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First approach to bone histology and skeletochronology of *Equus hemionus*



Première approche de l'histologie osseuse et de la squeletochronologie chez *Equus hemionus*

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ABSTRACT

Histological approaches to extant mammalian life histories (LHs) provide the basis for reconstructing LHs of fossil mammals. They are of special interest in lineages such as perissodactyls that played a key role in fossil ecosystems. We studied an ontogenetic series of *Equus hemionus* (Asiatic wild ass), the most appropriate extant representative of fossil horses. We analyzed growth marks in femora of 10 specimens of different ontogenetic stages and habitats. Bone tissue types and vascular canal orientation vary both during ontogeny and within cross-sections. Skeletochronology generally fits previous age estimates from dental eruption patterns. Our wild adult female attained skeletal maturity at the age of four, the wild male at 5 years. Our results do not only contribute to our knowledge of Asiatic wild ass but they also show that bone histology is a valid tool in reconstructing LH evolution in the horse lineage.

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R É S U M É

L'approche des traits d'histoire de vie (LHs) des mammifères actuels par le biais de l'histologie osseuse fournit une base pour la reconstruction des traits d'histoire de vie (LHs) des mammifères fossiles. Cette étude est d'un intérêt particulier pour des lignées comme les Périssodactyles qui ont joué un rôle clé dans les écosystèmes du passé. Nous avons étudié une série ontogénétique d'*Equus hemionus* (l'âne sauvage d'Asie), le taxon actuel le plus représentatif des chevaux fossiles. Nous avons analysé les marques de croissance dans les fémurs de 10 spécimens parvenus à différents stades ontogénétiques et inféodés à différents habitats. Les types de tissu osseux et l'orientation des canaux vasculaires varient au cours de l'ontogenèse et à l'intérieur des sections. Les résultats de la squeletochronologie correspondent généralement aux estimations de l'âge, basées sur les modalités de l'éruption dentaire. Notre femelle adulte sauvage a atteint la maturité squelettique à l'âge de quatre

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ans, le mâle sauvage à cinq ans. Nos résultats ne contribuent pas seulement à la connaissance de l'âne sauvage d'Asie, ils montrent aussi que l'histologie osseuse est un outil valable pour l'étude de l'évolution des traits d'histoire de vie dans la lignée équine.

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1. Introduction

The histological analysis of bones and teeth has been shown to be an excellent approach when studying an animal's pace of life. Due to their characteristic growth, these structures record some of the most important life history traits of animals such as longevity, growth rate or age at maturity (Bromage et al., 2009; Castanet, 2006; Chinsamy-Turan, 2005; Klevezal, 1996). According to life history theory, these traits are modulated by environmental conditions, specifically by extrinsic mortality and resource availability (Brown and Sibly, 2006; Ricklefs, 2007; Stearns, 1992). For that reason, hard tissue histology provides not only information about a species' ecology, diversity, or vulnerability (Ricklefs, 2007; Stearns, 1992) but also about evolutionary trends (Jordana and Köhler, 2011; Köhler, 2010; Köhler and Moyà-Solà, 2009).

Traditionally, histological studies of bones have been developed to identify growth patterns in ectotherm animals because their cyclical growth leaves characteristic marks (cyclical growth marks [CGMs]) in their calcified tissue (Castanet et al., 1993; Chinsamy-Turan, 2005); however, these approaches are scarce in the study of endotherms since these are supposed to grow constantly. Recent studies (Castanet, 2006; García-Martínez et al., 2011; Köhler et al., 2012; Marín-Moratalla et al., 2013; Martínez-Maza et al., 2014), however, have shown that CGMs are not only present in many groups of mammals, but also that these structures are related to the physiology of these animals and record cyclical periods of growth (Köhler et al., 2012). These results confirm that skeletochronology, i.e. the study of CGMs in cortical bone, can provide valuable information about the life history strategy of extant and extinct mammals (Castanet, 2006), allowing inferences about longevity (García-Martínez et al., 2011), age at sexual maturity (Marín-Moratalla et al., 2013) or growth rate (de Margerie et al., 2002).

Although the number of histological studies aimed to reconstruct life history traits in many mammalian lineages has increased during the past years, equids are still poorly studied. Not only because horses are a classical group in paleontological research (MacFadden, 2005) but also because the delicate conservation status of many of the extant species of the genus *Equus* (Steiner and Ryder, 2011), the histological analysis of bones in this mammalian group is a potentially very important source of information to understand the evolution of their LHs and is relevant to various scientific fields such as Paleontology and Conservation Biology. Much previous work has been done on bone histology in extant horses aimed to differentiate either among horse breeds (Stover et al., 1992) or among species from archaeological sites (Cuijpers and Lauwerier, 2008). Additionally, some researches have focused on the histology of extinct horses (Enlow and Brown, 1958; Sander and

Table 1

Main life history traits of *Equus hemionus* (Tacutu et al., 2013).

Tableau 1

Les principaux traits d'histoire de vie d'*Equus hemionus* (Tacutu et al., 2013).

Traits	Data
Longevity	Up to 26.2 years (captivity)
Adult weight	200–260 kg
Sexual maturity	3–4 years ♀; 3–4 years ♂
Gestation	11 months
Weaning	1–1.5 years
Litter size	1 foal
Breeding	April–October

Andrássy, 2006) but only the recent research by Martínez-Maza et al., 2014 on the species *Hipparion concudense* proposed a life history perspective.

In the present study, we analyze bone histology and apply skeletochronology in the Asiatic wild ass or kulan (*Equus hemionus* Pallas, 1775). The primary objective of this work is to reconstruct the histological development of bones in an ontogenetic series and correlate periodic and other growth marks with age and certain LH events. The information obtained in this study could be useful not only for the conservation policy of the endangered Asiatic wild ass, but it also provides a basis for future research on the evolution of life histories of fossil horses in their environmental context.

