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Fetal bone development in the lowland paca (*Cuniculus paca*, Rodentia, Cuniculidae) determined using ultrasonography

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Abstract

Studying the timing of the main events of embryonic and fetal development may clarify the strategies adopted by species to maximize neonatal survival and the consequences of these events for their life history. This study describes bone development during the fetal phase of the lowland paca (*Cuniculus paca*), comparing it with other precocial or altricial species, and its relationship with the species' adaptive strategies. A total of 102 embryos/fetuses obtained over the course of 17 years through collaboration with local subsistence hunters in the Amazon were analyzed. Measurements of mineralization 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) was: skull (TDL = 4.1 cm); vertebral bodies (TDL = 4.6 cm); scapula, humerus, radius, ulna, ilium, ischium, femur, tibia, and fibula (TDL = 6.7 cm); ribs (TDL = 7.8 cm); clavicle (TDL = 8.5 cm); metacarpi/metatarsi (TDL = 11 cm); phalanges (TDL = 15 cm); tarsus (TDL = 18 cm); patella (TDL = 23 cm); and carpus (TDL = 27.2 cm). Secondary ossification centers first appeared in the femoral distal epiphysis (TDL = 16.6 cm) and tibial proximal epiphysis (TDL = 18.4 cm). Advanced fetuses (TDL > 30 cm, 97% gestational period) presented mineralization in all primary and most secondary centers. Compared to other species, paca neonates have a well-developed skeletal system at birth, which is important for their independent postnatal locomotion. Our results may contribute to the monitoring of bone development in other wild species, helping us to understand their life history, and serving as parameters for comparisons between precocial and altricial mammals.

KEYWORDS
bone, fetal development, hystricomorph, locomotor system, mineralization, precociality, Rodentia

1 | INTRODUCTION

Studying the timing of the main events that occur during embryonic and fetal development may clarify the strategies adopted by species to maximize neonatal survival and the consequences of these events for their life history (Derrickson, 1992). For instance, studies on the development of the locomotor system during the fetal phase are crucial to evaluate and compare the level of neonatal independence among species. Although recent studies on the fetal development of wild mammals have been developed (Andrade et al. 2018a,b; El Bizri et al. 2017; Mayor et al. 2019a,b), little is known about the development of the musculoskeletal system during fetal growth (Berendsen and Olsen, 2015).

The lowland paca (*Cuniculus paca*) is a hystricomorph rodent that can reach up to 80 cm in length, with females and males weighing approximately 8–10 kg and 9–12 kg, respectively (Pachaly et al. 2001). In captivity, pacas reproduce throughout the year and have an average pregnancy length of 149 days, delivering one offspring per parturition, with a mean parturition interval of 224.5 days (Guimarães et al. 2008). In the wild, the parturition interval is between 247 and 266 days and prolificacy is of 1.41–1.52 pups/year/female (Mayor et al. 2013). Paca is considered a precocial species, generating newborns with morphological structures suitable for survival with low parental dependence (El Bizri et al. 2017), reaching puberty at 4 months of age (El Bizri et al. 2019).

The lowland paca is amongst the most hunted Neotropical species, both for consumption (Cajaiba et al. 2015; Mayor et al. 2015; El Bizri et al. 2018) and sport (El Bizri et al. 2015); however, the reproductive capacity of this species apparently cannot sustain this high hunting pressure (Mayor et al. 2013; El Bizri et al. 2019). Several aspects of its biology and ecology must, therefore, be understood in order for *in situ* management plans to be effectively established. As this knowledge is still incipient, the aim of the present study was to improve the understanding of the level of precociality in wild species through description of gestational bone development in the lowland paca using ultrasonography, comparing it with that of mice and domestic carnivores. The results of this study may also be useful to assist in the monitoring of gestation and improve our knowledge of the species' life history.

2 | MATERIALS AND METHODS

2.1 | Study sites

This study was conducted in two locations in the Amazon forest. The first site was the Yavarí-Mirín River (YMR, S 04°19.53;

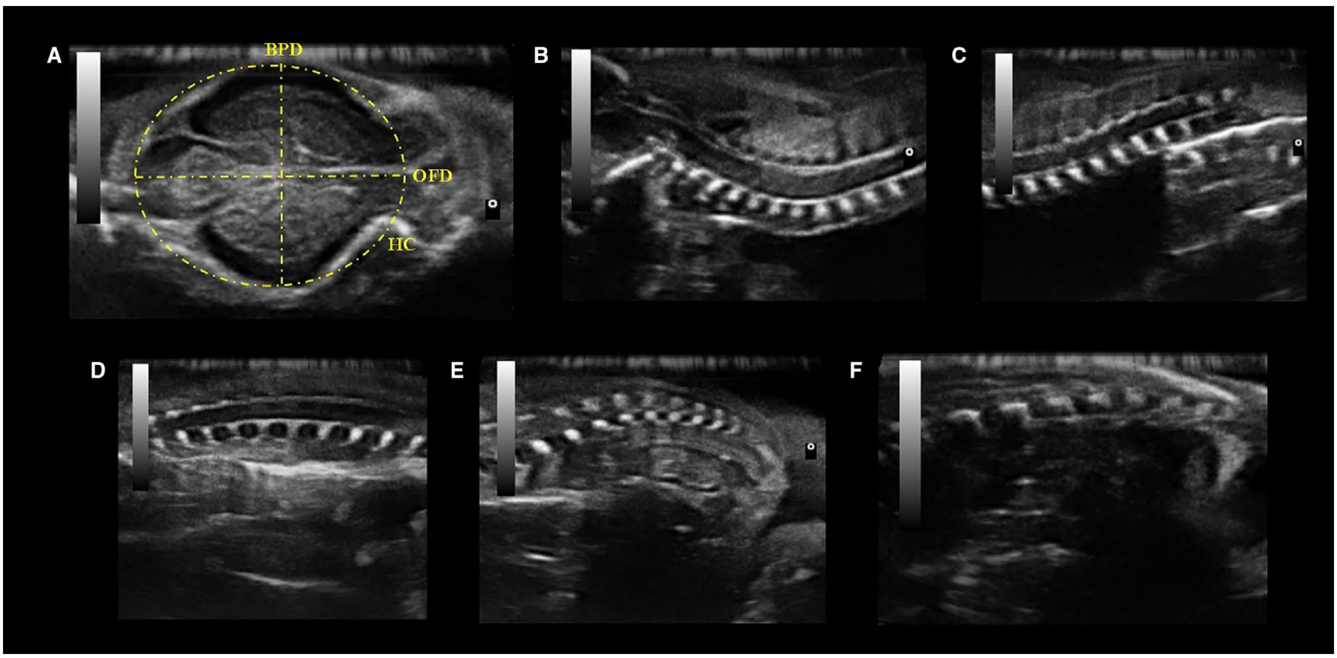

FIGURE 1 Ultrasonographic planes used for the examination of the axial skeleton of lowland paca (*Cuniculus paca*) fetuses: (A) biparietal diameter (BPD), occipitofrontal diameter (OFD), and head circumference (HC) measurements; (B–F) sagittal fetal spine sections, (B) cervical, (C) thoracic, (D) lumbar, (E) sacral, and (F) caudal portion of spine and (F) caudal vertebrae.

