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Histological variability in the limb bones of the Asiatic wild ass and its significance for life history inferences

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The study of the bone growth marks (BGMs) and other histological traits of the bone tissue provides insights into the life history of present and past organisms. Important life history traits like longevity or age at maturity, which could be inferred from the analysis of these features, form the basis for estimations of demographic parameters that are essential in ecological and evolutionary studies of vertebrates. Here, we study the intraskeletal histological variability in an ontogenetic series of Asiatic wild ass (*Equus hemionus*) in order to assess the suitability of several skeletal elements to reconstruct the life history strategy of the species. Bone tissue types, vascular canal orientation and BGMs have been analyzed in 35 cross-sections of femur, tibia and metapodial bones of 9 individuals of different sexes, ages and habitats. Our results show that the number of BGMs recorded by the different limb bones varies within the same specimen. Our study supports that the femur is the most reliable bone for skeletochronology, as already suggested. Our findings also challenge traditional beliefs with regard to the meaning of deposition of the external fundamental system (EFS). In the Asiatic wild ass, this bone tissue is deposited some time after skeletal maturity and, in the case of the femora, coinciding with the reproductive maturity of the species. The results obtained from this research are not only relevant for future studies in fossil *Equus*, but could also contribute to improve the conservation strategies of threatened equid species.

1 **Article Title**

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ABSTRACT

The study of the bone growth marks (BGMs) and other histological traits of the bone tissue provides insights into the life history of present and past organisms. Important life history traits like longevity or age at maturity, which could be inferred from the analysis of these features, form the basis for estimations of demographic parameters that are essential in ecological and evolutionary studies of vertebrates. Here, we study the intraskeletal histological variability in an ontogenetic series of Asiatic wild ass (*Equus hemionus*) in order to assess the suitability of several skeletal elements to reconstruct the life history strategy of the species. Bone tissue types, vascular canal orientation and BGMs have been analyzed in 35 cross-sections of femur, tibia and metapodial bones of 9 individuals of different sexes, ages and habitats. Our results show that the number of BGMs recorded by the different limb bones varies within the same specimen. Our study supports that the femur is the most reliable bone for skeletochronology, as already suggested. Our findings also challenge traditional beliefs with regard to the meaning of deposition of the external fundamental system (EFS). In the Asiatic wild ass, this bone tissue is deposited some time after skeletal maturity and, in the case of the femora, coinciding with the reproductive maturity of the species. The results obtained from this research are not only relevant for future studies in fossil *Equus*, but could also contribute to improve the conservation strategies of threatened equid species.

KEYWORDS

Equus hemionus, bone histology, bone growth marks, skeletochronology, external fundamental system, life history

1. INTRODUCTION

The study of bone growth marks (BGMs) is nowadays the focus of many investigations due to its potential to reconstruct many aspects of the life history of present and past vertebrates (Amson et al., 2015; Kolb et al., 2015a; Woodward et al., 2015; Jordana et al., 2016; Moncunill-Solé et al., 2016; Nacarino-Meneses, Jordana & Köhler, 2016; Orlandi-Oliveras et al., 2016). These histological features, which record cyclic variation in bone growth rate, can take the form of "lines of arrested growth" (LAGs) or of "annuli" within the cortical bone (Castanet et al., 1993). ~~The first ones~~ appear as thin dark lines in bone cross-sections and are considered to represent moments of cessation of growth (Francillon-Vieillot et al., 1990; Chinsamy-Turan, 2005). Annuli, on the other hand, are poorly vascularized rings of lamellar or parallel-fibered bone within the bone cortex (Francillon-Vieillot et al., 1990; Chinsamy-Turan, 2005) that indicate periods of growth rate decrease. From Peabody (1961) to the present, it has been repeatedly demonstrated that most of the BGMs found in the bone tissue record annual cycles of growth (cyclical growth marks – CGMs) reflecting physiological cycles (Köhler et al., 2012) that match environmental cycles (Castanet et al., 1993; Chinsamy-Turan, 2005). Nevertheless, BGMs are also suggested to register biological events that entail moments of physiological stress in the organism (Woodward, Padian & Lee, 2013) instead of periodical growth (Castanet, 2006).

From dinosaurs to mammals, the periodicity of the CGMs is the basis for inferences of life history strategies in many groups of fossil organisms (e.g. Klevezal, 1996; Horner, de Riqclès & Padian, 2000; Köhler & Moyà-Solà, 2009). The number of CGMs within a bone cortex allows researchers to calculate important life history traits such as longevity (Castanet et al., 2004; Köhler & Moyà-Solà, 2009; Köhler, 2010) or age at maturity (Chinsamy & Valenzuela, 2008; Horner, de Riqclès & Padian, 2000; Köhler & Moyà-Solà 2009; Köhler 2010; Marín-Moratalla, Jordana & Köhler, 2013; Jordana et al., 2016) by means of a technique called skeletochronology (Castanet et al., 1993). This method also provides information about other biological aspects of the animals such as their growth strategy or physiology (Horner, de Riqclès & Padian, 2000; Padian, de Ricqlès & Horner, 2001; Köhler et al., 2012; Woodward et al., 2015). However, skeletochronology has some limitations that are particularly important when dealing with mammals. Firstly, the remodelling process (haversian systems) and the expansion of the medullary cavity that accompany the increase in age can hide the presence of previous CGMs

and, thus, give an underestimated value of longevity (Woodward, Padian & Lee, 2013). The inference of this important trait could also be altered if non-cyclical BGMs are erroneously counted as cyclical ones. On the other hand, CGMs are difficult to identify if they are located in the lamellar and avascular bone tissue deposited in the outermost cortex of adult individuals (external fundamental system – EFS) (Woodward, Padian & Lee, 2013), because of the structural similarity between LAGs and the lamellae of this tissue (Horner, de Ricqlès & Padian, 1999). Finally, several authors had reported a variable number of CGMs depending on the bone analyzed within an individual (García-Martínez et al., 2011; Woodward, Horner & Farlow, 2014). Thus, it is important to select the most appropriate bone for skeletochronological studies in each taxon before making general assessments about the life history of the species (Horner, de Riqlès & Padian, 1999).

The histological analysis of bones for this kind of studies in mammals is still little explored in comparison with other vertebrate groups (Castanet et al., 2004; Kolb et al., 2015a; Jordana et al., 2016). However, since the study of Köhler et al. (2012) that demonstrated the correlation between cyclical bone growth and seasonal physiology in a wide sample of ruminants, the number of histological works in extant (Marín-Moratalla, Jordana & Köhler, 2013, Marín-Moratalla et al., 2014; Jordana et al., 2016; Nacarino-Meneses, Jordana & Köhler, 2016) and extinct mammals (Martínez-Maza et al., 2014; Kolb et al., 2015b; Amson et al., 2015; Moncunill-Solé et al., 2016; Orlandi-Oliveras et al., 2016) has considerably increased. Among all mammalian clades, members of the family Equidae play a key role in extant and fossil ecosystems (MacFadden, 1992; Downer, 2014). Besides, they are a classical group of research in Paleontology due to their characteristic evolution (MacFadden, 2005). Nevertheless, histological studies in equids are scarce and only a few aimed to infer the life history strategies of some fossil (Sander & Andrássy, 2006; Martínez-Maza et al., 2014) or extant representatives (Nacarino-Meneses, Jordana & Köhler, 2016) of the group.

For the reasons set out above, the main objective of the present work is to study the histological variability (BGMs, pattern of vascularization, bone tissue types) between different limb bones of the same individual in the Asiatic wild ass (*Equus hemionus* Pallas, 1775). With this study, we aim to find out what life history information can be inferred from the histological study of equids and to try to determine which is the best skeletal element to develop skeletochronological studies

in this mammal. The kulan or Asiatic wild ass, a mammal endemic to the Gobi desert, is one of the eight extant species of the family Equidae (Steiner & Ryder, 2011) and presents nowadays a delicate conservation status (Kaczensky et al., 2015). Because previous studies pointed out the potential of histological analyses in conservation management of wild populations (Chinsamy & Valenzuela, 2008; García-Martínez et al., 2011; Marín-Moratalla, Jordana & Köhler, 2013), we have considered this species as the most appropriate to conduct this study. Moreover, its extant habitat – the steppe and semi-desert plains of Mongolia, Iran, Turmekistan, India and China (Feh et al., 2001; Reading et al., 2001; Kaczensky et al., 2015) – make this extant taxon the most similar to fossil stenoid horses (Forstén, 1992) extending the importance of our research form Conservation Biology to Palaeontology.

2. MATERIAL AND METHODS

Thin sections from femur, tibia, metatarsus and metacarpus were analyzed in an ontogenetic series of 9 specimens of *E. hemionus* (Table 1). Only specimen IPS83154 lacks metacarpal bone, totaling 35 the cross-sections studied. As shown in Table 1, the sample includes individuals from different habitats, sex and ages. Sex data were provided by curators while age at death was estimated according to dental eruption pattern of the species (Lkhagvasuren et al., 2013) and corroborated with the analysis of cementum layers in adult individuals (R. Schafberg, pers. comm.). Wild specimens (IPS83876 – IPS83877) were collected during the Mongolian-German Biological Expeditions in the Gobi desert (Schöpke et al., 2012) and are housed at the Natural History Collections of the Martin-Luther-University Halle-Wittenberg (Halle, Germany). Captive individuals (IPS83149 – IPS83155) lived in the Hagenbeck Zoo (Hamburg, Germany) and belong to the collections of the Zoological Institute of Hamburg University (Hamburg, Germany).

