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Fetal bone development in the black agouti (*Dasyprocta fuliginosa*) determined by ultrasound

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ABSTRACT

This study describes the bone development during the intrauterine phase of the black agouti (*Dasyprocta fuliginosa*), discussing its relationship with the species' adaptive strategies and comparing it with other precocial and altricial species. We analyzed 33 conceptuses (four embryos and twenty-nine fetuses) obtained through collaboration with local hunters in the Amazon. Mineralization measurements of the axial and appendicular skeletons were performed by ultrasonography using a 10–18 MHz linear transducer. The chronological order of occurrence of mineralization in relation to the total dorsal length (TDL) and to the percentage of the total gestational period (GP) was: skull, ribs, vertebral bodies, clavicle, scapula, humerus, radius, ulna, ilium, ischium, femur, tibia, and fibula (TDL = 8.2 cm, 48 % GP); metacarpi, metatarsi and pubis (TDL = 9 cm, 51 % GP); thoracic and pelvic limb phalanges (TDL = 13.2 cm, 65 % GP); carpus (TDL = 15.10 cm, 72 % GP) and distal row of tarsus (TDL = 19.6 cm, 87 % GP). Mineralization of the patella was not observed in any advanced fetus (fetus with > 80 % GP). Regarding secondary ossification centers, the first signs of mineralization were observed in the distal epiphysis of the radius, distal epiphysis of the femur, and proximal and distal epiphysis of the tibia (TDL = 13.2 cm, 65 % GP). Fetuses at birth (TDL > 21.5 cm, 93.5 % GP) showed mineralization in all primary centers, and in most secondary ossification centers. Black agouti neonates have a high level of precociality with well-developed skeletal system at birth, which promotes independent postnatal locomotion and dexterity to manipulate and forage in search of food. Our results can contribute to the monitoring of bone development in other wild species, providing parameters for the identification of gestational age and serving as a model for comparisons between precocial and altricial mammals, ultimately helping understand life history strategies in different species.

Introduction

In mammals, the pre-natal life involves the period from fecundation to parturition, when fetuses' bones grow in preparation for their post-natal, aerial life [1]. The knowledge of embryological and fetal development processes can help interpret differences and similarities in life-history strategies for the survival of the neonates among mammals. Different degrees of fetal development during pregnancy may produce neonates that are well-developed and relatively independent (precocial) or poorly developed and more dependent on parental care (altricial) [2]. Analyses of the gestational period of precocial and altricial species can offer important insights on the

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adaptive strategies adopted by different species for the survival of their newborns and the perpetuation of the species [3]. However, there is a great lack of information on physiological, biological, and behavioral development processes aspects of wild species, from the gestational period through adulthood.

Researchers have developed *in situ* studies on the fetal development of several Amazonian wild species, including the lowland paca (*Cuniculus paca*) [3,4] red brocket deer (*Mazama americana*) [5], white-lipped peccary (*Tayassu peccary*) [6,7], woolly monkey (*Lagothrix poeppigii*) [8] and collared peccary (*Pecari tajacu*) [7,9], but so far little is known about the intrauterine development in agoutis.

The agouti (*Dasyprocta* spp.) is a taxon of hystricomorph rodents [10] that inhabits Neotropical forests, occurring from the southern portion of Mexico to Central and South America. Agouti species can reach a length of 54–76 cm and a body mass of 3.5–6 kg [11]. They reproduce throughout the year, usually reaching maturity after six months of age [12]. The gestational period for the genus lasts approximately 104 days [13,14], generating up to four neonates per gestation, with an average of 2.5 infants per year [15].

Agoutis are among the main target species of subsistence hunting by Amazonian communities, who harvest the species mostly to obtain meat as a protein source for their diet [15,16]. Some Amazonian communities also trade by-products from hunted agoutis to raise extra income [17]. In parallel, although not in large scale, agoutis are also raised and bred in zoos and other captive facilities, which fosters the possibility of research with these animals, making them experimental models for wild species that are at risk of extinction [12,18].

Ultrasonography is one of the most commonly-employed diagnostic imaging techniques due to its practicality, portability, durability, and low invasiveness, which allows conducting exams even in rural and remote areas [19]. It is currently used for early pregnancy diagnosis [20], monitoring and identification of possible changes during pregnancy [21], assessment of gestational bone development [4,7], and fetal age estimation in many mammals [7,22,23]. Analyzing the gestational bone development gives us evidence of the adaptive strategies adopted during the intrauterine period that may be essential for the survival of neonates, including hystricomorph rodents, such as lowland paca and agouti [4].

The black agouti (*Dasyprocta fuliginosa*) is a species classified as Least Concern on the IUCN Red List [24]. Despite that, this species plays an important ecological role as seed dispersers [25,26], and human factors such as habitat fragmentation [27] and hunting [16,17] can cause severe population declines. Therefore, it is important to develop studies on the black agouti to understand reproductive aspects as well as the habits of these animals. In this study, we conducted ultrasonography in black agouti conceptuses collected using an innovative and collaborative process involving subsistence hunters in the Peruvian and Brazilian Amazon with the aim of evaluating the intrauterine bone development in the species, discussing its implications in terms of the species' life history strategies for the survival of neonates and comparing these results with other altricial and precocial species.

Material and methods

Study sites

The research was conducted in two locations in the Amazon Rainforest. The first site was the Yavarí-Mirín River (YMR, S 04°19.53; W 71°57.33), located in the northeastern Peruvian Amazon, composed mainly of upland forests, and which has a single indigenous community of 307 inhabitants. The second site was the Amanã Sustainable Development Reserve (ASDR, S 01°54.00; W 64°22.00), located in the central Brazilian Amazon, between the Negro and Japurá rivers, with a population of approximately 4000 people distributed into 23 communities and some isolated settlements.

Biological sample collection and processing

The samples were obtained through voluntary donations by hunters between 2007 and 2015. Hunters were trained to remove the viscera of the specimens hunted for subsistence, extract all abdominal and pelvic organs, including the perineal region and store them in gallons containing formaldehyde solution of 4 % (v/v) [15]. Because hunters do not consume these extracted materials, all invasive procedures, and additional mortalities for the purpose of this study were avoided. The research protocol was approved by the Research Ethics Committee for Experimentation in Wildlife at the Dirección General de Flora y Fauna Silvestre from Peru (Licenses 041-2007-INRENA-IFFS-DCB, 108-2008-INRENA-IFFS-DCB, 0127-2010-AG-DGFFS-DGEFFS, 0229-2011-DGFFS-DGEFFS, 0350-2012-AG-DGFFS-DGEFFS; 0249-2013-MINAGRI-DGFFS/DGEFFS), by the Chico Mendes Institute for Biodiversity Conservation from Brazil (License SISBIO no. 29092-1), and by the Committee on Ethics in Research with Animals of the Federal Rural University of the Amazon (UFRA CEUA protocol 008/2016). Samples were sent to UFRA - Belém, Pará, Brazil, using the export license CITES /IBAMA (No. 14BR015991/DF).

