the matrix. These features reflect a rapid rate of bone deposition. Because of a lack of spatial organization of the collagen fibers, woven matrix appears

isotropic (dark) under cross-polarized light. Typically this bone matrix also contains randomly distributed rounded osteocytes. Woven bone is found in embryonic bone tissues. However, during postnatal growth, the spaces of woven bone are filled by primary osteons, incorporating the lamellar fraction that surrounds the vascular canal, forming the *fibrolamellar complex (FLC) bone* (Francillot-Vieillot et al., 1990). Under cross-polarized light, woven matrix appears dark whereas lamellar matrix is bright. This bone matrix is generally highly vascularised with different vascular orientations. Vascular orientation can be classified into longitudinal canals (that run parallel to the bone diaphysis), circular canals (that run roughly parallel to the bone periphery), radial canals (that run roughly orthogonal to the bone periphery) or oblique canals (that are deposited between the parallel and orthogonal planes to the bone periphery) (Francillot-Vieillot et al., 1990; de Margerie et al., 2002). Because of the high abundance and different types of vascularisation within the FLC, different sub-typologies have been proposed to compile all the information.

Laminar bone tissue: This type of FLC contains numerous circular primary osteons. Laminar bone is formed rapidly and deposited during the phase of active growth in large land vertebrates, such as many mammals (for instance artiodactyls) and dinosaurs (Francillot-Vieillot et al., 1990; Castanet et al., 1996).

Plexiform bone tissue: This tissue type is similar to laminar bone tissue, but it comprises different vascular orientations. Plexiform bone tissue is formed by radial and oblique canals that generally connect the circular ones. This tissue type is formed slightly slower than laminar bone (Francillot-Vieillot et al., 1990; Castanet et al., 1996).

Reticular bone tissue: In this case, the numerous primary osteons show an oblique orientation and they are rather irregularly anastomosed. This bone tissue type seems to be associated with relatively modest amounts of primary compact bone, under slower rates than plexiform or laminar bone (Francillot-Vieillot et al., 1990; Castanet et al., 1996).

Radial bone tissue: In this tissue type, radially oriented primary osteons take prevalence over circular osteons to organize the tissue. Radial bone has the fastest growth of all vascular orientation types (Francillot-Vieillot et al., 1990; Castanet et al., 1996; de Margerie et al., 2004).

Growth marks:

Also known as incremental growth layers or growth rings, growth marks represent any variation in growth rate recorded at the morphological or histological levels in hard tissues (e.g. chitinous insect cuticles, keratinous turtle scales, sheep horns, mineralized mollusc shells, vertebrate bones and teeth among others). In bone, growth marks may constitute annuli or rest lines (Klevezal, 1996; Chinsamy-Turan, 2005).

Zones correspond to the tissue either lamellar, PFB or FLC bone, that forms between growth marks (annuli or rest lines), which is deposited during active osteogenesis and it is therefore broader than an annulus or a rest line. Conversely, annuli are narrow bands that correspond to periods of decrease (slow) growth, formed by lamellar bone. If present, the cells are flattened. On the other hand, rest lines or Lines of Arrested Growth (LAGs) correspond with temporary but complete cessations of growth (arrested osteogenesis) and may occur alone or within an annulus. LAGs, or annuli when present, are used for individual age estimations (Francillot-Vieillot et al., 1990; Woodward et al., 2013).

Accordingly, if primary bone tissue shows periodic growth layers then two kinds of bone can be distinguished, (1) *Lamellar-zonal bone*, when zones are composed by lamellar or parallel-fibered bone matrix (Francillot-Vieillot et al., 1990) and (2) *Fibrolamellar-zonal bone,* when zones are composed by woven matrix with abundant primary osteons (Castanet, 2006).

3.2.2.2. Quantitative analyses: data acquisition

The data obtained for the quantitative studies compiled in this PhD Thesis are grouped into vascular (orientation and density) and cellular parameters (density), based on the method described by Cubo et al. (2012). All measurements are carried out using the software ImageJ.

Vascular parameters:

Only primary osteons within the FLC bone are considered because they are formed during bone appositional growth. Secondary osteons (Haversian systems) are excluded as they are remodelling structures (Francillot-Vieillot et al., 1990). Polarized microscopy is essential to quantify vascular parameters because it allows to determine the lamellar bone that surrounds vascular canals as well as to differentiate primary from secondary osteons (a cementum line, which is more evident under polarized light, is

deposited surrounding the secondary osteon). Vascularity is quantified using medium magnification (objective: 10x).

Vascular orientation (circular, oblique and radial) is based on the method of Cubo et al. (2012) incorporating longitudinal canals. First, the roundness of the canals is measured dividing the minor by the major axis of de canal. If the value of this index is > 0.75 (i.e. the shape of the canal approximates a circle), the canal is considered as longitudinal (running parallel to the bone diaphysis). By contrast, when the value of canal roundness is < 0.75, the orientation of the canal is classified in circular, oblique or radial, following the method described by Cubo et al. (2012). According to this method, the orientation of the canals is computed as the angle between the major axis of the vascular canal tangent to bone periphery. Vascular canal orientation is a continuous trait that is transformed into discrete orientation classes using according to the following criteria shown in Figure 3.4. Vascular orientation is expressed as the proportion of a given vascular orientation respect to the other ones.

Vascular density is quantified by counting the number of vascular canals (primary osteons) on a given surface.

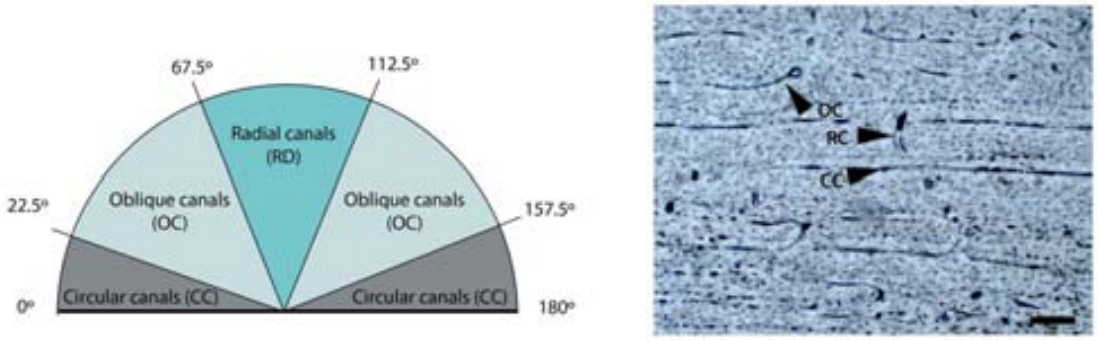

Figure 3.4: *Method for determining vascular orientation. Left: once the angle (continuous data) is obtained (calculated as the angle formed by the major axis of the canal tangent to bone periphery), it is transformed into three discrete types of orientation (circular, oblique and radial). Longitudinal orientation cannot be determined using this classification. Right: Example of vascular orientation classification in* Capreolus capreolus *(IPS 73885). Scale bar: 100 µm.*

Cellular density:

This parameter is quantified in the woven matrix as the number of osteocyte lacunae divided by the surface in mm^2. That is, the number of these structures in a single focused plane of osteocytes. Because polarized microscopy limits the quantity of light that

passes, the best option to analyse osteocyte lacunae is using transmitted light. First, the sampling zone (woven matrix) is selected using polarized microscopy allowing differentiation between primary and secondary bone and between woven and lamellar tissue. When a woven region is focused, then the polarized filters are removed, enhancing the quantity of light, which is importantly limited under high magnifications (objective used: 63x).

Osteocytes are quantified within primary bone matrix outside primary osteons (i.e. woven bone), taking into account whether osteocytes are derived from stationary osteogenesis (SO) or from derived osteogenesis (DO) (Marotti, 2010). Stationary osteogenesis forms the first bony framework, conforming the typical woven bone. Conversely, dynamic ostoegenesis thickens the SO-bony framework and/or filling the primary osteons, forming a more highly oriented parallel fibered bone. SO and DO osteocytes can be distinguished within FLC bone matrix (Figure 3.5). Osteocytes originated from SO are relatively large, globous (plump), retaining features of their incipient osteoblast morphology (Palumbo et al., 2004). DO-derived osteocytes, on the other hand, are smaller, more elongated and flattened (Franz-Odendaal et al., 2006; Kerschnitzki et al., 2011). Only SO osteocytes (enclosed within the woven bone) were quantified in order to compare exactly the same type of bone cells (Figure 3.5).

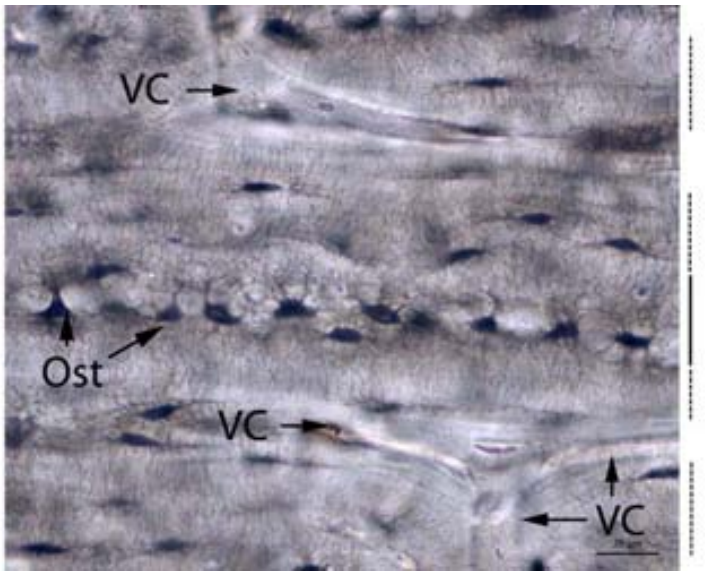

Figure 3.5: *Detail of FLC bone tissue with osteocytes of the bovid* Tragelaphus scriptus *(IPS 56206). Straight lines show the region that corresponds to the SO osteoctyes whereas dotted lines show the area corresponding to the DO osteocytes. VC: vascular canals; Ost: osteocytes. Scale bar: 20 µm.*

LAG retrocalculation:

Unlike other recording structures such as dentin or enamel in teeth, bone may undergo internal destruction (resorption) and redeposition, a process called bone remodelling. During remodelling, primary bone is deleted and substituted by secondary bone (Currey, 1984, 2003). Primary bone is the original tissue of a bone, while secondary bone, such as secondary osteons, is newly deposited in areas where primary bone tissue has been resorbed. Bone remodelling is associated with the phenomenon of bone drift and bone resorption (Francillot-Vieillot et al., 1990) and secondary remodelling (Currey et al., 2003).

Secondary remodelling results in the production of Haversian bone, in which most of the bone is occupied by secondary osteons (Haversian systems). The main reasons that have been suggested for secondary remodelling are: (1) storage of calcium and phosphate; (2) mechanical competence (both tension and compression remodelled bone); (3) changing the grain (under large muscle insertions and during fracture repair); (4) taking out microcracks (in bones under strong loaded in fatigue are linked to large number of microcracks, which in turn are associated with secondary remodelling) and (5) with increasing age (bone became hypermineralised and weaker with aging) (Figure 3.6) (Currey, 2003). Secondary remodelling hides primary bone matrix, which complicates some histological studies as quantitative analyses or skeletochornology (some LAGs can be hidden).

Bone drift involves a relocation process through bone resorption and bone reconstruction during growth (Francillot-Vieillot et al., 1990). This process is a response to biomechanical strains and /or stresses that continuously change during ontogeny, providing the best bone shape depending on the current demands. During bone growth, the medullar cavity expands causing the deletion of the inner cortical bone and subsequent deposition of secondary (lamellar) bone.

Bone resorption (without bone deposition) is not considered as a remodelling process but it can also remove the inner cortical bone (Figure 3.6) (Francillot-Vieillot et al., 1990). This process is related to aging (Hall, 2005).

Therefore, remodelling processes, either secondary bone remodelling or bone drift, and bone resorption are important factors to take into account in studies on primary

bone tissue as well as when skeletochronology is applied (Horner and Padian, 2004; Woodward et al., 2013).

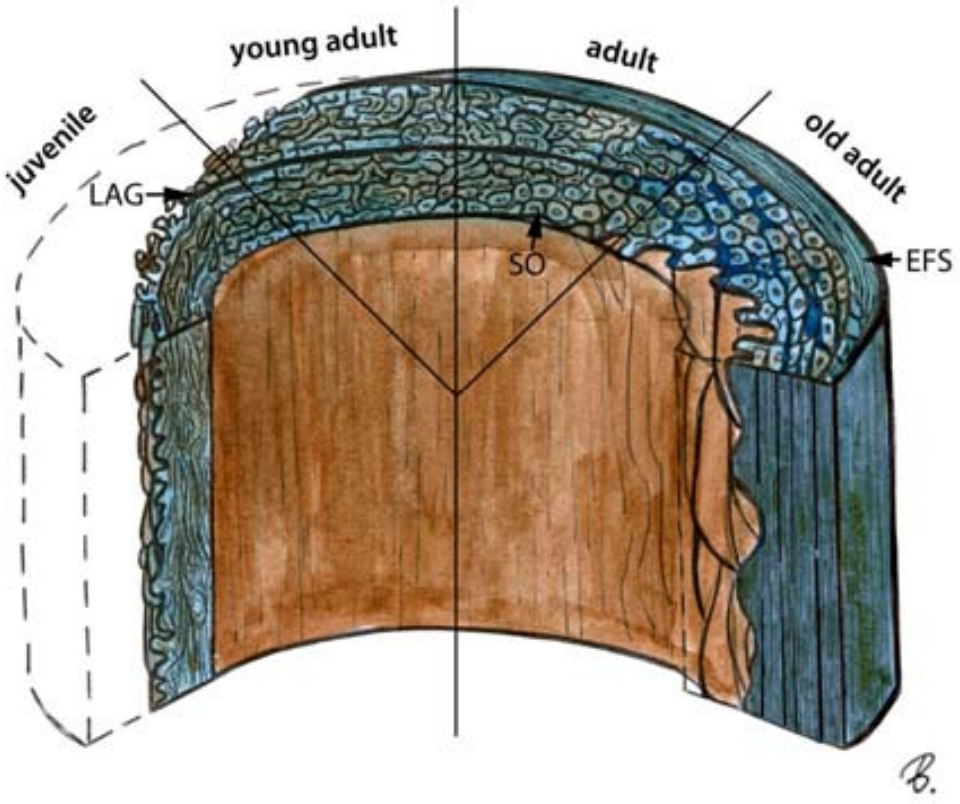

Figure 3.6: *The effects of secondary remodelling and bone resorption on primary bone matrix with increasing age. Juvenile, bone matrix is formed basically by FLC, the first LAG is formed and then FLC is deposited again. Young adult, the EFS is formed, indicating that sexual maturity has been reached at their second year. Adult, secondary osteons (SO) can appear in the innermost part of the cortical area, hiding the inner part of the primary bone matrix. Old adult, secondary remodelling and bone resorption make impossible the analysis of primary bone tissue. Note that bone drift is not reflected in this figure.*

In order to reconstruct the information lost by remodelling processes or bone resorption, the analysis of ontogenetic series become essential (Figure 3.6). To estimate the age of an individual, the antero-posterior diameters of successive growth rings in bone cross-sections are measured, assuming that these diameters are similar in similar-aged individuals. Consequently, the LAGs lost are reconstructed by comparing successive diameters of growth cycles between individuals of different ontogenetic stages in a given species. In artiodactyls, the latero-posterior region is that mostly affected by secondary

remodelling since this region supports the mechanical loadings of important muscle attachments (Currey, 2003). Conversely, the anterior-posterior axis is less prone to bone drift and secondary remodelling (Figure 3.7). This analysis has been carried out whenever an ontogenetic series was available.

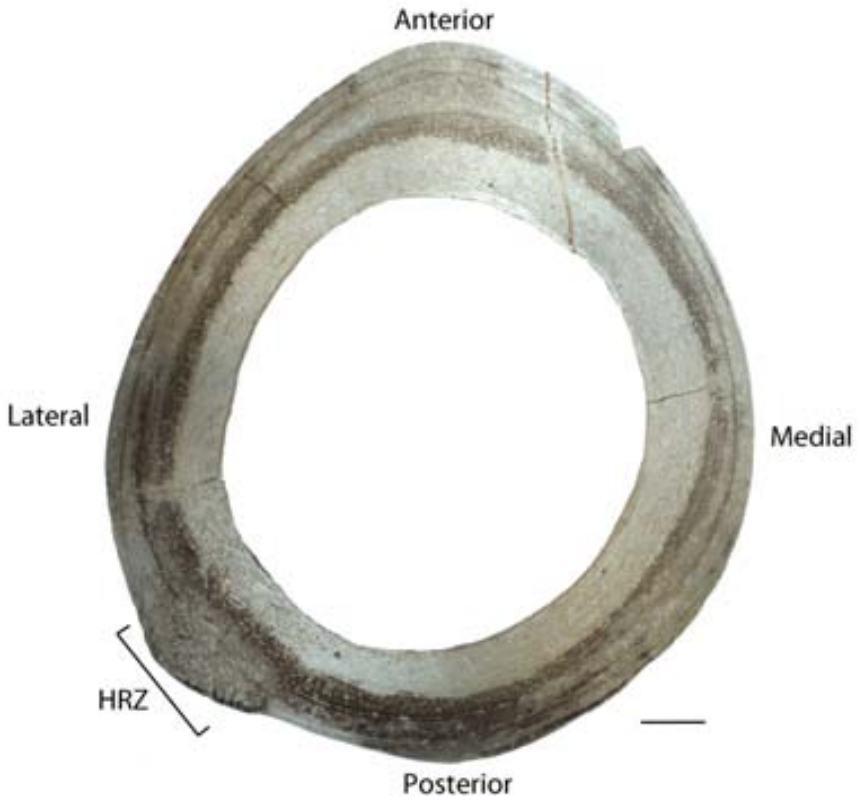

Figure 3.7: *Thin section orientation of* Tragelaphus scriptus *(IPS 56206), showing the four planes. HRZ, highly remodelled zone is lateralized in the latero-posterior axis. Scale bar: 4 mm.*

CHAPTER 4. THE ONTOGENY OF BONE GROWTH IN TWO SPECIES OF DORMICE:

RECONSTRUCTING LIFE HISTORY TRAITS

dx.doi.org/10.1016/j.crpv.2011.03.011

CHAPTER 5. SEASONAL BONE GROWTH AND PHYSIOLOGY IN ENDOTHERMS SHED LIGHT ON DINOSAUR PHYSIOLOGY

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CHAPTER 6. EXPLORING ONTOGENETIC SIGNAL IN THE FIBROLAMELLAR BONE OF MAMMALS WITH IMPLICATIONS FOR LIFE HISTORY INFERENCES.