W 71°57.33), which is located in the northeast 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

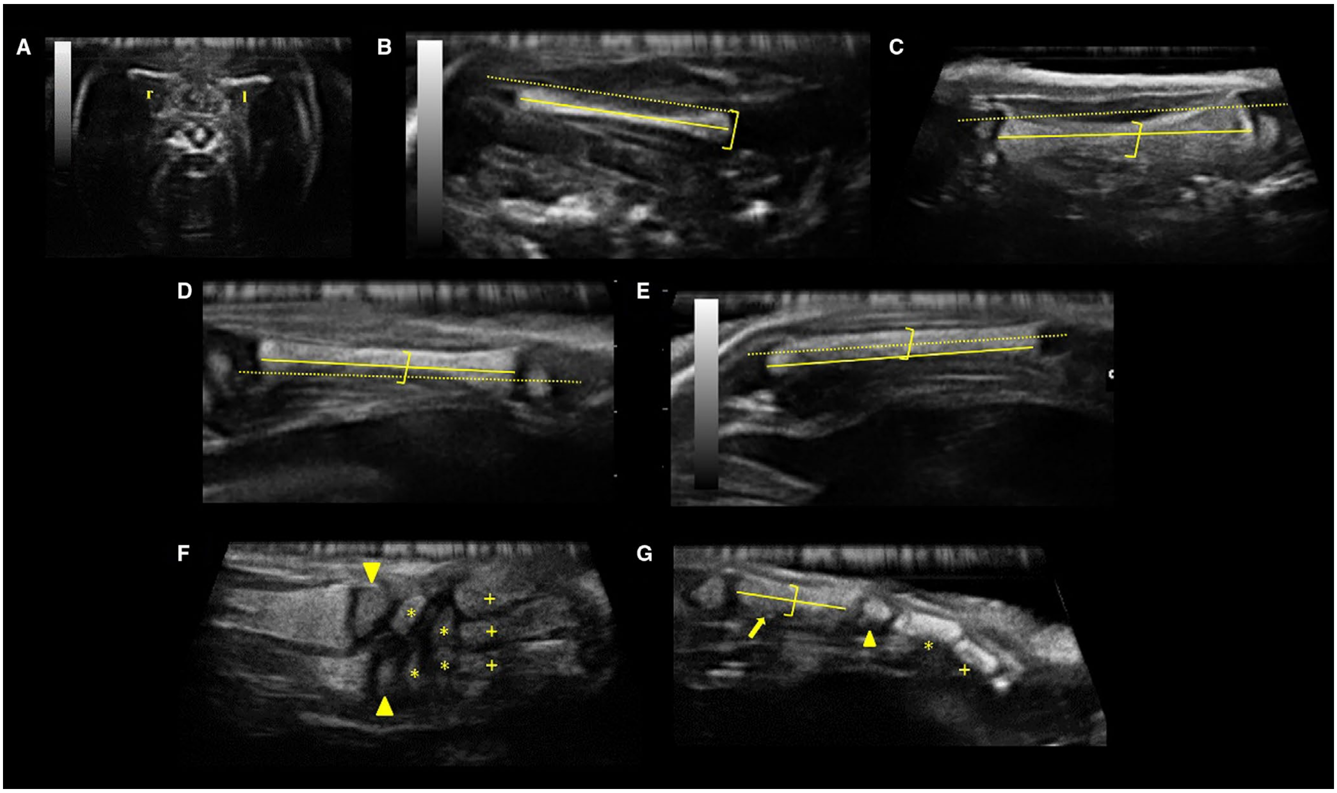

FIGURE 2 Ultrasonographic planes used for the examination and measurement of the thoracic limb of lowland paca (*Cuniculus paca*) fetuses: (A) length of clavicle (r-right, l-left), transversal scan, sagittal scan, (B) scapula, (C) humerus, (D) radius, (E) ulna, (F) distal ossification center of the radius (below) and ulna (above; arrowhead), carpal (*) and metacarpal bones (+); (G) metacarpi (arrow) with distal ossification center (arrowhead), proximal (*) and middle phalanx (+). Ossified portion/diaphysis (solid line), total length (dotted line), width (]).

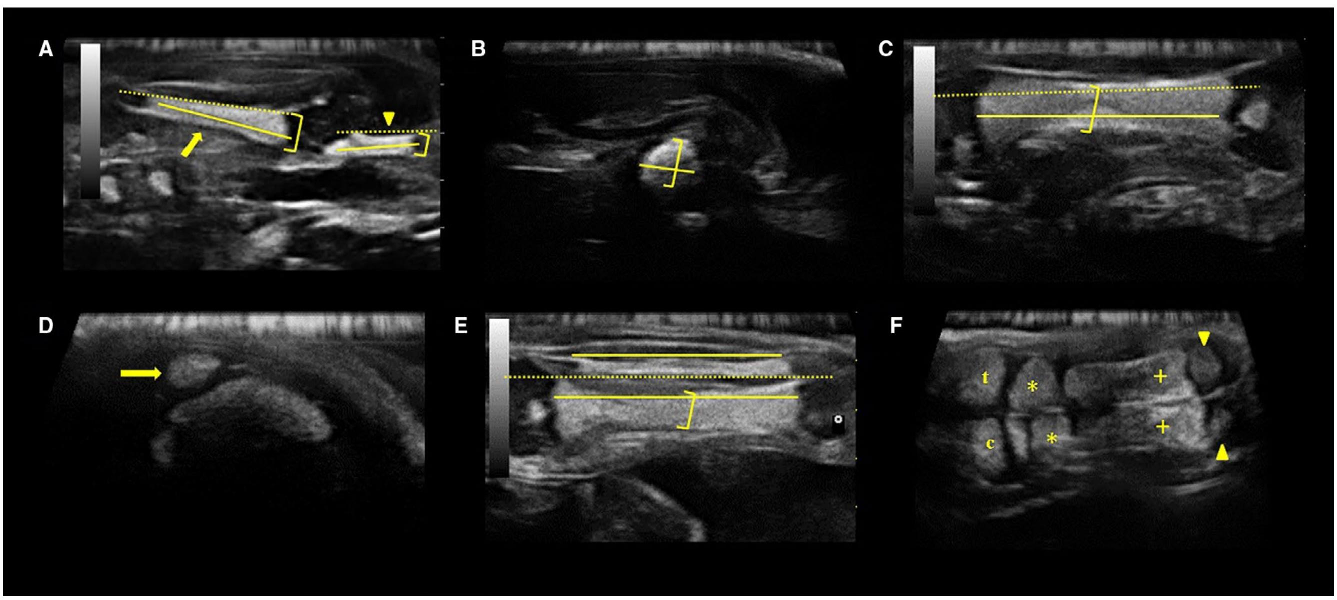

FIGURE 3 Ultrasonographic planes used for the examination and measurement of the pelvic limb in lowland paca (*Cuniculus paca*) fetuses: (A) ilium (arrow) and ischium (arrowhead) measurements, (B) pubis, (C) femur, (D) patella (arrow), (E) tibia and fibula, (F) calcaneus (c), talus (t), distal tarsal bones (*), and metatarsal bones (+), with distal ossification centers (arrowheads). Mineralized portion/diaphysis (solid line), total length (dotted line), width (]).