Genital organs of the hunted black agouti females were dissected to remove all conceptuses. A total of 33 conceptuses (four embryos and 29 fetuses) were analyzed. The total dorsal length (TDL) of all conceptuses were measured using a measuring tape (0.1 mm precision) from the frontal bone to the tailhead following the contour of the dorsal midline of each conceptus [28]. We removed each conceptus from formaldehyde, and the excess liquid was absorbed with the aid of absorbent wipes. Conceptuses were then weighted using a scale (Anyload Weigh & Measure). The embryonic/fetal stage was determined according to the International Committee on Embryonic Veterinary Nomenclature [29].

Ultrasound examination

The ultrasonographic analyses of the conceptuses' bones were performed using the Esaote® ultrasound equipment, model MyLab™ 30Gold VET (Genoa, Italy), using a 10–18 MHz B-mode linear and multifrequencial electronic transducer. Conceptuses were immersed in water and positioned in a way that the transducer was in contact with the evaluated tissue to detect and measure the mineralized (hyperechoic) and non-mineralized (anechoic) portions of the bones. A video portraying the method of ultrasound examination is presented as Supplementary material.

Ultrasonography has been used for the measurement of fetal bones and ossification centers [30-32]; however, the accuracy of this methodology has yet not been assessed for bone measurements. For the US set-up, we assessed the accuracy of ultrasonography to detect bone mineralization. We conducted the concomitant biometry assessment of the femur through ultrasonography and direct macroscopic measurement after dissection in six individuals from four different species (*Tayassu pecari, Cuniculus paca, Lagothrix poeppigii* and *Alouatta seniculus*) at different ages of the gestational development (TDL ranging from 13 to 32 cm; Fig. S8). The average agreements were 1.81 ± 14.93 [SD] % and 8.93 ± 8.34 [SD] % for the length and width of the mineralized diaphysis, respectively, 8.62 ± 7.79 [SD] % for the total bone length, and 15.06 ± 24.26 [SD] % and 14.67 ± 16.20 [SD] % for the proximal and distal extremities, respectively. Regarding the mineralization of epiphysis, there was 100 % (6/6) accuracy level in diagnosing the absence of mineralization. Ultrasonography was more accurate (100 %, 6/6) to identify the presence of early mineralization through the change of the US echogenicity, whereas macroscopic measurement after dissection detected 66.7% (4/6). Mineralization might be occurring in some locations, but the amount of mineral might not be sufficient to be identified macroscopically after dissection.

Ultrasound examinations were performed according to Silva et al. [4] and Pereira et al. [7]. Bone measurements were conducted in the

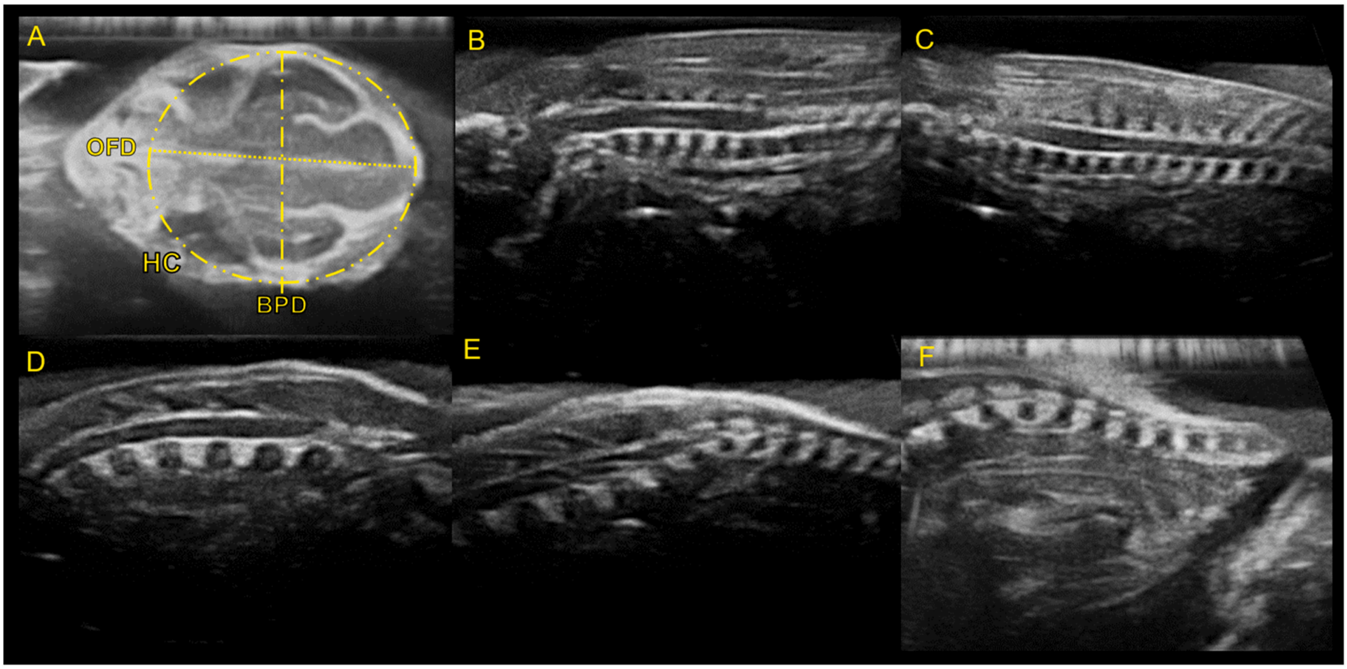

Fig. 1. Ultrasonographic planes used for the examination of the axial skeleton of black agouti (*Dasyprocta fuliginosa*): (A) biparietal diameter (BPD), occipitofrontal diameter (OFD), and head circumference (HC) measurements; (B–F) sagittal fetal spine sections, (B) cervical, (C) thoracic, (D) lumbar, (E) sacral, and (F) coccygeal vertebrae.