From the mid-shaft of each bone, we prepared histological slices following standard procedures in our laboratory (Nacarino-Meneses, Jordana & Köhler, 2016). After measuring and photographing each bone, three centimeters of its mid-shaft were cut and embedded in an epoxy resin (Araldite 2020). This block was later cut into two halves (ISO Met, Biometa) and the exposed surface was polished with carborundum powder to be fixed to a frosted glass with an UV curing glue (Loctite 358). Afterwards, it was cut with a diamond saw (Petrothin, Buehler) up

to a thickness of 100-120 microns and polished again with carborundum powder. Finally, a mix of oils (Lamm, 2013) was spread over the slice before being sheltered with a cover slip. The thin-sections obtained were observed in a Leica DM 2500P microscope under polarized light with a 1/4λ filter and photographed with the camera incorporated in the microscope.

To analyze the histological variability between skeletal elements, bone tissue types and BGMs were studied. The histological descriptions follow the classification of Francillon-Vieillot et al., 1990 and de Margerie et al., 2002. The terminology proposed by Prondvai et al., 2014 was employed to describe the different components of the fibrolamellar complex (FLC) (a special case of woven-parallel complex for this authors): "fibrous" or woven bone (WB) and "lamellar" or parallel-fibered bone (PFB). Because the femoral bone histology of the Asiatic wild ass has been previously described in detail (Nacarino-Meneses, Jordana & Köhler, 2016), only descriptions of the bone tissue of tibiae, metacarpi and metatarsi will be detailed in the present work. Regarding growth marks, we have generally used the term "bone growth mark – BGM", interchangeably for LAGs or annuli, instead of "cyclical growth mark – CGM" because not all the marks identified in the samples have proved to be periodical. Double LAGs or LAGs that split were considered as a single event. BGMs were traced along the cross-sections and superimposition of individuals was performed to identify growth marks that have been erased by the remodeling process or the expansion of the medullary cavity (Woodward, Padian & Lee, 2013). Each BGM circumference was measured with ImageJ® software to estimate the bones' perimeter at different times during ontogeny and the results were plotted to obtain growth curves for each sample (Bybee, Lee & Lamm, 2006). The perimeter of the cross-section was also calculated with ImageJ® software in those animals that are still growing (subadult individuals) to estimate its bone perimeter at the time of death. The perimeter of adult individuals was not determined and only the length of the BGMs identified within the EFS is shown. Because it is generally considered that the presence of EFS indicates the cessation of radial growth in long bones (Huttenlocker, Woodward & Hall, 2013), the length of the BGMs located in this bone tissue and the perimeter of the cross-section are almost the same value. Thus, the estimation of the cross-section's perimeter in adult specimens does not provide relevant information about the growth of the animal. Furthermore, we calculated the size variation per year of each bone in yearling and adult specimens as the difference of BGMs' perimeters of consecutive annual

158 growth cycles and interpreted it as a proxy of growth rate. Finally, several life history traits were
159 calculated in each bone from the study of CGMs. Longevity was determined as the total number
160 of CGMs present in the bone cortex (Castanet et al., 2004) and compared with the age estimated
161 from teeth. Age at maturity was calculated by counting the CGMs before the deposition of the
162 EFS (Chinsamy & Valenzuela, 2008; Marín-Moratalla, Jordana & Köhler, 2013) and contrasted
163 with literature data.

164 **3. RESULTS**

165 3.1. Bone tissue types

166 All bones of *E. hemionus* present a well-vascularized FLC that is progressively remodeled during
167 ontogeny. However, the arrangement of the vascular canals embedded in the FLC varies among
168 the bones sampled and in the course of ontogeny. An ontogenetic change in the proportion of the
169 different components of the bone matrix (WB and PFB) has also been noted in some of the limb
170 bones studied.

171 The histology of kulan's femora was previously described in Nacarino-Meneses, Jordana &
172 Köhler (2016). It consists of a highly vascularized FLC that presents an ontogenetic change in
173 the orientation of the vascular canals to a predominantly circumferential arrangement, along with
174 a decrease in the proportion of the WB of the matrix. The EFS was only indentified in adult
175 stages and remodeling was associated to the course of ontogeny and to mechanical loading.

176 Tibial cortices consist of laminar bone (Fig. 1A) and remodeling begins early in ontogeny, as
177 indicates the high number of secondary osteons (SO) identified in yearling specimens (Fig. 1B).
178 Regarding primary bone tissue, the cortical bone of the perinatal individual presents FLC with a
179 high proportion of PFB in the bone matrix (Fig. 1C). The cortex of foals, as well as those of
180 yearling and juvenile individuals, is divided into two well-defined areas that differ in the
181 proportion of this bone matrix component. In these specimens, the laminar bone of the internal
182 cortex presents a higher proportion of PFB than the outer one (Fig. 1A). The EFS is not
183 identified in any of the tibiae analyzed. Instead, several packages of a poorly vascularized
184 lamellar bone that interrupt the FLC matrix, can be recognized in the mid-outer cortex of adult

specimens (Fig. 1D). This bone tissue differs from the real EFS (Huttenlocker, Woodward & Hall, 2013) because it is not restricted to the outermost cortex.

Bone tissue and vascular arrangement is very similar in metatarsi and metacarpi. In both skeletal elements, the bone cortex is mainly composed of a FLC with primary osteons (POs) oriented in circular rows (Fig. 2A). The vascular canals of these POs present a larger diameter in the outer half of the cortex than in the inner half (Fig. 2A). Some radial canals are situated in the proximity of the medullary cavity in metacarpal bones (Fig. 2B) whereas metatarsi present several areas with laminar bone (Fig. 2C). Haversian bone is restricted to the posterior side of the cortex in immature kulans but it is more generalized in adult ones. The EFS is identified in the outermost cortex of adult individuals (Fig. 2D).

3.2. Bone growth marks

Table 2 shows the number of BGMs identified in the different bones of each individual. From foals to adults, all samples present these features, although its number varies among skeletal elements of the same individual and between individuals of the same age category.

The presence of a BGM in the middle cortex of tibia, metacarpus and metatarsus (Fig. 3, Table 2) of foals (IPS83153 and IPS83154) is surprising. LAGs and annuli are known to be annual and deposited during the unfavorable season (i.e. winter for *E. hemionus*) in mammals (Köhler et al., 2012). Because kulans tend to give birth in summer (Zuckerman, 1952; Nowak, 1999; Feh et al., 2001; Feh et al., 2002) and our foals are around six months old (Table 1), the CGM corresponding to the first winter should be observed in the outermost cortex, not in the mid-cortex (Fig. 3). Therefore, this feature is interpreted as a non-cyclical growth mark and will not be taken into account for age estimation.

Yearling specimens (IPS83149, IPS83150 and IPS83151) present a variable number of LAGs. As it is shown in Table 2, one BGM is identified in all skeletal elements of IPS83151, while IPS83149 and IPS83150 present two (Fig. 4). Such variability might be explained by the fact that the first permanent molar is totally unworn in IPS38151 but presents initial wear in IPS83149 and IPS83150. Thus, the former might be somewhat younger than the others. Because these specimens are aged as one year, we interpret the most external BGM identified in all bones of

IPS83149 and IPS83150 (Fig. 4B, C) as CGM deposited during the first year of life. However, we consider the internal BGM observed in these individuals (Fig. 4B, C), as well as the single BGM identified in the mid-cortex of all bones of IPS83151 (Fig. 4A, D), as a non-cyclical growth mark.

Two BGMs are identified in the tibia and the metatarsus of the juvenile individual (IPS83155) while the femur and the metacarpus present only one (Table 2, Fig. 5). In these latter bones, the growth mark appears in the outer cortex (Fig. 5A, C). Because this individual is aged around 2 years, we consider that this external BGM is representing the winter growth arrest during its second year of life. The second BGM in the tibia and metatarsus is also found in the external part of the cortex (Fig. 5B, C), so we interpret it as the CGM corresponding to the second winter. On the other hand, superimposition of individuals reveals that the first BGM of these bones (Fig. 5B, C) does not correspond to the CGM identified in yearlings, as it appears more internally within the cortex. This fact suggests that the first winter has not been recorded in this animal and that such internal BGM could be considered as non-cyclical.

Wild adult individuals (IPS83876 and IPS83877) also present differences in the number of BGMs between limb bones (Table 2, Fig. 6). Femur, metatarsus and metacarpus of the wild female (IPS83876) show five BGMs while only four BGMs are identified in its tibia (Table 2, Fig. 6A, B, E, F). In the femur, four BGMs lie within the FLC and one within the avascular and highly organized lamellar tissue deposited in the periphery of the bone (EFS) (Table 2, Fig. 6A, B). Metapodial bones, however, present three BGMs within the FLC and two BGMs in the EFS (Table 2, Fig. 6E, F). The four BGMs found in the tibia are within the FLC, as an EFS is not identified in this bone. On the other hand, the wild male (IPS83877) presents six BGMs in femur and metapodial bones, whereas five BGMs are found in the tibia (Table 2, Fig. 6C, D, G, H). Superimposition of both adult individuals reveals that one BGM has been lost in the femur of the wild male due to bone remodeling (Nacarino-Meneses, Jordana & Köhler, 2016). This process, however, has not erased the presence of any BGM in the other limb bones studied. Thus, a total of seven BGMs should be counted in the femur of the wild male: five in the FLC (one hidden by secondary osteons) and two in the EFS. Five BGMs, all located in the FLC, are identified in the tibia of this wild male (IPS83877; Table 2, Fig. 6C, D). Finally, four BGMs are found in the FLC and two in the EFS of its metatarsus and metacarpus (Table 2, Fig. 6G, H). The correspondence

243 between the age of both adults and the number of BGMs identified in their limb bones indicates
244 that all these features could be considered as CGMs. However, superimposition suggests that the
245 most internal BGM observed in metapodial bones of wild adults might be a non-cyclical feature,
246 as they are deposited previously to the CGM identified in yearlings.