6. EXPLORING ONTOGENETIC SIGNAL IN THE FIBROLAMELLAR BONE OF MAMMALS WITH IMPLICATIONS FOR LIFE HISTORY INFERENCES.

6.1. INTRODUCTION

Because bone tissue, as most biological hard tissues, is a recording structure, inferences about the life history strategies of extinct and extant vertebrates can be based on bone histology (Klevezal, 1996; Chinsamy-Turan, 2005; Padian et al., 2013). Recording structures are those that, during formation, respond to changes in the physiological condition of an organism by changing their morphological characteristics (Klevezal, 1996). Bone cross-sections preserve incremental growth layers indicating periods of active and arrested osteogenesis that correspond to annual growth cycles (Castanet and Smirina, 1990; Woodward et al., 2013). It has long been thought that annual cycles of bone growth arrest are exclusive to ectotherms, which depend largely on elevated ambient temperatures to fuel their metabolic processes and stop growing at lower temperatures (Pough, 1980). Some few studies, however, have shown that annually deposited lines of arrested growth (LAGs) may also be present in endotherms, though they have been thought to be restricted to heterothermic taxa (Klevezal, 1996; Chinsamy-Turan, 2005) and early mammals (Chinsamy and Hurum, 2006). During the last years, however, it has increasingly been observed that also homeothermic mammals may deposit LAGs (Sander and Andrássy, 2006; Köhler and Moyà-Solà, 2009; Köhler, 2010; Marín-Moratalla et al., 2011, 2013; Köhler et al., 2012, Straehl et al., 2013). Most recently, Köhler et al. (2012) (see Chapter 5) performed a comprehensive global study of wild ruminants from tropical to polar environments providing strong evidence that annual cycles of growth arrest in bones are a universal trait of homoeothermic endotherms. In this study, we presented a general model for the correlation between cyclical bone growth and seasonal physiology in endotherms. Following this model, growth is arrested during the unfavourable season (low resource supply) forming part of a plesiomorphic thermometabolic strategy for energy conservation. Conversely, active osteogenesis during the favourable season reflects the capability to efficiently exploit and allocate resources to growth. We concluded that bone growth is regulated by an endogenous rhythm that is synchronized with seasonal resource availability rather than by environmental stresses.

Annually deposited lines of arrested growth (LAGs) in mammals, the same as in ectotherms, can be used to reconstruct the timing and duration of certain life history events (i.e. age at maturity, lifespan, growth rate). This approach, known as 'skeletochronology', has been widely applied mainly in non-avian archosaurs (Chinsamy-Turan, 2005; Woodward et al.,

2013 and references therein), but only sporadically to mammals (Castanet et al., 2004; Köhler et al., 2012; Marín-Moratalla et al., 2011, 2013 –Chapter 8 and 9 respectively-; Straehl, 2013).

The structural classification of primary bone tissue (bone matrix types and vascularity) deposited during active growth provides information on growth rates (Amprino, 1947; Castanet et al., 1996; de Margerie et al., 2002, 2004). Amprino (1947) was the first to suggest that the discrete variation in periosteal bone tissues is primarily the expression of different rates of bone deposition, e.g. slow-growing lamellar or parallel-fibered bone *vs* fast-growing fibrolamellar bone. This relationship between bone tissue structure and growth rate has been coined *Amprino's rule*. Subsequently, experimental fluorescent labelling allowed accurate quantification of periosteal deposition rates in growing bone. These studies evidenced that *Amprino's rule* applies well to major categorical tissues types. That is, *Amprino's rule* has limited value in the estimation of absolute values of periosteal growth rates, but it is a powerful tool in understanding the relative changes. This work expanded *Amprino's rule* by including new histological features related to the vascular and cellular network pattern in primary bone tissue (Castanet et al., 1996, 2000; de Margerie et al., 2002, 2004; Cubo et al., 2012).

Despite the increasing interest in bone histology as an approach to make inferences about life history in vertebrates, and specifically in mammals, some important issues remain poorly studied (Castanet et al., 1996; Padian et al., 2013). Currently, bone microstructure is considered to record four signals, namely ontogeny, phylogeny, mechanics and environment (Padian, 2013; Padian et al., 2013). Among these, ontogeny plays a fundamental role in the reconstruction of life histories of vertebrates. However, it is still poorly understood how this factor influences bone development, specially the fibrolamellar complex (FLC) bone tissue. Because ectotherms do not show the whole spectrum of histological patterns but only lamellar or parallel-fibered bone, and because other taxonomic groups that do show it are extinct (as non-avian dinosaurs), mammals represent the only group that can provide insights into these relationships.

This study aims to explore how ontogenetic changes influences primary bone tissue. Using 85 wild individuals of almost all ruminant tribes from a wide range of habitats, including environmental extremes, some histological features such as the number of LAGs, the cellular and vascular density, and the vascular orientation in primary bone are quantified. This study, hence, provides valuable data on how ontogeny is reflected in mammalian FLC.

Ruminants are an excellent group on which to conduct such a study. They are a geologically young group of modern large endotherms mitigating the phylogenetic signal in

bone tissue. They share locomotor behaviour removing the effect of biomechanical properties in bone histology. Finally ruminants dwell in a wide array of climatic regimes, allowing assessing the environmental correlates of bone histology. Most ruminants are habitat specialists for which variables such as rainfall, temperature, and vegetation for food and shelter must remain within specific limits (Gentry et al., 1999). Thus, they strongly respond to environmental changes, whether natural or anthropogenic (Vrba and Schaller, 2000).

6.2. MATERIAL AND METHODS

The material studied here corresponds to 85 wild individuals (right femora) that belong to 3 species of cervids and 27 species of bovids (see Appendix 6A). Most of red deer (*Cervus elaphus hispanicus*) and roe deer (*Capreolus capreolus*) material comes from hunters, who donated the skeletons for scientific study. The sample of red deer is composed of individuals killed during the hunting season in February 2012 in Badajoz and La Rioja as well as by two carcasses collected in Huesca (all localities from Spain). Roe deer (*Capreolus capreolus*) were killed during the hunting season in May 2013 in Lleida (Spain). Fresh carcasses of Svalbard reindeer (*Rangifer tarandus platyrhynchus*) were collected and sexed by researches from Norwegian Polar Institute (NPI) during a field campaign in Svalbard archipelago (Norway) during July 2010. All skeletal remains of these deer are housed at the Institut Català de Paleontologia Miquel Crusafont (ICP). The species of bovids were hunted in Africa between the fiftieth and the seventies by H. Oboussier (Köhler et al., 2008). The skeletal remains with associated individual data (body mass, sex, site and date of death) are housed at the scientific collections of the Zoological Museum of University of Hamburg.

Histological slides of femora were made at midshaft following standard procedures (García-Martínez et al., 2011, Marín-Moratalla et al., 2013, see also Chapter 3). Slides were examined under transmitted or polarized light with a 1λ filter. Micrographs were taken with polarization microscope with camera (Nikon, Elipse E600POL and Leica D; 2500P). For quantitative analysis we used the software Image J.

6.2.1. Histological features

Overall, the cyclical rate of bone deposition is recorded in a cross-section by alternating bands of bone tissue, i.e. a growth zone and a zone of slowdown growth (annulus) or arrested growth (LAG) (Chinsamy-Turan, 2005). In ruminants, the fibrolamellar-zonal complex is composed of alternating zones of fibrolamellar bone and rest lines that determine

an annual growth cycle (in few cases we can observe two LAGs running together within an annulus or an annulus without LAGs) (Köhler et al., 2012). This conformation of long bone tissue allows estimating the chronological age of certain life history events, such as the age at reproductive maturity and at death (Köhler and Moyà-Solà, 2009; Marín-Moratalla et al., 2011, 2013 -Chapter 8 and 9 respectively-; Köhler et al., 2012 -Chapter 5-).

Bone remodelling may hide and remove early ontogenetic stages and consequently the first growth cycles (Woodward et al., 2013, see Chapter 3 for further information). Hence, for age estimations, LAG retrocalculation is essential to find out whether or not LAGs deposited early during ontogeny were missing in the sample analyzed here. For that, the antero-posterior diameters of successive growth rings in a single bone were measured and compared among individuals of the same species, under the assumption that cortical diameters are similar in similar-aged individuals following Marín-Moratalla et al. (2013) (see Chapter 9) (Figure 6.1A). Retrocalculation of LAGs was conducted only for those species represented by at least two ontogenetic stages, a juvenile and an adult individual (see Appendix 6A).

The age at reproductive maturity of the sample analysed here was estimated by bone histology. The age at first reproduction (AFR) can be theoretically determined by counting the number of growth cycles within the FLC bone, before the beginning of the lamellar tissue deposited in the outermost cortical layer, known as External Fundamental System (EFS). The transition from the fast-growing fibrolamellar complex (FLC) bone to slow-growing lamellar tissue (EFS) is classically believed to record the reproductive maturity of an animal (Figure 6.1B and Figure 6.1C). The distinction between FLC and EFS is qualitatively possible due to the nature of their bone matrix (woven bone and lamellar bone, respectively) (Francillot-Vieillot et al., 1990).

In order to estimate the age at reproductive maturity only the adult sample was used (see Appendix 6A). The species-averages of AFR were estimated separately for males and females, and were compared with data on age at reproductive maturity available from literature (see Appendix 6B), as well as among species taking into account the body mass. The antero-posterior diameter at the femora's midshaft (APD) was used as a proxy for body mass (Carrano, 2001).

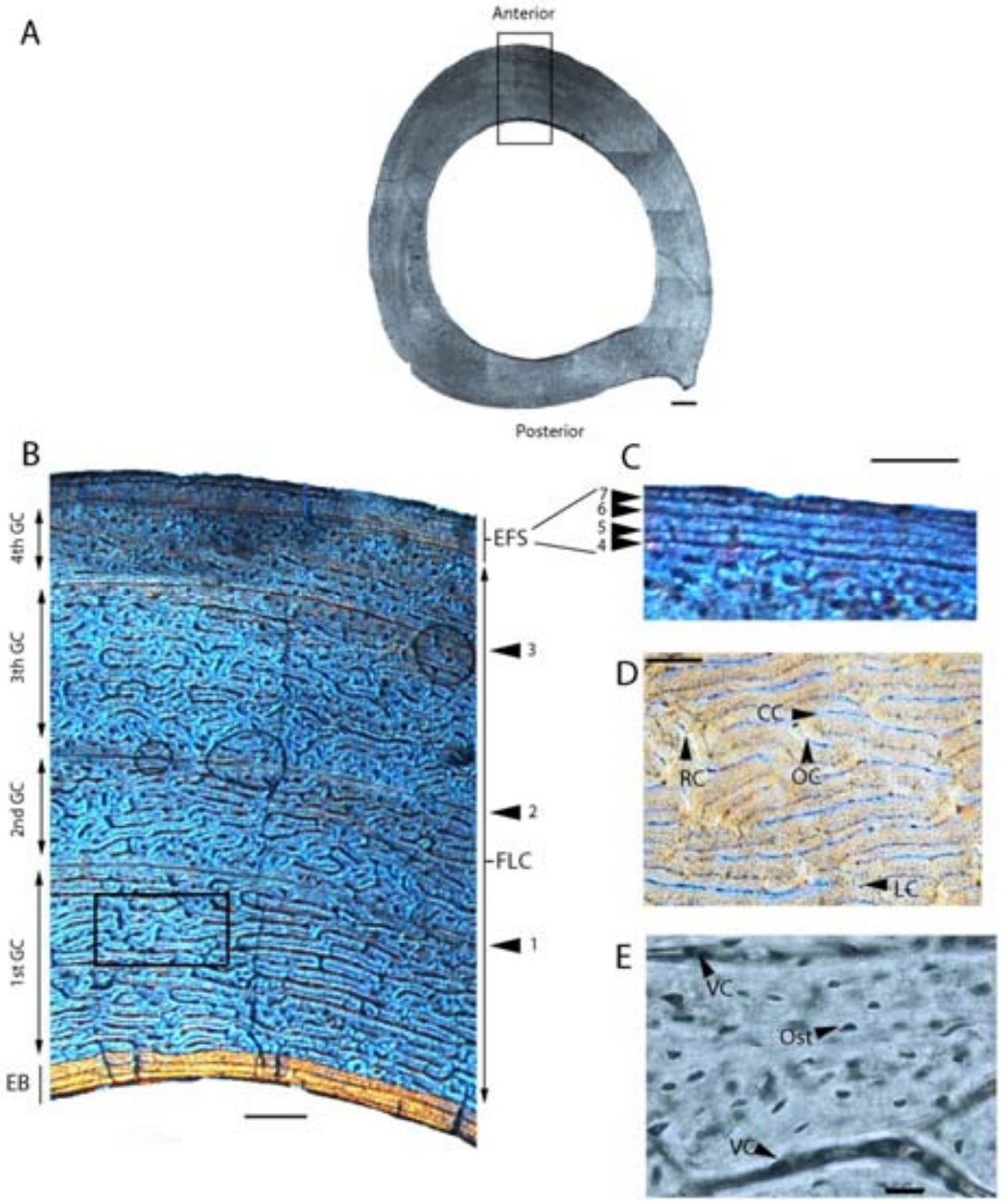


Figure 6.1: *Femora cross-section of* C. elaphus hispanicus *(IPS 60873) under different magnifications. A. The whole section showing the zone where histological features were analyzed. Scale bar: 2 mm. B. Detail of anterior region. We have determined three growth marks (arrowheads) and four annual growth cycles within FLC. The presence of an EFS indicates that this specimen reached maturity. Secondary deposition of endosteal bone (EB). Scale bar: 500 µm. C. Detail of the EFS showing three LAGs (arrow heads), Scale bar: 50 µm. D. Detail of the vascular orientation within the first growth cycle. CC: circular canals, OC: oblique canals, RC: radial canals, LC: longitudinal canals. Scale bar: 200 µm. E. Detail of the zone where osteocyte density is quantified. VD: vascular canal, Ost: Osteocytes. Scale bar: 20 µm.*

Vascular and cellular quantitative data were obtained by measuring the structures that contained the blood vessels and nerves during life (vascular canals, Figure 6.1D) and the osteocytes (osteocyte lacunae, Figure 6.1E) based on methods described in Cubo et al. (2012). We quantified the canals dividing them into four categories by their orientation: longitudinal,

circular, oblique and radial (see Chapter 3). Moreover, vascular and cellular densities were measured by counting the number of these structures on a given surface.

As commented above, the FLC-zonal bone (juvenile phase) is composed of alternating zones of active and arrested osteogenesis and is bordered by the EFS (maturity). The above mentioned features were measured in the growth zone of each annual cycle throughout the ontogeny of each individual. Because of the sample analysed is scarce in female individuals, this analysis was conducted only in males in those species represented by a minimum of two specimens showing at least two growth cycles within the FLC (see Appendix 6A). Because histological variation may exist within a single bone (Castanet et al., 2000), all measurements were taken in the anterior region of the femoral cross-section (Figure 6.1A and 6.1B).

6.3. RESULTS

Primary bone tissue of immature ruminants consists of woven bone with primary osteons (FLC). The main vascular type that prevails within the FLC bone is the circular canal, forming the laminar or plexiform bone tissue (Francillot-Vieillot et al., 1990; de Ricqlès, 1991) and bone remodelling is negligible in those individuals (Figure 6.2A,C). It is not unusual to observe open canals at the outermost part of the cortex of immature individuals (Figure 6.2B) as well as LAG's embedded in the primary matrix forming the fibrolamellar-zonal complex (Castanet, 2006).

Adult ruminants deposit highly organized lamellar bone with rest lines in the outermost cortical layer forming the EFS (Figure 6.2D,E). With increasing age, secondary osteons (Haversian systems) are gradually deposited in the cortical bone from the inner towards the outer cortical area, whereas bone resorption and remodelling around medullar cavity removes the inner primary bone deposited during the earlier stage of ontogeny (Figure 6.2F).

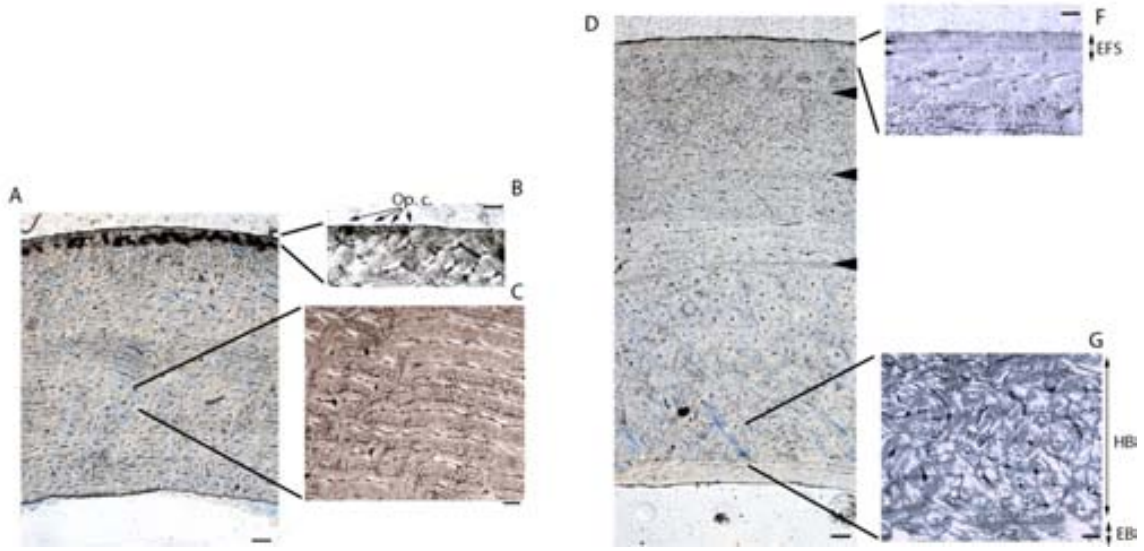


Figure 6.2: *Differences between juvenile and adult bone histology. A. Femora cross-section of a juvenile deer (*Cervus elaphus hispanicus*, IPS 74310) showing the FLC bone tissue. Scale bar: 200 µm. B. Detail of the outermost part of the section, showing a plexifom bone tissue with open vascular canals (Op. c.) in the bone's periphery. Scale bar: 100 µm. C. Detail of the innermost part of the immature femur, showing the plexiform bone tissue without bone remodelling. Scale bar: 100 µm. D. Femora cross-section of an adult deer (*Cervus elaphus hispanicus*, IPS 60875), showing the fibrolamellar-zonal complex with three LAGs (arrowheads). Scale bar: 200 µm. E. Detail of the outermost part of the adult bone, showing the EFS with three LAGs (arrowheads). Scale bar: 100 µm. F. Detail of the innermost part of an adult femur, showing bone remodelling (haversian bone, HB; and the endosteal bone, EB). Scale bar: 100 µm.*

In order to explore whether or not the first LAGs were missing (deposited during early ontogeny), the antero-posterior diameters of successive growth rings were plotted against estimated age in those species with at least two ontogenetic stages (juvenile and adult) in the sample analysed (see Appendix 6A). To estimate age from LAGs, it is assumed that cortical diameters are similar in similar-aged individuals. For most of these species (47 individuals comprising 10 species), femora cross-sections record the whole of ontogeny (see Appendix 6C), except for two males of *C. elaphus hispanicus* and a male of *T. strepsiceros*, in which the first LAG was deleted by remodelling (Figure 6.3).