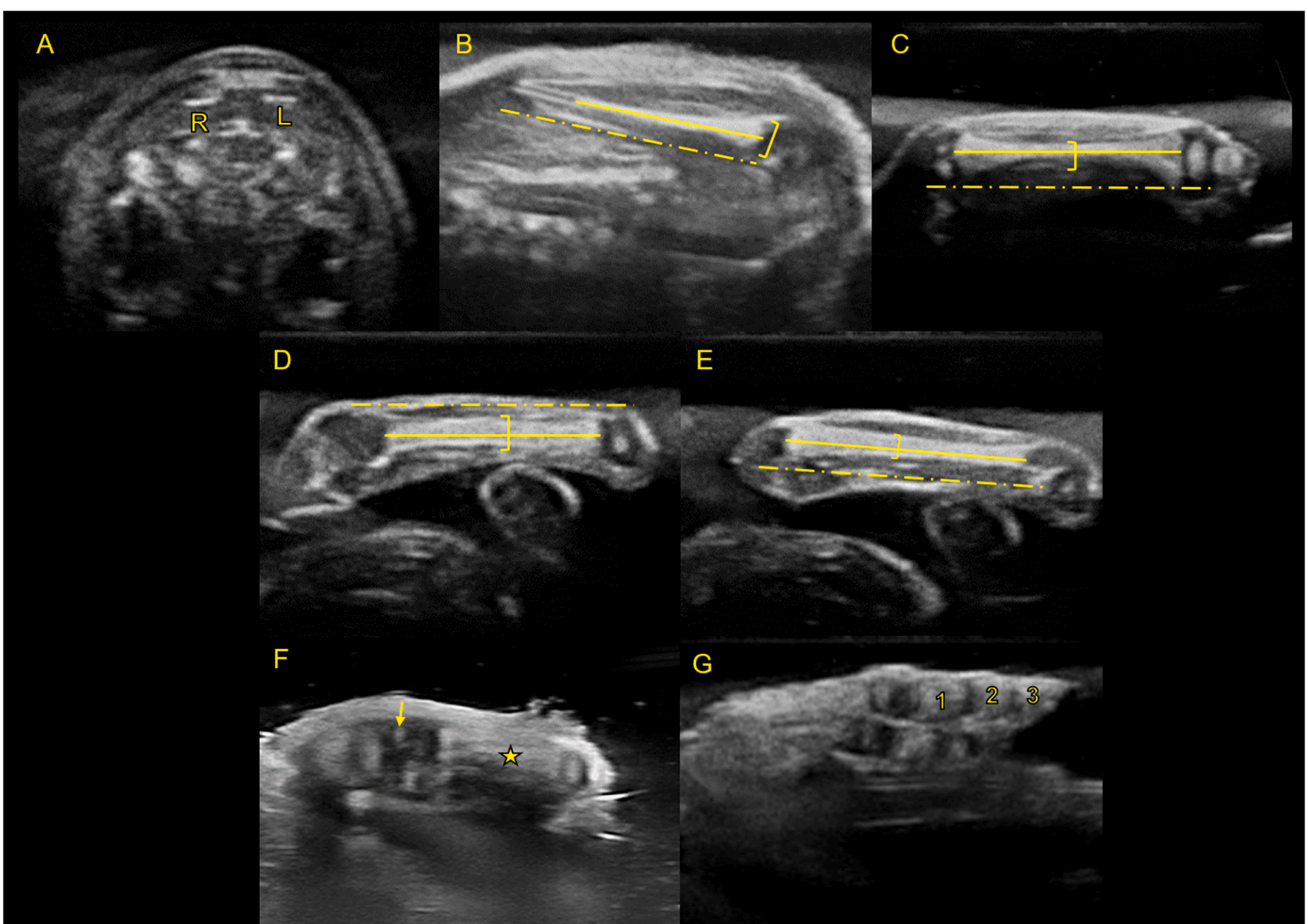

Fig. 2. Ultrasonographic planes used for the examination and measurement of the thoracic limb of black agouti (*Dasyprocta fuliginosa*) fetuses: (A) clavicle (R-right, L-left), (B) scapula, (C) humerus, (D) radius, (E) ulna, (F) metacarpal bone (star), carpal bone (arrow); (G) (1) proximal, (2) middle, and (3) distal phalanges. Ossified portion/diaphysis (solid line), total length (dotted line), width ([]).