247 3.3. Growth curves

248 Based on the ontogenetic time schedule obtained from the study of the BGMs, we represented
249 the growth curve for the different bones of each specimen (Fig. 7A-D). In these graphs, the
250 perimeter of the bone (outline of the BGM) at different years is plotted against the estimated age.
251 Because the non-cyclical BGM identified in several bones is deposited sometime before the six
252 months of life (Table 2, Fig. 3), it has been considered as time "zero" in the growth curves. The
253 amount of growth in successive years, calculated as a proxy of growth rate, is also represented
254 for yearlings and adult kulans (Fig. 7E-H).

255 In adult individuals, the growth curves, as well as the plots of growth rate estimations, indicates a
256 change in the pace of growth during ontogeny. Figure 7A shows that in both adults, growth of
257 the femur slows down at the fourth year of life and from this time onwards growth is minimal
258 (Fig. 7E). However, this decrease in growth rate takes place at the age of two in tibia, metatarsus
259 and metacarpus (Fig. 7B-D), followed by only minimal growth (Fig. 7F-H).

260 Figure 7 also reveals differences in growth between captive and wild kulans. The results
261 obtained from the analysis of bone growth cycles of the femur indicate two different growth
262 tendencies with wild specimens growing more slowly than captives (Fig. 7A). While this
263 difference is not perceived in the growth curves of the other limb bones studied (Fig. 7B-D),
264 growth rates of captive individuals are always higher than those of wild kulans in the first year of
265 life (Fig. 7E-H).

4. DISCUSSION

In the present research, we analyzed for the first time the histological variability between limb bones in the extant species *Equus hemionus*. Previous studies have addressed this issue in isolated bones of fossil vertebrate species (Horner, de Ricqlès and Padian, 2000; Sander & Andrássy, 2006; Cullen et al., 2014; Martínez-Maza et al., 2014), but only a few have studied the histological variation of bone tissue within the same individual (Horner, de Ricqlès and Padian, 1999; García-Martínez et al., 2011; Woodward, Horner & Farlow, 2014; Cambra-Moo et al., 2015). Our thorough analysis of kulan's bone histology contributes to the knowledge of intraskeletal variability in mammals, providing new and important results that are of interest in different scientific areas. The applicability of histological studies to describe the life history of past animals and their evolutionary trends is well known (Köhler & Moyà-Solà, 2009; Marín-Moratalla et al., 2011; Martínez-Maza et al., 2014; Woodward et al., 2015). However, many researchers claim that more studies in living taxa are needed to truly understand the correlation between bone histology and the life history strategy of past organisms (Martínez-Maza et al., 2014; Woodward, Horner & Farlow, 2014; Cambra-Moo et al., 2015; Kolb et al., 2015a; Jordana et al., 2016). The results obtained from the present research will serve as a basis for inference of life history parameters from the histology of extinct vertebrate species. Even more, skeletochronological studies of extant species are also of interest in related biological disciplines like Conservation Biology (Chinsamy & Valenzuela, 2008; García-Martínez et al., 2011; Marín-Moratalla, Jordana & Köhler, 2013). Nowadays, most of the wild species of the genus *Equus* are threatened and conservation policies are usually focus on genetic studies of captive individuals (Orlando, 2015). Here, key life history traits such as longevity or age at sexual maturity inferred from the study of the BGMs in the bone tissue of wild specimens (Castanet et al., 2004; Marín Moratalla, Jordana & Köhler, 2013; Jordana et al., 2016) help calculating demographic parameters (e. g. life expectancy, generation time) that are essential to carry out conservation programs in the wild (Feh et al., 2001).

The detailed analysis of LAGs and annuli performed in the present research reveals that the number of BGMs recorded by the different limb bones varies within the same specimen (Table 2), a fact that has previously been reported for other vertebrate groups (Horner, de Ricqlès & Padian, 1999; García-Martínez et al., 2011; Cullen et al., 2014; Woodward, Horner & Farlow,

297 2014). Our results show that the femur registers the highest total number of BGMs, as well as the
298 highest number of these features within the FLC (Table 2). This observation, which has been
299 previously observed in mammals (García-Martínez et al., 2011), is likely related with the fact
300 that the femur is the bone that more tightly correlates with the final size of the individual because
301 it fuses its epiphyses late in ontogeny (Silver, 1969). Horner, de Ricqlès & Padian, 1999, in their
302 study of *Hypacrosaurus stebingeri*, suggested that also the tibia is suitable for
303 skeletochronology. However, the presence of many haversian systems in the tibial cortices of
304 hemionus yearlings (Fig. 1B) indicates that it does not provide accurate skeletochronological
305 results in the Asiatic wild ass. The use of metapodial bone tissues in skeletochronology is still a
306 controversial issue. While Horner, de Ricqlés & Padian, (1999) do not recommend it, for
307 perissodactyls, Martínez-Maza et al. (2014) obtained acceptable results in their histological
308 analysis of the fossil species *Hipparion concudense*. In artiodactyls, however, it does not work
309 because of the ontogenetically late fusion of metatarsus III and IV that deletes growth structures
310 (M. Köhler, pers. observation). Our results show that these bones register a similar total number
311 of BGMs as the femur (Table 2), although the first BGM identified in these skeletal elements
312 seems to be a non-cyclical BGM (Table 2, Fig. 3-6), a fact that must be taken account when
313 calculating individual age. Moreover, adult metacarpi and metatarsi show a lower number of
314 BGMs than femora within the FLC (Table 2, Fig. 6), which contrasts with the results obtained by
315 Martínez-Maza et al. (2014). The presence of BGMs in the still growing fibrolamellar tissue
316 provides important information about the growth rate of the species and the timing of key life
317 history traits (Marín-Moratalla, Jordana & Köhler, 2013). Therefore, the results obtained from
318 metapodial bones should be used with caution. Despite these drawbacks, the
319 skeletochronological study of metacarpi and metatarsi still provides valuable longevity estimates
320 because they present a similar total number of BGMs as femora (Table 2). This result is
321 especially interesting for the study of fossil species, as these bones are the most abundant
322 remains of equids in paleontological sites.

323 Regarding bone tissue types, our results show that femora and tibiae present laminar bone (Fig.
324 1A) while the cortices of metapodial bones are mainly composed of longitudinal POs arranged in
325 circular rows (Fig. 2A) (Francillon-Vieillot et al., 1990). This histological variability, which
326 agrees with previous descriptions of the bone tissue of extant (Enlow & Brown, 1985; Stover et

al., 1992) and fossil (Sander & Andrássy, 2006; Martínez-Maza et al., 2014) equid species, is likely related with the specific growth rate and biomechanics of each bone (Horner, de Riqclès & Padian, 1999; de Margerie et al., 2002; de Margerie et al., 2004). Furthermore, ontogenetic histological changes regarding bone matrix have been noticed in the different limb bones studied. Our study shows a marked change in the proportion of PFB (Fig. 1A) within the FLC matrix in tibiae of subadult kulans. Bone matrix change, along with a modification of the orientation of the vascular canals, has also been observed in femora of *E. hemionus* (Nacarino-Meneses, Jordana & Köhler, 2016). These histological modifications are likely related to both the changes in loadings (Firth, 2006) and in growth rate (Peters, 1983) that foals experience at the moment of birth.

Amongst all bone tissue types, the occurrence of EFS in vertebrates is a controversial issue. Traditionally, its deposition has been interpreted as the attainment of skeletal maturity (Cormack, 1987; Chinsamy-Turan, 2005; Woodward, Padian & Lee, 2013; Martínez-Maza et al., 2014; Amson et al., 2015; Kolb et al., 2015b) but recent studies have shown that, at least in mammals, it might also records the onset of sexual maturity of the species (Klevezal, 1996; Marín-Moratalla, Jordana & Köhler, 2013; Jordana et al., 2016). Growth studies have been shown to provide good estimations of these traits in fossil species (Lee et al., 2013). Our results indicate that the EFS is deposited after epiphyseal fusion in all bones and at a later time in the male than in the female (Table 3, Fig. 7). Actually, in most of the bones analyzed, the time of fusion of both epiphyses agrees with an important drop in the rate of radial growth (inflection point in the growth curves, Fig. 7) and does not match the time of deposition of the EFS. Concretely in the femur, which epiphyses are fused at the age of three (Silver, 1969), the EFS of the wild female is deposited in the fourth year of life while in the wild male it appears at the age of six (Table 3; Fig. 7). In metapodials, the EFS appears after the third year in the female and after the fourth year in the male (Table 3, Fig. 7). These skeletal elements are completely fused at the age of two (Silver, 1969). The correspondence between the pronounced decrease in periosteal growth rate and the age of epiphyseal fusion (Silver, 1969) (Table 3) suggests the decrease in periosteal growth rate to be a good indicator of the end of longitudinal growth in the respective bone. However, the deposition of the EFS some time after growth decline (Fig. 7) indicates that the bone shaft continues growing at minimal rates over some time until full radial growth is achieved (Huttenlocker, Woodward & Hall, 2013). This decoupling between longitudinal and radial

growth suggests that inferences of skeletal maturity from the time of deposition of the EFS in equids might be incorrect. However, the presence of the EFS in femora agrees fairly well with the age at first reproduction reported for *E. hemionus* (Table 3; Kaczensky et al., 2015). In general terms, the femur in mammals presents the longest time of development with the latest epiphyseal fusion (Silver, 1969). Thus, its histological structure should provide the best record of life history events. It is known that although kulans are sexually mature at their second or third year of life (Nowak, 1999), they delay some years its first mating (Kaczensky et al., 2015). Hence, our results provide histological evidence for this well-known behavior in equids (Fielding, 1988; Monfort, Arthur & Wildt, 1994).