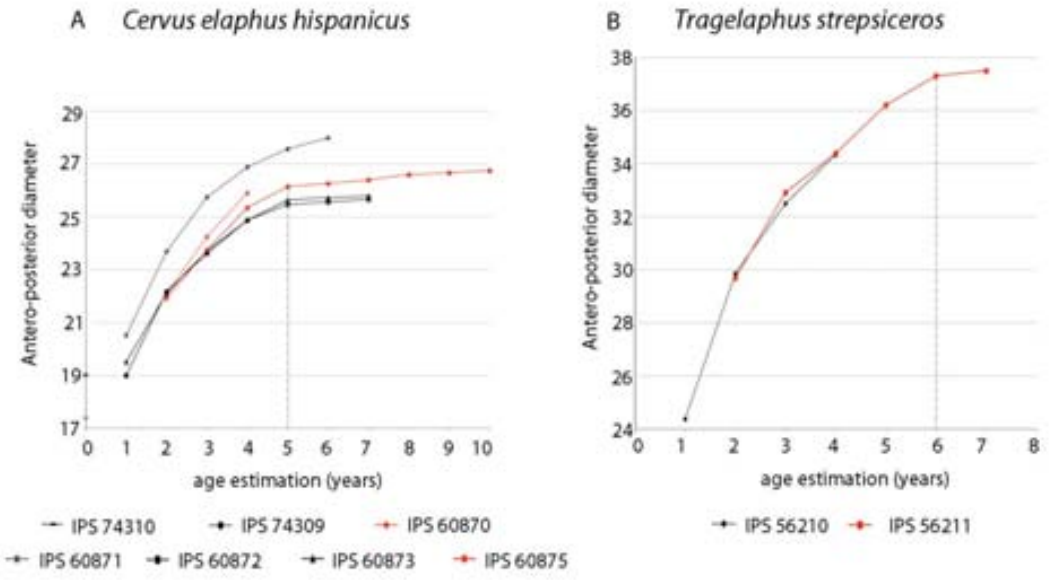


Figure 6.3: *LAG retrocalculation method. Plot of the antero-posterior diameter (APD) of successive growth cycles against the corresponding estimated age. Juvenile individuals (dashed lines). Adult individuals (solid lines). Individuals that lack their first LAG (red lines). Note that the APD of the first growth cycle in these individuals fits the APD of the second growth cycle of other specimens. A. Males of* C. elaphus hispanicus*. Note that adult individuals attained reproductive maturity at 5 years of age (vertical dashed line). B. Males of* T. strepsiceros*. Note that the adult male attained reproductive maturity at 6 years of age*.

Both species belong to the largest species of the sample with the longest growth period. In *C. elaphus hispanicus* (ICP 60875) and *T. strepsiceros* (ICP 56211), the advanced age of both male specimens may explain the lack of their first rest lines by medullar reabsorption. A four years young deer (ICP 60870) also lost its first LAG in the anterior region of the femoral cross-section, though it is still visible in other regions. The results, hence, indicate that the largest cervids and bovids (male *C. elaphus hispanicus* ≈180 kg, Gaspar-López et al., 2009; male *T. strepsiceros* 280 kg, see Appendix 6A) may lose their first rest line during ontogeny. In the present study, however, is considered that the first LAG was not missing in those species of the sample that are represented by adult individuals only.

When distinguishing between males and females, all adult individuals within a species, show equal numbers of growth cycles in the FLC-zonal bone, except for males of *C. capreolus* where the number of growth cycles varies between 3-4. The number of growth cycles in the FLC-zonal bone in the ruminant species analysed here is broadly consistent with ethological data previously published on age at reproductive maturity (AFR) (see Appendix 6B). The figure 6.4 shows the plots between the number of growth cycles within FLC-zonal bone for each species (as a proxy for AFR) and their APD (within-species average), distinguishing between

males (Figure 6.4A) and females (Figure 6.4B). Considering the regression line for all ruminant species, cervids (*C. elaphus hispanicus, R. tarandus platyrhynchus* and *Capreolus capreolus*) show a larger number of growth cycles than expected from their size (suggesting delayed growth) while most bovids are placed below the regression line. Interestingly, addax (*Addax nasomaculatus*), the blue duiker (*Philatomba monticola*) as well as most species of Tragelaphini are placed above the regression line like cervids.

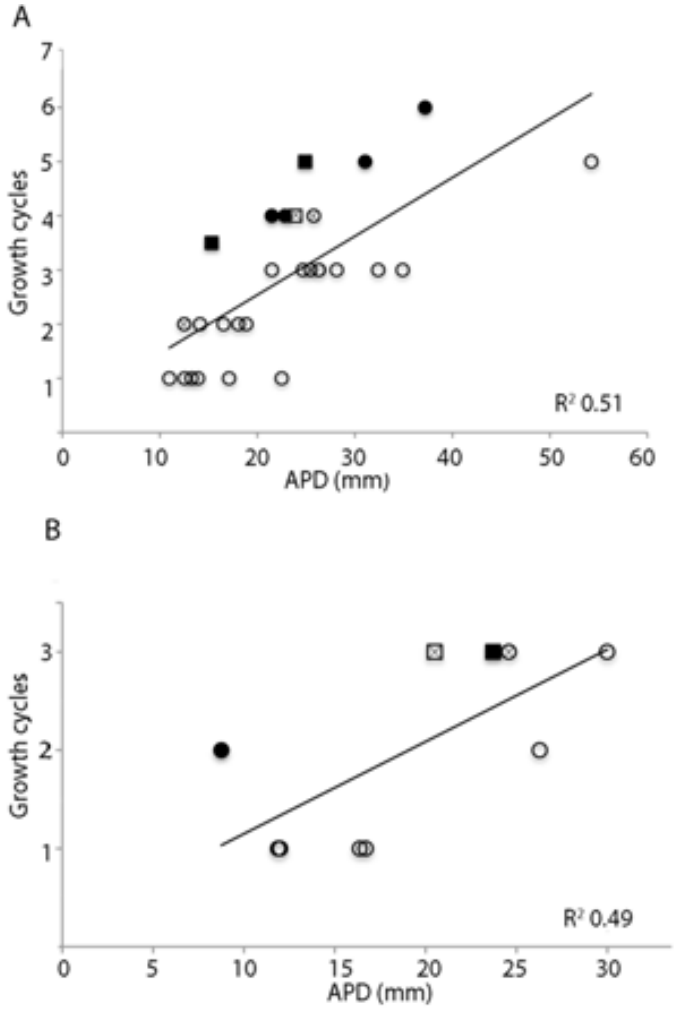


Figure 6.4: *Plot of the number of growth cycles within the FLC-zonal bone against APD of the adult sample (species average). A. males, B. females. Empty-circles: bovids from open habitats under more suitable conditions, crossed-circles: bovids from open habitats under resource limitation, filled circles: bovids from closed habitats, crossed-squares: cervids from open landscapes but under resource limitation, filled-squares: cervids from closed environments*

Ontogenetic changes of quantitative histological features (cellular and vascular density and vascular orientation) have been explored for male specimens (see Appendix 6A). Overall, quantitative features of FLC-zonal bone change along the growth period (Table 1).

Table 6.1: *Ontogenetic variation in quantitative histological features (species-average).*

Family	Species	CD	VD	C	L
Bovidae	*A. melampus*	↓(3/3)	↓(2/3)	↓(2/3)	↑(3/3)
	G. dorcas	↓(2/2)	↓(2/2)	↓(2/2)	↑(2/2)
	T. scriptus	↓(4/4)	↓(4/4)	↓(2/4)	↑(2/4)
	T. spekii	↓(3/4)	↓(3/4)	↓(3/4)	↑(4/4)
	T. strepsiceros	↓(4/6)	↓(4/6)	↓(3/6)	↑(3/6)
Cervidae	*C. elaphus*	↓(4/6)	↓(5/6)	↓(3/6)	↑(3/6)
	R. t. platyrhynchus	=	=	↓(4/4)	↑(2/4)

Symbols (arrows and equal symbol) show whether the parameter analyzed drop, rise or remains constant along ontogeny, as well as the growth cycle at which the most significant changes occur in relation to the total number of growth cycles recorded in the FLC-zonal bone. CD: cellular density, VD: vascular density, C: circular canals, L: longitudinal canals.

During early ontogeny, circular canals are predominant forming laminar bone (Francillot-Vieillot et al., 1990), whereas later in ontogeny, circular canals decrease and longitudinal canals increase in frequency. This pattern is more evident in *T. strepsiceros*, *G. dorcas,* and *A. melampus*, in which the proportion is reversed in the latest stages, becoming longitudinal canals more abundant than circular ones. As regards cellular and vascular density, the results indicate that both features decrease significantly throughout ontogeny (Table 6.1, and see Appendix 6D). However, ontogenetic changes in vascular orientation and vascular or cellular density are not always synchronized. Vascular orientation tends to change before vascular and cellular densities drop. For example, the proportion of circular canals drops earlier than do vascular density in *T. scriptus, T. strepsiceros* and *C. elaphus hispanicus* (Table 6.1, and see Appendix 6D). Despite this, the significant changes in these quantitative histological features generally occur during the last or close to last year of juvenile growth (Table 6.1, and see Appendix 6D). These results are consistent with the decrease of growth rate when maturity approaches (Maiorana, 1990). Therefore, the missing of the first growth cycle in large ruminants such as in *C. elaphus hispanicus* and *T. strepsiceros,* is a minor issue when comparing quantitative histological features among species (see Appendix 6D). Interestingly, the arctic reindeer from Svalbard archipelago shows no significant changes neither in vascular nor in cellular density throughout ontogeny, which would require further research (Table 6.1, and see Appendix 6D).

6. 4. DISCUSSION

Age at first reproduction (AFR) is a critical trait that marks the moment in an individual's life at which resources are shifted away from growth toward reproduction (Stearns, 1992). It is one of the most important traits that determine the species' fitness (Ricklefs, 2007). Predation is considered to exert strong selection on age at first reproduction (Stearns, 1992; Roff, 2002) with increasing extrinsic mortality rates triggering evolutionary shifts towards reduced age and size at maturity (Palkovacs, 2003, 2011).

Reproductive maturity of ruminant long bones is recorded as the transition between fast growing 'FLC' to slow growing EFS bone (Köhler, 2010; Marín-Moratalla et al., 2011, 2013; see Chapter 8 and 9) due to the trade-off between growth and reproduction. Life history theory postulates that energy and time are limited and hence, individuals should allocate resources optimally among growth, maintenance, and reproduction to maximize fitness (Stearns, 1992). As long as an organism needs to grow in order to attain a minimum size for successful reproduction, resources are channelled towards growth (and maintenance). As soon as this size is attained, resources are channelled away from growth towards reproduction (Stearns, 1992; Roff, 2002; Ricklefs, 2007).

The results presented in this study show that the age at which ruminants reach the reproductive maturity could be reasonably estimated by counting the number of growth cycles (or LAGs), that records the FLC bone in femoral cortices. However, in a few cases obvious discrepancies exist with the data from literature, as in the case of *K. ellipsiprymnus, N.granti* or *A. nasomaculatus* (see Appendix 6B). Different reasons could be behind these discrepancies. We estimated that the addax female specimen reached reproductive maturity almost two year later than described for wild addax in the literature (Kingdon, 1997). It is, however, no clear whether these data refer to physiological or reproductive maturity (see later in discussion). Also population variability may explain some of the discrepancies. The results show that *N. granti* reached reproductive maturity within the first year of life; however, Estes (1991) reported an age at sexual maturity of three years for this gazelle. *N. granti* typically dwell in arid to semi-arid habitats (Estes, 1991). In these ecosystems, water restrictions affect reproductive patterns (Cain III, 2006) and gazelles attain maturity according to the water availability. Under favourable conditions gazelles may attain sexual maturity earlier than when under water restriction (Baharav, 1983). The *N. granti* analysed in this study comes from Lolkisale Conservation Area (Tanzania), characterized by a tropical savanna climate where water is less limited than in the typical habitat of this gazelle.

Other important factor to take into account is that bone resorption and secondary remodelling are common features in mammalian bones (Enlow and Brown, 1957), specifically in ruminant bone because their fast growth (Georgiadis, 1985). This study shows that, in large ruminants (>150kg, approximately), the first growth cycle could be missing in older animals, as we have shown for *Cervus elaphus hispanicus* and *Tragelaphus strepsiceros.* This could also be the case for the large antelope *K. ellipsiprymnus* (>200kg, >7 years old), for which we estimated an age at reproductive maturity of 3 years, i.e. to two years earlier than that recorded in literature (Owen-Smith, 1993). However, the lack of an ontogenetic series of this species does not allow us to verify this hypothesis.

Age at reproductive maturity in mammals, the same as other life history traits, correlates positively with body mass (Stearns, 1992; Roff, 2002; Ricklefs, 2007). As expected, the estimations of reproductive maturity using bone histology correlate well with the antero-posterior diameter at femoral midshaft 'APD', as a proxy for body size. Moreover, in agreement with life history theory, these results further reflect co-variation between the AFR with environment regardless of body mass. The age at reproductive maturity is often the demographic trait most sensitive to environmental variation and density-dependence (Clutton-Brock et al., 1982; Gaillard et al., 2000). This study shows that among ruminants, deer species and some bovid species such as the blue duiker (*P. monticola*), addax (*A. nasomaculatus*) and most of Tragelaphine antelopes (*T. imberbis, T. scriptus, T. spekii, T. strepsiceros*) display delayed AFR (estimated by bone histology) relative to body size. All these species but addax live in wooded and closed habitats, which are characterized by low predation pressure (Clutton-Brock et al., 1982; Owen-Smith, 1993; Köhler, 1993; Wronski et al., 2009). Addax dwells in deserts, characterized by resource limitation and scarcity of predators (Mallon and Kingswood, 2001). Following life history theory, low extrinsic mortality, generally caused by low predation, is the fundamental force in shifting life histories towards the slow end of the fast-slow continuum (Stearns, 1992; Roff, 2002; Festa-Bianchet et al., 2006; Ricklefs, 2007). On the other hand, 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 (Stearns, 1992; Gaillard et al., 2000; Festa-Bianchet, 2006). In agreement with this, the results of this study show that bovids dwelling in open habitats, such as Antilopinae, Aepycerotinae, Hippotraginae, Alcelaphinae, attain AFR early relative to their body mass. Hence, this study shows that reproductive maturity, the life history trait that is most sensitive to environmental conditions, is reliably recorded in the bone tissue of all ruminants here analysed.

Vascularity and cellular (osteocyte lacunae) density are histological features strongly related with bone growth rates (Mullender, 1996; Bromage et al., 2009, 2012; Cubo et al., 2012). Growth rate is a life history trait that varies greatly during ontogeny (Maiorana, 1990; Castanet et al., 1996). The results on vascular orientation show an important variation during ontogeny. Circular canals are predominant in early ontogeny forming the laminar bone. In later ontogeny, the number of circular canals drops and that of longitudinal canals increase. The results further show an important ontogenetic variation in vascular and cellular density with both features decreasing throughout bone development. According with previous works, high proportion of circular canals and high cellular and vascular densities are related with high bone deposition rate (Castanet et al., 1996; Mullender et al., 1996; de Margerie et al., 2002; Montes et al., 2007, 2010; Bromage et al., 2009; Cubo et al., 2012). Vascular and cellular network patterns, hence, reflect the decrease in growth rate, as maturity approaches (Maiorana, 1990).

Overall, the most significant change in the FLC bone features occurs a short time before reproductive maturity (EFS formation), indicating that the most significant decrease in periosteal deposition rate throughout the FLC-zonal bone occurs at the latest stages. A hypothesis to explain this trend is that the change in the vascular and cellular network pattern could be related with physiological maturity (Georgiadis, 1985; Clutton-Brock et al., 1982; Vanpé et al., 2009), whereas the major change in tissue typology from FLC to EFS bone is related to reproductive maturity (Klevezal, 1996; Marín-Moratalla et al., 2011, 2013). The decoupling between the age at physiological and reproductive maturity is widely found 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 (Geist, 1971; Clutton-Brock et al., 1982; Lincoln, 1998; Stewart et al., 2000; Festa-Bianchet et al., 2004; Vanpé et al., 2009). For example, in wild goats, sheep and deer, males continue increasing body size and weight for more than half the full life-span, and there is a progressive development of the male secondary sexual traits (Geist, 1971; Schaller, 1977; Clutton-Brock et al., 1982; Lincoln, 1998). Because most ungulates are polygamous and males compete with each other for access to females for mating, the acquisition of adult physical traits is a prerequisite for high reproductive success (Clutton-Brock et al., 1982; Lincoln, 1998). Certain data in literature provide support to this suggestion. According to Garde et al. (1998) some red deer (*Cervus elaphus hispanicus*) reach the spermatic maturity after the third year of age (Garde et al., 1998), coinciding with the age when significant changes in the vascular and cellular network occur in the red deer sample. Likewise, it was described that the bushbucks (*T. scriptus*) reach physiological maturity at 1.5 years (Apio et al., 2007), which could be related to the significant change in vascular orientation during the second year.

Summarizing, the results on the bone histology of ruminants provide evidence that changes in ontogeny play a prominent role in shaping the FLC-zonal bone of mammals. This study shows that in ruminants, the growth cycles recorded within the FLC-zonal bone are good proxies for one of the most important life history traits, namely the age at reproductive maturity. Moreover, the results indicate that the vascular and cellular pattern of the FLC bone suffers an important variation along ontogeny, reflecting a decrease in growth rate when maturity approaches. The most important changes in the vascular and cellular pattern within the FLC-zonal bone may be coupled to the physiological maturity. It is worth to note that, in large ruminants, the record of the first years within the FLC-zonal bone may be missed due to the important bone remodelling and reabsortion.