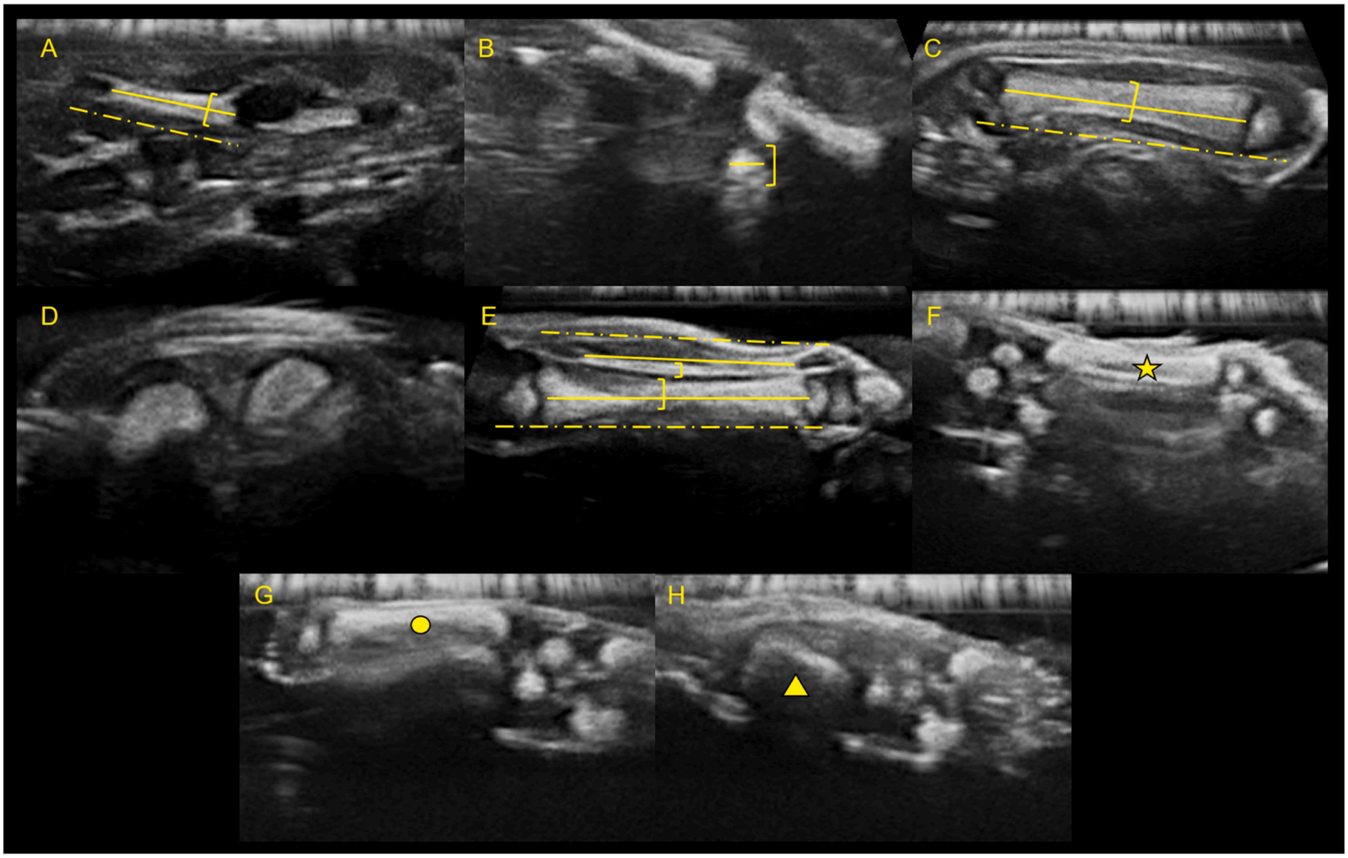

Fig. 3. Ultrasonographic planes used for the examination and measurement of the pelvic limb of black agouti (*Dasyprocta fuliginosa*) fetuses: (A) ilium and ischium, (B) pubis, (C) femur, (D) knee joint (patella not observed), (E) tibia and fibula, (F) metatarsal bone (star), (G) calcaneus (yellow dot), (H) talus (arrowhead). Ossified portion/diaphysis (solid line), total length (dotted line), width ([]).

axial skeleton (Fig. 1) and thoracic (Fig. 2) and pelvic limbs (Fig. 3). For the axial skeleton, the largest transversal diameter between the two parietal bones was considered as the biparietal diameter (BPD), whereas the occipitofrontal diameter (OFD) was measured as the distance perpendicular to the BPD, respecting the limits of the occipital and frontal bones. Head circumference (HC) was obtained in the maximum region at the same position (sagittal projection) by a circumference around the outer margin of the hyperechoic contour at the border of the conceptuses' skulls. The vertebrae, divided into cervical, thoracic, lumbar, sacral and caudal, were examined using longitudinal cuts, and the presence of a mineralized vertebral body was qualitatively assessed (presence vs absence). The ribs were also qualitatively examined for mineralization through longitudinal and transverse planes (presence vs absence).

Appendicular skeleton bones were measured from a proximal-distal perspective (Fig. 2). The clavicle was examined in cross section at the level of the first thoracic vertebra, with the measurement of the longest length of the mineralized part. In relation to the scapula, the total length (including mineralized and non-mineralized parts), the length and the width of the mineralized portion (lateromedial diameter, measured in the distal portion of the bone that is in contact with the scapula-humeral joint) were measured. For the humerus, radius, and ulna, we measured the following: length and width (lateromedial diameter) of the mineralized diaphysis (hyperechoic part); proximal and distal extremities (epiphysis, hypoechoic part); total length (diaphysis and epiphysis) and ossification secondary centers.

Projections were chosen in which the largest areas of the secondary ossification centers were visualized. The length and width of each bone were measured, and subsequently each measurement was recorded. All measurements were performed in triplicate and mean values were calculated. All measurements were done by the same observer.

For the femur, tibia and fibula, we measured the length and width (lateromedial diameter) of the mineralized diaphysis (hyperechoic part); proximal and distal extremities (epiphysis, hypoechoic part); total length (diaphysis and epiphysis) and ossification secondary centers. In the ilium and ischium (Fig. 3), the total length (mineralized and non-mineralized parts), the length of the mineralized parts and the width (near the acetabulum) were measured. The length and width of the mineralized parts of the pubis, metacarpi, metatarsi and phalanges of the thoracic and pelvic limbs were evaluated. The length and width of the talus and calcaneus were measured. The absence or presence of the other carpal and tarsal bones were verified and recorded. To locate the patella, transverse and longitudinal cuts of the knee joint were performed.

Data analysis

The gestational age (GA) was calculated according to the formula $\sqrt[3]{W} = a\,(t - t0)$, described by Huggett and Widdas [28], in which "W" is the fetal weight, "a" is the constant of specific fetal growth, "t" is the gestational age in days and "t0" is the intercept on the time axis, which corresponds to 20 % of the gestational length. The relationship between GA with TDL and body mass of all conceptuses was established through linear regressions. To refer to the main events of bone development, the terms GA (days) and total gestational period (GP) were represented in percentage. Therefore, an estimated delivery date was used for these calculations, considering 104 days of gestational period [14] and a mean weight of 274.5 g at birth, based on the average of male and female weights at birth [33].

Logistic regressions were applied to estimate the probability of the occurrence of mineralization in the studied structures in relation to TDL using Statistica 8.0 software (StatSoft Inc., Tulsa, USA). Multiple

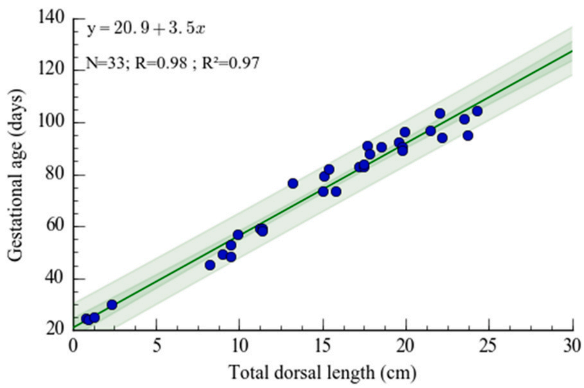

Fig. 4. Relationship between total dorsal length (TDL) and gestational age in 33 conceptuses of black agouti (*Dasyprocta fuliginosa*).

regressions were used to model the relationships between TDL and biometric measures using the software CurveExpert Professional 2.6 (© Copyright 2017, Daniel G. Hyams) to define which functions were best fitted for each measure evaluated. The regressions started on day 0 to represent the beginning of gestational development, and those with the highest coefficients of determination (r^2) were selected.

Results

The mean value of the total dorsal length (TDL) of the 33 conceptuses studied was 14.62 ± 6.78 cm (0.8–24.3 cm), and the mean body mass was 105.10 ± 86.54 g (0.02–278 g). GA and TDL showed a highly positive linear relationship ($r^2 = 0.97$, $P < 0.001$ - Fig. 4). Thus, the formula used to calculate the gestational age was $\sqrt[3]{W} = 0.078\,(t - 20.8)$. The measurements obtained in relation to TDL, conceptus weight, GA and GP are available in Table S1, in Supplementary material.

Axial skeleton

Mineralization of the skull bones (occipital, frontal and parietal), ribs and vertebrae (cervical, thoracic, lumbar, sacral and coccygeal) were first observed in fetuses with TDL ≥ 8.20 cm (50 days of gestation, 48 % of GP), having a high probability (approximately 100 %) of presenting mineralization of the skull bones, vertebrae and ribs (TDL ≥ 8.20 cm). The data regarding the probability curves of occurrence of mineralization in the axial skeleton in relation to the TDL are presented in Fig. 5A and Table 1.