Finally, the growth analysis has also revealed a high inter-individual variability in size (Fig. 7) that should be taken into account when retrocalculating lost CGMs. Our results show different femoral growth tendencies between wild and captive individuals (Fig. 7A) and a higher growth rate in captive exemplars than in wild ones during the first year of life (Fig. 7E-H). These differences, that reflect the influence of the habitat in the life history of the species, have been previously reported for mammals (Marín-Moratalla, Jordana & Köhler, 2013) and alligators (Woodward, Horner & Farlow, 2014) and are related with the constant food supply and care that captive animals experience during their life (Asa, 2010). To obtain the most accurate data, we propose to study wild animals when possible to avoid overestimation of growth rates for the species under study.

5. CONCLUSIONS

Our study analyzes the histological variation between different limb bones of the Asiatic wild ass. Our research provides evidence that the femur is the most reliable bone for skeletochronological studies in equids, although metapodial bones also provide good individual age estimations. The use of tibiae, however, is not recommended for this group due to the high presence of secondary osteons observed in early ontogenetic stages. Furthermore, all bones present histological changes regarding the proportions of bone matrix components and / or the arrangement of vascular canals in the course of ontogeny. Finally, the presence of an EFS in the outermost cortex of adult femora is likely related to the reproductive maturity of the species (first

reproduction) than to skeletal maturity. Skeletal maturity, however, is recorded in growth curves as a significant drop in periosteal growth rate.

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7. REFERENCES

Asa CS. 2010. Reproductive physiology. In: Kleiman DG, Thompson KV, Kirk-Baer C, eds. *Wild Mammals in Captivity*. London: The University of Chicago Press, 219-252.

Amson E, Kolb C, Scheyer TM, Sánchez-Villagra MR. 2015. Growth and life history of Middle Miocene deer (Mammalia, Cervidae) based on bone histology. *Comptes Rendus Palevol* 14:637-645. DOI: 10.1016/j.crpv.2015.07.001.

Bybee PJ, Lee AH, Lamm ET. 2006. Sizing the Jurassic theropod dinosaur *Allosaurus*: assessing growth strategy and evolution of ontogenetic scaling limbs. *Journal of Morphology* 267:347-35.9 DOI: 10.1002/jmor.10406.

Cambra-Moo O, Nacarino-Meneses C, Díaz-Güemes I, Enciso S, García Gil O, Llorente Rodríguez L, Rodríguez Barbero MA, de Aza AH, González Martín A. 2015. Multidisciplinary characterization of the long-bone cortex growth patterns through sheep's ontogeny. *Journal of Structural Biology* 191:1-9. DOI: 10.1016/j.jsb.2015.06.013.

Castanet J. 2006. Time recording in bone microstructures of endothermic animals; functional relationships. *Comptes Rendus Palevol* 5:629-636. DOI: 10.1016/j.crpv.2005.10.006.

Castanet J, Francillon-Vieillot H, Meunier FJ, de Ricqlès A. 1993. Bone and individual aging. In: Hall BK, ed. *Bone, vol. 7*. London: CRC Press, 245-283.

410 Castanet J, Croci S, Aujard F, Perret M, Cubo J, de Margerie E. 2004. Lines of arrested growth
411 in bone and age estimation in a small primate: *Microcebus murinus*. *Journal of Zoology* 263:31-
412 39. DOI: 10.1017/S0952836904004844.

413 Chinsamy-Turan A. 2005. *The Microstructure of Dinosaur Bone*. Baltimore and London: The
414 Johns Hopkins University Press.

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

417 Cormack D. 1987. *Ham's Histology*. Philadelphia: JC Lippincott Williams and Wilkins.

418 Cullen TM, Evans DC, Ryan MJ, Currie PJ, Kobayashi Y. 2014. Osteohistological variation in
419 growth marks and osteocyte lacunar density in a theropod dinosaur (Coelurosauria:
420 Ornithomimidae). *BMC Evolutionary Biology* 14:231. DOI: 10.1186/s12862-014-0231-y.

421 Downer CC. 2014. The horse and burro as positively contributing returned natives in North
422 America. *American Journal of Life Sciences* 2:5-23. DOI: 10.11648/j.ajls.20140201.12

423 Enlow DH, Brown SO. 1958. A Comparative Histological study of Fossil and Recent Bone
424 Tissues. Part III. *Texas Journal of Science* 10:187-230.

425 Feh C, Munkhtuya B, Enkhbold S, Sukhbaatar T. 2001. Ecology and social structure of the Gobi
426 khulan *Equus hemionus* subsp. in the Gobi B National Park, Mongolia. *Biological Conservation*
427 10: 51-61. DOI:10.1016/S0006-3207(01)00051-9.

428 Feh C, Shah N, Rowen M, Reading R, Goyal SP. 2002. Status and Action Plan for the Asiatic
429 Wild Ass (*Equus hemionus*). In: Mohelman PD, ed. *Zebras, Asses and Horses. Status Survey and*
430 *Conservation Action Plan*. Gland and Cambridge: IUCN/SS Equid Specialist Group, 62-71.

431 Fielding, D. 1988. Reproductive characteristics of the jenny donkey-*Equus asinus*: A review.
432 *Tropical Animal Health and Production* 20:161-166. DOI: 10.1007/BF02240085.

433 Firth EC. 2006. The response of bone, articular cartilage and tendon to exercise in the horse.
434 *Journal of Anatomy* 208:513-526. DOI: 10.1111/j.1469-7580.2006.00547.x.

435 Forstén A. 1992. Mitochondrial-DNA time-table and the evolution of *Equus*: comparison of
436 molecular and paleontological evidence. *Annales Zoologici Fennici* 28:301-309.

437 Francillon-Vieillot H, de Buffrénil V, Castanet J, Géraudie J, Meunier FJ, Sire JY, Zylberberg L,
438 de Ricqlès A. 1990. Microstructure and mineralization of vertebrate skeletal tissues. In: Carter
439 JG, ed. *Skeletal Biomineralization: Patterns, Processes and Evolutionary Trends*. New York:
440 Van Nostrand Reinhold, 471-530.

441 García-Martínez R, Marín-Moratalla N, Jordana X, Köhler M. 2011. The ontogeny of bone
442 growth in two species of dormice: Reconstructing life history traits. *Comptes Rendus Palevol*
443 10:489-498. DOI: 10.1016/j.crpv.2011.03.011.

444 Horner JR, de Ricqlès A, Padian K. 1999. Variation in dinosaur skeletochronology indicators:
445 implications for age assessment and physiology. *Paleobiology* 25:295-304.

446 Horner JR, de Ricqlès A, Padian K. 2000. Long bone histology of the hadrosaurid dinosaur
447 *Maiasaura peeblesorum*: growth dynamics and physiology based on an ontogenetic series of
448 skeletal elements. *Journal of Vertebrate Paleontology* 20:115-129. DOI: 10.1671/0272-
449 4634(2000)020[0115:LBHOTH]2.0.CO;2

450 Huttenlocker AK, Woodward HN, Hall BK. 2013. The biology of Bone. In: Padian K, Lamm
451 ET, eds. *Bone Histology of Fossil Tetrapods*. Berkeley, Los Angeles, London: University of
452 California Press, 13-34.

453 Jordana X, Marín-Moratalla N, Moncunill-Solé B, Nacarino-Meneses C, Köhler M. 2016.
454 Ontogenetic changes in the histological features of zonal bone tissue of ruminants: a quantitative
455 approach. *Comptes Rendus Palevol* 15:265-276. DOI: 10.1016/j.crpv.2015.03.008.

456 Kaczensky P, Lkhagvasuren B, Pereladova O, Hemami M, Bouskila, A. 2015. *Equus hemionus*.
457 *The IUCN Red List of Threatened Species* 2015:e.T7951A45171204. DOI:
458 10.2305/IUCN.UK.2015-4.RLTS.T7951A45171204.en

459 Klevezal G.A. 1996. *Recording Structures of Mammals: Determination of Age and*
460 *Reconstruction of Life History*. Rotterdam: AA Balkema.

461 Köhler M, Moyà-Solà S. 2009. Physiological and life history strategies of a fossil large mammal
462 in a resource-limited environment. *Proceedings of the National Academy of Sciences of the*
463 *United States of America*. 106:20354-20358. DOI: 10.1073/pnas.0813385106

464 Köhler M. 2010. Fast or slow? The evolution of life history traits associated with insular
465 dwarfing. In: Pérez-Mellado V, Ramon MM, eds. *Islands and Evolution*. Maó: Institut Menorquí
466 d'Estudis, Recerca 19, 261-279.

467 Köhler M, Marín-Moratalla N, Jordana X, Aanes R. 2012. Seasonal bone growth and physiology
468 in endotherms shed light on dinosaur physiology. *Nature* 487, 358-361. DOI
469 10.1038/nature11264.

470 Kolb C, Scheyer TM, Veitschegger K, Forasiepi AM, Amson E, Van der Geer AAE, Van den
471 Hoek Ostende LW, Hayashi S, Sánchez-Villagra MR. 2015a. Mammalian bone paleohistology: a
472 survey and new data with emphasis on island forms. *PeerJ* 3:e1358 DOI 10.7717/peerj.1358.

473 Kolb C, Scheyer TM, Lister AM, Azorit C, De Vos J, Schlingemann MAJ, Rössner GE,
474 Monaghan NT, Sánchez-Villagra MR. 2015b. Growth in fossil and extant deer and implications
475 for body size and life history evolution. *BMC Evolutionary Biology* 15:1–15 DOI
476 10.1186/s12862-015-0295-3.

477 Lamm ET. 2013. Preparation and Sectioning of Specimens. In: Padian K, Lamm ET, eds. *Bone*
478 *Histology of Fossil Tetrapods*. Berkeley, Los Angeles, London: University of California Press,
479 55-160.

480 Lee AH, Huttenlocker AK, Padian K, Woodward HN. 2013. In: Padian K, Lamm ET, eds. *Bone*
481 *Histology of Fossil Tetrapods*. Berkeley, Los Angeles, London: University of California Press,
482 217-251.