4000 people distributed into 23 communities and some isolated settlements.

2.2 | Biological sample collection and processing

The samples used in the present study were obtained through voluntary donations by hunters who collected the material from 2000 to 2016. These hunters received training to remove the viscera from hunted specimens, extracting all abdominal and pelvic organs, including the perineal region, and to store them in formaldehyde-buffered 4% solution (v/v; Mayor et al. 2017). Because the 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 (License 0350-2012-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 007/2016). Samples were sent to UFRA, Belém, Pará, Brazil, using the export license CITES/IBAMA (No 14BR015991/DF).

Genital organs of the hunted females were dissected to remove all conceptuses. A total of 102 conceptuses (seven embryos and 95 fetuses) of lowland paca were analyzed. The crown-rump length (CRL) and total dorsal length (TDL) were measured in all conceptuses using a measuring tape (0.1-mm accuracy). The embryonic/fetal phase was determined according to the International Committee on Veterinary Embryological Nomenclature (2017).

2.3 | Ultrasound examination

An ultrasonographic evaluation of the conceptuses was performed using Esaote® ultrasound equipment, model MyLab™ 30Gold VET (Genova, Italy), using a 10–18-MHz B-mode linear and multifrequency electronic transducer. The embryos/fetuses were immersed in water and positioned so that the transducer would be in contact with the evaluated tissue. Ultrasound examinations were performed on all conceptuses to detect and measure the mineralized (echoic) and non-mineralized (anechoic) portion of the bones. A video related to the ultrasound methodology is provided in the Supporting Information.

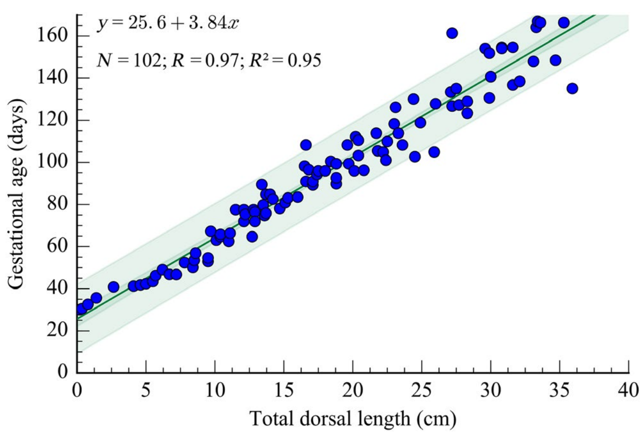

FIGURE 4 Relationship between total dorsal length and gestational age in 102 lowland paca (*Cuniculus paca*) embryos/fetuses. Prediction intervals (light green band) and confidence intervals (green dark band). SE = 8.16; CI = 1.58.

The studied bone measurements and planes of the axial skeleton and thoracic and pelvic limbs are shown in Figures 1–3, respectively. For the axial skeleton, the largest transversal diameter between both parietal bones was classified as the biparietal diameter (BPD); the occipitofrontal diameter (OFD) was measured as the perpendicular distance from the BPD, respecting the limits of the occipital and frontal bones. The head circumference (HC) was obtained in the maximum region in the same position by a circumference around the outer margin of the hyperechogenic contour at the border of the conceptus skull. The vertebrae were divided into cervical, thoracic, lumbar, sacral, and caudal; they were examined using longitudinal sections and the presence of mineralized vertebral body was qualitatively evaluated. Likewise, the ribs were qualitatively examined for mineralization through longitudinal and transverse planes.

The bones of the appendicular skeleton were measured from a proximal-distal perspective. The clavicle was examined in a cross section at the level of the first thoracic vertebra, and the longest length of the mineralized part was measured. Regarding the scapula, we measured the total length (including mineralized and non-mineralized parts), the length of the mineralized part, and the width (lateromedial diameter, measured at the distal portion of the bone that is in contact with the scapulohumeral joint). For the humerus, radius, ulna, femur, tibia, and fibula, the following were measured: the length and width (lateromedial diameter) of the mineralized diaphysis (hyperechoic part); the proximal and distal extremities (epiphysis, hypoechoic part); total length (diaphysis and epiphysis); and the secondary centers of ossification. To measure the patella, the mean measures between the lateromedial and longitudinal planes were calculated.

In the ilium and ischium, the total length (comprising 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 were evaluated in the pubis, metacarpi, metatarsi, and phalanges of the thoracic and pelvic limbs. We identified and quantified the mineralization in the seven carpal bones and eight tarsal bones; only the length and width of the talus and calcaneus were measured.

The largest and smallest perpendicular diameters of the secondary ossification centers studied were recorded. All measurements were performed in triplicate and the mean values were calculated.

2.4 | Data analysis

Gestational age (GA) of conceptuses was estimated using the formula crown-rump length (CRL) = 0.179 × age − 5.28, presented by El Bizri et al. (2017). We then established the relationship between

GA and TDL in all conceptuses. The timing of the main development was expressed in terms of GA (days) and in percentage of the total gestational period (GP).

Logistic regressions were applied to estimate the probability of the occurrence of ossification in the studied structures in relation to TDL using the software Statistica 8.0 (StatSoft Inc.). Multiple regressions modeling relationships between TDL and biometric measures, and between the length and width (robustness index) of the humerus, radius, ulna, femur, and tibia were conducted using CurveExpert Professional 2.6 software (© Copyright 2017, Daniel G. Hyams), which defined the best-fitted function for each plot. Regressions were forced to zero to represent day 0 of gestational development, and regressions with higher determination coefficients were selected.

3 | RESULTS

The mean (range) TDL value of the 102 conceptuses studied was 18.1 ± 9.1 (0.2–35.9) cm. GA and TDL had a high positive linear relationship [$r^2 = 0.95$, $P < 0.001$ (Figure 4)].

TABLE 1 Logistic equations [y=exp(Intercept+Estimate*x)/(1+exp(Intercept+Estimate*x)] for the axial and appendicular bone parameters in 102 embryo/fetuses of lowland paca (*Cuniculus paca*).