The Asiatic wild ass, one of the eight extant species of the family Equidae (Oakenfull et al., 2000; Steiner and Ryder, 2011), is classified as endangered by IUCN (Moehlman et al., 2008) because of the important decline of its population due to illegal hunting and habitat loss, degradation, and fragmentation (Clark et al., 2006). It is endemic to steppe and desert plains of Iran, Turmekistan, India and China (Feh et al., 2001; Reading et al., 2001) and has been reintroduced in several countries of Asia (Kazakhstan, Uzbekistan, Ukraine, Israel and Saudi Arabia; see Moehlman et al., 2008). Although its diet has not been thoroughly studied, most observations suggest that it is a habitual grazer but tends to browse during the dry season (Feh et al., 2002), when it is forced to travel long distances to find resources (Nowak, 1999). Its main life history traits are provided in Table 1.

2. Material and methods

The sample comprises a total of 10 femurs of *E. hemionus* of different ontogenetic stages and habitats (Table 2). Sex data are known for several specimens and were kindly provided by curators. The captive individuals (IPS83148a–IPS83155a) were born in the Hagenbeck Zoo

Table 2

Samples studied and measurements taken on the different femora analyzed.

Tableau 2

Échantillonnage étudié et mesures des différents fémurs étudiés.

Code	Estimated age	Age group	Sex	Habitat	Collection	LMax	DAP	DT	PER	EF
IPS83148a	–	Newborn	–	Hagenbeck Zoo	Hamburg	188	21.07	17.5	92	N
IPS83149a	1–2 years	Yearling	–	Hagenbeck Zoo	Hamburg	301	37.5	28.95	134	N
IPS83150a	1–2 years	Yearling	–	Hagenbeck Zoo	Hamburg	305	35.43	38.4	147	N
IPS83151a	1–2 years	Yearling	–	Hagenbeck Zoo	Hamburg	287	33.59	27.02	130	N
IPS83152a	< 3 weeks	Newborn	–	Hagenbeck Zoo	Hamburg	151 ^a	24.09	21.79	104	N
IPS83153a	0.5–1 year	Foal	M	Hagenbeck Zoo	Hamburg	272	33.49	28.5	128	N
IPS83154a	0.5–1 year	Foal	M	Hagenbeck Zoo	Hamburg	273	33.69	26.6	127	N
IPS83155a	2 years	Juvenile	F	Hagenbeck Zoo	Hamburg	324	39.19	37.4	153	N
IPS83876a	4.5 years	Adult	F	Gobi desert	Halle	329	39.48	28.46	110	Y
IPS83877a	8 years	Adult	M	Gobi desert	Halle	331	35.05	30.72	115	Y

M: male; F: female; N: epiphyses unfused; Y: epiphyses fused; LMax: maximal length (mm); DAP: anteroposterior diameter at the midshaft (mm); DT: transversal diameter at the midshaft (mm); PER: maximal perimeter at the midshaft (mm).

^a The epiphyses of this individual were not present so the measurement indicates the length of the diaphysis.

(Hamburg, Germany) and are housed at the Zoological Institute of Hamburg University (Hamburg, Germany). Wild exemplars (IPS83876a and IPS83877a) were collected in the Gobi desert during the Mongolian-German Biological Expeditions from 2001 to 2006 after being found killed by poachers (Schöpke et al., 2012) and belong to the Natural History Collections of Martin-Luther-University Halle-Wittenberg (Halle, Germany).

The age at death in captive individuals was estimated following the work of Lkhagvasuren et al. (2013) who established the eruption pattern for *E. hemionus*. The age at death of individual IPS83148a could not be calculated due to the lack of the mandible, although we estimated an age similar to IPS83152a based on different measures (Table 2) and the unfused epiphyses. Age at death of the wild specimens has been kindly provided by the Museum of Domesticated Animals of the Martin-Luther-University Halle-Wittenberg (Halle, Saale, Germany). In both specimens, it was estimated following the eruption pattern of the species and later corroborated by analysis of cementum layers of their teeth (R. Schafberg, pers. comm.). These previous estimations are compared with the number of CGMs identified in the femoral cortex of our individuals.

To study bone histology, thin sections of bone were prepared following the standard protocol of our laboratory (García-Martínez et al., 2011; Marín-Moratalla et al., 2011, 2013). A chunk of approximately 3 cm from the middle of the diaphysis was extracted from each femur (from 1.5 cm above to 1.5 cm below the exact midshaft) and embedded in an epoxy resin (Araldite 2020). This block was cut into two halves with a low speed diamond saw (ISO Met low speed saw, Biometa). The cut surfaces were polished with carborundum powder and fixed to a frosted glass with ultraviolet curing glue (Loctite 358). Once the sample was fixed, it was cut with a diamond saw (Buehler, Petrothin) up to a thickness of 100–120 microns and finely polished again to perfect the slide. Finally, it remained 5 minutes with a special mix of oils (Lamm, 2013) in a vacuum chamber to improve the visualization under the microscope. The

obtained slides were studied under polarized light and circularly polarized light with a 1/4λ filter under a Leica DM 2500P microscope.

For each specimen, we described the different bone tissue types following the classification of Francillon-Vieillot et al., 1990 and de Margerie et al., 2002. Bone tissue is classified as primary when it is the first tissue laid down, or secondary when it is deposited after resorption of primary tissue (Currey, 2002). Regarding the disposition of the collagen fibres within the bone matrix and its vascularization, primary bone tissue is classified under different typologies that, amongst others, reflect the growth rate of the animal (de Margerie et al., 2002). In mammals, there is commonly an early deposited fast-growing primary tissue (fibrolamellar complex [FLC] or parallel-fibered bone [PFB]) filling the central part of the cortex and a slow-growing primary tissue (lamellar bone [LB]) later in ontogeny making up the endostium and periostium layers (Martin et al., 1998; Padian and Lamm, 2013). Lamellar bone in the outer cortex (external fundamental system [EFS]) appears when growth rate suddenly decreases because the animal is attaining its adult body size (Chinsamy-Turan, 2005; Huttenlocker et al., 2013). On the other hand, the remodeling process forms secondary bone to repair the bone tissue damaged by biomechanical stresses or to change the shape of the bone in response to changing biomechanical strains and stresses during ontogeny (Currey, 2002). Secondary bone appears as Harvesian systems (or secondary osteons) within the central cortex, or as endosteal bone (EB) surrounding the medullary cavity (Chinsamy-Turan, 2005).