This study provides valuable tools to reconstruct life history traits (age at maturity, growth rate) of medium to large sized mammals from bone histology. Mammalian bone histology can be particularly useful in conservation biology to obtain key life history traits when applied to bones collected from the wild, such as in the case of carcasses from death assemblages (Marín-Moratalla et al, 2013, see Chapter 9). Moreover, bone histology of fossil species can provide data on the evolution of life history traits in mammals. The study of life history evolution in fossil communities can potentially provide important insights into processes that cannot be studied in extant populations, such as evolution of fitness components under different selective regimes, long-term trends in population dynamics, the causes underlying extinction, among others (McKinney, 1997; Isaac, 2009). Hence, mammalian bone histology will have major implications in the field of conservation biology, paleobiology as well as in the evolutionary biology.

Appendix 6A. Material used in this study and the data associated with each individual: specimens with fused epiphyses are marked with x. The analysis conducted for each individual is indicated with X. APD: antero-posterior diameter of femora midshaft.

Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Bovidae	Bovinae	*Tragelaphus imberbis*	x	male	140	31.05	Tanzania, Longido	56199		X	
		Tragelaphus scriptus		female	27.6	16.51	Angola, Tumba Grande	56205	X		
			x	male	45.5	20.43	S. Africa, Umfolozi	56204	X	X	X
			x	male	52	21.50	Ethiopia, Cunni	56206	X	X	X
			x	male	47.5	22.53	S. Africa, Umfolozi	56203	X	X	X
		Tragelaphus spekii		male	47.5	14.08	Angola, Camata	56208	X		X
			x	male	73	22.83	Angola, Culele	56207	X	X	X
		Tragelaphus strepsiceros		male	181.5	35.37	Angola, Ruacaná	56210	X		X
			x	male	280	37.10	Namibia, Otjiseva	56211	X	X	X

Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Bovidae	Bovinae	*Tragelaphus derbianus*	x	male	900	54.25	C. Afr. Republic, Tiri	56212		X	
	Aepycerotinae	*Aepyceros melampus*		male	60	20.77	Angola, uacaná	56215	X		X
			x	male	78	21.41	Mozambique, Massingir	56213	X	X	X
			x	male	62	22.30	Angola, Ruacaná	56214	X	X	X
	Antilopinae	*Oreotragus oreotragus*	x	male	14.12	14.12	Angola, Capolopopo	56218			
			x	female	10.5	12.60	Angola, Capolopopo	56217		X	
		Raphicerus campestris	x	male	10	10.70	Angola, Capolopopo	56221		X	
			x	male	10	12.13	Angola, Capolopopo	56222		X	
			x	female	12	11.99	Angola, Dirico	56223		X	
		Antidorcas marsupialis		female	30.5	16.39	Angola, Capolopopo	56225	X		
			x	female	36	17.02	Angola, Capolopopo	56226		X	

Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Bovidae	Antilopinae	*Antidorcas marsupialis*	x	male	30.2	17.19	S. Africa, Bredasdorp	56224	X	X	
			x	male	37	17.22	Angola, Capolopopo	56227	X	X	
		Eudorcas rufifrons	x	male	25	17.05	Chad, Abéché	56228		X	
			x	male	25	16.01	Chad, Abéché	56229		X	
		Eudorcas thomsonii	x	male	21	13.91	Tanzania, Lolkisale	56230		X	
		Nanger granti	x	male	75	22.50	Tanzania, Lolkisale	56231		X	
		Gazella dorcas		female	-	10.80	Nigeria	56233	X		
			x	male	16.7	12.65	Chad, Biltine	56232	X	X	X
			x	male	16	12.50	Niger, Agadez	56235	X	X	X
	Cephalophinae	*Sylvicapra grimmia*		female		12.32	Angola, Sanguengue	56238	X		
			x	male	16	13.26	Angola, Sanguengue	56237	X	X	

Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Bovidae	Cephalophinae	*Sylvicapra grimmia*	x	male	9	11.56	Sudan, Yambio	56236	X	X	
		Philatomba monticola	x	female	-	8.76	C. Afr. R., Bakota	56241		X	
		Cephalophus natalensis	x	male	10.5	13.23	S. Africa, False Bay	56245		X	
			x	female	12.5	11.88	S. Africa, False Bay	56244		X	
		Cephalophus callipygus	x	male	15	13.95	C. Afr. R., Mbata	56243		X	
			x	female	20	16.38	C. Afr. R., Mbata	56242		X	
	Reduncinae	*Redunca fulvorufula*	x	male	33.5	18.06	S. Africa, Giants Castle	56248		X	
		Redunca redunca	x	male	50	19.08	Uganda, Lake Mburo	56249		X	
			x	male	42	18.65	Sudan, Tonj	56250		X	
		Kobus kob	x	male	125	23.45	Uganda, Semliki	56251		X	

Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Bovidae	Reduncinae	*Kobus kob*	x	male	99	25.45	Sudan, Yambio	56253		X	
		Kobus ellipsiprymnus	x	male	250	36.33	Uganda, Lake Mburo	56255		X	
			x	male	225	33.60	Angola, Lake Mburo	56254		X	
		Kobus megaceros	x	male	84	24.68	Sudan, Tonj	56256		X	
	Alcelaphinae	*Connochaetes gnou*	x	male	160	28.85	S. Africa, W. Pret. Park	56259		X	
			x	male	150	27.41	S. Africa, W. Pret. Park	56260		X	
		Damaliscus lunatus	x	male	150	28.50	Uganda, Lake Mburo	56263	X	X	
			x	female	145	26.28	Angola, Cambembe	56262		X	
				female	90	24.43	Angola, Cambembe	56261	X		
	Hippotraginae	*Addax nasomaculatus*	x	male	120	28.78	Chad, Oum Chalouba	56264		X	
			x	female	107	24.58	Chad, Ouadi Achim	56265		X	

Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Bovidae	Hippotraginae	*Oryx damma*	x	male	135	27.28	Chad, Ouadi Achim	56269		X	
			x	male	124	26.32	Chad, Ouadi Achim	56270		X	
		Oryx gazella	x	male	200	24.4	Namibia, Otjiseva	56275		X	
			x	female	190	20.21	Namibia, Otjiseva	56271		X	
			x	female	140	20.78	Uganda, Komolo	56273		X	
Cervidae	Cervinae	*Cervus elaphus hispanicus*		male	-	19.23	Huesca, Spain	74309	X		X
				male	-	17.89	Huesca, Spain	74310	X		X
				male	-	26.22	Badajoz, Spain	60870	X		X
				male	-	28.28	Badajoz, Spain	60871	X		X
			x	male	-	24.60	Badajoz, Spain	60872	X	X	X
			x	male	-	24.80	Badajoz, Spain	60873	X	X	X
			x	male	-	25.47	Badajoz, Spain	60875	X	X	X

Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Cervidae	Cervinae	*Cervus elaphus hispanicus*		female	-	22.59	Badajoz, Spain	60876	X		
			x	female	-	24.09	Badajoz, Spain	60869	X	X	
			x	female	-	23.69	Badajoz, Spain	60874	X	X	
			x	female		23.37	La Rioja, Spain	62075	X	X	
	Capreolinae	*Rangifer tarandus platyrhynchus*		male	-	16.51	Svalbard, Norway	56293	X		X
				male	-	18.10	Svalbard, Norway	56294	X		X
				male	-	16.72	Svalbard, Norway	56295	X		X
				male	-	22.94	Svalbard, Norway	56286	X		X
			x	male	-	24.20	Svalbard, Norway	56288	X	X	X
			x	male	-	24.07	Svalbard, Norway	56282	X	X	X
			x	male	-	22.98	Svalbard, Norway	56283	X	X	X

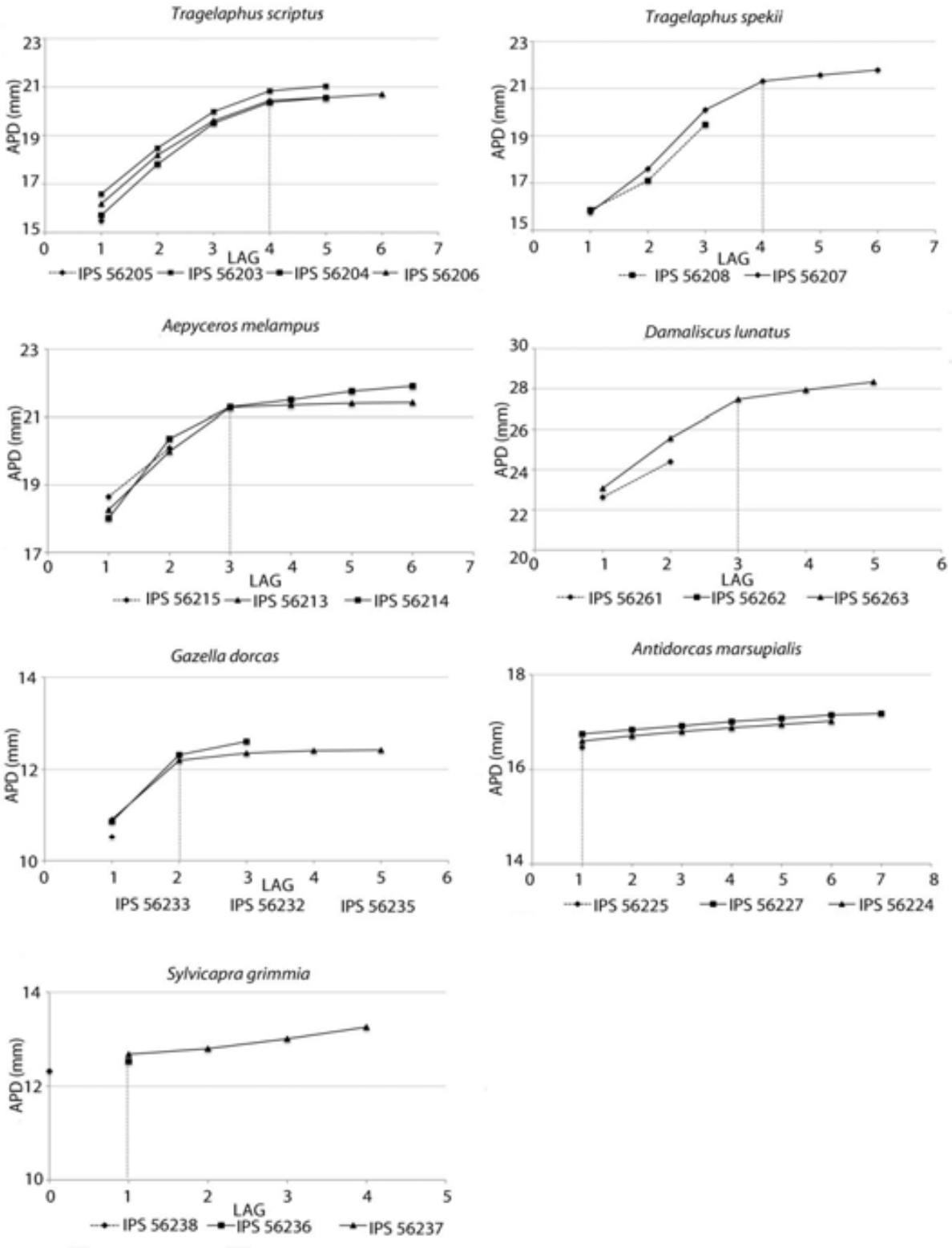
Family	SubFamily	Species	Fused epiphysis	Sex	Mass (kg)	DAP (mm)	Site of death	ID (IPS)	LAG retrocalculation	Growth cycles	Ontogenetic variation
Cervidae	Capreolinae	*Rangifer tarandus platyrhynchus*	x	male	-	23.59	Svalbard, Norway	56287	X	X	X
				female	-	16.69	Svalbard, Norway	56296	X		
				female	-	15.49	Svalbard, Norway	56300	X		
			x	female	-	21.10	Svalbard, Norway	56289	X	X	
			x	female	-	20.32	Svalbard, Norway	56292	X	X	
			x	female	-	20.08	Svalbard, Norway	56291	X	X	
		Capreolus capreolus	x	male	-	16.22	Lleida, Spain	73885		X	
			x	male	-	15.42	Lleida, Spain	73886		X	
			x	male	-	14.29	La Rioja, Spain	62076		X	

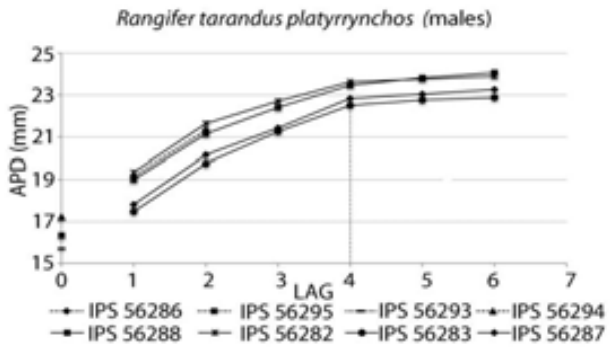
Appendix 6B. *Specimens used to compare the age at reproductive maturity estimated from bone histology with data from literature. All individuals are adult, most of them males except if it is specified female (♀). GR: the number of growth cycles within the FLC; RM: reproductive maturity reported from literature; *: those species that is not clearly specified if the age at maturity corresponds to physiological or reproductive maturity.*

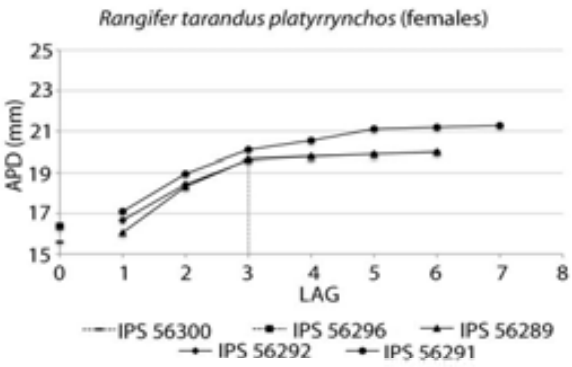
Family	Subfamily	Species	n	GC	RM	References
Bovidae	Bovinae	*Tragelaphus imberbis*	1	5	4	Kingdon, 1997.
		Tragelaphus scriptus	3	4	3-4	Kingdon, 1997; Wronski, 2009.
		Tragelaphus spekii	1	4	4*	Kingdon, 1997.
		Tragelaphus strepsiceros	1	6	6	Kingdon, 1997.
		Tragelaphus derbianus	1	5	4*	Pappas, 2002; Nowak, 1999 .
	Aepycerotinae	*Aepyceros melampus*	2	3	4	Estes, 1991.
	Antilopinae	*Oreotragus oreotragus*	1	2	1-2	Kingdon, 1997.
		Oreotragus oreotragus ♀	1	2		Kingdon, 1997.
		Raphicerus campestris	2	1		
		Raphicerus campestris ♀	1	1		Kingdon, 1997.
		Antidorcas marsupialis	2	1	2*	Cain III et al., 2004.
		Antidorcas marsupialis ♀	1	1	1*	Cain III et al., 2004.
		Eudorcas rufifrons	2	2	1-2	Nowak, 1999.
		Eudorcas thomsonii	1	1	1	Estes, 1991.
		Nanger granti	1	1	3	Estes, 1991.
		Gazella dorcas	2	2	2*	Dittrich, 1972.
	Cephalophinae	*Sylvicapra grimmia*	2	1	1*	Kingdon, 1997.
		P. monticola ♀	1	2	2*	Kingdon, 1997.
		Cephalophus natalensis	1	1		
		Cephalophus natalensis ♀	1	1		
		Cephalophus callipygus	1	1		
		Cephalophus callipygus ♀	1	1		

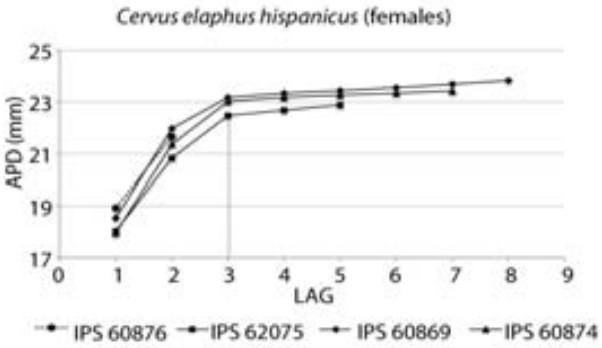
	Reduncinae	*Redunca fulvorufula*	1	2	3	Estes, 1991.
		Redunca redunca	2	2	3	Estes, 1991.
		Kobus kob	2	3	3	Estes, 1991; Kingdon, 1997.
		Kobus ellipsiprymnus	2	3	5	Owen-Smith, 1993.
		Kobus megaceros	1	3	4	Estes, 1991.
	Alcelaphinae	*Connochaetes gnou*	2	3	≥4	von Richter, 1974.
		Damaliscus lunatus	1	3	>3*	Kingdon, 1997.
		Damaliscus lunatus ♀	1	2	2.5*	Kingdon, 1997.
	Hippotraginae	*Oryx dammah*	2	3		
		Oryx gazella	1	3	2*	Kingdon, 1997.
		Oryx gazella ♀	2	3	2*	Kingdon, 1997.
		Addax nasomaculatus	1	4	>3*	Kingdon, 1997.
		Addax nasomaculatus♀	1	3	1.5*	Kingdon, 1997.
Cervidae	Cervinae	*Cervus elaphus*	3	≥5*	5-7	Clutton-Brock et al., 1982.
		Cervus elaphus ♀	3	3	3-4	Clutton-Brock et al., 1982.
	Capreolinae	*Rangifer tarandus platyrhynchus*	4	4		
		Rangifer tarandus platyrhynchus ♀	3	3		
		Capreolus capreolus	3	3-4	>3	Vanpé et al., 2009.