Bones	Intercept	Estimate	Chi-square (Df)	*P*
Axial bones (skull; spine; ribs)				
Skull	−4.1952	1.14522	36.753 (1)	*P* < 0.001
Cervical	−4.7491	1.17328	41.283 (1)	*P* < 0.001
Thoracic	−4.7491	1.17328	41.283 (1)	*P* < 0.001
Lumbar	−4.7491	1.17328	41.283 (1)	*P* < 0.001
Sacral	−4.7491	1.17328	41.283 (1)	*P* < 0.001
Caudal	−9.6568	1.29808	72.270 (1)	*P* < 0.001
Ribs	−9.6568	1.29808	72.280 (1)	*P* < 0.001
Appendicular bones (forelimb)				
Clavicle	−11.061	1.33411	75.380 (1)	*P* < 0.001
Scapula	−9.0526	1.28897	67.124 (1)	*P* < 0.001
Humerus	−9.0526	1.28897	67.123 (1)	*P* < 0.001
Radius	−9.0526	1.28897	67.124 (1)	*P* < 0.001
Ulna	−9.0526	1.28897	67.124 (1)	*P* < 0.001
Proximal carpal bones	−110.4	4.10649	102.611 (1)	*P* < 0.001
Distal carpal bones	−26.554	0.963848	81.2371 (1)	*P* < 0.001
Metacarpi	−16.546	1.49446	103.409 (1)	*P* <0.001
Proximal phalanx	−28.203	1.77307	126.393 (1)	*P* < 0.001
Middle phalanx	−32.062	1.93078	128.297 (1)	*P* < 0.001
Distal phalanx	−26.281	1.53037	124.133 (1)	*P* < 0.001
Appendicular bones (hind limb)				
Ilium	−9.0526	1.28897	67.124 (1)	*P* < 0.001
Ischium	−10.444	1.31641	70.989 (1)	*P* < 0.001
Pubis	−10.406	0.525871	94.559 (1)	*P* < 0.001
Femur	−9.0526	1.28897	67.124 (1)	*P* < 0.001
Patella	−71.886	3.03984	118.431 (1)	*P* < 0.001
Tibia	−9.0526	1.28897	67.124 (1)	*P* < 0.001
Fibula	−9.0526	1.28897	67.124 (1)	*P* < 0.001
Calcaneus	−36.552	2.05517	135.721 (1)	P < 0.001
Talus	−46.41	2.32295	131.905 (1)	*P* < 0.001
Distal tarsal bones	33.846	1.26849	93.209 (1)	*P* < 0.001
Metatarsi	−15.822	1.47786	101.286 (1)	*P* < 0.001
Proximal phalanx	−19.262	1.23548	119.011 (1)	*P* < 0.001
Middle phalanx	−18.76	1.09246	118.026 (1)	*P* < 0.001
Distal phalanx	−36.488	2.0424	130.458 (1)	*P* < 0.001

3.1 | Axial skeleton

Figure 5A and Table 1 show the probability curves for the occurrence of mineralization in axial bones in relation to TDL. The occipital, frontal, and parietal bones were the earliest mineralized skull bones and occurred in fetuses with TDL ≥ 4.1 cm (42 gestation days, 28% GP). At this GP, the mineralization of cervical, thoracic, lumbar, and sacral vertebral bodies was also observed. The mineralization of ribs and caudal vertebral bodies was identified in fetuses with TDL ≥ 7.8 cm (56 gestation days, 37% GP).

Fetuses with TDL ≥ 8.0 cm (56 gestation days, 37% GP) had a 100% probability of skull mineralization, while those with TDL ≥ 12.0 cm (72 gestation days, 48% GP) had a 100% probability of mineralization of vertebrae and ribs.

3.2 | Appendicular skeleton

The probability curves for the occurrence of mineralization of the appendicular skeleton bones in relation to the TDL are presented in Figure 5B,C and Table 2. Fetuses with TDL ≥ 13.0 cm (75 gestation days, 50% GP) had a 100% probability of mineralization in bones of thoracic limbs (clavicle, scapula, humerus, radius, and ulna) and pelvis (ilium and ischium). In relation to the pelvic limbs (femur, tibia, and fibula), this probability occurred in fetuses with TDL ≥ 11.0 cm (68 gestation days, 46% GP). First signs of mineralization in long bones were observed in the diaphysis.

The onset of mineralization in the thoracic limbs (scapula, humerus, radius, and ulna) and pelvic limbs (ilium, ischium, femur, tibia, and fibula) was observed in fetuses with TDL ≥ 6.7 cm (51 gestation days, 34% GP). Mineralization in the clavicle was first observed in fetuses with TDL ≥ 8.5 cm (58 gestation days, 39% GP), and mineralization in the metatarsi and metacarpi was first observed in fetuses with TDL ≥ 11.0 cm (68 gestation days, 46% GP).

The pubis, carpal and tarsal bones, and phalanges of the forepaw and hindpaw showed delayed mineralization (Figure 5B,C). Mineralization in the proximal phalanges of the forepaw and hindpaw were identified in fetuses with TDL ≥ 14.7 cm (82 gestation days, 55% GP) and 15.1 cm (84 gestation days, 56% GP), respectively; in the pubis, mineralization was observed in fetuses with TDL ≥ 16.0 cm (87 gestation days, 58% GP). Regarding the tarsal bones, mineralization in the calcaneus, talus, and eight tarsal bones was observed in fetuses with TDL ≥ 18.0 cm (95 gestation days, 64% GP), TDL ≥ 20.2 cm (103 gestation days, 69% GP), and TDL ≥ 30.8 cm (144 gestational days, 97% GP), respectively. A mineralized patella was observed in fetuses with TDL ≥ 23.0 cm (114 gestation days, 76% GP); first mineralization in carpal bones was observed in fetuses with TDL ≥ 27.2 cm (130 gestation days, 87% GP), and seven carpal bones were observed in fetuses with TDLs ≥ 30.0 cm (140 gestation days, 94% GP). Advanced fetuses with TDL 35.3 cm, at birth, presented mineralization in primary ossification centers in all evaluated bones of the appendicular skeleton, in both thoracic and pelvic limbs.