To estimate the different life history traits from the femur bone histology of our *E. hemionus* individuals, CGMs (both annuli and LAGs) were traced along the cortex of each individual and retrocalculation by superimposition (Woodward et al., 2013) was performed when the growth record was incomplete. The age at maturity was estimated by counting the number of CGMs before the beginning of the EFS, while the age at death was calculated from the total number of CGMs across the whole cortex (Castanet et al.,

2004; Chinsamy and Valenzuela, 2008; Marín-Moratalla et al., 2013). Although Horner et al., 1999 indicated that LAGs must be identified on the whole cortex, sometimes it was difficult to do it because of the presence of secondary osteons that eroded the primary bone. Also, the higher proportion of lamellar bone (parallel-fibered bone, following Prondvai et al., 2014) within the FLC of these animals (Mori et al., 2003) complicated the identification of CGMs on the femoral cortices. Besides, caution is necessary with closely spaced or double CGMs, usually LAGs, which might represent only 1 year or event. Therefore, we considered double LAGs or LAGs that split to belong to the same annual cycle and counted them as only one CGM. Growth curves were reconstructed from our sample plotting the accumulative distance of CGMs on those individuals that present more than one of these structures.

3. Results

3.1. Skeletochronology

Assuming a similar age for IPS83148a and IPS83152a because of their femoral dimensions (Table 2), we estimated an age at death of less than 3 weeks for them based on teeth eruption of IPS83152a. At that age, all deciduous incisors and premolars are erupted in *E. hemionus* (Lkhagvasuren et al., 2013). However, the second deciduous incisor (di2) is emerging and the third deciduous incisor (di3) is still inside alveoli in IPS83152a (Fig. 1a). Thus, we conclude that it was less than 3 weeks old and categorized both of them as newborn. In the case of IPS83153a and IPS83154a, all deciduous premolars are emerged as well as the first and second deciduous incisors (Fig. 1b). Nonetheless, the third deciduous incisor is still emerging (Fig. 1b), indicating an age at death between 6 months and 1 year. This stage corresponds to the foal stage. As might be expected, there are no CGMs in the femoral cortex of newborn individuals (IPS83148a and IPS83152a) and foals (IPS83153a and IPS83154a), as they did not survive the first year of life.

In the yearling mandibles (IPS83149a, IPS83150a and IPS83151a) deciduous incisors, deciduous premolars and first molar (M1) are emerged, indicating an age at death between 1 and 2 years (Fig. 1c). However, M1 is unworn in IPS83151a while it presents initial wear in IPS83149a and IPS83150a. Regarding bone histology, yearlings present disparity in the number of growth marks in their femoral cortical bone: IPS83149a and IPS83150a show two CGMs within the cortex while IPS83151a shows only one (Fig. 2a–c). The CGM in IPS83151a fits the first one in IPS83150a but its cortical perimeter does not reach the second CGM of IPS83150a. This suggests that IPS83151a had not reached the second year of life. Besides, it was not possible to find the correspondence between the CGMs in IPS83150a and those in IPS83149a, which is also smaller (Table 2). Therefore, we can conclude that in this age group both methodologies to estimate age at death are in agreement.

The mandible of the juvenile individual (IPS83155a) shows that deciduous incisors, deciduous premolars and first molar are emerged, whereas second molar is erupting

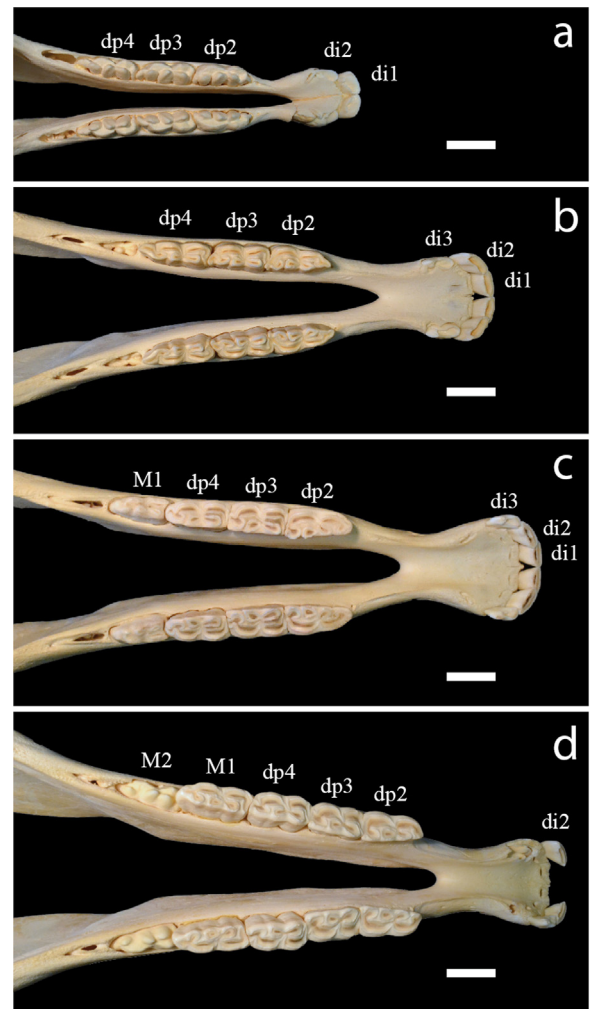


Fig. 1. (Color online.) Mandibles of immature individuals of *Equus hemionus*: a: mandible of newborn IPS83152a; b: mandible of the foal IPS83153a; c: mandible of the yearling IPS83150a; d: Mandible of the juvenile IPS83155a. di1: first deciduous incisor; di2: second deciduous incisor; di3: third deciduous incisor; dp2: second deciduous premolar; dp3: third deciduous premolar; dp4: fourth deciduous premolar; M1: first molar; M2: second molar. Scale bar: 5 millimeters.