Appendicular skeleton

Fetuses with TDL ≥ 8.2 cm (50 days of gestation, 48 % GP) had a high probability (≥ 99 %) of mineralization in the bones of the thoracic limbs (clavicle, scapula, humerus, radius and ulna), pelvis (ilium and ischium) and pelvic limbs (femur, tibia and fibula). The probability curves for the occurrence of appendicular skeletal bone mineralization relative to TDL are shown in Figs. 5B and 5C.

The mineralization of the pubis, metacarpal and metatarsal bones was first observed in fetuses with TDL ≥ 9 cm (53 days of gestation, 51 % GP), followed by first signs of mineralization in the phalanges (proximal, middle and distal of the thoracic and pelvic limbs) and calcaneus (fetus with TDL ≥ 13.2 cm, 68 days of gestation, 65 % GP), talus (fetus with TDL ≥ 15 cm, 74 days of gestation, 71 % GP), carpus (TDL ≥ 15.10 cm, 75 days of gestation, 72 % GP) and distal row of tarsus (fetus with TDL ≥ 19.6 cm, 90 days of gestation, 87 % GP). The mineralization of patella was not observed in any of the analyzed fetuses.

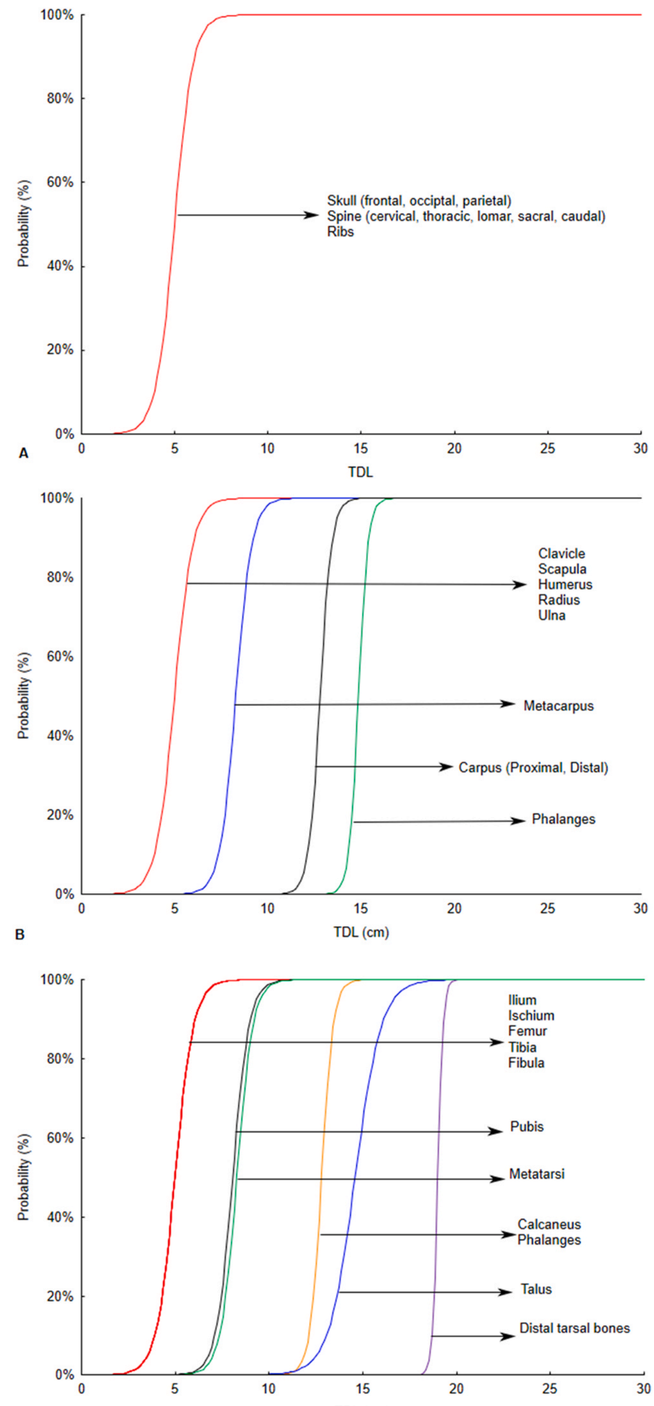

Fig. 5. Probability curves for the occurrence of skeletal bone mineralization in 33 conceptuses of black agouti (*Dasyprocta fuliginosa*) in relation to the total dorsal length (TDL): (A) in the axial skeleton, (B) thoracic limb, and (C) pelvic limb.

Secondary ossification centers

Among the secondary ossification centers, the distal epiphysis of the radius and femur and the proximal and distal epiphysis of the tibia were the first to present mineralization, being observed in fetuses with TDL ≥ 13.2 cm (68 days of gestation, 65 % GP). This was followed by mineralization of distal epiphysis of the humerus, head of the femur and the greater trochanter (femur), observed in fetuses with TDL ≥ 15.40 cm (76 days of gestation, 73 % GP).

Table 1
Logistic equations [y = exp (Intercept + Estimative * x)/(1 + exp (Intercept + Estimative * x)] for the axial and appendicular bones parameters in 33 conceptuses of black agouti (*Dasyprocta fuliginosa*).

Bones	Intercept	Estimate	Chi-square (Df)	P
Axial bones (skull; spine; ribs)				
Skull	-10.0933	2.02485	24.36036 (1)	< 0.001
Cervical	-10.0933	2.02485	24.36036 (1)	< 0.001
Thoracic	-10.0933	2.02485	24.36036 (1)	< 0.001
Lumbar	-10.0933	2.02485	24.36036 (1)	< 0.001
Sacral	-10.0933	2.02485	24.36036 (1)	< 0.001
Caudal	-10.0933	2.02485	24.36036 (1)	< 0.001
Ribs	-10.0933	2.02485	24.36036 (1)	< 0.001
Appendicular bones (thoracic limb)				
Clavicle	-10.0933	2.02485	24.36036 (1)	< 0.001
Scapula	-10.0933	2.02485	24.36036 (1)	< 0.001
Humerus	-10.0933	2.02485	24.36036 (1)	< 0.001
Radius	-10.0933	2.02485	24.36036 (1)	< 0.001
Ulna	-10.0933	2.02485	24.36036 (1)	< 0.001
Proximal carpal bones	-58.4889	3.93808	42.02607 (1)	< 0.001
Distal carpal bones	-58.4889	3.93808	42.02607 (1)	< 0.001
Metacarpi	-19.4691	2.35032	26.25753 (1)	< 0.001
Proximal Phalanx	-41.35490	3.21041	42.72076 (1)	< 0.001
Middle Phalanx	-41.35490	3.21041	42.72076 (1)	< 0.001
Distal Phalanx	-41.35490	3.21041	42.72076 (1)	< 0.001
Appendicular bones (pelvic limb)				
Ilium	-10.0933	2.02485	24.36036 (1)	< 0.001
Ischium	-10.0933	2.02485	24.36036 (1)	< 0.001
Pubis	-18.6099	2.30926	25.29340 (1)	< 0.001
Femur	-10.0933	2.02485	24.36036 (1)	< 0.001
Tibia	-10.0933	2.02485	24.36036 (1)	< 0.001
Fibula	-10.0933	2.02485	24.36036 (1)	< 0.001
Calcaneus	-41.3549	3.23134	42.72076 (1)	< 0.001
Talus	-20.8479	1.42911	38.49698 (1)	< 0.001
Distal tarsal bones	-135.087	7.10934	40.38337 (1)	< 0.001
Metatarsi	-19.46291	2.35032	26.25753 (1)	< 0.001
Proximal phalanx	-41.35490	3.21041	42.72076 (1)	< 0.001
Middle phalanx	-41.35490	3.21041	42.72076 (1)	< 0.001
Distal phalanx	-41.35490	3.21041	42.72076 (1)	< 0.001