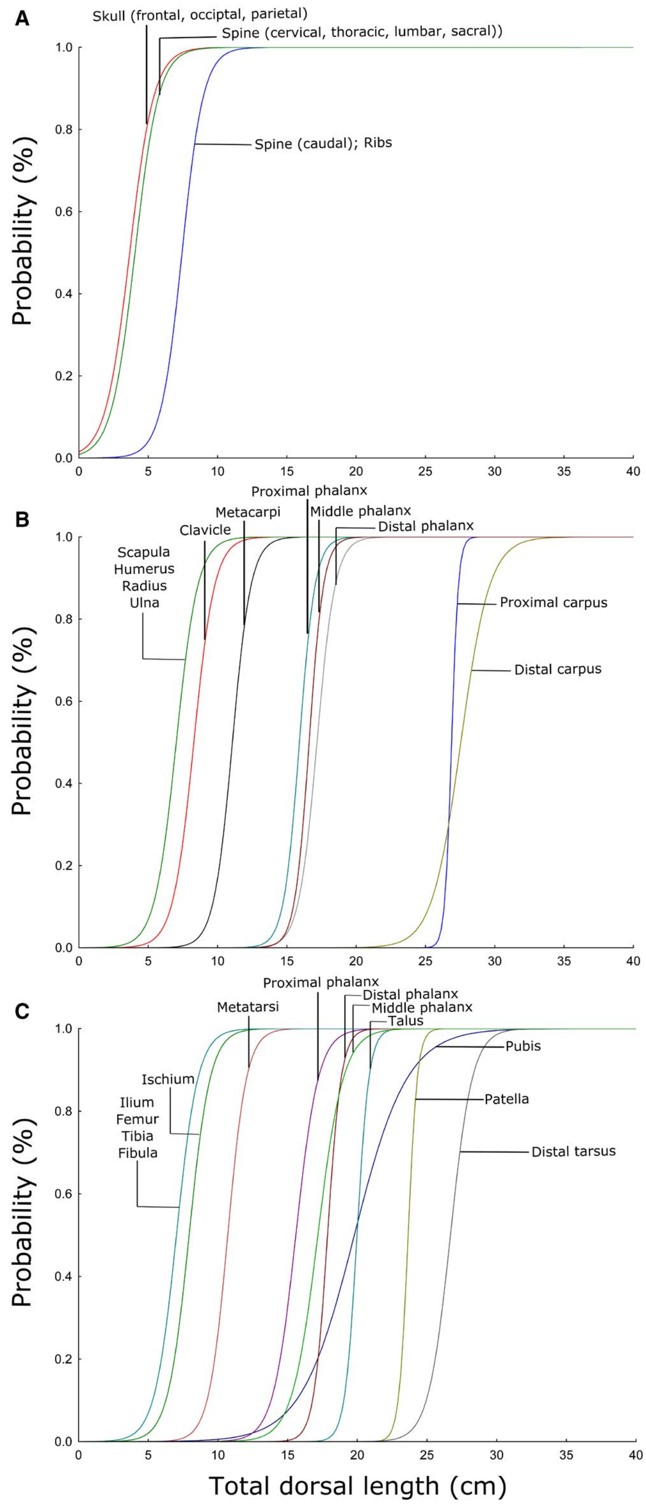

FIGURE 5 Probability curves for the occurrence of skeletal bone mineralization in 102 embryos/fetuses of lowland paca (*Cuniculus paca*) in relation to the total dorsal length (TDL): (A) in the axial skeleton, and (B) in the thoracic and (C) pelvic limb.

As the fetuses grew, the anechoic area between the diaphysis and secondary ossification centers, the epiphyseal cartilage, was reduced in long bones such as the humerus (proximal epiphysis, $r = -0.599$, $r^2 = 0.359$, $P < 0.001$; distal epiphysis, $r = -0.732$, $r^2 = 0.535$, $P < 0.001$),

TABLE 2 Logistic equations [y=exp(Intercept+Estimate*x)/(1+exp(Intercept+Estimate*x)] for the secondary ossification centers in forelimb and hindlimb parameters in 102 embryo/fetuses of lowland paca (*Cuniculus paca*).

Bones	Intercept	Estimate	Chi-square (Df)	*P*
Ossification center (forelimb)				
Humerus (head)	−31.424	1.48417	118.87 (1)	*P* < 0.001
Humerus (distal)	−46.686	2.04784	115.26 (1)	P < 0.001
Humerus (greater tubercle)	−49.497	2.10065	78.56 (1)	*P* < 0.001
Radius (proximal)	−31.711	1.31197	106.18 (1)	*P* < 0.001
Radius (distal)	−37.509	1.66346	114.30 (1)	*P* < 0.001
Ulna (proximal)	−20.161	0.67678	57.79 (1)	*P* < 0.001
Ulna (distal)	−53.448	2.02685	101.30 (1)	*P* < 0.001
Metacarpi	−32.694	1.26471	98.79 (1)	*P* < 0.001
Proximal phalanx	−53.448	2.02685	101.30 (1)	*P* < 0.001
Middle phalanx	−27.913	1.03211	87.29 (1)	*P* < 0.001
Ossification Center (hindlimb)				
Femur (head)	−27.294	1.23958	112.61(1)	*P* < 0.001
Femur (distal)	−23.59	1.29657	121.91 (1)	P < 0.001
Femur (greater trochanter)	−33.222	1.46926	104.39 (1)	*P* < 0.001
Tibia (proximal)	−24.498	1.18783	116.58 (1)	*P* < 0.001
Tibia (distal)	−41.28	1.83131	115.35 (1)	*P* < 0.001
Tibia (tuberosity)	−34.794	1.65359	88.16 (1)	*P* < 0.001
Fibula (distal)	−31.286	1.09512	76.39 (1)	*P* < 0.001
Calcaneus	−23.806	0.806132	62.16 (1)	*P* < 0.001
Metatarsi	−35.091	1.35721	99.30 (1)	*P* < 0.001
Proximal phalanx	−33.845	1.26847	89.64 (1)	*P* < 0.001
Middle phalanx	−13.511	0.482793	60.53 (1)	*P* < 0.001

radius (proximal epiphysis, r = −0.429, r^2 = 0.183, P = 0.02; distal epiphysis, r = −0.400, r^2 = 0.160, P = 0.02), femur (proximal epiphysis, r = −0.324, r^2 = 0.105, P = 0.066; distal epiphysis, r = −0.334, r^2 = 0.112, P = 0.02), and tibia (proximal epiphysis, r = −0.393, r^2 = 0.154, P = 0.01; distal epiphysis, r = −0.351, r^2 = 0.123, P = 0.045).

3.3 | Secondary ossification centers

The probability curves for the occurrence of mineralization of the secondary centers of ossification of the appendicular skeleton are presented in Figure 6. During the GP, the stylopod (femur/humerus) was the segment that mineralized first and more rapidly; all the epiphyseal centers, initially the distal femoral epiphysis (TDL ≥ 16.6 cm, 89 gestation days, 60% GP) and then the greater tubercle of the humerus (TDL ≥ 23.0 cm, 114 gestation days, 76% GP), mineralized during the fetal period. Similarly, all autopod centers (forepaw/hindpaw) were still identified in the fetal period; the appearance of epiphyseal centers of the metacarpal and metatarsal bones occurred in fetuses with TDLs ≥ 24.5 and 24.9, respectively (approximately 121 gestation days, 81% GP), and epiphyseal centers of the medial phalanx appeared in fetuses with TDL ≥ 26.0 cm (126 days of gestation, 84% GP). The zeugopod (radius and ulna/tibia and fibula) began to develop a few days after the stylopod. We identified the proximal tibial epiphysis center in fetuses with TDL ≥ 18.4 (96 gestation days, 64% GP), whereas almost all epiphyseal centers were mineralized in fetuses with TDL ≥ 27.2 cm (130 gestation days, 87% GP). However, the center of the proximal fibular epiphysis was not observed, not even in the more developed fetuses.

Regarding the pelvis, ossification centers of the iliac crest and the ischial tuberosity were not observed. Advanced fetuses with 35.3 cm TDL, at birth, presented mineralization in most secondary ossification centers (Figures 7 and 8).

Tables 3 shows the secondary ossification centers that were observed in the most advanced fetuses included in the present study compared to those in fetuses and infants of altricial species such as the laboratory mouse, dog, and cat. In the lowland paca, most secondary ossification centers appeared during the GP, whereas, for altricial species, the secondary ossification centers appear in the postnatal period.

3.4 | Allometric relationships

The growth of long bones was not isometric (Figuire 9). In both thoracic and pelvic limbs, when mineralization of the diaphysis began, humerus and tibia were the longest bones, followed by femur and radius. However, at the end of pregnancy, the femur and the tibia had a similar length and were larger than the humerus and radius. During the gestational development, the humerus and radius had a similar growth ($P = 0.19$), which resulted in a humerus/radius length ratio of 1.20 ± 0.03 in most advanced fetuses. Although the femur was shorter than the tibia in earlier fetuses, its larger growth ($r = 0.702$, $r^2 = 0.493$, $P < 0.001$) explained the femur/tibia length ratio of 0.99 ± 0.05 in most advanced fetuses. In contrast, the femur had a larger growth than the humerus ($r = 0.702$, $r^2 = 0.492$, $P < 0.001$), and in most advanced fetuses, the humerus/femur length ratio was 0.93 ± 0.04. The radius and the tibia had a similar growth ($P = 0.629$), and the tibia was constantly longer than the radius, with a constant ratio of radius/tibia length of 0.77 ± 0.04 throughout gestation. In advanced fetuses, the stylopod and the zeugopod in the thoracic member were less developed than in the pelvic member. The humerus, femur, and tibia had a large width/length ratio (robustness index) and were thus considered robust bones (Figure S11).