Fig. 1. (Couleur en ligne.) Mandibules d'individus immatures de *Equus hemionus*: a: mandibule de l'individu nouveau-né IPS83152a; b: mandibule du poulain IPS83153a; c: mandibule de l'individu IPS83150a; d: mandibule de l'individu juvénile IPS83155a. di1: première incisive primaire; di2: deuxième incisive primaire; di3: troisième incisive primaire; dp2: deuxième prémolaire primaire; dp3: troisième prémolaire primaire; dp4: quatrième prémolaire primaire; M1: première molaire; M2: deuxième molaire. Barre d'échelle: 5 millimètres.

(Fig. 1d). This indicates an age at death around 2 years. Its femoral cortex presents only one CGM (Fig. 2d) that fits, because of its size, the second CGM of IPS83150a. Taking into account this premise, the age estimated from the eruption pattern agrees with skeletochronology. The identification of the CGM was restricted to the lateral side of the cortex and it has not been possible to recognize any other one within its cortex. Moreover, IPS83155a shows the largest diameter and perimeter, which indicates that it grew at a faster rate than the other specimens.

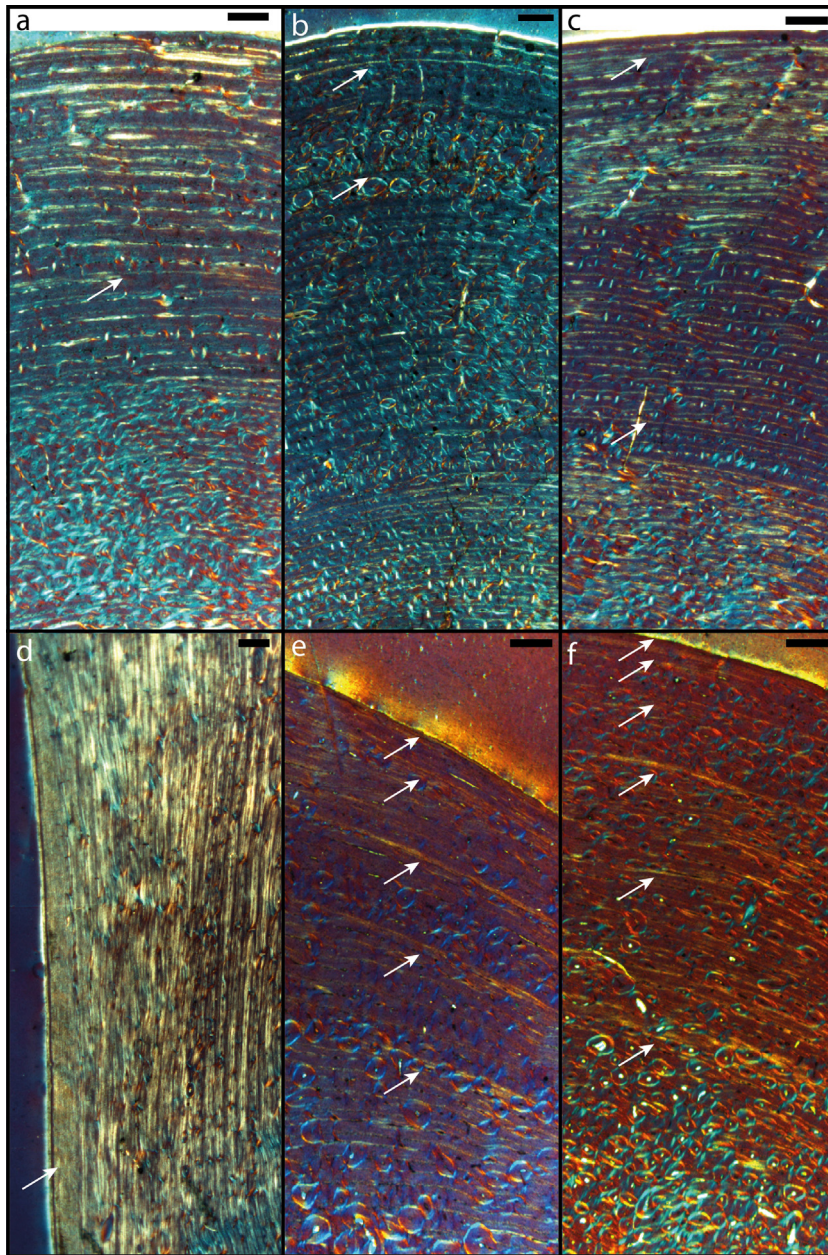


Fig. 2. (Color online.) Cyclical growth marks (CGMs) in the femoral cortex of *Equus hemionus*: a: one CGM in the anterior area of the cortex of the captive yearling IPS83151a; b: two CGMs in the anterior area of the cortex of the captive yearling IPS83149a; c: two CGMs in the anterior area of the cortex of the captive yearling IPS83150a; d: one CGM in the medial area of the cortex of the captive juvenile IPS83155a; e: five CGMs in the anterior area of the cortex of the wild female IPS83876a; f: six CGMs in the anterior area of the cortex of the wild male IPS83877a. White arrows indicate CGM. Scale bar: 500 micrometers.

Fig. 2. (Couleur en ligne.) Marques de croissance cyclique (CGMs) sur le cortex du fémur de *Equus hemionus* : a : une CGM dans la zone antérieure du cortex du yearling captif IPS83151a ; b : deux CGM dans la zone antérieure du cortex du yearling captif IPS83149a ; c : deux CGMs dans la zone antérieure du cortex du yearling captif IPS83150a ; d : une CGM dans la zone médiale du cortex de l'individu juvénile captif IPS83155a ; e : cinq CGMs dans la zone antérieure du cortex de la femelle sauvage IPS83876a ; f : six CGMs dans la zone antérieure du cortex du mâle sauvage IPS83877a. Les flèches blanches indiquent les CGMs. Barre d'échelle : 500 micromètres.

Especially remarkable is the size of the anteroposterior diameter (Table 2) that might be reflecting a directionality of the growth.

The age at death established from the dentition (all permanent teeth erupted and counting of cementum layers) of the wild female (IPS831876a) was about 4 and a half years.

Four CGMs are present in the FLC and one within the EFS, totalling five CGMs in the femur cortex (Fig. 2e). Thus, we can consider that both methodologies agree in that case. The presence of the EFS after the fourth growth mark indicates that this individual attained skeletal maturity at the age of four.