483 Lkhagvasuren D, Ansorge H, Samiya R, Schafberg R, Stubbe A, Stubbe M. 2013. Age
484 determination of the Mongolian wild ass (*Equus hemionus*, Pallas 1775) by the dentition patterns
485 and annual lines in the tooth cementum. *Journal of Species Research* 2:85-90. DOI:
486 10.12651/JSR.2013.2.1.085.

MacFadden BJ. 1992. *Fossil horses. Systematics, Paleobiology, and Evolution of the Family Equidae*. Cambridge, New York, Melbourne, Madrid: Cambridge University Press.

MacFadden BJ. 2005. Fossil Horses – Evidence of evolution. *Science* 307:1728-1730. DOI: 10.1126/science.1105458.

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. DOI: 10.1016/S1631-0691(02)01429-4.

de Margerie E, Robin JP, Verrier D, Cubo J, Groscolas R, Castanet J. 2004. Assessing a relationship between bone microstructure and growth rate: a fluorescent labeling study in the king penguin chick (*Aptenodytes patagonicus*). *Journal of Experimental Biology* 207:869-879. DOI: 10.1242/jeb.00841.

Marín-Moratalla N, 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. DOI: 10.1016/j.crpv.2011.03.007.

Marín-Moratalla N, 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. DOI: 10.1016/j.mambio.2013.07.079.

Marín-Moratalla N, 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. DOI: 10.1111/bij.12302.

Martínez-Maza C, Alberdi MT, Nieto-Diaz M, Prado JL. 2014. Life-History Traits of the Miocene *Hipparion concudense* (Spain) Inferred from Bone Histological Structure. *Plos One* 9:e103708. DOI: 10.1371/journal.pone.0103708.

Moncunill-Solé B, Orlandi-Oliveras G, Jordana X, Rook L, Köhler M. 2016. First approach of the life history of *Prolagus apricenicus* (Ochotonidae, Lagomorpha) from Terre Rosse sites (Gargano, Italy) using body mass estimation and paleohistological analysis. *Comptes Rendus Palevol* 15:235-245. DOI: 10.1016/j.crpv.2015.04.004.

514 Monfort, SL, Arthur NP, Wildt DE. 1994. Reproduction in the Przewalski's horse. In: Boyde L,
515 Houpt KA, eds. *Przewalski's horse. The history and biology of an endangered species*. Albany:
516 State University of New York Press, 173-194.

517 Nacarino-Meneses C, Jordana X, Köhler M. 2016. First approach to bone histology and
518 skeletochronology of *Equus hemionus*. *Comptes Rendus Palevol* 15:277-287. DOI:
519 10.1016/j.crpv.2015.02.005.

520 Nowak RM. 1999. *Walker's Mammals of the World, 6th ed*. Baltimore and London: The Johns
521 Hopkins University Press.

522 Orlandi-Oliveras G, Jordana X, Moncunill-Solé B, Köhler M. 2016. Bone histology of the giant
523 fossil dormouse *Hypnomys onicensis* (Gliridae, Rodentia) from Balearic Islands. *Comptes*
524 *Rendus Palevol* 15:247-253. DOI: 10.1016/j.crpv.2015.05.001.

525 Orlando L. 2015. Equids. *Current biology* 25:R973-R978. DOI: 10.1016/j.cub.2015.09.005.

526 Padian K, de Ricqlès A, Horner JR. 2001. Dinosaurian growth rates and bird origins. *Nature*
527 412:405-408. DOI: 10.1038/35086500.

528 Peabody FE. 1961. Annual growth zones in living and fossil vertebrates. *Journal of Morphology*
529 108:11-62. DOI: 10.1002/jmor.1051080103.

530 Peters RH. 1983. *The ecological implications of body size*. Cambridge, New York, Victoria:
531 Cambridge University Press.

532 Prondvai E, Stein KHW, de Ricqlès A, Cubo J. 2014. Development-based revision of bone tissue
533 classification: the importance of semantics for science. *Biological Journal of the Linnean Society*
534 112:799-816. DOI: 10.1111/bij.12323.

535 Reading RP, Mix HM, Lhagvasuren B, Feh C, Kane DP, Dulamtseren S, Enkhbold S. 2001.
536 Status and distribution of khulan (*Equus hemionus*) in Mongolia. *Journal of Zoology* 254:381-
537 389. DOI: 10.1017/S0952836901000887.

Sander PM, 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 A* 277:143-159.

Schöpke K, Stubbe A, Stubbe M, Batsaikhan N, Schafberg R. 2012. Morphology and variation of the Asiatic wild ass. *Erforschung biologischer Ressourcen der Mongolei*. 12:77-84.

Silver IA. 1969. The ageing of domestic mammals. In: Brothwell D, Higgs E, eds. *Science in Archaeology*. New York: Basic Books, 250-268.

Steiner CC, Ryder OA. 2011. Molecular phylogeny and evolution of the Perissodactyla. *Zoological Journal of the Linnean Society* 163:1289-1303. DOI: 10.1111/j.1096-3642.2011.00752.x.

Stover SM, Pool RR, Martin RB, Morgan P. 1992. Histological features of the dorsal cortex of the third metacarpal bone mid-diaphysis during postnatal growth in thoroughbred horses. *Journal of Anatomy* 181:455-469.

Woodward HN, Padian K, Lee AH. 2013. Skeletochronology. In: Padian K, Lamm ET, eds. *Bone Histology of Fossil Tetrapods*. Berkeley, Los Angeles, London: University of California Press, 195-216.

Woodward HN, Horner JR, Farlow JO. 2014. Quantification of intraskeletal histovariability in *Alligator mississippiensis* and implications for vertebrate osteohistology. *PeerJ* 2:e422. DOI: 10.7717/peerj.422.

Woodward HN, Freedman Fowler EA, Farlow JO, Horner JR. 2015. *Maiasaura*, a model organism for extinct vertebrate population biology: a large sample statistical assessment of growth dynamics and survivorship. *Paleobiology* 41:503-527. DOI: 10.1017/pab.2015.19.

Zuckerman S. 1952. The breeding season of mammals in captivity. *Proceedings of the Zoological Society of London* 122:827-950. DOI: 10.1111/j.1096-3642.1952.tb00251.x.

Table 1(on next page)

Sample studied.

M: male; F: female; Fe: femur; Ti: tibia; Mc: metacarpus; Mt: metatarsus.

Individual	Estimated age	Age group	Habitat	Sex	Bones studied	Collection
IPS83152	< 3 weeks	Perinatal	Hagenbeck Zoo	-	Fe, Ti, Mc, Mt	Zoological Institute of Hamburg University (Hamburg, Germany)
IPS83153	0.5 years	Foal	Hagenbeck Zoo	M	Fe, Ti, Mc, Mt	Zoological Institute of Hamburg University (Hamburg, Germany)
IPS83154	0.5 years	Foal	Hagenbeck Zoo	M	Fe, Ti, Mc	Zoological Institute of Hamburg University (Hamburg, Germany)
IPS83149	1 year	Yearling	Hagenbeck Zoo	-	Fe, Ti, Mc, Mt	Zoological Institute of Hamburg University (Hamburg, Germany)
IPS83150	1 year	Yearling	Hagenbeck Zoo	-	Fe, Ti, Mc, Mt	Zoological Institute of Hamburg University (Hamburg, Germany)
IPS83151	1 year	Yearling	Hagenbeck Zoo	-	Fe, Ti, Mc, Mt	Zoological Institute of Hamburg University (Hamburg, Germany)
IPS83155	2 years	Juvenile	Hagenbeck Zoo	F	Fe, Ti, Mc, Mt	Zoological Institute of Hamburg University (Hamburg, Germany)
IPS83876	4.5 years	Adult	Gobi desert	F	Fe, Ti, Mc, Mt	Museum of Domesticated Animals (Halle, Germany)
IPS83877	8 years	Adult	Gobi desert	M	Fe, Ti, Mc, Mt	Museum of Domesticated Animals (Halle, Germany)

1

Table 2(on next page)

Number of bone growth marks (BGMs) identified in each cross-section.

M: male; F: female; FLC: number of BGMs identified within the fibrolamellar complex; EFS: number of BGMs identified within the external fundamental system. Asterisk (*) indicates that the most internal BGM has been considered as a non-cyclical BGM.

Individual	Estimated age	Age group	Sex	Femur			Tibia			Metacarpus			Metatarsus		
				FLC	EFS	Total	FLC	EFS	Total	FLC	EFS	Total	FLC	EFS	Total
IPS83152	< 3 weeks	Perinatal	-	0	-	**0**	0	-	**0**	0	-	**0**	0	-	**0**
IPS83153	0.5 years	Foal	M	0	-	**0**	1	-	**1***	1	-	**1***	1	-	**1***
IPS83154	0.5 years	Foal	M	0	-	**0**	1	-	**1***	1	-	**1***	-	-	**-**
IPS83149	1 year	Yearling	-	2	-	**2***	2	-	**2***	2	-	**2***	2	-	**2***
IPS83150	1 year	Yearling	-	2	-	**2***	2	-	**2***	2	-	**2***	2	-	**2***
IPS83151	1 year	Yearling	-	1	-	**1***	1	-	**1***	1	-	**1***	1	-	**1***
IPS83155	2 years	Juvenile	F	1	-	**1**	2	-	**2***	1	-	**1**	2	-	**2***
IPS83876	4.5 years	Adult	F	4	1	**5**	4	-	**4**	3	2	**5***	3	2	**5***
IPS83877	8 years	Adult	M	4	2	**6**	5	-	**5**	4	2	**6***	4	2	**6***

1

Table 3(on next page)

Age of deposition of the external fundamental system (EFS) in the limb bones of adult kulans and time of several biological traits in equids obtained from the literature.