Appendix 6C. *LAG retrocalculation by plotting the antero-posterior diameter (APD) of successive growth cycles. Dashed lines correspond to juvenile individuals whereas solid ones correspond to adult. Vertical dashed lines indicate the attainment of age at reproductive maturity (EFS formation).*

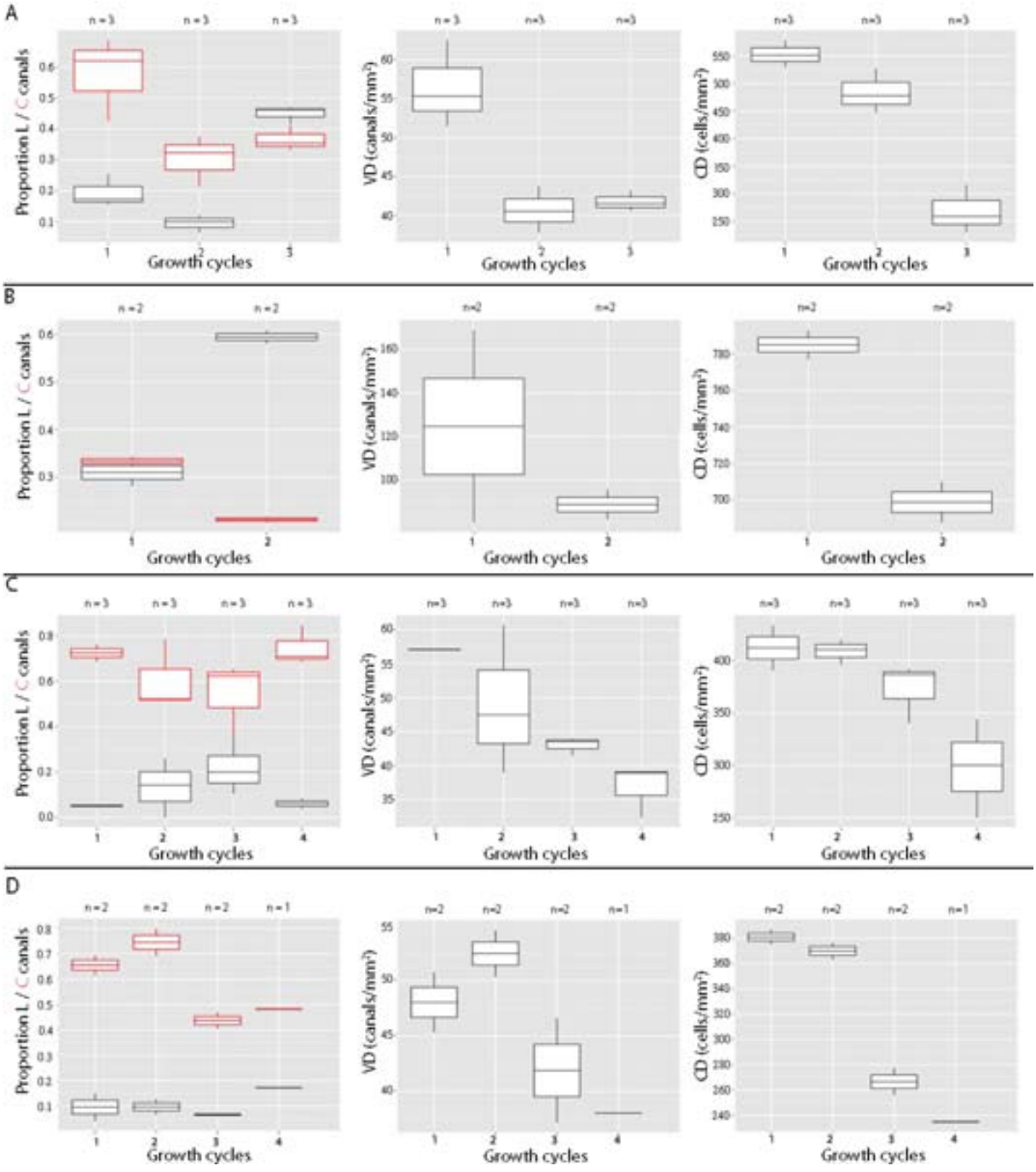


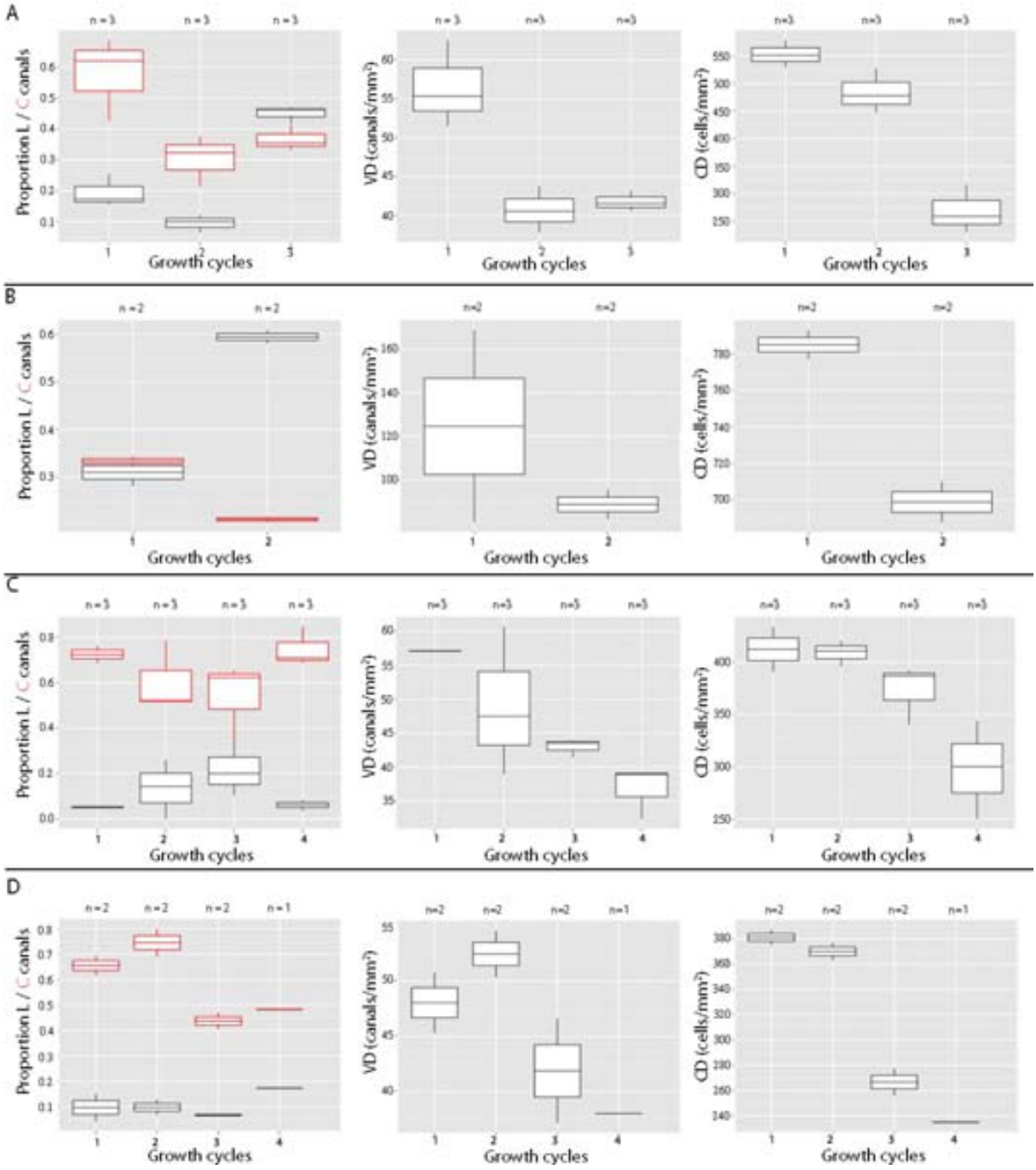

Rangifer tarandus platyrrynchos (males)
APD (mm)
LAG
IPS 56286
IPS 56295
IPS 56293
IPS 56294
IPS 56288
IPS 56282
IPS 56283
IPS 56287


Rangifer tarandus platyrrynchos (females)
APD (mm)
LAG
IPS 56300
IPS 56296
IPS 56289
IPS 56292
IPS 56291


Cervus elaphus hispanicus (females)
APD (mm)
LAG
IPS 60876
IPS 62075
IPS 60869
IPS 60874

Appendix 6D. *Plots of the histological along ontogeny, all individuals are males. A.* Aepyceros melampus. *B.* Gazella dorcas. *C.* Tragelaphus scriptus. *D.* Tragelaphus spekii. *E.* Tragelaphus strepsiceros. *F.* Rangifer tarandus platyrhynchus.




A
B
C
D
Proportion L / C canals
VD (canals/mm²)
CD (cells/mm²)
Growth cycles

CHAPTER 7. CORRELATION OF QUANTITATIVE BONE HISTOLOGY DATA WITH LIFE HISTORY AND CLIMATE: A PHYLOGENETIC APPROACH

dx.doi.org/10.1111/bij.12302

CHAPTER 8. TRACING THE EVOLUTION OF FITNESS COMPONENTS IN FOSSIL BOVIDS UNDER DIFFERENT SELECTIVE REGIMES

dx.doi.org/10.1016/j.crpv.2011.03.007

CHAPTER 9. BONE HISTOLOGY AS AN APPROACH TO PROVIDING DATA ON CERTAIN KEY LIFE HISTORY TRAITS IN MAMMALS: IMPLICATIONS FOR CONSERVATION BIOLOGY

dx.doi.org/10.1016/j.mambio.2013.07.079

CHAPTER 10. DISCUSSION

10. GENERAL DISCUSSION

The general aim of the research presented in this work is to contribute to the foundations of bone histology as a tool for inferring life history strategies in mammals. To achieve this goal, bones of 225 individuals belonging to five families of eutherian mammals, including extant dormice (Gliridae) and fossil and living ruminants (Bovidae, Cervidae, Moschidae and Tragulidae), have been analysed. This large sample allows exploration of bone histology in mammals of a wide range of body sizes (from 45 g to 900 kg) and dwelling in very different environments (from the Equator to near the poles).

All these mammals have shown some similarities in bone tissue, such as the presence of rest lines as well as a transition between main primary tissues when maturity is reached. However, remarkable differences in the type of primary bone matrix and vascularity and also in the degree of bone remodelling have been documented in this study between dormice and ruminants.

Dormice (Gliridae, Sciuromorpha) range from 15 to 180 g and inhabit the southern Sahara and the Palearctic regions, i.e. Europe, northern Africa, central and southwestern Asia and Japan (Nowak, 1999). In this work, long bones and mandibles of 16 wild individuals belonging to two different species of dormice, *Glis glis* and *Eliomys quercinus*, have been analysed (see Chapter 4). Primary bone of immature individuals (unfused epiphyses) consists of either parallel-fibered (PFB) or woven bone with rounded osteocytes, and presents scarce vascularisation, mainly longitudinal primary osteons. Some juvenile individuals show a single LAG in their cortical bones (see Figure 4a in Chapter 4). The bone tissue deposited in immature individuals is greatly reabsorbed in adulthood and substituted by the deposition of an inner and outer cortical layer of lamellar bone with rest lines. In the outermost cortical layer, adult individuals deposit a lamellar-zonal bone (with rest lines) with flattened osteocytes and sporadic vascularisation. The incidence of secondary osteons is very low and they are found in the innermost cortical area formed during the juvenile stage. All long bones of dormice show similar bone tissue pattern, except for the ulna that shows alternating PFB or woven bone and lamellar bone tissue in the outermost cortical layer of adult individuals. The ulna pattern is likely to result from the important change in shape experienced during ontogeny (bone drift).

The femora cross-sections of 131 extant ruminants (120 wild and 11 captive individuals) belonging to 4 families (83 bovids, 45 cervids, 2 moschids and a chevrotain) have been histologically examined. In general, the primary bone tissue of immature individuals consists

of woven bone with primary osteons (i.e. fibrolamellar complex, FLC, bone). The main vascular type that prevails within the FLC bone is the circular canal, forming the laminar or plexiform bone tissue (Francillot-Vieillot et al., 1990; de Ricqlès, 1991) (see Figure 2i and 2k in Chapter 5; Figures 6.1B, 6.1C, 6.2A and 6.2C in Chapter 6; Figure 1b and 1c in Chapter 7; Figure 3a in Chapter 8 and Figures 2 and 5 in Chapter 9). It is not unusual to observe rest lines embedded in the primary matrix of immature individuals forming the fibrolamellar-zonal bone (see Figures 6.1B and 6.2D in Chapter 6, Figures 1b and 1c in Chapter 7 and Figure 2 in Chapter 9).

Adult ruminants deposit highly organized lamellar bone with rest lines in the outermost cortical layer (see Figure S2 in Chapter 5; Figures 6.1B, 6.1C, 6.2D and 6.2F in Chapter 6; Figure 1a, 1b in Chapter 7; Figure 3c and 3d in Chapter 8 and Figure 5 in Chapter 9), known as External Fundamental System 'EFS' (Cormack, 1987). With increasing age, secondary osteons (Haversian systems) are gradually deposited in the cortical bone from the inner towards the outer cortical area, whereas bone reabsorption around medullar cavity removes the inner primary bone deposited during the earlier stage of ontogeny (Figure 6.2D and 6.2G in Chapter 6 and Figure 4 in Chapter 8). Unlike dormice, adult ruminants keep a large portion of the primary bone tissue deposited during juvenile growth. This may be explained in part because of their more extended growth period (1-6 years) relative to dormice (3-6 months).

The patterns of bone tissue described in this work agree with previous studies on bone histology in both dormice and ruminants. In fact, laminar and plexiform bone tissues were already described in artiodactyls (Francillot-Vieillot et al., 1990; Horner et al., 1999; Sander and Andrássy, 2006). Furthermore, PFB with longitudinal vascularisation was described as the principal bone type in rodents (Singh et al., 1974; Weiner et al., 1997). FLC bone has been described in other large mammals such as elephantids (Curtin et al., 2012), perissodactyls (Sander and Andrássy, 2006), and some xenarthrans (large Folivora, >20 kg; Vermilingua, 5-22 kg) (Straehl et al., 2013), whereas PFB was documented in the small primate *Microcebus murinus* (60-80 g) (Castanet et al., 2004) and also in some small xenarthrans (small Folivora, <20 kg and small Cingulata, <20 kg) (Straehl et al., 2013). Because bone tissue typology is known to be related to bone deposition rate (Amprino, 1947; Castanet et al., 1996; de Margerie et al., 2002; Montes et al., 2010), differences between the primary bone matrix of ruminants and dormice could be attributed to different growth strategies. While dormice are small mammals that reach maturity no later than 6 months of age (Nowak, 1999), ruminants can attain large sizes in a few years (Georgiadis, 1985).

Below, the main results and the integrated discussion following the order of the specific objectives established for this work (see Chapter 2) are presented.

10.1. RELIABILITY OF THE SKELETOCHRONOLOGY IN MAMMALS

Dormice are an especially suitable group to test the reliability of skeletochronology in mammals. This is because of their long life span for their body size, their pronounced biological rhythms that closely matches the seasonal changes in their environment (Moreno, 2005; Pilastro et al., 2003), and their negligible secondary remodelling. Previous work had already denoted a LAG deposition in hibernating mammals matching the unfavourable season (Klevezal, 1996). Dormice bones analysed in this work (see Chapter 4) show LAGs in adults and in some juvenile individuals. However, the number of rest lines was not always consistent throughout the bones of a single specimen (jaw, ulna, radius, femur, tibia). Different degrees of bone drift, including long bones and jaws, were found in dormice. Femora exhibited the greatest number of growth marks, and hence they are clearly the most reliable bones for skeletochronology analyses. This result suggests that in small mammals, femora are less affected by bone drift during ontogeny than other bones. The presence of a single LAG in long bones of some immature dormice (defined by unfused epiphyses) may be related to a hibernation period experienced before maturity.

The same as in dormice, rest lines (LAGs) or annuli are universally present in the femora of all species of ruminants studied in this work, regardless of their climatic regime (see Chapters 5, 6, 8 and 9). These growth layers are not only confined to the outermost cortical layer, as previously believed (Klevezal, 1996; Chinsamy-Turan, 2005), but they can also appear within the fast-growing bone tissue forming the fibrolamellar-zonal bone. The number of LAGs in the femora of five addax (*Addax nasomaculatus*) specimens studied in this work was consistent with age estimates based on dental eruption and wear (see Chapter 9). Hence, this result indicates that also in ruminants, the total number of LAGs in femora bone tissue is a good proxy for chronological age at death.

Previous work also suggested the usefulness of skeletochronology in mammals. By using fluorescent labelling in long bones of the small primate *Microcebus murinus*, Castanet et al. (2004) demonstrated that LAGs are formed following the annual photoperiodic variation. This suggests that the number of LAGs in long bone cross-sections is a useful tool to estimate the age of an individual. However, the authors pointed that the age estimated by LAGs may underestimate the chronological age in animals from captivity, which live longer (11 years) than those from the wild (<4 years). More studies are needed, especially by using vital labelling, to check the reliability of skeletochronology.

Bone remodelling and reabsorption is a common feature in mammalian bones (Enlow and Brown, 1957), which can result in deletion of the earliest ontogenetic record. It can be especially important in ruminant bones because of their fast growth (Georgiadis, 1985).

Different methods have been proposed for LAG retrocalculation (i.e. to reconstruct the growth marks missed due to above-mentioned reasons) in non-avian dinosaurs and other extinct vertebrates using ontogenetic series (Chinsamy, 1993; Erickson and Tumanova, 2000; Horner et al., 2000). However, because of the scarcity of bone histology studies in mammals, the effect of bone remodelling in this group has never been exhaustively studied. The analysis of the number of growth cycles within the FLC bone in ontogenetic series of 10 ruminant species (47 individuals) showed that the remodelling of medullar cavity is an important factor to take into account for age estimations in large ruminants. In these ruminants (>120 kg, approximately), the first growth cycle deposited in femora cross-sections can be missing (Marín-Moratalla et al., 2013, see Chapters 6 and 9). Hence, despite the fact that the femur is the long bone with a lower degree of bone drift, the findings of this work indicate that methods of LAGs retrocalculation are necessary when skeletochronology is applied to large mammals.

10.2. ASSOCIATION OF BONE GROWTH CYCLES WITH ENVIRONMENTAL AND PHYSIOLOGICAL TRAITS

The study of 115 ruminants (102 wild individuals and 13 captive ones) from tropical to polar environments was carried out to explore the association of bone growth cycles with environmental and physiological traits (see Chapter 5). Ruminants were selected for this study because most species have a relatively long juvenile period with osteogenesis continuing beyond at least one year, a basic condition for the formation of fibrolamellar-zonal bone. Furthermore, they dwell in a wide array of climatic regimes, allowing the identification of the environmental and physiological correlates of rest lines.