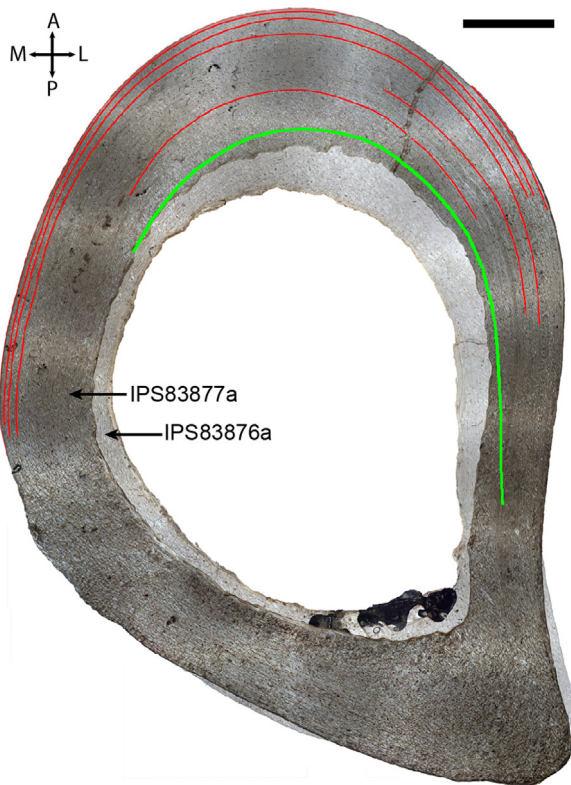


Fig. 3. (Color online.) Retrocalculation of the skeletal age from femoral cross-sections of wild adult individuals. The superimposition of the wild female (IPS83876a) and the wild male (IPS83877a) shows that first cyclical growth mark (green) of IPS83877a has been eroded by secondary osteons. A: anterior; L: lateral; M: medial; P: posterior. Scale bar: 5 millimeters.
Fig. 3. (Couleur en ligne.) Rétrocalcul de l'âge squelettique sur les cortex des fémurs d'individus sauvages adultes. La superposition des résultats relatifs à la femelle sauvage (IPS83876a) et au mâle sauvage (IPS83877a) montre que la première marque de croissance cyclique (vert) de IPS83877a a été érodée par les ostéons secondaires. A: antérieur; L: latéral; M: médial; P: postérieur. Barre d'échelle: 5 millimètres.

The age previously calculated from the teeth (cementum layers; literature) of the wild male (IPS83877a) is around 8 years. Its femoral cortex shows a total of six CGMs, four in the FLC matrix and two in the EFS (Fig. 2f). However, superposition of the two wild kulans (Fig. 3) indicates that one LAG has been eroded by secondary osteons. Thus, assuming that one growth mark has been lost, this individual attained skeletal maturity during its fifth year of live and was 7 years old when it died. On this individual, the estimated age provided by bone histology agrees fairly well with the one calculated from cementum layers.

Finally, from the information obtained from CGMs present in the cortex, we represent the growth curves of several of the exemplars studied (Fig. 4). The results obtained show a high degree of variability in growth rate between captive individuals: while IPS83149a seems to follow a similar growth rate as wild animals, IPS83150a grows at a higher rate. Besides, although both wild exemplars slow down their growth at the fourth age of live, the wild male (IPS83877a) grows faster than the female (IPS83876a).

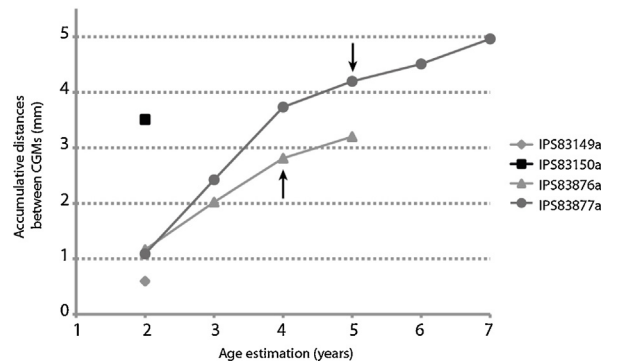


Fig. 4. Growth curves obtained for *Equus hemionus*. The estimated age is plotted against accumulative distances between cyclical growth marks of the femoral cortices of two captive individuals (IPS83150a and IPS83149a), the wild female (IPS83876a) and the wild male (IPS83877a). Black arrows indicate the occurrence of EFS in each adult individual.

Fig. 4. Courbes de croissance obtenues pour *Equus hemionus*. L'âge estimé est tracé en fonction des distances cumulées entre les marques de croissance cyclique des cortex fémoraux de deux individus en captivité (IPS83150a et IPS83149a), la femelle sauvage (IPS83876a) et le mâle sauvage (IPS83877a). Les flèches noires indiquent l'apparition d'EFS dans chaque individu adulte.

3.2. Bone tissue types

Generally, the femoral bone tissue of *E. hemionus* consists of a FLC at early ontogenetic stages that is progressively remodeled by secondary osteons and eroded by the expansion of the medullary cavity. However, the proportion of parallel-fibered (PFB) and woven-fibered (WFB) components in the bone matrix (Prondvai et al., 2014) varies within the same cortex. Also, there is an ontogenetic variation in the orientation of vascular canals.

In both newborn specimens (IPS83148a and IPS83152a), the entire femoral cortex consists of FLC with the same proportion of PFB and WFB components. However, especially in the anterior part of the cortex, the orientation of the vascular canals is different in each individual: while the cortex of IPS83148a (Fig. 5a) is composed only of circular primary osteons (POs) configuring a lamellar bone, the cortical bone of IPS83152a (Fig. 5b) presents a larger proportion of longitudinal POs within the FLC. Both present longitudinal primary vascular canals on the posterior and medial sides of the cortex, areas where tendons and muscles are attached.