3.5 | Bone measurements and gestational age

Skull measurements showed a strong association with GA ($r^2 > 0.86$), with BPD being the measure with the highest coefficient of determination ($r^2 = 0.96$, $P < 0.001$). For long bones, length of mineralized diaphysis was strongly associated with GA ($r^2 > 0.86$, $P < 0.01$). The strongest association with GA was observed in the mineralized diaphysis length of long bones of thoracic and pelvic limbs ($r^2 \geq 0.94$, $P < 0.001$; Figures S2 and S5). All associations are shown in the Supporting Information (Figures S1–S11).

4 | DISCUSSION

In the present study we describe the precocial skeletal development of the lowland paca and compare it with that of mice and other altricial mammals. Paca fetuses at an advanced stage of development presented signs of mineralization in all primary and most secondary ossification centers. Thus, the early development of the skeletal system is consistent with previous studies, which have described other precocial external morphological features in pacas (El Bizri et al. 2017; Figures 10-13).

The first evidence of bone mineralization in paca fetuses was observed in the skull at 42 gestation days (28% GP), which is considerably earlier than in altricial species such as the laboratory rat (approximately 15 days, 75% GP) and the marmoset [*Callithrix jacchus*; 111 days, 74% GP (Wright et al. 1958; Phillips, 1976)]. The first signs of mineralization in the paca occurred just before the differentiation of limbs and genitalia, fusion of eyelids, and formation of the outer ear [46–47 days, 30.7–31.4% GP (El Bizri et al. 2017)]. At this stage, the paca conceptus goes from being an embryo to becoming a fetus.

Vertebral bodies and ribs were observed in paca fetuses at 44 (29.5% GP) and 56 gestation days (37.8% GP), respectively, which is earlier than in the laboratory rat [17 days, 81% GP (Wright et al. 1958; Phillips, 1976)].

Regarding the appendicular skeleton, the onset of mineralization of the pelvis and the diaphysis of long bones in paca fetuses (49 days, 33% GP) was similar to that described for *Thrichomys laurentinus* [31 days, 32.6% GP (Favaron et al. 2016)]. In the laboratory rat, an altricial rodent, the mineralization of bones in the thoracic limb, pelvic limb, and pelvis started at 15 (75% GP), 16 (80% GP), and 17 days [85% GP (Patton and Kaufman, 1995)], respectively; and in marmosets, the mineralization of the appendicular skeleton was first identified at 109 days [72.6% GP (Phillips, 1976)].

From 86 days of gestation (58% GP) onwards, a higher growth rate and robustness of the femur, tibia, and humerus were observed. These characteristics are likely to be maintained in postnatal life since in adult pacas the humeral shaft is more rigid when compared to that of *Dasyprocta*; this may be explained by the paca's use of the thoracic limbs in digging movements (Biknevicius, 1993).

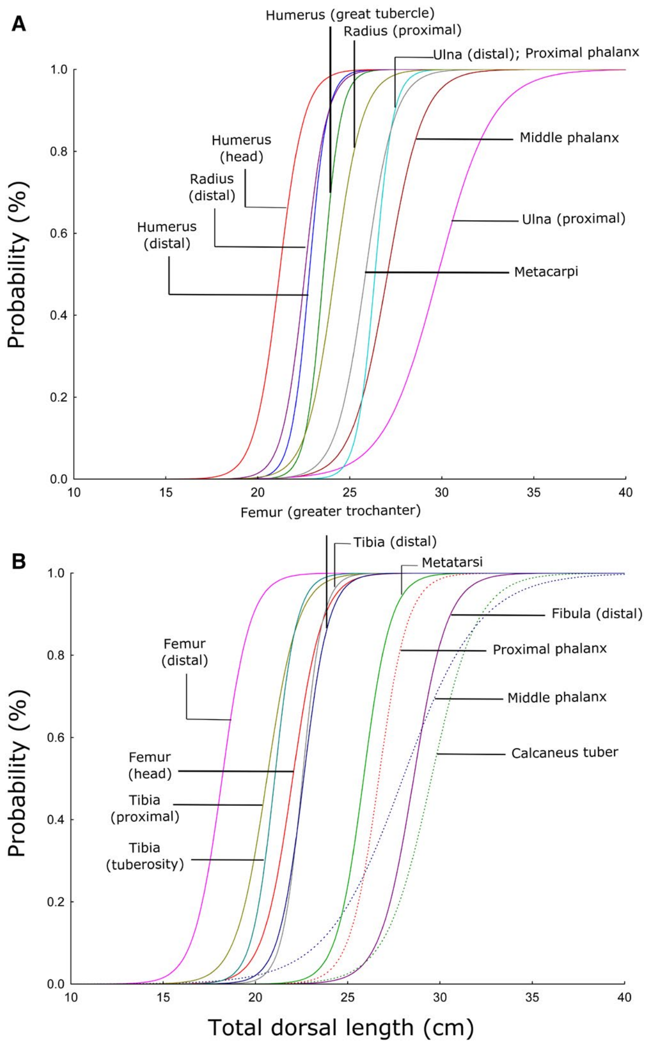

FIGURE 6 Probability curves for the occurrence of mineralization of the secondary centers of ossification in 102 embryos/fetuses of lowland paca (*Cuniculus paca*) in relation to the total dorsal length (TDL): (A) in the thoracic and (B) pelvic limb.