Age of epiphyseal fusion (Silver, 1963) is indicated for the closely related species *Equus caballus* while age at sexual maturity (Nowak, 1999) and age at first reproduction (Kaczensky et al., 2015) is reported for *Equus hemionus*. All data are expressed in years. F: femur; T: tibia; Mc: metacarpus; Mt: metatarsus.

	EFS				Epiphyseal fusion				Sexual maturity	Age at first reproduction
	F	T	Mc	Mt	F	T	Mc	Mt		
Female	4	-	3	3	3-3,5	3-3,5	1,25-1,5	1,3-1,6	2	3
Male	6	-	4	4	3-3,5	3-3,5	1,25-1,5	1,3-1,6	3	5

1

Figure 1

Tibial bone histology of the Asiatic wild ass.

A) Detail of the lateral cortex of the foal IPS83153, showing two areas that differ in the proportions of the parallel-fibered component (PFB) of the bone matrix. B) Haversian systems in the anterior cortex of the yearling IPS83150. C) Anterior cortex of the newborn individual (IPS83152) with a high proportion of parallel-fibered component (PFB) in its bone matrix. D) Packages of lamellar bone within the fibrolamellar complex in the anterior cortex of the wild male (IPS83877). PFB: parallel-fibered bone; LB: lamellar bone. Scale bars: 1 millimeter.

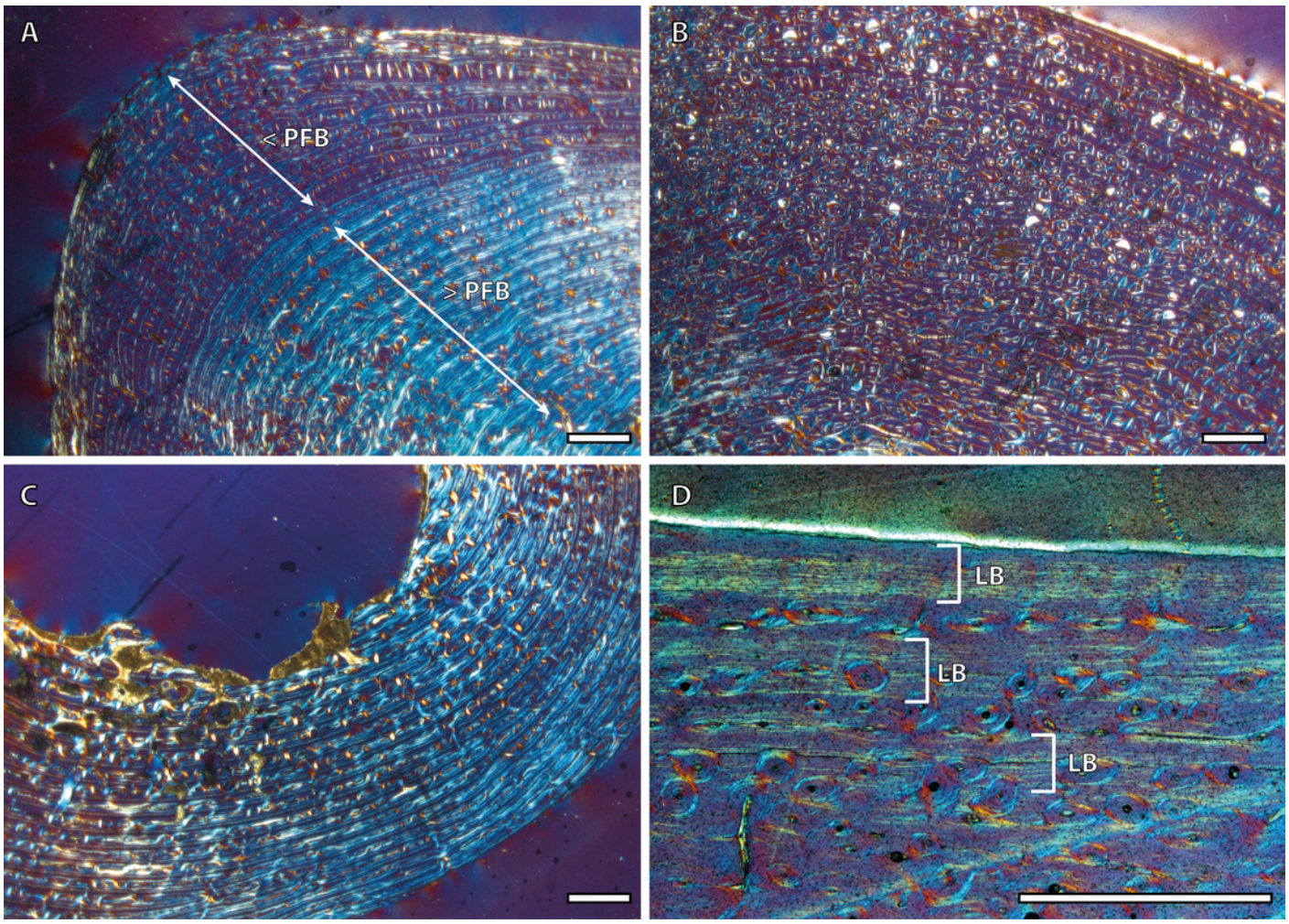

Figure 2

Metapodial bone histology of the Asiatic wild ass.

A) Anterior metatarsal cortex of the yearling IPS83149, showing a fibrolamellar complex with primary osteons oriented in circular rows. B) Radial canals in the metacarpus of the yearling IPS83150. C) Circular canals in the metatarsus of the foal IPS83153. D) Detail of the external fundamental system in the metatarsus of the wild female IPS83876. EFS: external fundamental system. Scale bars: 1 millimeter.

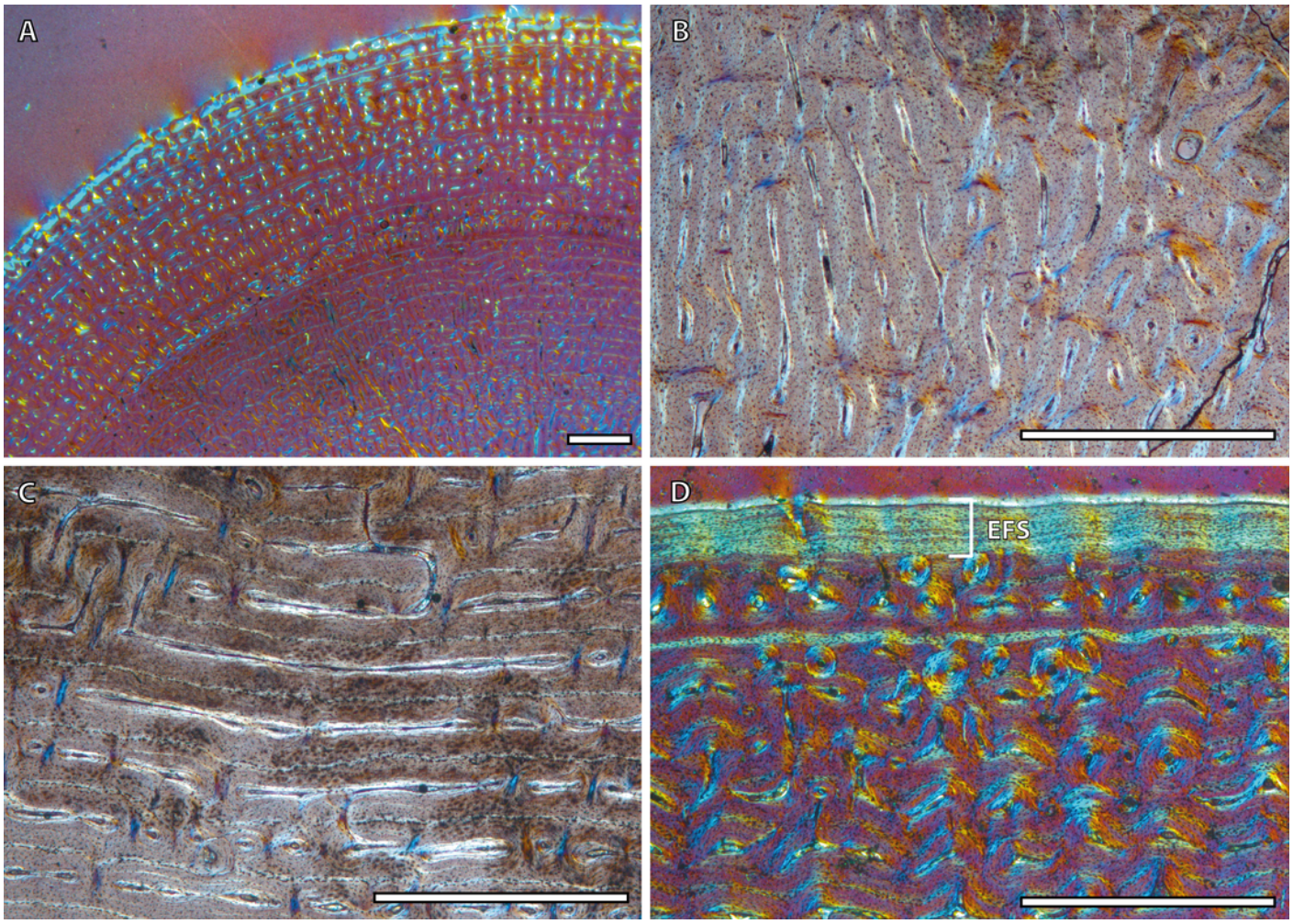

Figure 3

Bone growth marks in foal kulans.

A) BGM in the lateral side of the tibia (IPS83154). B) BGM in the anterior cortex of the metacarpus (IPS83153). C) BGM in the anterior side of the metatarsus (IPS83153). White arrows indicate bone growth marks. Scale bar: 1 millimeter.