The cortices of foals (IPS83153a and IPS83154a) show a characteristic pattern that is also present in following ontogenetic stages. In these individuals, two different types of vascular organization divide the femoral cortex: an early "ring" of FLC with POs in the inner cortex followed by a FLC with circular POs (lamellar bone) (Fig. 5c). The bone matrix of the first ring seems to be less organized (less proportion of PFB component, Fig. 5d) than that of the lamellar bone (Fig. 5e). It is especially remarkable that there is a correspondence between form and dimension of the innermost "ring" of the foals and the whole cortex of IPS83152a (Fig. 6). The lateral side is composed of FLC with a higher proportion of PFB component (Fig. 5f). Isolated secondary osteons (SO) are mainly present on the caudal and medial sides of the cortex.

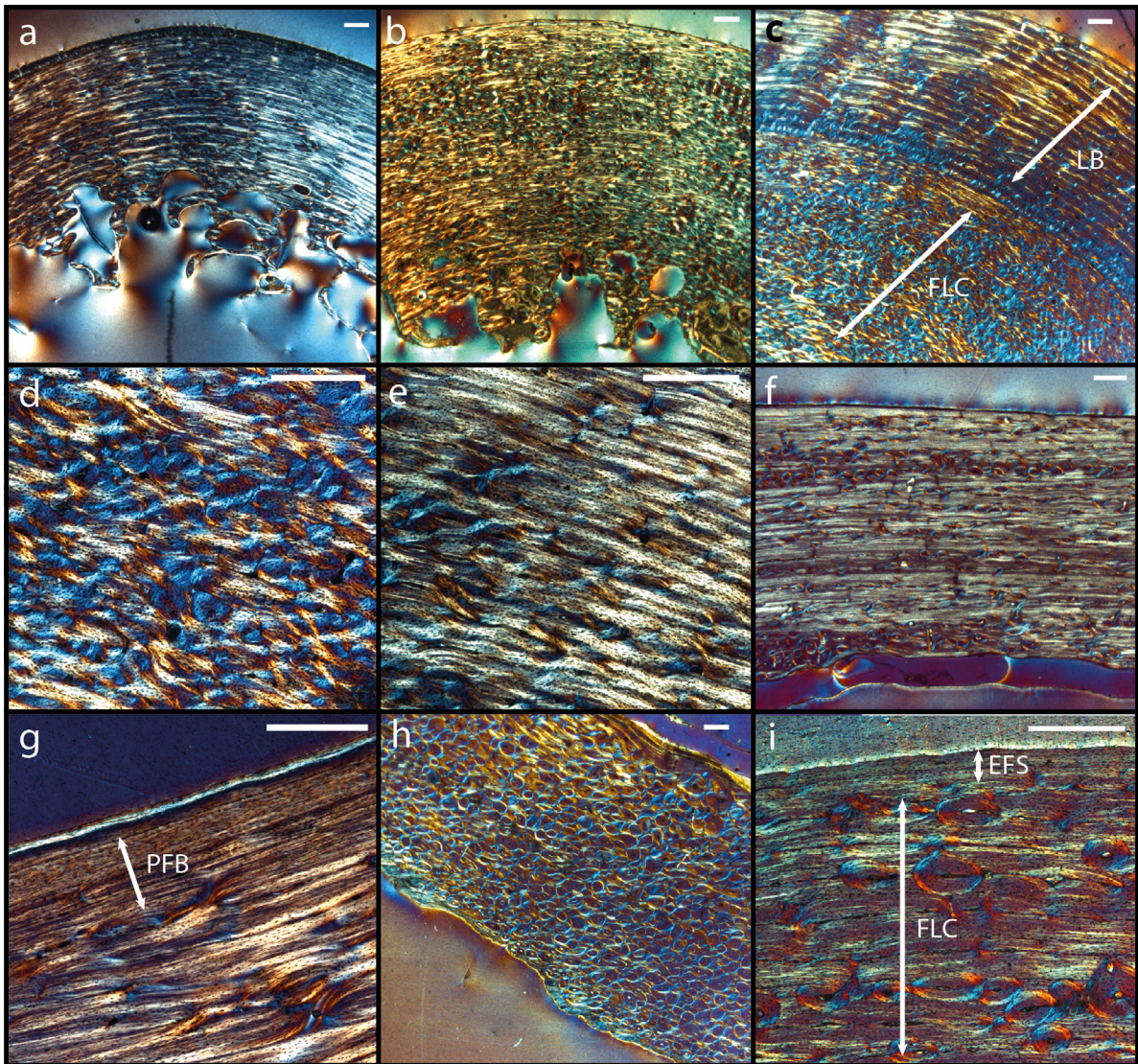


Fig. 5. (Color online.) Bone tissue types in the ontogenetic series of *Equus hemionus*: a: laminar bone in IPS83148a; b: disorganized fibrolamellar complex in the IPS83152a; c: transition between the disorganized FLC and laminar bone in the foal IPS83153a; d: bone matrix of FLC with a high proportion of parallel-fibered bone (PFB) and circular primary osteons in the foal IPS83153a; e: bone matrix of FLC with a high proportion of woven-fibered bone (WFB) in the foal IPS83153a; f: highly-organised fibrolamellar complex on the lateral side of the femoral cortex of the wild male IPS83877a; g: parallel-fibered bone on the lateral side of the juvenile IPS83155a; h: high remodeled area in wild female IPS83876a; i: external fundamental system in wild female IPS83876a. EFS: external fundamental system; FLC: fibrolamellar complex; LB: laminar bone; PFB: parallel-fibered bone. Scale bar: 500 micrometers.

Fig. 5. (Couleur en ligne.) Types de tissus osseux dans la série ontogénétique de *Equus hemionus* : a : tissu osseux laminaire chez l'individu IPS83148a ; b : complexe fibrolamellaire désorganisé chez l'individu IPS83152a ; c : transition entre le FLC désorganisé et l'os laminaire chez le poulain IPS83153a ; d : matrice osseuse du FLC avec une forte proportion de *woven-fibered bone* (WFB) chez le poulain IPS83153a ; e : matrice osseuse du FLC avec une forte proportion de *parallel-fibered bone* (PFB) et canaux vasculaires circulaires chez le poulain IPS83153a ; f : complexe fibrolamellaire très ordonné dans la zone latérale du cortex fémoral du mâle sauvage IPS83877a ; g : tissu osseux à fibres parallèles dans la zone latérale de l'individu juvénile IPS83155a ; h : ostéons de remplacement chez la femelle sauvage IPS83876a ; i : système fondamental externe chez la femelle sauvage IPS83876a. EFS : système fondamental externe ; FLC : complexe fibrolamellaire ; LB : os laminaire ; PFB : os à fibres parallèles. Barre d'échelle : 500 micromètres.