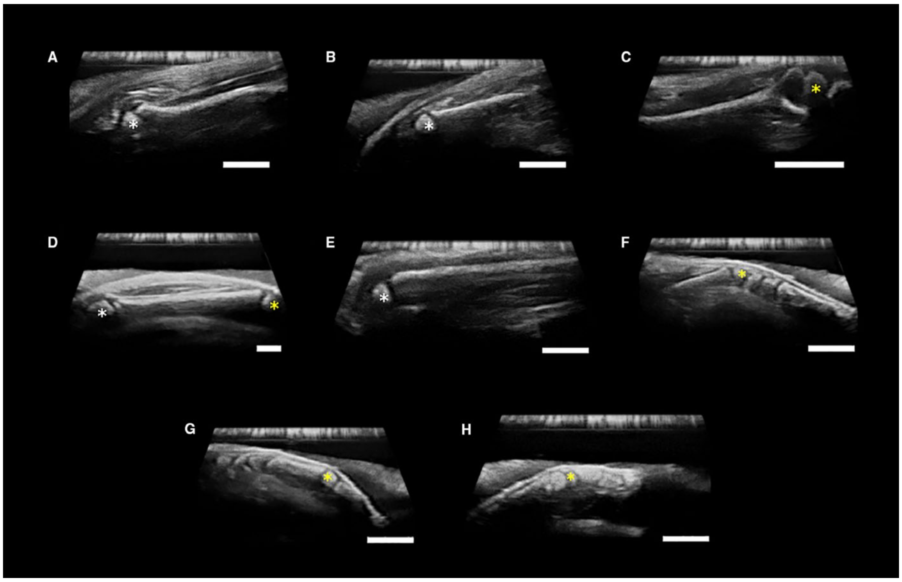

FIGURE 7 Ultrasound evaluation of the bone development in the thoracic limb of an advanced (total dorsal length = 35.3 cm) fetus of lowland paca (*Cuniculus paca*): (A) head of the humerus (scale bar: 1 mm), (B) great tubercle of the humerus (scale bar: 1 mm), (C) distal epiphysis of the humerus (scale bar: 1.5 mm), (D) proximal (white) and distal (yellow) epiphysis of the radius (scale bar: 0.5 mm), (E) proximal epiphysis of the ulna (scale bar: 1 mm), (F) distal epiphysis of the ulna (scale bar: 1 mm), (G) distal epiphysis of the metacarpi (scale bar: 1 mm), and (H) proximal epiphysis of the proximal phalanx (scale bar: 1 mm). Asterisks (*) represent ossification centers.

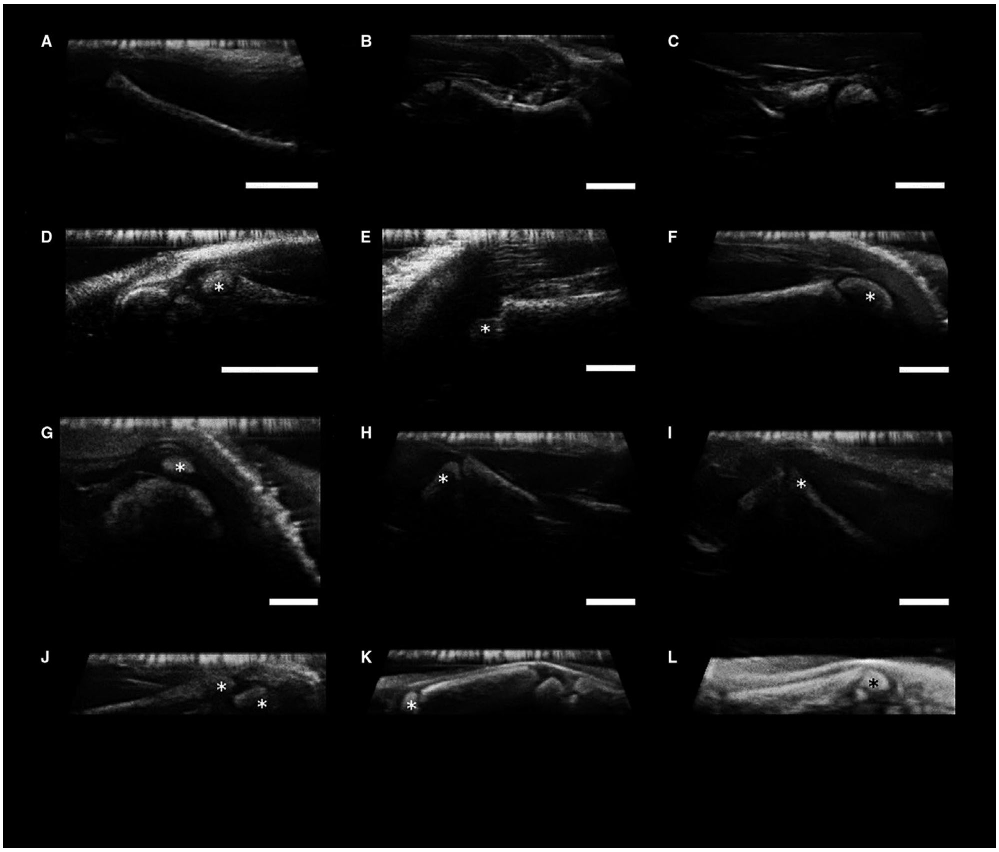

FIGURE 8 Ultrasound evaluation of the bone development in the pelvic limb of an advanced (total dorsal length = 35.3 cm) fetus of lowland paca (*Cuniculus paca*): (A) ilium (scale bar: 1.5 mm), (B) ischium (scale bar: 1 mm), (C) pubis (scale bar: 1 mm), (D) head of the femur (scale bar: 2 mm), (E) great trochanter of the femur (scale bar: 1 mm), (F) distal epiphysis of the femur (scale bar: 1 mm), (G) patella (scale bar: 1 mm), (H) proximal epiphysis of the tibia (scale bar: 1 mm), (I) tuberosity of the tibia (scale bar: 1 mm), (J) distal epiphysis of the tibia and the fibula (scale bar: 1 mm), (K) calcaneal tuber (scale bar: 1 mm), and (L) metatarsal distal epiphysis (scale bar: 1 mm). Asterisks (*) represent ossification centers.

TABLE 3 Secondary ossification centers during prenatal development in fetuses of lowland paca (precocial species; present study) and postnatal development in fetuses and infants of laboratory mice (Patton and Kaufman, 1995) dogs and cats [altricial species (Dyce, 2010)].

		Precocial (prenatal development)	Altricial (postnatal development)	
Bones	Ossification center	Lowland paca	Laboratory mouse	Dog and cat
Humerus	Head	15 weeks (66-70%)	1 week (100-135%)	1–2 weeks (100-123%)
	Great tubercle	16 weeks (70-75%)	1 week (100-135%)	1–2 weeks (100-123%)
	Distal epiphysis	15 weeks (66-70%)	1 week (100-135%)	2–8 weeks (111-190%)
Radius	Proximal epiphysis	16 weeks (70-75%)	2 weeks (135-170%)	3–5 weeks (123-156%)
	Distal epiphysis	15 weeks (66-70%	1 week (100-135%)	2–4 weeks (111-145%)
Ulna	Proximal epiphysis	18 weeks (80-85%)	1 week (100-135%)	6–8 weeks (156-190%)
	Distal epiphysis	17 weeks (75-80%)	1 week (100-135%)	6–8 weeks (156-190%)
Metacarpi/metatarsi	Distal epiphysis	17 weeks (75-80%)	1 week (100-135%)	4 weeks (134-156%)
Proximal and middle phalanx (thoracic and pelvic)	Proximal epiphysis	17 weeks (75-80%)	1 week (100-135%)	4–5 weeks (134-168%)
Ilium	Crest	Absence (>100%)*	> 2 weeks (>135%)	16 weeks (269-292%)
Isquium	Tuberosity	Absence (>100%)*	> 2 weeks (>135%)	12 weeks (224-247%)
Femur	Head	14 weeks (61-66%)	2 weeks (135-170%)	2 weeks (111-134%)
	Greater trochanter	16 weeks (70-75%)	Not analyzed	8 weeks (190-202%)
	Distal epiphysis	12 weeks (52-56%)	1 week (100-135%)	3 weeks (123-145%)
Patella	-	16 weeks (70-75%)	2 weeks (>135-170%)	9 weeks (190-213%)
Tibia	Proximal epiphysis	13 weeks (56-61%)	1 week (100-135%)	3 weeks (123-145%)
	Tuberosity	14 weeks (61-65%)	Not analyzed	8 weeks (179-202%)
	Distal epiphysis	15 weeks (66-70%)	1 week (100-135%)	3 weeks (123-145%)
Fibula	Proximal epiphysis	Absence (>100%)*	> 2 weeks (>135%)	9 weeks (190-213%)
	Distal epiphysis	18 weeks (80-85%)	1 week (100-135%)	2–7 weeks (111-179%)
Calcaneus	Tuber	18 weeks (80-85%)	2 weeks (135-170%)	6 weeks (156-179%)

Values <100% indicate the percentage of prenatal development in relation to the total gestation period, while >100% indicate postnatal development
*Absence in all fetuses. Pregnancy length was 149 days in the paca (Guimarães et al. 2008), 21 days in the laboratory mouse (Patton and Kaufman, 1995), and 62 days in the dog/cat (Colville and Bassert, 2015).