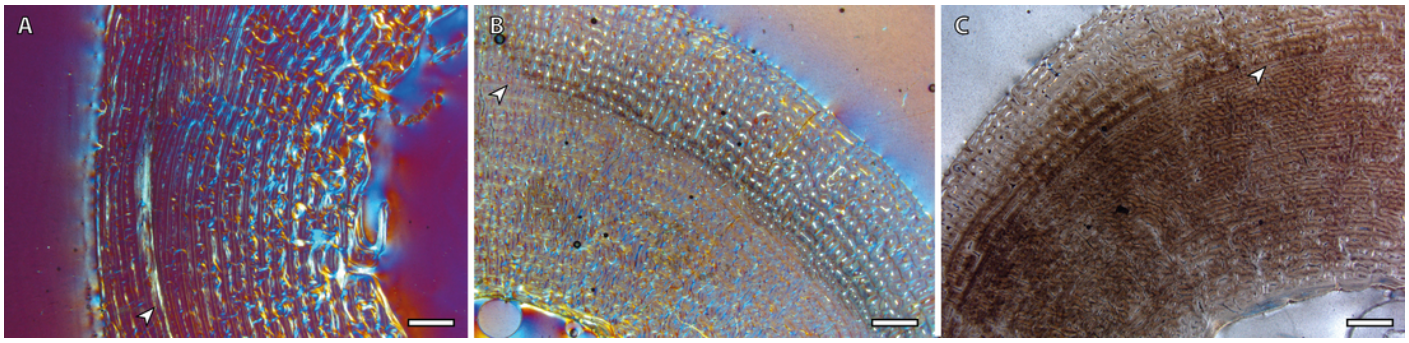

Figure 4

Bone growth marks in yearling kulans.

A) Femoral bone cortex of IPS83151 showing one BGM in its anterior side. B) Tibial bone cortex of IPS83150 showing two BGMs in its lateral side. C) Metacarpal bone cortex of IPS83151 showing one BGM in its lateral side. D) Metatarsal bone cortex of IPS83149 showing two BGMs in its anterior side. White arrows indicate bone growth marks. Scale bar: 1 millimeter.

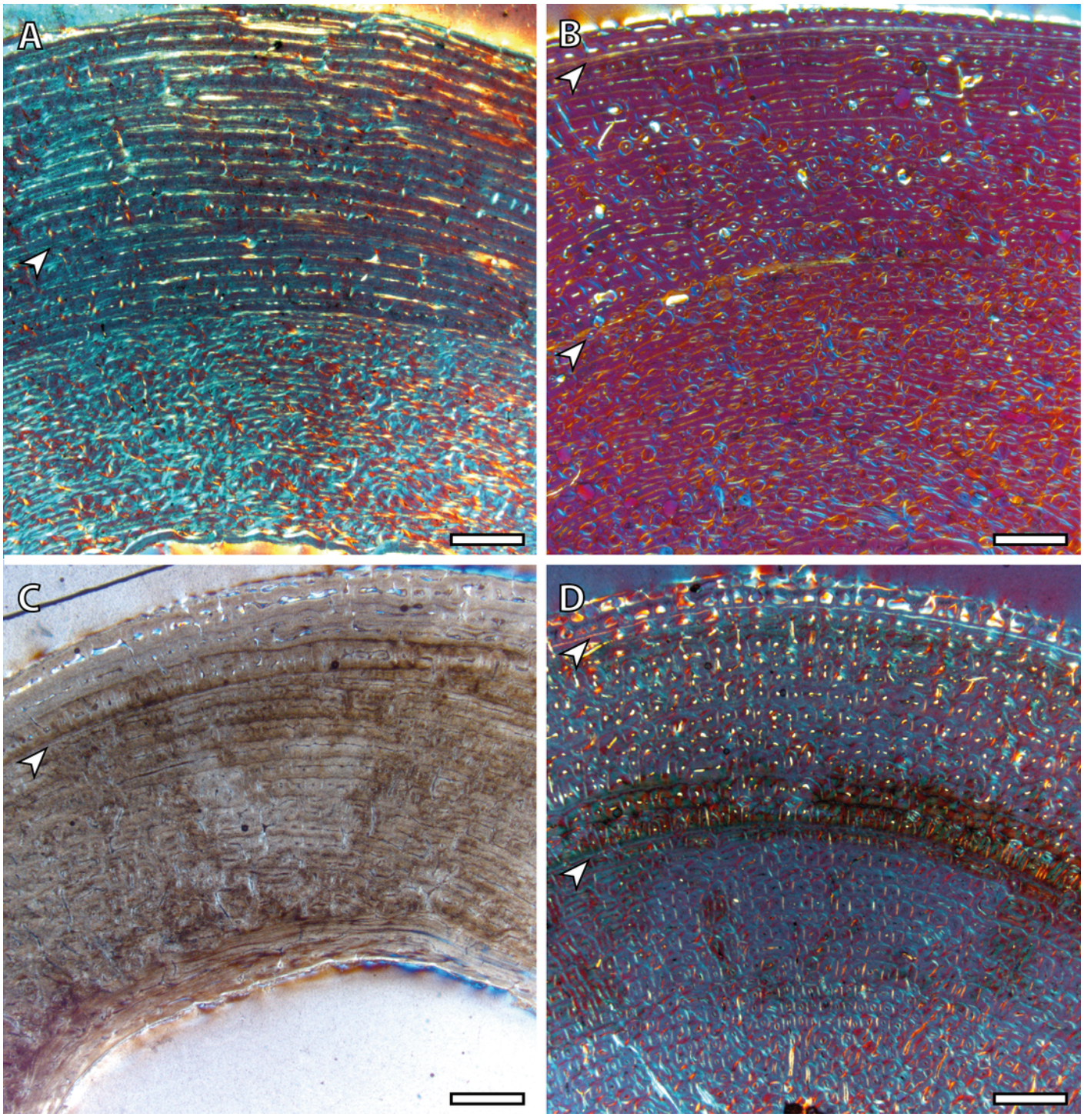
A
B
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Figure 5

Bone growth marks in the juvenile kulan (IPS83155).

A) Femoral bone cortex showing one BGM in its anterior side. B) Tibial bone cortex showing two BGMs in its lateral side. C) Metacarpal bone cortex showing one BGM in its anterior side. D) Metatarsal bone cortex showing two BGMs in its anterior side. White arrows indicate bone growth marks. Scale bar: 1 millimeter.

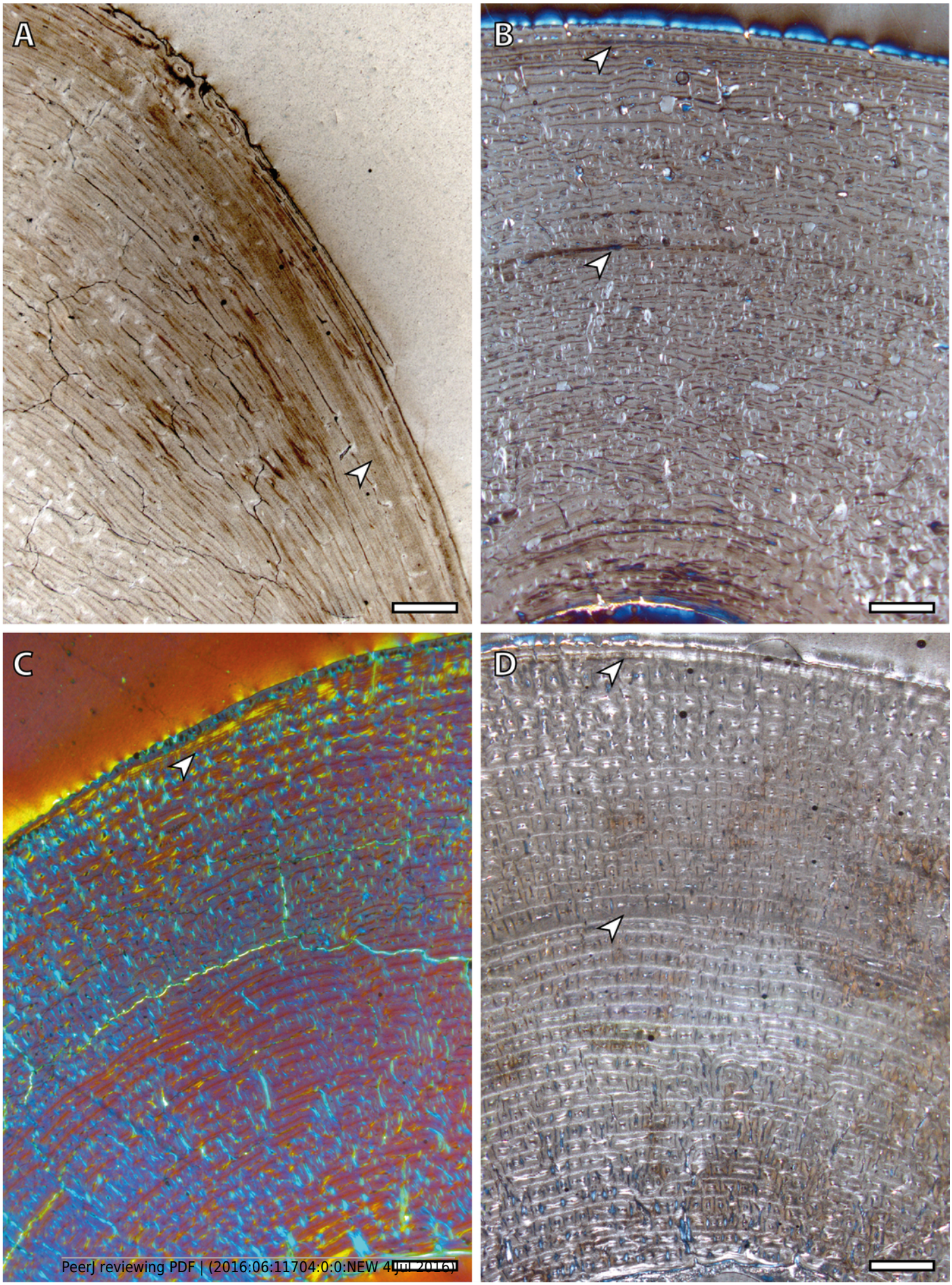
A
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Figure 6

Bone growth marks in adult kulans.