The humerus' head and greater tubercle (humerus), proximal epiphysis of the radius as well as the distal epiphysis of the metatarsi presented first signs of mineralization in fetuses with TDL ≥ 17.2 cm (82 days of gestation, 79 % GP). Sequentially, we observed mineralization of tibia's tuberosity (TDL ≥;17.5 cm, 83 days of gestation, 80 % GP); distal epiphysis of the metacarpi and calcaneus' tubercle (TDL ≥ 17.7 cm, 84 days of gestation, 81 % GP); and then in the proximal and distal epiphysis of ulna (TDL ≥ 19.6 cm, 90 days of gestation, 87 % GP).

Fetuses with TDL ≥ 19.8 cm (91 days of gestation, 88 % GP) showed mineralization in the center of the distal epiphysis of the hindlimb proximal phalanx. First signs of mineralization in the fibula's distal epiphysis, secondary centers of proximal and middle phalanges (forelimb) and middle phalanx (hind limb) were first observed in fetuses with TDL ≥ 21.5 cm, (97 days of gestation, 93 % GP). Advanced fetuses with TDL 23.7 cm at birth had a 100 % probability of having mineralization in all abovementioned secondary centers. We did not observe the ossification centers of the iliac crest and the ischial tuberosity. Fig. 6 and Table 2 show the probability curves for the occurrence of mineralization of the secondary ossification centers of the appendicular skeleton.

Allometric relationships

The growth of long bones was not isometric and as such did not show similar development in their proportions (Fig. 7). In fetus with GP < 60 %, the tibia was the bone with the longest diaphyseal length, followed by the humerus, radius, and femur. The tibia remained the longest bone in fetuses at advanced gestational ages, however, the femur showed a more accentuated growth throughout pregnancy, being the second longest bone, followed by the humerus and radius. By evaluating the stylopod of both limbs in advanced fetuses, we observed that the femur presented a slightly longer length than the humerus (ratio femur/humerus of 1.02 ± 0.04 cm).

The long bones of the stylopod and zeugopod (humerus, radius, femur, and tibia) of black agoutis had a greater width/length ratio at the beginning of pregnancy but presented a decrease during fetal development, showing a slightly greater length and reducing their robustness (Fig. 7).

Bone measurements and gestational age

There was a strong association between skull measurements and TDL ($r^2 > 0.94$), with the occipito-frontal diameter and head circumference measurements having the highest determination coefficient ($r^2 = 0.96$, $P < 0.001$). In terms of long bones, the length of the mineralized diaphysis was also strongly associated with TDL ($r^2 > 0.95$, $P < 0.001$). A strong association of mineralized diaphysis length with TDL was observed in the long bones of the thoracic (radius and ulna) and pelvic limbs (tibia and fibula; $r^2 \geq 0.96$, $P < 0.001$). The relationships of the bones of the axial and appendicular skeleton are shown in Figs. S9–S25, in Supplementary material.

Discussion

The assessment of bone growth in different intrauterine development phases contributes to the understanding of the level of precociality of the black agouti in comparison to other wild animals, such as lowland paca (*Cuniculus paca*) [4], collared peccary (*Pecari tajacu*) [7], white-lipped peccary (*Tayassu pecari*) [7], and punare (*Thrichomys laurentinus)* [34], all precocial mammals; or the laboratory mouse [35,36] and marmoset (*Callithrix jacchus*) [37], both altricial species. In

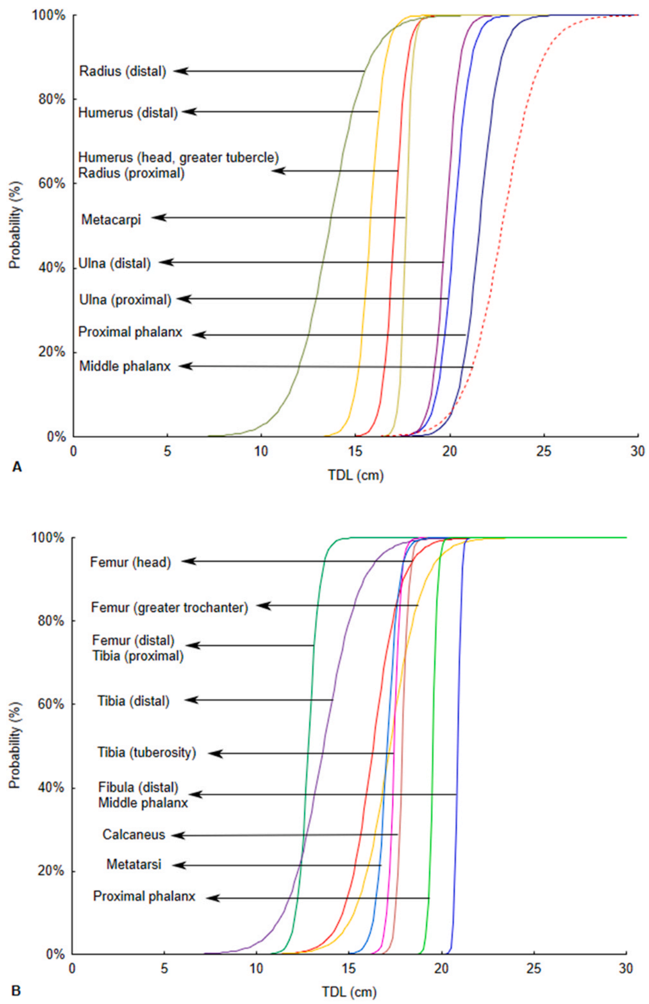

Fig. 6. Probability curves for the occurrence of mineralization in secondary ossification centers in 33 conceptuses of black agouti (*Dasyprocta fuliginosa*) in relation to the total dorsal length (TDL): (A) thoracic limb, and (B) pelvic limb.