The femora cross-sections of the still growing individuals were histological analysed in order to assess the growth stage at death, either arrested (a LAG is near to bone periphery) or active growth (high amount of vascularised bone tissue in bone periphery). The correlations between the growth stage with date of death and habitat indicate that bone growth is arrested during the energetically challenging periods (low resource supply), usually the dry season. Conversely, at the beginning of the favourable (usually humid) season, when resources with high nutritive values become plentiful, ruminants maximize bone growth rates, reflected by the deposition of fibrollamelar bone. Both hemispheres show inverted seasonal patterns of growth and growth arrest in accordance with the inverted patterns of precipitation and hence, resources availability. The seasonal bone growth in ruminants correlates with seasonal variation of their metabolism and endocrine system, indicating that growth arrest forms part of a plesiomorphic thermometabolic strategy for energy

conservation. All together suggest that bone growth is regulated by an endogenous clock and synchronized with seasonal resource availability rather than by environmental stresses.

Seasonal growth cycles have been reported for almost all ectotherms and are considered to be a plesiomorphy (Horner et al., 1999, 2000; de Ricqlès, 2000). The presence of such cycles in modern homeothermic endotherms and their fine-tuning with seasonal fluctuations in resource availability suggest that they form part of an ancient inheritance that is still operative today (Köhler et al., 2012, see Chapter 5). These findings debunk the classical assumption that homeothermic endotherms grow continuously until they attain maturity because of their constant high body temperature and sustained metabolic rate (see Chapter 5). Indeed, the azonal bone classically attributed to endotherms (Chinsamy and Hillenius, 2004; Chinsamy-Turan, 2005), is restricted to those specimens that attain maturity fast, within the first year of life, as most small mammals. The absence of LAGs within the FLC bone in mammals hence can be attributed to primary causes, such as a short time of growth, but also to secondary causes associated to bone remodelling or reabsorption (Castanet et al., 2006). One of the reasons why this classical assumption has persisted for years may be likely explained by the fact that small mammals, which reach maturity in a few months, are usually used in experimental studies. Moreover, seminal works on hard tissues (Klevezal, 1996; Chinsamy-Turan, 2005) have focused on adult individuals, which can be strongly affected by bone reabsorption and remodelling.

10.3. ONTOGENETIC CHANGES IN PRIMARY BONE TISSUE

Life History Theory postulates that energy and time are limited and hence, individuals should allocate resources optimally among growth and reproduction to maximize fitness (Stearns, 1992). As long as an organism needs to grow in order to attain a minimum size for successful reproduction, resources are channelled towards growth. As soon as this size is attained, resources are channelled away from growth towards reproduction (Stearns, 1992; Roff, 2002; Ricklefs, 2007). The life history trade-off between growth and reproduction is recorded in the bone tissue of mammals as a single-event, the deposition of the External Fundamental System (EFS). Taking it into account, the age at first reproduction (AFR) can be theoretically determined by counting the number of growth cycles within the fibrolamellar complex (FLC) bone, before the EFS (Klevezal, 1990; Chinsamy, 2005; Köhler and Moyà-Solà, 2009; Köhler, 2010; Marín-Moratalla et al., 2011, 2013; see Chapters 6, 8 and 9).

All long bones of adult ruminant specimens (considered adults when epiphyses are fused) analysed in this work (65 individuals belonging to 30 species, see Chapters 6 and 9) show a transition between two main primary tissues: a fast-growing fibrollamelar bone (FLC) and a slow-growing lamellar bone deposited in the outermost cortical layer (EFS).

The number of growth cycles in the FLC-zonal bone of femora of 65 wild extant ruminants (30 species) analyzed in Chapter 6 is broadly consistent with ethological data previously published on age at reproductive maturity (AFR) (Appendix 6B). The discrepancies found can be attributed to misunderstanding between physiological or reproductive maturity from the literature data, to population variability and/or to bone reabsorption. In this sense, it is worth to highlight again the need to analyse ontogenetic series in order to reconstruct the missing growth cycles.

The age at reproductive maturity is often the demographic trait most sensitive to environmental variation (Clutton-Brock et al., 1982; Gaillard et al., 2000). Following Life History Theory, low extrinsic mortality, generally caused by low predation, is the fundamental force in shifting life histories towards the slow end (delayed maturity, long lifespan) of the fast-slow continuum. Conversely, 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 (Stearns 1992; Gaillard et al., 2000; Roff, 2002; Festa-Bianchet et al., 2006; Ricklefs, 2007). The histological estimations of reproductive maturity in 64 wild ruminants (see Chapters 6 and 9) correlate well with those expected according to Life History Theory. Among the ruminants analysed in this work, species dwelling in wooded and closed habitats, which are characterized by low predation pressure, display delayed AFR (estimated by bone histology) relative to body size. The same applies to the desert-dwelling addax, characterized by resource limitation and scarcity of predators (Mallon and Kingswood, 2001). On the other hand, bovids dwelling in open habitats, characterized by high predation pressure, attain AFR early relative to their body mass.

Growth rate is a life history trait that varies greatly during mammalian ontogeny (Maiorana, 1990; Castanet et al., 1996). Precisely, the vascular and cellular network of FLC bone (vascular orientation and vascular and cellular densities) is strongly related to bone deposition rates (Francillot-Vieillot et al., 1990; Castanet et al., 1996; Mullender, 1996; Montes et al., 2007, 2010; Bromage et al., 2009, 2012). Hence, taking it into account, vascular and cellular parameters have been analysed throughout the FLC-zonal bone in 8 ruminant species (27 individuals). As expected, vascular and cellular parameters quantified in successive growth cycles in the ruminant sample undergo important variation. Specifically, along the ontogeny, vascular and cellular density drops, and the proportion of longitudinal

canals increases whereas that of circular canals decreases. According to previous work (Castanet et al., 1996; de Margerie et al., 2002; Montes et al., 2007, 2010; Cubo et al., 2012), these changes are strongly related to the decrease of growth rate as maturity approaches (Maiorana, 1990; Castanet et al., 1996). The results of this work (see Chapter 6) further show that the most significant variation in the vascular and cellular parameters occurs few growth cycles before the deposition of the EFS (reproductive maturity). This finding raises the hypothesis that this significant variation in the vascular and cellular network is related to physiological maturity. Physiological (gonad maturation) and reproductive maturity (AFR) are not always synchronized in ruminants; some of them are only reproductive years after physiological maturity, when they have almost reached their full adult body mass (Jarman, 1983; Bon and Campan, 1996; Pettorelli et al., 2002; Vanpé et al., 2009). This hypothesis is supported by the concordance between the age at physiological maturity of red deer (*Cervus elaphus hispanicus*) and bushbuck (*Tragelaphus scriptus*) reported in the literature (Garde et al., 1998; Apio et al., 2010) with the age when the most significant decrease in vascular and cellular density takes place in the same species of the sample analysed here. More research, however, is needed to explore whether or not vascular and cellular features of primary bone may provide information on physiological maturity.

The abovementioned ontogenetic changes on vascular and cellular parameters make it essential to standardize the sampling region when individuals are compared among them.

10.4. ENVIRONMENTAL AND LIFE HISTORY CORRELATES OF HISTOLOGICAL FEATURES OF PRIMARY BONE TISSUE

The vascular and cellular parameters of FLC bone were quantified in the anterior region of the first growth cycles (the phase of highest growth rate) in 51 wild individuals of 27 ruminant species, all of them bovids except for a chevrotain species. These parameters were correlated with climate regimes (extrinsic factors) and some life history traits (intrinsic factors) such as body mass at birth, adult body mass and relative age at reproductive maturity (relative to body mass) as a proxy of the species' pace of life. These analyses were conducted under a phylogenetic control. The results show that phylogenetic signal explains the variation of some bone tissue traits such as the proportion of longitudinal and circular canals as well as the cellular density among extant taxa. These results support the use of phylogenetic comparative methods when bone histology is studied in a variety of taxa (Cubo et al., 2008, 2012).

Laminar or plexiform bone (FLC bone with high proportion of circular canals) is the widespread bone typology among the bovids analysed in this study. However, forest duikers (*Philantomba monticola*, *Cephalophus natalensis, C. callypigus*) and the chevrotain (*Moschiola meminna*) show higher proportion of longitudinal canals than circular ones. Chevrotains and forest duikers share the same ecological niche: they live in tropical dense forests and present a frugivorous component on their diet (Estes, 1991; Kingdon, 1997). Because of forest duikers adapted secondarily to a forest-frugivore niche (Estes, 1991; Kingdon, 1997), the confluent bone histology (predominance of longitudinal canals) may reflect an adaptation to this ecological niche.

Despite these latter findings, no histological variables studied here differ significantly among ruminants living under the three main climates of the Köppen-Geiger climate classification (tropical, temperate and dry); though, it can not be ruled out that some relationship could exist using more detailed categories. More samples would be needed to explore this issue statistically under a phylogenetic control.

The results of this work show that vascular and cellular parameters are related to body mass. Specifically, the proportion of circular canals increases while that of longitudinal canals decreases as body mass increases. Following Amprino's rule (Amprino, 1947), the higher the proportion of circular canals in primary bone tissue, the faster the bone deposition rate (Francillot-Vieillot et al., 1990; Castanet et al., 1996, 2000; de Margerie et al., 2002; Montes et al., 2007, 2010; Cubo et al., 2012). Our results thus suggest that the increase in the proportion of circular canals and the decrease in the proportion of longitudinal ones with increasing body mass reflects a positive correlation between bone deposition rate and body mass, congruent with the positive correlation between growth rate and body mass (Case, 1978).

On the other hand, vascular and cellular densities show a negative allometry with body mass. With regard to cellular density, previous studies have suggested that osteocyte density is related to rates of osteoblast proliferation and obeys scaling laws related to body mass and metabolic rate (Bromage et al., 2009, 2012). Specifically, the drop of cellular density with increasing body mass in the bovid sample may be linked to mass-specific metabolic rate rather than to basal metabolic rate, in agreement with previous works (Mullender et al., 1996; Bromage et al., 2009). Mass-specific metabolic rate, i.e. the power required for sustaining a unit mass of an organism, decreases with increasing body mass at a scale factor of 0.25. Similar scaling factor for the allometric relationship between cellular density and body mass is found in the present work. These findings provide support that within metabolic constraints, a high rate of bone growth could be associated with higher rate of bone matrix

deposition per osteoblast rather than simply a higher density of osteoblasts. This mechanism of bone growth would correspond to a relatively lower density of osteocytes because they would each be surrounded by more bone matrix (Padian et al., 2013). This may explain why large mammals tend to show lower osteocyte densities despite their higher absolute growth rates.

As occurs with cellular density, vascular density in the bovid sample decreases with body mass. However, this result is at variance with findings in living sauropsids (Cubo et al. 2005), though obtained using different methods of quantifying vascular density. Hence, the correlation between vascular density and body mass requires further research.

Finally, there is no relationship between the vascular and cellular parameters and the relative age at first reproduction when the effect of body mass is removed. Despite the fact that life history traits are intrinsic factors, the age at first reproduction is often the demographic trait most sensitive to environmental variation (Sæther, 1996; Gaillard et al., 2000).

Altogether suggest that extrinsic factors appear not to be critical in shaping FLC bone in bovids, indicating low developmental plasticity in these histological features.

10.5. INSIGHTS IN LIFE HISTORY EVOLUTION

The study of life history evolution in fossil communities can potentially provide important insights into processes that cannot be studied in extant populations, such as the evolution of fitness components under different selective regimes, long-term trends in population dynamics, the causes underlying extinction, among others (McKinney, 1997).

In Chapter 8 (Marín-Moratalla et al., 2011), some life history traits of two Pleistocene fossil bovids from different selective regimes, *Gazella borbonica* from a continental ecosystem and *Myotragus balearicus* from an insular ecosystem, were reconstructed from bone histology. Insular ecosystems are characterized by lower predation pressure than continental ones, as well as by resource limitation (McArthur and Wilson, 1967).

Similar to the general pattern of extant bovids, *G. borbonica* shows a plexiform bone comprising most of the cortical area and an EFS deposited when maturity is reached (Figures 3a and 3b in Chapter 8). The absence of LAGs within the fast-growing plexiform bone suggests that this fossil gazelle attained reproductive maturity within its first year of life. This result fits the predictions of extant gazelles of similar body mass (Nowak, 1999).

The insular fossil bovid *M. balearicus* shows, during its early ontogeny (neonates), a plexiform bone tissue indicating fast bone deposition rates. However, juvenile individuals (unfused epiphyses) show high amounts of PFB or lamellar bone separated by annulus and/or LAGs (Figure 4 in Chapter 8), indicating a sudden decrease in the rate of bone deposition in young individuals. This bone tissue type that forms most of the cortical area of *M. balearicus* is not found in any extant ruminant studied throughout this work. When older than 8-10 years, *M. balearicus* presents a decrease in the inter-LAG distance forming an EFS, with an important variability among individuals. This suggests that *M. balearicus* displayed an extremely delayed age of maturity, around the 8 years of age. This result does not fit the predictions if it is compared with extant bovids with similar body size, which attain maturity within the first or second year of life (Walther, 1990; Nowak, 1999). The high plasticity showed in *M. balearicus* in this life history trait is likely related to environmental variability, which commonly underlies variability in this trait in ungulates (Sæther, 1997; Gaillard et al., 2000), and which is accentuated on small islands (MacArthur and Wilson, 1967).

A longevity of 34 years was estimated in *M. balearicus* by bone histology, which is 17 years longer than the extant similar-sized continental caprine *Rupicapra rupicapra* (AnAge database; Tacutu et al., 2013). The generation time obtained for *M. balearicus* is about 17 years, whereas for other similar-sized living bovids is about 10 years. The value of generation time of *M. balearicus* is only comparable with long-lived and slow-maturing mammals such as large primates (Oates, 2006). A long generation time (K-species) makes sense in the context of resource limitation under insularity (MacArthur and Wilson, 1967). However, as in the case of primates, it makes the species vulnerable to those factors that increase mortality rate, such as predation pressure or hunting, because it imposes long time periods of recovery from declines in population size (Isaac, 2009). That long generation time of *M. balearicus* was the likely cause underlying its fast extinction after the arrival of man at Majorca some 3000 years ago (Alcover et al., 1981). Hence, under environments such as islands, where resources are scare and extrinsic mortality is low, species shift towards the slow end of the fast-slow life history continuum, as occurred in *M. balearicus*. Conversely, under conditions of high extrinsic mortality, i.e. continental ecosystems, species invest in rapid growth and early maturation that entails higher probabilities of surviving to first reproduction (Stearns, 1992; Gaillard et al., 2000; Festa-Bianchet, 2006) as shown by the life history strategy of the fossil *Gazella borbonica*. These results highlight the potential of bone histology in the fields of paleobiology, evolutionary biology and conservation biology.

Despite it has been unexplored in this work, the use of quantitative bone histology to obtain vascular and cellular parameters can be potentially interesting in the case of insular fossil ruminants, such as *Myotragus* from the Balearic Islands. The evolution of body size on

islands is a controversial issue (Palkovacs, 2003; Lomolino, 2005; Köhler and Moyà-Solà, 2009; Meiri and Raia, 2010; Jordana and Köhler, 2010; Köhler, 2010). Large mammals evolve into dwarfs whereas small ones evolve into giants over short evolutionary times, a rule coined *The island rule* (Foster, 1964; Van Valen, 1973). Two contradictory hypotheses are aimed to explain the causes underlying insular dwarfing. One of them states that insular dwarfs accelerate (fast end) to increase their reproductive investment (fast growth rate, early age at maturity and increase in production rate) (Bromage et al. 2003; Raia et al., 2003; Raia and Meiri, 2006). Conversely, other authors (Palkovacs, 2003; Köhler and Moyà-Solà, 2009; Köhler, 2010; Jordana and Köhler, 2011) suggested that insular dwarfs shift resource allocation from reproduction to growth, shifting towards the slow end of the life history continuum. The quantification of bone parameters, hence, could shed light on this controversial issue by comparing the insular fossil taxa with their extant continental relatives.

10.6. APPLICATION TO CONSERVATION BIOLOGY

Mammalian bone histology can also be particularly useful in the field of conservation biology to obtain key life history traits when applied to bones collected from the wild, such as the case of carcasses from death assemblages (Capurro et al., 1997; Mduma et al., 1999; Lubinski, 2001; Marín-Moratalla et al., 2013). Successful conservation and management of endangered species depends on the knowledge of the life history traits that determine the species' demography (Stearns, 1992; Rolandsen et al., 2008). For mammals, a variety of studies have demonstrated that species displaying certain life history traits (e.g. slow rates of development) are more vulnerable to extinction than others under environmental changes (McKinney, 1997; Isaac, 2009). Such species (K-selected species) are likely to be initially resistant to perturbations but will have little capacity to recover from long-term change due to their low reproductive potential. Conversely, *r*-selected species that display faster generation times can better adapt to climatic changes, increasing their chance of persistence. Thus, the life history of a certain species will be crucial in deciding its fate under future climatic conditions (Isaac, 2009). However, the assessment of critical changes in the schedule of life history traits is a very difficult enterprise in studies of wild populations (Ballou et al., 2010).

The results presented in this work on the endangered species *Addax nasomaculatus* (IUCN, 2013) (Marín-Moratalla et al., 2013, see Chapter 9), the near threatened *Eliomys quercinus* (IUCN, 2013) and the least concern *Glis glis* (IUCN, 2013) (García-Martínez et al., 2011, see Chapter 4), confirm that bone histology is indeed a complementary tool to field studies to obtain some key life history traits (the age at first reproduction, age at death, birth

season and growth rates). Age at death determination in wild mammals is habitually based on dental features such as eruption, wear and cementum layers (Lieberman, 1993; Klevezal, 1996; Lubinski, 2001; Azorit et al., 2002a, b, 2004). While skeletochronology (cementum layers in teeth and LAGs in bones) provide information on the chronological age (absolute age) of an individual, dental eruption/wear estimates the biological age. The analysis of two *A. nasomaculatus* (addax) from the wild (Chad) exemplifies the potential of skeletochornology in chronological age at death estimations. Based on the external appearance of the Chad individuals (dental wear and subcutaneous fat), Oboussier (scientist and hunter of these individuals) inferred that the female was older than the male (Köhler et al., 2008). However, the number of LAGs in the midshaft of femora indicates that both individuals had approximately the same age when culled, denoting differences between chronological age *vs*. biological age estimations.

The sample of dormice, which was obtained from carcasses collected from the wild, provides insights in the longevity of the species analysed. Specifically, the oldest individual of *G. glis* studied was 3 years old, which is close to the mean age at death (2.2 for males, 3.3 for females) previously reported (Krystufek et al., 2005; Ruf et al., 2006; Lebl et al., 2011). The maximum age at death obtained by skeletochronology of *E. quercinus* is 6 years, which slightly surpasses the maximum life span in captivity (5.5 years) hitherto reported (Anage database, Tacutu et al., 2013), adding new data on wild individuals of this species.