A) Femoral bone cortex of the wild female (IPS83876) showing five BGMs in its anterior side. B) Detail of the most external BGMs identified in the femur of IPS83876. Fifth BGM is located within the external fundamental system. C) Tibial bone cortex of the wild male (IPS83877) showing five BGMs in its lateral side. D) Detail of the most external BGMs identified in the tibia of IPS83877. E) Metacarpal bone cortex of the wild female (IPS83876) showing five BGMs in its anterior side. F) Detail of the most external BGMs identified in the metacarpus of IPS83876. Fourth and fifth BGMs are located within the external fundamental system. G) Metatarsal bone cortex of the wild male (IPS83877) showing six BGMs in its anterior side. H) Detail of the most external BGMs identified in the metacarpus of IPS83877. Fifth and sixth BGMs are located within the external fundamental system. White dashed rectangles indicate areas of image magnifications. White arrows indicate bone growth marks. White scale bar: 1 millimeter; black scale bar: 500 microns.

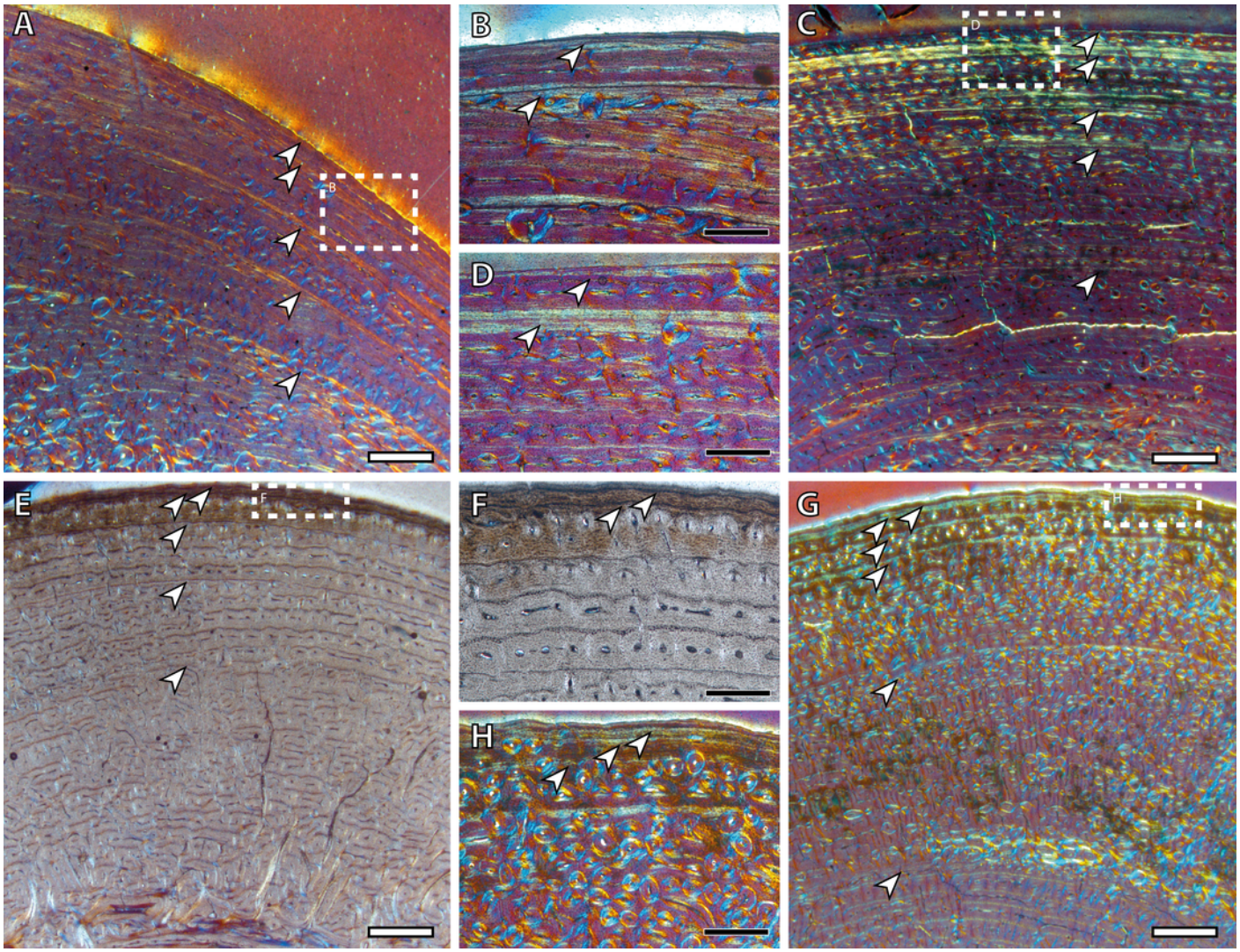
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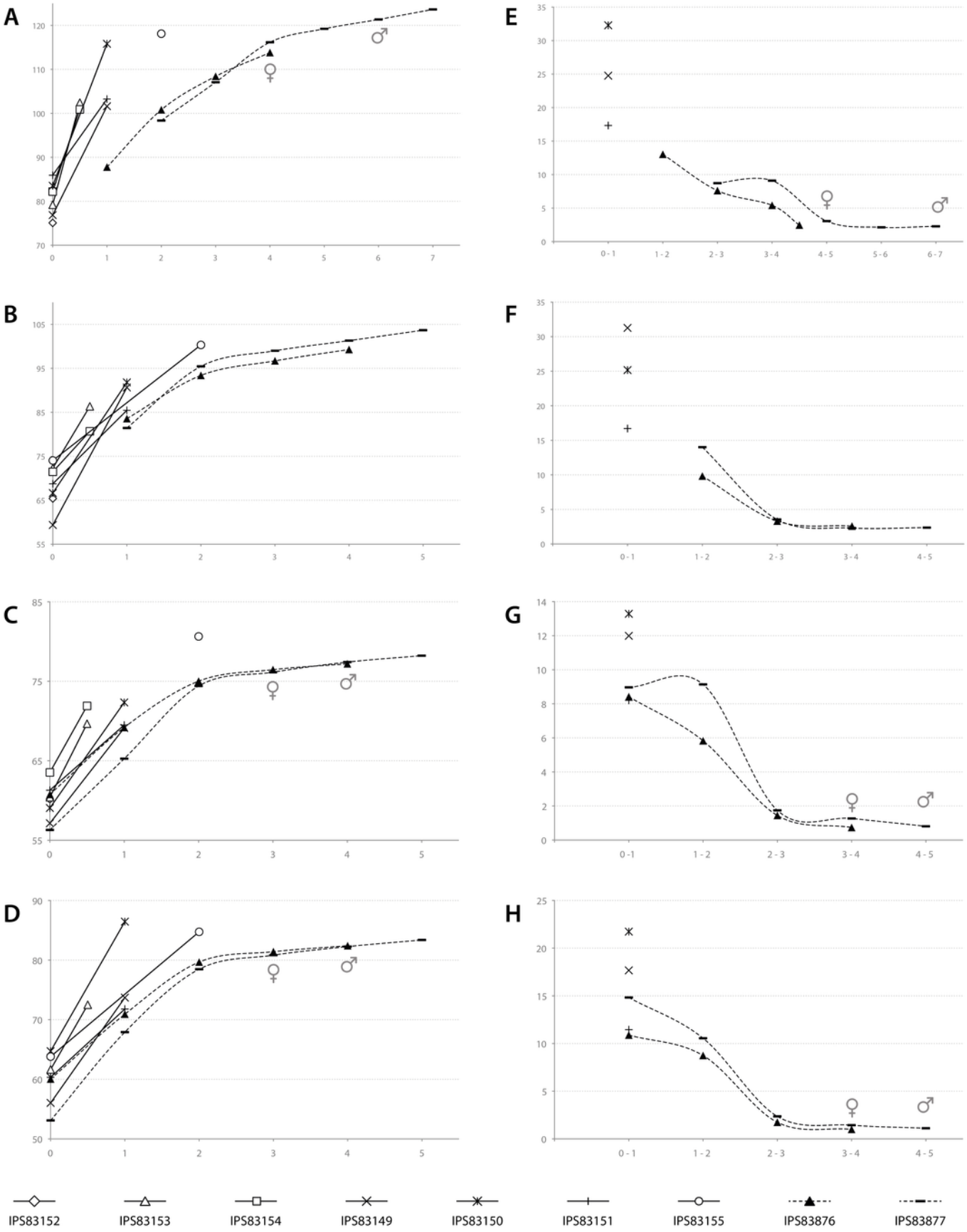
Figure 7

Bone growth of the Asiatic wild ass.

From A to D, bone perimeter (mm, ordinate axis) is plotted against estimated age (years, abscissa axis) to obtain growth curves. From E to F, variation of bone perimeter (mm, ordinate axis) is plotted against estimated age (years, abscissa axis) as a proxy of growth rate. A) Growth curves obtained from the femora. B) Growth curves obtained from the tibiae. C) Growth curves obtained from the metacarpi. D) Growth curves obtained from the metatarsi. E) Femoral growth rate. F) Tibial growth rate. G) Metacarpal growth rate. H) Metatarsal growth rate. Legend is shown in the bottom of the figure. Dashed lines indicate wild animals while continuous lines represent captive ones. Male and female symbols indicate the time of deposition of the external fundamental system (EFS) in each wild adult respectively.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