Table 2
Logistic equations [y = exp (Intercept + Estimative * x)/(1 + exp (Intercept + Estimative * x)] for the secondary ossification centers in thoracic limb and pelvic limb parameters in 33 conceptuses of black agouti (*Dasyprocta fuliginosa*).

Bones	Intercept	Estimate	Chi-square (Df)	P
Ossification Center (thoracic limb)				
Humerus (head)	-53.161	3.11545	40.303(1)	< 0.001
Humerus (distal)	-41.440	2.62752	40.984(1)	< 0.001
Humerus (great tubercle)	-53.161	3.11545	40.303(1)	< 0.001
Radius (proximal)	-53.161	3.11545	40.303(1)	< 0.001
Radius (distal)	-13.613	0.99975	35.698(1)	< 0.001
Ulna (proximal)	-46.974	2.32225	28.531(1)	< 0.001
Ulna (distal)	-54.857	2.77259	30.538(1)	< 0.001
Metacarpi	-100.408	5.68145	40.799(1)	< 0.001
Proximal Phalanx	-39.987	1.85299	22.524(1)	< 0.001
Middle Phalanx	-23.024	1.01025	12.197(1)	< 0.001
Ossification Center (pelvic limb)				
Femur (head)	-21.609	1.32595	36.038(1)	< 0.001
Femur (distal)	-41.355	3.23133	42.721(1)	< 0.001
Femur (greater trochanter)	-19.277	1.12029	32.438(1)	< 0.001
Tibia (proximal)	-41.355	3.23133	42.721(1)	< 0.001
Tibia (distal)	-13.613	0.99975	35.698(1)	< 0.001
Tibia (tuberosity)	-95.734	5.48967	40.989(1)	< 0.001
Fibula (distal)	-232.385	11.1305	31.291(1)	< 0.001
Calcaneus	-102.498	5.72990	39.066(1)	< 0.001
Metatarsi	-53.161	3.11545	40.302(1)	< 0.001
Proximal Phalanx	-157.459	8.06025	36.146(1)	< 0.001
Middle Phalanx	-232.385	11.1305	31.291(1)	< 0.001

addition, ultrasonography offers standardized measures to estimate the gestational age of the species. We observed that agouti fetuses showed mineralization in all primary centers and major secondary centers of ossification. This fact suggests that the black agouti is a precocial animal, which agrees with studies on the morphology during the gestational period of other agouti species [23,38].

Ultrasonography is a technique widely used for monitoring pregnancy, and the crown-rump length (CRL) is frequently used as a surrogate of the gestational age in different species [8,39,40]. Unfortunately, the limitations in measuring CRL by ultrasonography may cause errors in age estimations. The incorrect classification of fetal growth can cause severe consequences for both short-term and long-term fetal monitoring [41] Therefore, the use of TDL can be seen as an alternative to estimate fetal age, given the high correlation between GA and TDL ($r^2 = 0.97$, $P < 0.001$, Fig. 4), as well as bone measurements with TDL (total length of radius $r^2 = 0.97$, $P < 0.001$, Fig. S12; occipitofrontal diameter cranial circumference and head circumference $r^2 = 0.96$, $P < 0.001$, Fig. S9). In addition, TDL values are not influenced by handling, avoiding possible measurement errors due to fetal positioning [7].

Skull mineralization in the black agouti (50 days of gestation, 48 % GP) occurs at a more advanced stage in gestation compared to other precocial wild species, such as lowland paca (42 days of gestation, 28 % GP) [4], collared peccary (42 days of gestation, 30 % GP) [7], white-lipped peccary (51 days of gestation, 32 % GP) [7], and *T. laurentinus* fetuses (31 days, 33 % GP) [34]. In contrast, black agouti's skull mineralizes earlier than in altricial species such as the laboratory rat (16 days, 76 % GP) [36] and marmoset (111 days, 76 % GP) [37]. In all of these species, the skull was the first bone to show mineralization, in the earlier stages of gestation. This fact demonstrates the importance of this structure to protect the soft tissues of the fetal brain [41,42], even during the intrauterine phase.

Mineralization of the vertebrae and ribs were observed later in the agouti (50 days, 48 % GP) compared to the same bones in lowland paca (56 days, 37 % GP) [4], collared peccary (50 days, 36 % GP) [7], and white-lipped (54 days, 34 % GP) [7], in which mineralization was observed in the first third of pregnancy. Concomitantly with the mineralization of these portions, in the black agouti the spinal canal surrounded by the vertebral was visualized at 55 days, and the internal organs identified (such as heart, lung, stomach, and liver) might receive protection from the ribs [23].

Black agouti's thoracic limbs (clavicle, scapula, humerus, radius, and ulna), pelvis (ilium and ischium), and pelvic limbs (femur, tibia, and fibula) mineralized at 48 days (50 % GP), which is similar to the age at mineralization in the lowland paca (75 gestation days, 50 % GP) [4], collared peccary (64 gestation days, 46 % GP), and white-lipped peccary (71 gestation days, 45 % GP) [7]. The pubis in these species presented a later mineralization when compared to the other pelvic portions (black agouti 51 % GP; lowland paca 58 % GP; collared peccary 55 % GP; white-lipped 59 % GP) [4,7]. In contrast to these species, altricial animals such as the marmoset and the laboratory rat showed late mineralization in the portions thoracic limbs' bones (*C. jacchus*: 73 % GP; rat: 76 % GP) [36,37], and pelvis, mainly ischium and pubis (*C. jacchus*: 87 % GP; laboratory rat: 80 % GP) [36,37].

The accentuated mineralization of the appendicular skeleton in the mid-gestational phase confirms the precociality of black agoutis, since well-established bones may receive muscle insertions and promote their maturation, making the limbs capable of withstanding traction forces