The femoral histology of yearlings (IPS83149a, IPS83150a and IPS83151a) and juvenile individual (IPS83155a) is the same as described for the previous group. Scattered SO are remodeling various areas of the primary cortex, although they are mainly concentrated on the attachment sites (posterior and medial regions). Especially remarkable is the presence of parallel-fibered

bone, instead of FLC, on the lateral side of individual IPS83155a (Fig. 5g).

Finally, the femoral cortical bone of adult individuals (IPS83876a and IPS83877a) is highly remodeled by SO (Fig. 5h) although it is still possible to identify some primary bone tissue types. The bone tissue types and the proportion of PFB or WFB components within the FLC matrix of these

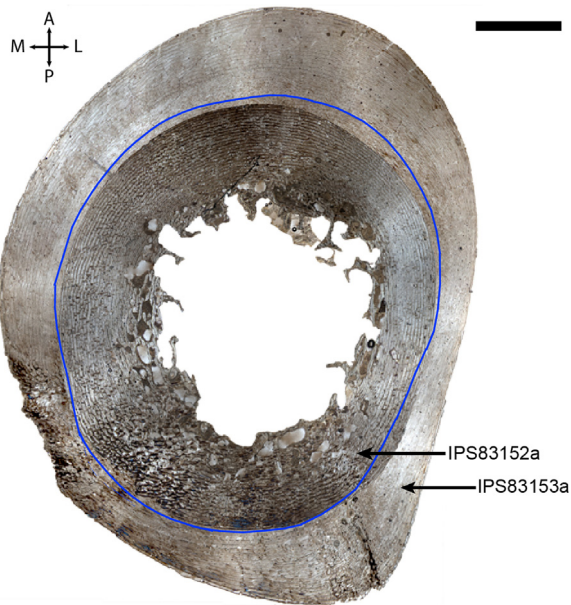


Fig. 6. Bone tissue correspondence between newborn individual (IPS83152a) and foal (IPS83153a). The blue line indicates the border between the fibrolamellar complex (FLC) with longitudinal vascular canals and the FLC with circular canals on the kulan foal. The cortex of IPS83152a matches almost exactly that line. A: anterior; L: lateral; M: medial; P: posterior. Scale bar: 5 millimeters.

Fig. 6. Correspondance du tissu osseux entre l'individu nouveau-né (IPS83152a) et le poulain (IPS83153a). La ligne bleue indique la frontière entre le complexe fibrolamellaire (FLC) et les canaux vasculaires longitudinaux et le FLC avec des canaux circulaires, chez le poulain kulan. Le cortex du fémur de l'individu IPS83152a correspond exactement à cette ligne. A : antérieur ; L : latéral ; M : médial ; P : postérieur. Barre d'échelle : 5 millimètres.

wild animals do not differ from those present in captive individuals. Apart from the laminar bone described for the previous stages, in the external cortex of both adults it is possible to identify a lamellar bone forming an external fundamental system (Fig. 5i).

4. Discussion

The main objective of our work was to analyze the bone tissue changes in an ontogenetic series of Asiatic wild ass and to relate CGMs with certain life history traits of the species. Over the last years, several authors have pointed out that the histological study of bones and teeth provides valuable information about the life history strategy of extant and extinct mammals (Jordana and Köhler, 2011; Jordana et al., 2012, 2014; Köhler and Moyà-Solà, 2009; Köhler et al., 2012; Marín-Moratalla et al., 2011, 2013, 2014). Our first results from the histology of the femur of *E. hemionus* support the previous results on mammals and offer a basis for future studies on both extant and extinct equids. Nowadays, most species of the genus *Equus* are classified by IUCN on different degrees of risk of extinction and the development of new conservation strategies is urgently needed. Therefore, the understanding of key life history traits of the species is essential to improve conservation policies. Previous studies have shown the high potential

of hard tissue analyses for this challenge (Chinsamy and Valenzuela, 2008; Marín-Moratalla et al., 2013). Moreover, it has been shown that life history theory offers a way to reconstruct past environments (Köhler, 2010; Palkovacs, 2003; Raia and Meiri, 2006) and to interpret evolutionary trends (Jordana and Köhler, 2011; Köhler, 2010; Marín-Moratalla et al., 2011). Forstén (1992) highlighted that the Asiatic wild ass is morphologically and ecologically similar to the extinct stenoid horses. For that reason, the results obtained in this work could be also of interest to understand the evolution of the different species of fossil horses.

This work describes for the first time the femoral bone histology of an ontogenetic series of *E. hemionus*. In general, our results agree with the bone histology described for other species of extant and extinct equids (Cuijpers and Lauwerier, 2008; Enlow and Brown, 1958; Martínez-Maza et al., 2014; Sander and Andrassy, 2006; Stover et al., 1992), where the cortex is built of FLC during early stages of growth. However, we have noted that this description does not correspond to all ontogenetic stages and that the primary bone tissue type and its vascularization also vary within the same cortex. Regarding vascularization, changes of vascular canal orientation from a disorganized FLC to a mainly circular arrangement are especially remarkable (Fig. 5c). We hypothesize that this transition, which fits the perimeter of the newborn IPS83152a (Fig. 6), could be related with the moment of birth. The other individual of the same age group (IPS83148a) does not show, however, this disorganized FLC and its entire cortex is constituted of laminar bone. As its mandible is not preserved and we do not have any more information about this exemplar, we cannot propose yet an explanation for the variability of vascular orientation within this age group and why the exemplar IPS83148a does not seem to follow the ontogenetic pattern. At birth, many aspects of the physiology and behaviour of an animal change and could be recorded in its bone histology. The arrangement of bone vascularization has been related to biomechanical factors (de Margerie, 2002; de Margerie et al., 2002, 2004). For instance, de Margerie (2002) suggests that laminar bone is the best bone tissue type to support torsional loads in mallards. Equids, as many other mammals, need to stand up and run almost immediately after birth to avoid predation (Firth, 2006). Although it has been traditionally thought that bending strains are the principal forces involved in the locomotion of terrestrial mammals, Gross et al. (1992) demonstrated that torsional loads are also significant in the limb bones of horses. Thus, the presence of laminar bone within the femoral cortex of the Asiatic wild ass might reflect the onset of locomotor activities in this species. However, further studies should be done to confirm this hypothesis. On the other hand, the disorganized FLC has a higher proportion of WFB component in its matrix than the one with circular canals (Fig. 5d and e), suggesting a deceleration in growth rate. This thesis agrees with the known growth rate of eutherian mammals, which establishes that animals grow very fast during the fetal period and decelerate its growth rate after birth (Peters, 1983). Thus, further studies will be required to understand the causes of that change in vascular canal orientation during this important event in the life history of the Asiatic wild ass.