Besides age at death estimations, the season of death can be determined in juvenile individuals by exploring their growth stage: active or arrested. That is, a rest line bordering the bone periphery suggests that the individual died during the unfavourable season. Conversely, a high amount of vascularised bone tissue from the last deposited LAG suggests that the individual died during the favourable season, when resources are plentiful (Köhler et al., 2012; Marín-Moratalla et al., 2013; see Chapter 5 and Chapter 9).

Moreover, the variation in the presence of a single LAG in juvenile dormice allows discerning between offspring from different reproductive events, (García-Martínez et al., 2011, see Chapter 4). That is, juveniles showing a LAG suggest that they were born before the hibernation period of that year. The lack of rest line in juvenile dormice indicates that they were born after hibernation of that year and they reached the adulthood before the next hibernation.

AFR provides reliable information about the position of a given species on the slow-fast life history continuum (Gaillard et al., 2000) and bone histology can be a useful tool to get this life history trait in wild specimens. AFR can provide insights on growth strategies either when different species are compared (see Chapter 6) and when the intra-specific variation is

considered (see Chapter 9). The results on the large mammal addax show that males and females attain reproductive maturity at different ages: males invest more time in growth attaining reproductive maturity later than females do (Marín-Moratalla et al., 2013, see Chapter 9).

Therefore, the results of these works confirm that mammalian bone histology can contribute to getting data for demographic models. These models require mean yearly estimates of fitness components, including reproductive parameters such as age at first reproduction, juvenile survival from birth to one year of age, and age-specific survival of yearlings and adults (Gaillard et al., 2003).

Quantification of osteocyte density as well as growth curves (computed from the diameters of successive growth rings in a single bone) in the male sample of the bovid addax, show differences in growth rate between captive and wild specimens. Specifically, individuals from zoos grow faster (higher osteocyte density and wider diameters of successive growth rings) than wild ones, suggesting an effect of care and constant food supply on physiology and metabolism (Asa, 2010). It reflects the capability of efficiently exploiting and allocating abundant resources to growth when food is plentiful (Piccione et al., 2009; Turbill et al., 2010; Signer et al., 2011; Köhler et al., 2012). Hence, bone histology may be useful to assess food variation between populations; for instance, between populations dwelling in different regions or born in different years, providing data about the harshness of a particular season.

CHAPTER 11. CONCLUSIONS

11. CONCLUSIONS

I. Juvenile ruminants (growing period) deposit mainly laminar or plexiform bone, while dormice deposit PFB or woven bone with longitudinal primary osteons. Both mammalian groups deposit a dense lamellar bone during the adult period. These bone typologies are common in mammals and the difference between these groups is most likely due to the different growth strategies of small and large mammals.

II. All mammalian species analysed in this work have shown bone growth marks, mainly LAGs, regardless of their climatic regime. LAGs were present in both the fast-growing bone tissue (PFB or FLC bone) deposited during the juvenile stage and the slow-growing lamellar bone (EFS) deposited in the adult stage. Overall, these growth marks correspond well to annual cycles of growth arrest.

III. Bone drift during the growing period potentially leads to the loss of cortical primary bone, and hence growth marks, in adult ages. In this sense, the femur is the bone that more suitably records the chronological age of the individual, since it preserves the greatest number of growth marks. Despite this, with increasing age, bone remodelling and resorption are important factors that may potentially hinder skeletochronology analyses, especially in large mammals.

IV. Bone growth is arrested during the energetically challenging (low resource supply) season, forming part of a plesiomorphic thermometabolic strategy for energy conservation in vertebrates. The evidence of cyclical bone growth in mammals debunks the classical assumption that homoeothermic endotherms grow continuously until they attain maturity, forming azonal bone in contrast to the zonal bone of ectotherms.

V. Strong ontogenetic variations in bone tissue occur in ruminants. The vascular and cellular pattern of the FLC bone suffers an important variation along ontogeny, reflecting a decrease in the growth rate when maturity approaches, which may be related to physiological maturity. Moreover, the transition from the fast-growing bone tissue (FLC) to the highly organized lamellar bone (EFS) reliably records the trade-off between growth and reproduction. Accordingly, the growth cycles recorded within the FLC-zonal bone may be good proxies for

one of the most important life history traits, namely the age at reproductive maturity.

VI. Some histological features of FLC bone tissue in bovids, such as the proportion of longitudinal and circular primary canals as well as the osteocyte density, are influenced by phylogeny. Vascular orientation and vascular and cellular densities are related to body mass rather than to environment or species' life history. According to Keiber's law, the FLC bone of larger bovids tends to show more circular canals (which may reflect higher rates of periosteal bone deposition) and lower cellular densities (which may reflect lower mass-specific metabolic rate) than that of smaller ones.

VII. The reconstruction of some life history traits in two Pleistocene fossil bovids using bone histology has provided insights into life history evolution under different selective regimes (continental and insular ecosystems). The mainland gazella *Gazella borbonica* conforms to the predictions for ungulates of similar body size. However, the insular caprine *Myotragus balearicus* completely differs from the generalized life cycle for extant ungulates, as it shows an important delay in all those fitness components that can be reconstructed. This variation in life history strategy makes sense in the context of resource limitation under insularity.

VIII. Finally, this PhD Thesis has shown the potential of using bone histology in endangered living mammals to obtain key life history traits as age at first reproduction, age at death, growth rates, as well as other parameters as birth season and death season, which are of great importance in the conservation biology field. Bone histology is a valuable complementary tool to monitoring programmes in demographic studies, providing useful data to calculate mortality and survival rates of wild populations, with multiple possible applications related to the conservation of endangered mammals.

REFERENCES

11. REFERENCES

Alcover, J.A., Moyà-Solà, S., Pons-Moyà, J., 1981 *Les quimeres del passat*. Editorial Moll, Palma de Mallorca, Spain.

Amprino, R. 1947 La structure du tissu osseux enviasagée comme expression de differences dans la vitesse de l'accroissement. *Archives de Biologie* **58**, 315-330.

Apio, A., Plath, M., Tiedemann, P., Wronski, T. 2007 Age-dependent mating tactics in male bushbuck (*Tragelaphus scriptus*). *Behaviour* **144**, 585-610.

Asa, C.S. 2010 Reproductive physiology. In: *Wild Mammals in Captivity* (ed. D.G. Kleiman, K.V. Thompson, C. Kirk-Baer), pp. 411-428. London: The University of Chicago Press.

Azorit, C., Analla, M., Hervas, J., Carrasco, R., Muñoz-Cobo, J., 2002a Growth marks observation: preferential techniques and teeth for aging of Spanish Red Deer (Cervus elaphus hispanicus). *Anatomia, Histologia, Embryologia* **31**, 303–307.

Azorit, C., Muñoz-Cobo, J., Analla, M., 2002b Seasonal deposition of cementum in first lower molars from Cervus elaphus hispanicus. *Mammalian Biology* **67**, 243–245.

Azorit, C., Muñoz-Cobo, J., Hervas, J., Analla, M., 2004 Aging through growth marks in teeth of Spanish red deer. *Wildlife Society Bulletin* **32**, 702–710.

Baharav, D. 1983 Reproductive strategies in female Mountain and Dorcas gazelles (*Gazella gazella gazella* and *Gazella dorcas*). *Journal of Zoology* **200**, 445-453.

Ballou, D., Lees, C., Faust, L.J., Long, S., Lynch, C., Lackey, L.B., Foose, T.J. 2010 Demographic and genetic management of captive populations. In: *Wild Mammals in Captivity.* (eds. D.G. Kleiman, K.V. Thompson, C. Kirk-Baer), pp. 219-252. London: The University of Chicago Press.

Bon, R., Campan, R. 1996 Unexplained sexual segregation in polygamous ungulates: a defense o fan ontogenetic approach. *Behavioural Processes* **38**, 131–154.

Bromage, T.G., Goldman, H.M., McFarlin, S.C., Warshaw, J., Boyde, A., Riggs, C.M. 2003 Circularly polarized light standards for investigations of collagen fiber orientation in bone. *The Anatomical Record* **274B**, 157-168.

Bromage, T.G., Lacruz, R.S., Hogg, R., Goldman, H.M., McFarlin, S.C., Warshaw, J., Dirks, W., Perez-Ochoa, A., Smoylar, I., Enlow, D.H., Boyde, A. 2009 Lamellar bone is an incremental tissue reconciling enamel rhythms, body size, and organismal life history. *Calcified Tissue International* **84**, 404-688.

Bromage, T. G., Rusell, T. H., Lacruz, R. S., Hou, C. 2012 Primate enamel evinces long period biological timing and regulation of life history. *Journal of Theoretical Biology* **305**, 131-144.

Bybee, P.J., Lee, A.H., Lamm, E-T. 2006 Sizing the Jurassic theropod dinosaur *Allosaurus*: Assessing growth strategy and evolution of ontogenetic scaling of limbs. *Journal of Morphology* **267**, 347-359.

Cain III J.W., Krausman, P.R., Germaine, H.L. 2004 Antidorcas marsupialis. *Mammalian Species* **753**, 1-7.

Cain III, J.W., Krausman, P.R., Rosenstock, S.S., Jack, C. 2006 Mechanisms of thermoregulation and water balance in desert ungulates. *Wildlife Society Bulletin* **34**, 570-581.

Capurro, A.F., Gatto, M., Tosi, G. 1997 Delayed and inverse density dependence in a chamois population of the Italian Alps. *Ecography* **20**, 37–47.

Carrano, M.T. 2001 Implications of limb bone scaling, curvature and eccentricity in mammals and non-avian dinosaurs. *Journal of Zoology* **254**, 41-55.

Castanet, J., Smirina, E. 1990 Introduction to the skeletochronological method in amphibians and reptiles. *Annales des Sciences Naturelles Zoologie* **11,** 191-196.

Castanet, J., Vieillot, H.F., Meunier, F.J., de Ricqlès, A. 1993 Bone and individual aging. In: *Bone*, vol. 7, *Bone Growth* (ed. B.K. Hall), pp. 245-283. Boca Ratón: CRC Press.

Castanet, J., Grandin, A., Abourachid, A., de Ricqlès, A. 1996 Expression of growth dynamic in th estructure of the periosteal bone in the mallard, *Anas platyrhynchos*. *Compte Rendus de l'Académie des sciences* **319**, 301-308.

Castanet , J. , Curry Rogers , K. , Cubo , J., Boisard , J.J. 2000 Periosteal bone growth rates in extant ratites (ostriche and emu). Implications for assessing growth in dinosaurs . *Comptes Rendus de l'Académie des sciences de la vie* **323** , 543- 550.

Castanet, J., Croci, S., Aujard, F., Perret, M., Cubo, J., de Margerie, E. 2004 Lines of arrested growth in bone and age estimations in a small primate: *Microcebus murinus*. *Journal of Zoology* **263**, 31-39.

Castanet, J. 2006 Time recording in bone microstructures of endothermic animals; functional relationships. *Comptes Rendus Palevol* **5**, 629-636.

Chinsamy, A. 1993 Bone histology and growth trajectory of the prosauropod dinosaur *Massospondylus carinatus* (Owen). *Modern Geology* **18**, 319-329.

Chinsamy, A., Hillenius, W.J., 2004. Physiology of non-avian dinosaurs. In: *The Dinosaurs* (ed. D.B. Weishampel, P. Dodson, I. Osmolska), pp. 643-659. Berkeley: University of California Press.

Chinsamy-Turan, A. 2005 *The microstructure of dinosaur bone*. Baltimore, Maryland: The Johns Hopkins University Press.

Chinsamy, A., Hillenius, J. 2006 Bone microstructure and growth patterns of early mammals. *Acta Paleontologica Polonica* **51**, 325-338.

Chinsamy, A., Valenzuela, N., 2008 Skeletochronology of the endangered side-neck turtle, Podocnemis expansa. *South African Journal of Science* **104**, 311–314.

Clutton-Brock, T.H., Guiness, F.E., Albon, S.D. 1982 *Red deer: Behaviour and ecology of two sexes*. Chicago: The University of Chicago Press.

Cooper, L.N., Lee, A.H., Taper, M.L., Horner, J. 2008 Relative growth rates of predator and prey dinosaur reflect effects of predation. *Proceedings of the Royal Society B* **275**, 2609-2615.

Cormack DH. 1987 *Ham's histology*. Philadelphia, PA: JG Lippincot Company.

Cubo, J., Ponton, F., Laurin, M., de Margerie, E., Castanet, J. 2005 Phylogenetic signal in bone microstructure of Sauropsids. *Systematic Biology* **54**, 562-574.

Cubo, J., Legendre, P., de Ricqlès, A., Montes, L., de Margerie, E., Castanet, J., Desdevises, Y. 2008 Phylogenetic, functional, and structural components of variation in bone growth rate of amniotes. *Evolution and Development* **10**, 217-227.

Cubo, J., Le Roy, N., Martinez-Maza, C., Montes, L. 2012 Paleohistological estimation of bone growth rate in extinct archosaurs. *Palebiology* **38**, 335-349.

Currey, J.D. 1984. *The Mechanical Adaptations of Bones*. Princenton, NH: Princeton University Press.

Currey, J. D. 2003 The many adaptations of bone. *Journal of Biomechanics* **36**, 1487–1495.

Curtin, A.J., MacDowell, A.A. Schaible, E.G., Roth, V.L. 2012 Non-invasive histological comparison of bone growth patterns among fossil and extant neonatal elephantids using synchrotron radiation X-Ra and microtomography. *Journal of Vertebrate Paleontology* **32**, 939-955.

de Margerie , E. 2002 Laminar bone as an adaptation to torsional loads in flapping flight . *Journal of Anatomy* **201**, 521-526.

de Margerie , E. , Cubo , J., Castanet , J. 2002 Bone typology and growth rate: testing and quantifying 'Amprino's rule' in the Mallard (*Anas platyrhynchos*) . *Comptes Rendus biologies* **325** , 221-230.

de Margerie , E. , Robin , J.P. , Verrier , D. , Cubo , J. , Groscolas , R., Castanet , J. 2004 Assessing a relationship between bone microstructure and growth rate: a fluorescent labelling study in the King Penguin chick (*Aptenodytes patagonicus*) . *Journal of Experimental Biology* **207**, 869-879.

de Ricqlès, A., Meunier, F. J., Castanet, J., Francillon-Vieillot, H. 1991. Comparative microstructure of bone. In: *Bone* (ed. B. K. Hall), pp. 1-78. CRC Press, Boca Raton, London.

de Ricqlès, A., Padian, K., Horner, J. R. 2003 On the bone histology of some Triassic pseudosuchian archosaurs and related taxa. *Annales de Paléontologie* **89**, 67-101.

de Ricqlès, A., Padian, K., Horner, J. R. 2008 On the origin of high growth rates in archosaurs and their ancient relatives: Complementary histological studies on Triassic archosauriforms and the problem of a 'phylogenetic signal' in bone histology. *Annales de Paléontologie* **94**, 57-76.

Dittrich, L. 1972 Gestation periods and age of sexual maturity of some African antelopes. *International Zoo Yearbook* **12**, 184–187.

Enlow, D.H., Brown, S.O. 1957 A comparative histological study of fossil and recent bone tissues, Part 2. *Texas Journal of Science* **9**, 186-214.

Erickson, G.M., Tumanova, T.A. 2000 Growth curve of *Psittacosaurus mongoliensis* Osborn (Ceratopsia: Psittacosauridae) inferred from long bone histology. *Zoological Journal of the Linnean Society* **130**, 551-566.

Estes, R.D., 1991 *The Behaviour Guide to African mammals*. Berkeley and Los Angeles: The University of California Press.

Festa-Bianchet, M., Coltman, D.W., Turelli, L., Jorgenson, J.T. 2004 Relative allocation to horn and body growth in bighorn rams varies with resource availability. *Behavioral Ecology* **15**, 305-312.

Festa-Bianchet, M., Coulson, T., Gaillard, J-M., Hogg, J.T., Pelletier, F. 2006 Stochastic predation events and population persistence in bighorn sheep. *Proceedings of the Royal Society B* **273**, 1537-1543.

Foster, J.B. 1964 Evolution of mammals on islands. *Nature* **202**, 234–235.

Francillot-Vieillot, H., Buffrénil, V.D., Castanet, J., Géraudie, J., Meunier, F.J., Sire, J.Y., Zylberberg, G., de Ricqlès, A. 1990 Microstructure and mineralization of vertebrate skeletal tissues. In: *Skeletal Biomineralization: Patters, Processes and Evolutionary trends* (ed. J.G. Carter), pp. 471-530. New York : Van Nostrand Reinhold.

Franz-Odendaal, T.A., Hall, B. K.; Eckhard Witten, P. 2006 Buried alive: how osteoblasts become osteocytes. *Developmental Dynamics* **235**, 176–190.

Gaillard, J. M., Festa-Bianchet, M., Yoccoz, N. G. Loison, A., Toïgo, C. 2000 Temporal variation in fitness components and population dynamics of large herbivores. *Annual Review of Ecology and Systematic* **31**, 367-393.

Gaillard, J.-M., Loison, A., Festa-Bianchet, M., Yoccoz, N. G., Solberg, E. 2003 Ecological correlates of life span in populations of large herbivorous mammals. In Life span: evolutionary, ecological, and demographic perspectives (eds. J. R. Carey, S. Tuljapurkar), pp. 39–56. New York, NY: Population and Development Review.

García-Martínez, R., Marín-Moratalla, N., Jordana, X., Köhler, M. 2011 The ontogeny of bone growth in two species of dormice: Reconstructing life history traits. *Comptes Rendus Palevol* **10**, 489-498.

Garde, J.J., Ortiz, N., García, A.J., Gallego, L., Landete-Castillejos, T., López, A. 1998 Postmortem assessment of sperm characteristics of the red deer during the breeding season. *Archives of Andrology* **41**, 195-202.

Gaspar-López, E., Estévez, J.A., Landete-Castillejos, T., Ceacero, F., Olguín, C.A., López-Parra, J.E., Gómez, J.A., Gallego, L., García, A. 2009 Crecimiento de la cuerna y variables biométricas en venado ibérico (*Cervus elaphus hispanicus*). In: *XXXIV Congreso nacional de la Sociedad Española de Ovinotecnia y Caprinotecnia.* (eds. L. Pardos, A. Abecia Martínez, L.F. de la Fuente Crespo, J.L. Olleta Castañer, P. González Redondo, M.J. Alcalde Aldea, F. Muñoz, J. Seva-alcaraz, C. Sañudo Astiz), pp. 613-617. Barbastro: Imprenta Moisés.

Geist, V., 1971 Mountain Sheep. A Study in Behaviour and Evolution. In: *Comparative Physiology* (eds. G. Gary, M. Mauri), pp. 441-445. Chicago: University of Chicago Press.