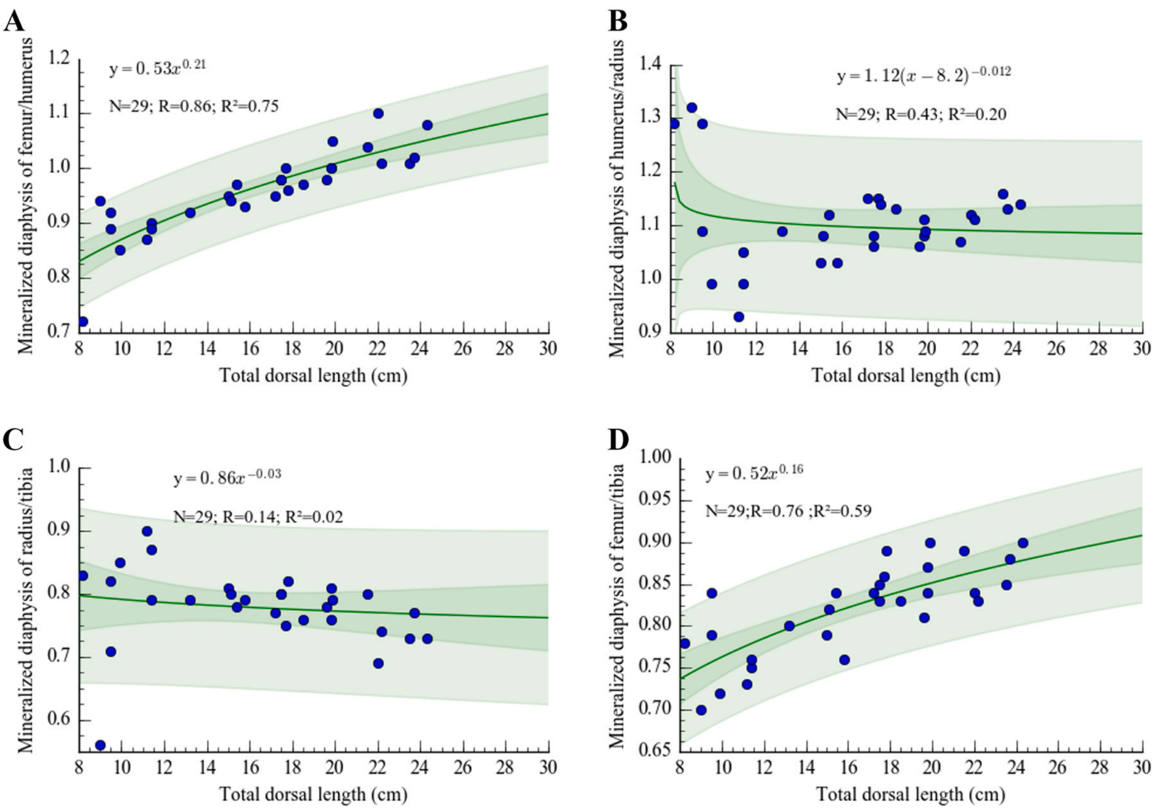

Fig. 7. Allometric relationships between diaphysis lengths of long bones in 29 conceptuses of black agouti (*Dasyprocta fuliginosa*), related to total dorsal length (TDL): (A) femur versus humerus, (B) humerus versus radius, (C) radius versus tibia and (D) femur versus tibia.

after birth [42,43]. The close links between the musculoskeletal system and the nervous system confers early capacity for locomotion and movement to neonates, providing them with the ability of standing, moving, and running right after birth [43]. These abilities are essential for the survival of these species in the wild, since agoutis are considered natural prey animals [44], in addition to being targets of sport [45] and subsistence hunting [15] in the Amazon.

The metacarpi/metatarsi and phalanges had an earlier mineralization in hystricomorph rodents (black agouti 65 % GP; and lowland paca 55 % GP) [4] compared to the laboratory rat (90 % GP) [35]. The slightly earlier mineralization of the thoracic and pelvic paws in the lowland paca may be related to the paca's necessity of early functional paws so they can perform the species' usual habits of digging and swimming. This is unlike the agouti, which is a cursorial species that needs the long bones to be well developed to run and jump [11]. The mineralization of the secondary centers (first signs of mineralization at 65 % GP), especially of the pelvic bones (distal epiphysis of the femur, and distal and proximal epiphysis of the tibia) and of the epiphyses of the long bones, corroborate with the cursorial habits of the species, since well-developed skeleton may provide support, firmness, and greater mobility and locomotion at birth [11]. This allows the neonates to run and move with dexterity to follow the mother in search of food, and escape from predators [43,46].

In the final third of pregnancy, agouti fetuses showed mineralization in most of the secondary phalange centers (93 % GP). Fetuses with more than 100 days of gestational age presented a high degree of development, with characteristic skin and hair pigmentation, presence of tactile hairs, palmar torus, and well-developed claws [38]. The mineralization of the secondary centers of the phalanges, concomitantly with the development of morphological characteristics, allows the neonate to forage in search of food, manipulate food (peel fruits), and bury seeds (a common habit among agoutis) since the introduction of food solids is made in the first weeks of the neonate's life [46].

Fetuses at 97 days of gestation (93 % GP) showed the first signs of mineralization in most of the secondary centers. Bone development of the other secondary centers are enhanced during post-natal life with the parental care usual in this species during the first weeks of life. During this period, due to the earlier development of the skeletal system, the neonate is also able to accompany the parturient in the daily activities of territorial exploration and foraging [46,47].

The patella and its ligaments are well developed in agouti adults, which gives them great movement capacity [49]. However, patella mineralization was not observed in advanced fetuses, similar to fetuses of *P. tajacu* and *T. pecari* [7]. This raises the hypothesis of mineralization of this bone only a few weeks after parturition. It is believed that the patella arises primarily as part of the femur due to the process of forming a patellofemoral joint, in which the interzonal cells lose their typical structure, becoming more flattened and elongated. This portion undergoes bone stress caused by walking or movement, causing this part of the femur to detach and form the patella [29].

Since the axial and appendicular skeletons were well developed in fetuses with more than 80% GP, as we observed, the neonates may have the capacity to perform characteristic habits of the species, such as leaning on the pelvic limbs so the animal can better observe its surroundings when they notice abnormal noises or adopting a typical feeding posture that facilitates better food manipulation by the thoracic limbs. In addition, well-developed thoracic paws allow the neonate to stimulate the parturient mammary glands during milk suction [48].

Agoutis are natural prey for wild felids, such as *Puma concolor* and *Leopardus pardalis* [44], so they need to adopt strategies for the survival not only of the adult but mainly of the neonates due to their higher vulnerability to predation. The presence of the parturient next to the neonate reduces attacks by predators or agoutis from other groups [46,47]. The adoption of adaptive strategies with a high degree of parental care and skeletal development, and consequently of the skeletal muscles, before birth provides greater chances of survival for the neonate.

By comparing the bone development of agoutis during the intrauterine phase to other species, it is possible to notice a marked and earlier preparation of the skeletal system, which concur with the high degree of precociality in this species compared especially to altricial mammals such as laboratory rat and marmosets. This ultimately provides earlier locomotor independence of neonates. However, it was clear that mineralization in several of the agouti's bones occurred at later ages than in other precocial species, such as lowland paca and peccaries, which shows that agoutis may need a longer time of parental care and post-natal bone development to reach its full musculoskeletal capacity. The results obtained here can contribute to the understanding of the chronology of fetal growth of the axial and appendicular skeleton of the agouti and other species, which can help improve gestational management and gain insights of the adaptive life history strategies adopted by precocial and altricial animals for neonatal survival.

Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2023.100029.

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