Our results also show differences in the proportion of PFB and WFB bone component within the FLC matrix in the same femoral cross-section. From foals to adult individuals, all specimens present a more organized FLC (higher proportion of PFB component) on the lateral side of the femoral cortex (Fig. 5f). According to Amprino's Rule, which relates the primary bone tissue type to growth rate, the higher proportion of PFB components within the bone matrix indicates slower rates of bone deposition (Amprino, 1947; Castanet et al., 2000; de Margerie et al., 2002, 2004). Thus, our results show that different areas on a whole cross-section grow at different rate. This fact, termed bone drift, is the result of the changes in size and shape that the diaphyses of long bones experience during growth and development (Chinsamy-Turan, 2005). Moreover, there are also differences in the primary bone tissue types within a femur cross-section, as we identified parallel-fibered bone (PFB) in the juvenile captive IPS83155a (Fig. 5g). The presence of this bone tissue type at that specific side of the femoral cortex might also be due to bone drift. Cuijpers and Lauwerier (2008) previously described PFB in the cortex of extant horses (*Equus caballus*), although the authors do not indicate the exact area where it was deposited and do not go deeper into the causes of its presence.

Several authors indicate that the transition from a fast-growing (FLC) to a slow-growing tissue (EFS) during ontogeny records the attainment of sexual maturity of the species (Marín-Moratalla et al., 2013). Our results show that EFS is only present in adult individuals (IPS83876a and IPS83877a, Fig. 5i). Following this previous work, the wild female would have attained sexual maturity in its fourth year of life while the wild male would have reached sexual maturity one year later, at the age of 5. Previous data (Tacutu et al., 2013) indicate that both sexes attain sexual maturity between the third and fourth year of life, so our results only agree for the wild adult female. However, data about age at first reproduction in *E. hemionus* is scarce and confused (Mohelman et al., 2008; Tacutu et al., 2013) and it seems to be studied in captive individuals only. Nowak (1999) noted that stallions of the subspecies *E. h. kurrh* attained sexual maturity one year later than the females, which agrees with our results. Nonetheless, he reported an age between 2 and 3 years for the females, and between 3 and 4 years for the males. On the other hand, and following life history theory (Stearns, 1992), an important change in growth rate occurs at reproduction because of an energetic trade-off between growth and reproduction. As Fig. 4 shows, the growth rates of both adults decrease at the age of four, possibly indicating the onset of sexual maturity, as previous data have shown (Tacutu et al., 2013). Sander (2000), in his study of sauropod bone histology also suggests that this decrease in growth rate is related to the attainment of sexual maturity. Another possible explanation is that these animals are sexually mature a year before final growth arrest (skeletal maturity, deposition of EFS) and that the deposition of EFS reflects the beginning of reproductive cycles. To solve these discrepancies, future studies on both captive and wild specimens will be necessary to finally conclude if EFS is related to the attainment of sexual maturity on the Asiatic wild ass.

Skeletochronology is a method widely used to assess longevity in many groups of animals (Castanet et al., 1993) although its accuracy has never been tested in extant equids. Our results support that this methodology is applicable to this group as the age obtained by counting CGMs in femoral cross-sections of *E. hemionus* agrees with the ages calculated by tooth eruption pattern. However, both methodologies do not exactly match in certain specimens. Firstly, we could only identify one CGM in the juvenile captive individual aged 2 years (IPS83155a, Fig. 2d). This exemplar, bigger than expected (Table 2), exceeds in size both adult kulans. Hence, its fast growth rate could have prevented the formation of CGMs (Horner et al., 1999). The different growth rate and its associated size (Table 2) between wild and captive *E. hemionus* could be the consequence of their different habitats (Horner et al., 1999; Marín-Moratalla et al., 2013), as it is known that constant care and food supply have effects on the metabolism of zoo animals (Asa, 2010). Generally, our results from the FLC matrix do not show differences between wild and captive specimens. Bromage et al. (2009) found a correspondence between osteocyte density and growth rate, and although this analysis should be very interesting to differentiate between both habitats, it was beyond the objectives of the present work. On the other hand, the results obtained from assessment of age at death from bone histology in both wild adults (IPS83876a and IPS83877a) present minimal differences with the ages provided by other techniques (cementum lines and eruption pattern). This slight difference could be related with the different capability of biological hard tissues to record the age of animals (Klevezal, 1996). Cementum, as a recording structure, is known to provide a good proxy of the age at death in mammals (Klevezal, 1996) and also in equids (Lkhagvasuren et al., 2013). As our results show that bone histology is also a useful tool to study longevity in equids, further studies must be developed to solve these small differences between both histological methodologies.

5. Conclusions

This work describes for the first time the histology of the femoral cortex in an ontogenetic series of extant *E. hemionus*. This first approach to the interpretation of the bone tissue and to the skeletochronology in the Asiatic wild ass has provided encouraging results that could be relevant for the study of life history strategies in extant and extinct equids. Firstly, differences in vascular canal orientation at specific ontogenetic stages seem to be related with key events during the animal's pace of life, such as the moment of birth. The results obtained from skeletochronology support the notion that important life history traits such as longevity or age at sexual maturity can be reliably inferred from histological analyses of bone tissue.

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