Gentry, A.W. 1999 Fossil pecorans from the Baynunah Formation, Emirate of Abu Dhabi, United Arab Emirates. In: *Fossil Vertebrates of Arabia* (eds. P.J. Whybrow, A. Hill), pp. 290-316. New Haven: Yale University Press.

Georgiadis, N. 1985 Growth patterns, sexual dimorphism and reproduction in African ruminants. *African Journal of Ecology* **23**, 75-87.

Hall, B.K. 2005 *Bone and Cartilage: Developmental and Evolutionary Skeletal Biology*. London: Elsevier Academic Press.

Horner, J.R., de Ricqlès, A., Padian, K. 1999 Variation in skeletochronological indicators of the hadrosaurid dinosaur *Hypacrosaurus*: implications for age assessment of dinosaurs. *Paleobiology* **25**, 295-304.

Horner, J.R., de Ricqlès, A., Padian, K. 2000 Long bone hisotology of the hadrosaurid dinosaur *Maiasaura peeblesorum*: growth dinanamics and physiology based on an ontogenetic series of skeletal elements. *Journal of Vertebrate Paleontology* **20**, 115-129.

Horner, J. R., Padian, K., de Ricqlès, A. 2001 Comparative osteohistology of some embryonic and perinatal archosaurs: developmental and behavioral implications for dinosaurs. *Paleobiology* **27**, 39-58.

Horner, J. R., Padian, K. 2004 Age and growth dynamics of Tyrannosaurus rex. *Proceedings of the Royal Society B* **271**, 1875-1880.

Huttenlocker, A.K., Woodward, H., Hall. B.K. 2013 The biology of bone. In: *Bone histology of fossil tetrapods. Advancing methods, analysis, and interpretation* (eds. K. Padian, E-T. Lamm), pp. 13-34. Berkeley and Los Angeles: University of California press.

Isaac, J.L. 2009 Effects of climate change on life history: implications for extinction risk in mammals. *Endangered Species Research* **7**, 115–123.

IUCN 2013 IUCN Red List of Threatened Species. Version 2013.1. <www.iucnredlist.org>.

Jakob, C., Seitz, A., Crivelli, A.J., Miaud, C., 2002 Growth cycle of the marbled newt (*Triturus marmoratus*) in the Mediterranean region assessed by skeletochronology. *Amphibia-Reptilia* **23**, 407–418.

Jarman, P. 1983 Mating system and sexual dimorphism in large, terrestrial, mammalian herbivores. *Biological Reviews* **58**, 485–520.

Jordana, X., Köhler, M. 2011 Enamel microstructure in the fossil bovid Myotragus balearicus (Majorca, Spain): Implications for life-history evolution of dwarf mammals in insular ecosystems. *Palaeogeography, Palaeoclimatology, Palaeoecology* **300**, 59-66.

Jordana, X., Marín-Moratalla, N., Moncunill-Solé, B., Bover, P., Alcover, A., Köhler, M. 2013 Teeth coupled with Life History Evolution along an anagenetic mammalian lineage. *PLoS ONE* **8,** e70743.

Kerschnitzki, M., Wagermaier,W., Roschger, P., Seto, J., Shahar, R., Duda, G. N., Mundlos, S., Fratzl, P. 2011 The organization of the osteocyte network mirrors the extracellular matrix orientation in bone. *Journal of Structural Biology* **173**, 303–311.

Kingdon, J. 1997 *The Kingdon Field Guide to African Mammals*. CA, USA: Academic Press.

Klevezal, G. 1996 *Recording structures of mammals*. Roterdam: A.A. Balkema Publishers.

Köhler, M. 1993 Skeleton and Habitat of Recent and Fossil Ruminants. *Munchner Geowissenschaftliche Abhandlungen. Reihe A. Geologie und Paläontologie* **25**, 1-88.

Köhler, M., Moyà-Solà, S., Esteban-Trivigno, S. 2008 Morphological variables and associated individual body weight for bovids. New equations for body mass predictions. *Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut* **105**, 103–136.

Köhler, M., Moyà-Solà, S. 2009 Physiological and life history strategies of a fósil large mammal in a resource-limited environment. *Proceedings of the National Academy of Science* **106**, 20354-20358.

Köhler, M., 2010. Fast or slow? The evolution of life history traits associated with insular dwarfing. In: *Islands and Evolution* (eds. V. Pérez-Mellado, M.M. Ramón, M.), pp. 261-279. Menorca: Institut Menorquí d'Estudis, Recerca 19.

Köhler, M., Marín-Moratalla, N., Jordana, X., Aanes, R. 2012 Seasonal bone growth and physiology in endotherms shed light on dinosaur physiology. *Nature* **487**, 358-361.

Krystufek, B., Pistotnik, M., Casar, K.S. 2005 Age determination and age structure in the edible dormouse Glis glis based on incremental bone lines. *Mammalian Review* **35**, 210–214.

Lebl, K., Bieber, C., Adamík, P., Fietz, J., Morris, P., Pilastro, A., Ruf, T. 2011 Survival rates in a small hibernator, the edible dormouse: a comparison across Europe. *Ecography* ***34***, 683-692.

Lee, A.H., Werning, S. 2008 Sexual maturity in growing dinosaurs does not fit reptilian growth models. *Proceedings of the National Academy of Science* **105**, 582-587.

Lee, A.H., Huttenlocker, A.K., Padian, K., Woodward, H. 2013 Analysis of growth rates. In: *Bone histology of fósil tetrapods. Advancing methods, analysis, and interpretation* (eds. K. Padian, E-T. Lamm), pp. 217-251. Berkeley and Los Angeles: University of California Press.

Legendre, L., Le Roy, N., Martinez-Maza, C., Montes, L., Laurin, M., Cubo, J. 2013 Phylogenetic signal in bone histology of amniotes revisited. *Zoologica Scripta* **42**, 44-53.

Lieberman, D.E. 1993 Life history variables preserved in dental cementum microstructure. *Science* **261**, 1162–1164.

Lincoln, G.A. 1998 Reproductive seasonality and maturation throughout the complete life-cycle in the mouflon ram (*Ovis musimon*). *Animal Reproduction Science* **53,** 87–105.

Lomolino, M.V. 2005 Body size evolution in insular vertebrates: generality of the island rule. *Journal of Biogeography* **32**, 1683-1699.

Lubinski, P.M. 2001 Observations on seasonality and mortality from recent catastrophic death assemblage. *Journal of Arqueological Science* **28**, 833–842.

MacArthur and Wilson. 1967. *The Theory of Island Biogeography*. Princeton University Press.

Maiorana, V. C. 1990 Evolutionary strategies and body in a Guild of mammals. In: *Body size in mammalian paleobiology. Estimation and biological implications* (eds. J. Damuth, B.J. MacFadden), pp. 69-103. Canada: Cambridge University Press.

Mallon, D.P., Kingswood, S.C. 2001 Antelopes. Global Survey and Regional Action Plans, Part 4: North Africa, the Middle East, and Asia. IUCN, Gland, Switzerland.

Marín-Moratalla, M., Jordana, X., García-Martínez, R., Köhler, M. 2011 Tracing the evolution of fitness components in fossil bovids under different selective regimes. *Comptes Rendus Palevol* **10**, 469-478.

Marín-Moratalla, M., Jordana, X., Köhler, M. 2013 Bone histology as an approach to providing data on certain key life history traits in mammals: Implications for conservation biology. *Mammalian Biology* **78**, 422– 429.

Marín-Moratalla, M., Cubo, J., Jordana, X., Moncunill-Solé, B., Köhler, M. 2014 Correlation of quantitative bone histology data with life history and climate: a phylogenetic approach. *Biological Journal of the Linnean Society* **112**, 678–687.

Marotti, G. 2010 Static and dynamic osteogenesis. *Italian Journal of Anatomy and Embryology* **115**, 123–126.

McKinney, M.L. 1997 Extinction vulnerability and selectivity: combining ecological and paleontological views. *Annual Review of Ecology, Evolution, and Systematics* **28**, 495–516.

Mduma, S.A.R., Sinclair, A.R.E., Hilborn, R. 1999 Food regulates the Serengeti wildebeest: a 40-year record. *Journal of Animal Ecology* **68**, 1101–1122.

Meiri, S., Raia, P. 2010 Reptilian all the way? *Proceedings of the National Academy of Science* E27–E.

Mina, M.V., Klevezal, G.A. 1970 The principles of investigation of recording structures. *Uspekhy Sovremennoi Biologii* **7**, 341-352.

Mitchell, C.D., Maher, C.R. 2006 Horn growth in male pronghorns *Antilocapra americana*: selection for precocialmaturation in stochastic environments. *Acta Theriologica* **51**, 405-409.

Montes L, Le Roy N, Perret M, de Buffrenil V, Castanet J, Cubo J. 2007 Relationship between bone growth rate, body mass and resting metabolic rate in growing amniotes: a phylogenetic approach. *Biological Journal of Linnean Society* **92**, 63–76.

Montes, L., Castanet, J., Cubo, J. 2010 Relationship between bone growth rate and bone tissue organization in amniotes: first test of Amprino's rule in a phylogenetic context. *Animal Biology* **60**, 25-41.

Moreno, S. 2005 Lirón careto – *Eliomys quercinus*. In: *Enciclopedia Virtual de los Vertebrados Españoles* (eds. L.M. Carrascal, A. Salvador, A.). Museo Nacional de Ciencias Naturales, Madrid.

Mullender MG, Huiskes R, Versleyen H, Buma P. 1996 Osteocyte density and histomorphometric parameters in cancellous bone of the proximal femur in five mammalian species. *Journal of Orthopaedic Research* **14**, 972–979.

Nowak, R.M. 1999 *Walker's Mammals of the World*, 6th ed. Baltimore and London: The Johns Hopkins.

Oates, J.F. 2006 Is the chimpanzee, *Pan troglodytes*, an endangered species? *Primates* **47**, 102–112.

Owen-Smith, N. 1993 Mortality rates of male and female Kudus: the costs of sexual size dimorphism. *Journal of Animal Ecology* **63**, 428–440.

Padian, K. 2013 Why study the bone microstructure of fossil tetrapods?. In: *Bone histology of fossil tetrapods. Advancing methods, analysis, and interpretation* (eds. K. Padian, E-T. Lamm), pp. 1-11. Berkeley and Los Angeles: University of California press.

Padian, K., de Boef Miara, M., Larsson, H.C.E., Wilson, L., Bromage, T. 2013 Research applicaations and integration. In: *Bone histology of fossil tetrapods. Advancing methods, analysis, and interpretation* (eds. K. Padian, E-T. Lamm), pp. 1-11. Berkeley and Los Angeles: University of California press.

Palkovacs, E. 2003 Explaining adaptive shifts in body size on islands: a life history approach. *Oikos* **103**, 37–44.

Palkovacs, E.P. 2011 The overfishing debate: an eco-evolutionary perspective. *Trends in ecology & evolution*, **26**, 616-617.

Palumbo, C., Ferretti, M., Marotti, G. 2004 Osteocyte dendrogenesis in static and dynamic bone formation: an ultrastructural study. *The Anatomical Record* **278A**, 474–480.

Pappas, L.A. 2002 *Taurotragus oryx*. *Mammalian Species* **689**, 1-5.

Peabody, F.E. 1958 A Kansas drought recorded in growth zones of a bullsnake. *Copeia* **2**, 91-94.

Peabody, F. E. 1961 Annual growth zones in living fossil vertebrates. *Journal of Morphology* **108**, 11-62.

Pettorelli, N., Gaillard, J.M., Van Laere, G., Duncan, P., Kjellander, P., Liberg, O., Delorme, D., Maillard, D. 2002 Variations in adult body mass in roe deer: the effects of population density at birth and of habitat quality. *Proceedings of the Royal Society B* **269**, 747–753.

Petter-Rousseaux, A. 1953 Recherches sur la croissance et le cycle d'activité testiculaire de *Natrix natrix Helvetica* (Lacépède). *Terre Vie* **4**, 175-223.

Pianka, E.R. 1970 On *r*- and K-Selection. *The American Naturalist* **104**, 592-597.

Pianka, E.R. 2000 Evolutionary ecology, 6th ed. San Francisco: Benjamin-Cummings, Addison-Wesley-Longman.

Piccione, G., Giannetto, C., Casella, S., Caola, G. 2009 Annual rhythms of some physiological parameters in *Ovis aries* and *Capra hircus*. *Biological Rhythm Research* **40**, 455–464.

Pilastro, A., Tavecchia, G., Marin, G. 2003 Long living and reproduction skipping in the fat dormouse. *Ecology* **84**, 1784–1792.

Pough, F.H. 1980 The Advantages of Ectothermy for Tetrapods. *The American Naturalist* **115**, 92-112.

Raia, P., Meiri, S. 2006 The island rule in large mammals: paleontology meets ecology. *Evolution*, **60**, 1731-1742.

Reznick et al., 2002. The evolution of senescence in fish. *Mechanism of ageing and development*. **123**, 773-789.

Rho, J-Y., Kuhn-Spearing, L., Zioupos, P. 1998 Mechanical properties and the hierarchical structure of bone. *Medical Engineering and Physics* **20**, 92-102.

Ricklefs, R.E. 2007 *The economy of the Nature*. 5th ed. New York: Freeman.

Roff. 2002. *Life History Evolution*. INC: Sinauer Associates.

Rolandsen, C.M., Solberg, E.J., Heim, M., Holmstrøm, F., Solem, M.I., Sæther, B. 2008 Accuracy and repeatability of moose (*Alces alces*) age as estimated from dental cement layers. *European Journal of Wildlife Research* **54**, 6–14.

Ross, M. H., Pawlina, W. 2011 *Histology: A text and Atlas*. Baltimore: Macmillan Company.

Ruf, T., Fietz, J., Schlund, W., Bieber, C. 2006. High survival in poor years: Life history tactics adapted to mast seeding in the edible dormouse. *Ecology* **87**, 372–381.

Sæther, B.E., Andersen, R., Hjeljord, O., Heim, M. 1996 Ecological correlates of regional variation in life history of the moose *Alces alces*. *Ecology* **77**, 1496–1500

Sander, P. M. 2000 Long bone histology of the Tendaguru sauropods: implications for growth and biology. *Paleobiology* **26**, 466–488.

Sander, P.A., Andrássy, P. 2006 Lines of arrested growth and long bone histology in Pleistocene large mammals from Germany: What do they tell us about dinosaur physiology? *Palaeontographica Abteilung* **277**, 143 - 159.

Schaller, G.B. 1977 *Mountain Monarchs: Wild Sheep and Goats of the Himalaya*. Chicago: University of Chicago Press.

Signer, C., Ruf, T., Arnold, A. 2010 Hypometabolism and masking: the strategies of Alpine ibex to endure harsh over-wintering conditions. *Functional Ecology* **25**, 537–547.

Singh, I.J., Tonna, E.A., Gandel, C.P. 1974 A comparative histological study of mammalian bone. *Journal of Morphology* **144**, 421–438.

Stearns, S.C. 1992 *The evolution of life histories*. New York: Oxford University Press.

Wallis, K. 1928 Zur Knochenhistologie und Kallusbildung beim Reptil (*Clemmys leprosa schweigg*). *Zeitschrift für Zellforschung* **6**, 1-26.

Stewart, K.M., Bowyer, R.T., Kie, J.G., Gasaway, C.G. 2000 Antler size relative to body mass in moose: tradeoffs associated with reproduction. *ALCES* **36**, 77-83.

Straehl, F.R., Scheyer, T.M., Forasiepi, A.M., MacPhee, R.D., Sánchez-Villagra, M.R. 2013 Evolutionary Patterns of Bone Histology and Bone Compactness in Xenarthran Mammal Long Bones. *PLoS ONE* **8**, e69275.

Tacutu, R., Craig, T., Budovsky, A., Wuttke, D., Lehmann, G., Taranukha, D., Costa, J., Fraifeld, V.E., de Magalhães, J.P. 2013 Human ageing genomic resources: integrated databases and tools for the biology and genetics of aging. *Nucleic Acids Research* **41**, D1027–D1033.

Tuljapurkar, S., Caswell, H., 1996 *Structured population models in marine, terrestrial, and freshwater systems.* New York: Chapman & Hall.

Turbill, C., Ruf, T., Mang, T., Arnold, W. 2011 Regulation of heart rate and rumen temperature in red deer: effects of season and food intake. *The Journal of Experimental Biology* **214**, 963-970.

Turmarkin-Deratzian, A.L. 2007 Fibrolamellar bone in wild adult *Alligator mississippiensis*. *Journal of Herpetology* **41**, 341-345.

Van Valen, L.M. 1973 Pattern and the balance of nature. *Evolutionary Theory* **1**, 31–49.

Vanpé, C. Gaillard, J-M., Morellet, N., Kjellander, P., Liberg, O., Delorme, D., Hewison, A.J.M. 2009 Age-specific variation in male breeding success of a territorial ungulate species, the European roe deer. *Journal of Mammalogy* **90**, 661-665.

Von Richter, W. 1974 *Connochaetes gnou. Mammalian Species* **50**, 1-6.

Vrba, E.S., Schaller, G.B. 2000 Introduction. In: *Antelopes, Deer, and Relatives: fossil record, behavioural ecology, systematics and conservation* (eds. E.S. Vrba, G.B. Schaller), pp. 1-8. USA: Yale University Press.

Walther, F.R., 1990. Gazelles and related species. In: *Grzimek's encyclopedia of mammals* (ed. S.P. Parker), pp. 462-484. New York: McGraw-Hill.

Weiner, S., Arad, T., Sabanay, I., Traub, W. 1997 Rotated plywood structure of primary lamellar bone in the rat: orientations of the collagen fibril array. *Bone* **20**, 509-514.

Wilbur, H.M., Tinkle, D.W., Collins, J.P. 1974 Environmental certainty, trophic level, and resource availability in life history evolution. *The American Naturalist* **108**, 805-817.

Williams, B., Waddington, D., Murray, D. H., Farquharson, C. 2004 Bone trength during growth: influence of growth rate on cortical porosity and mineralization. *Calcified Tissue International* **74**, 236-245.

Woodward, H.N., Padian, K., Lee, A.H. 2013 Skeletochronology. In: *Bone histology of fossil tetrapods. Advancing methods, analysis, and interpretation* (eds. K. Padian, E-T. Lamm), pp. 195-216. Berkeley and Los Angeles: University of California press.

Wronski, T., Apio, A., Plath, M., Averbeck, C. 2009 Do ecotypes of bushbuck differ in grouping patterns? *Acta ethologica* **12**, 71-78.

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