The presence of longer pelvic limbs and greater robustness of humerus and femur are locomotor adaptations of ancient rodents for swimming and digging (Samuels and Van Valkenburgh, 2008).

The onset of the mineralization of most secondary ossification centers occurred earlier in lowland pacas (130 days, 87% GP) than in altricial species, whose secondary ossification centers initiate mineralization after birth. The onset of mineralization in marmoset neonates, for instance, occurs only in ossification centers of the distal epiphysis of the radius and femur (Phillips, 1976), and in the laboratory mouse and domestic carnivores, the secondary ossification centers appear only 1–2 weeks after birth (Patton and Kaufman, 1995; Dyce et al. 2010). The paca fetus in this advanced gestational stage has developed most important external characteristics, except for the pre-birth eyelid opening (El Bizri et al. 2017).

Regarding the thoracic limbs of lowland pacas, although the ossification center of the olecranon is one of the last to occur (130 days, 87% GP), it shows a constant growth curve during the GP. When compared to *Dasyprocta punctata* (Smythe, 1978), the lowland paca has shorter limbs but a relatively long olecranon, which may be related to ambulatorial and locomotion habits. In addition, the paca is a nocturnal, elusive animal (Gómez et al. 2005), has a small living area of approximately 2.26 ha (Benavides et al. 2017), and builds shelters under roots and fallen trunks. Thus, this difference between agoutis and pacas is possibly related to its adaptation for excavation, since other hystricomorph rodents that use the anterior limbs for excavation movements also have a more elongated olecranon (Biknevicius, 1993).

In relation to the pelvis, the coxofemoral joint began to form with the appearance of the ilium and ischium (49 days, 33% GP) and was followed by the formation of the femoral head center (104 days, 70% GP). In the laboratory rat, as well as in domestic carnivores, the ilium and ischium appear in the fetal period and continue to grow until the second postnatal week with the formation of the femoral head (Patton and Kaufman, 1995; Dyce et al. 2010).

The stifle joint is the first joint in the long bones where all epiphyseal ossification centers appear mineralized, including the distal epiphyseal center of the femur (89 days, 59.7% GP), the proximal epiphyseal center

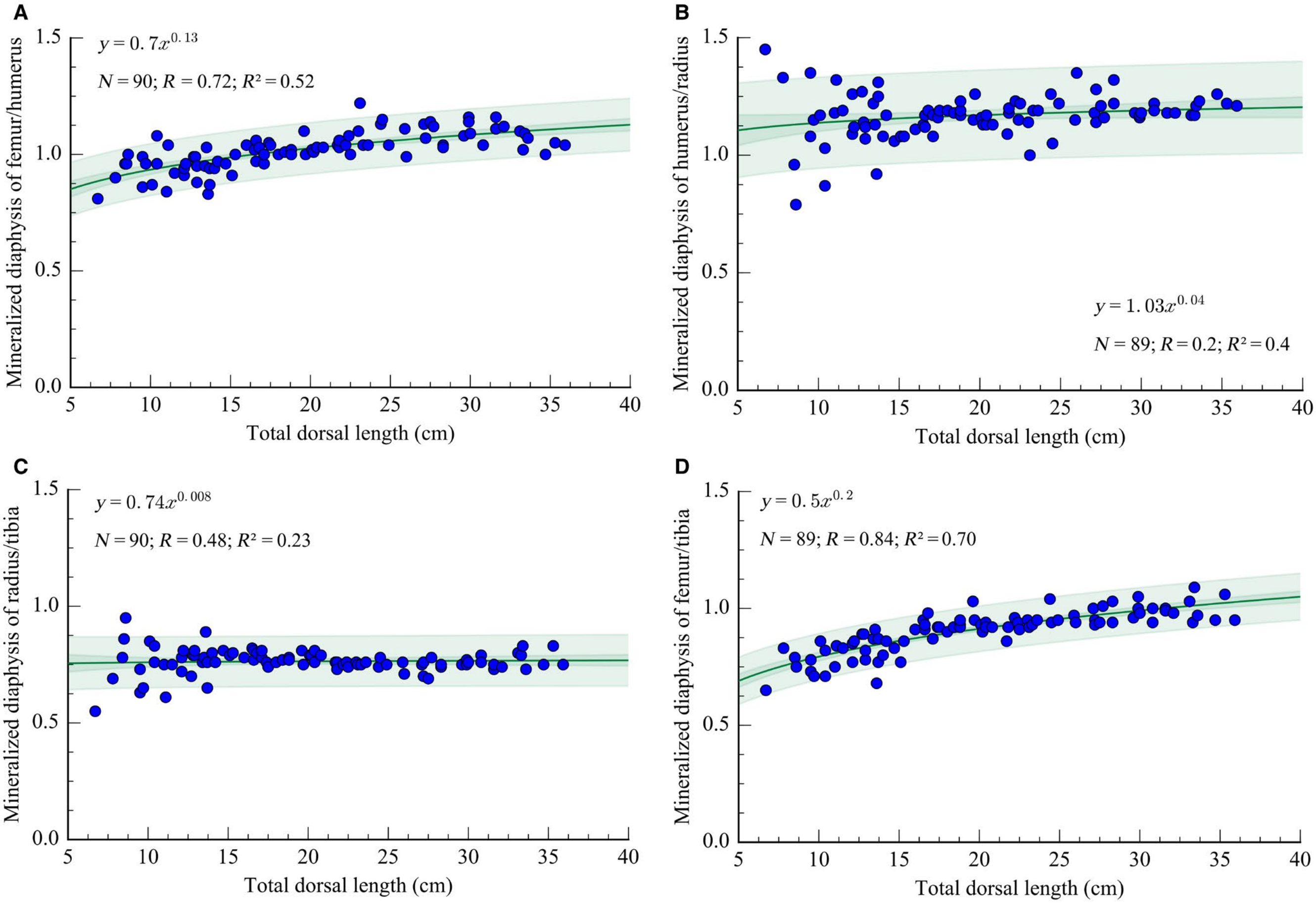

FIGURE 9 Allometric relationships between ratios of diaphyseal lengths of long bones in lowland paca (*Cuniculus paca*) embryos/fetuses (n = 102), correlated with the total dorsal length: (A) femur vs. humerus, (B) humerus vs. radius, (C) radius vs. tibia, and (D) femur vs. tibia. Prediction intervals (light green band) and confidence intervals (green dark band).

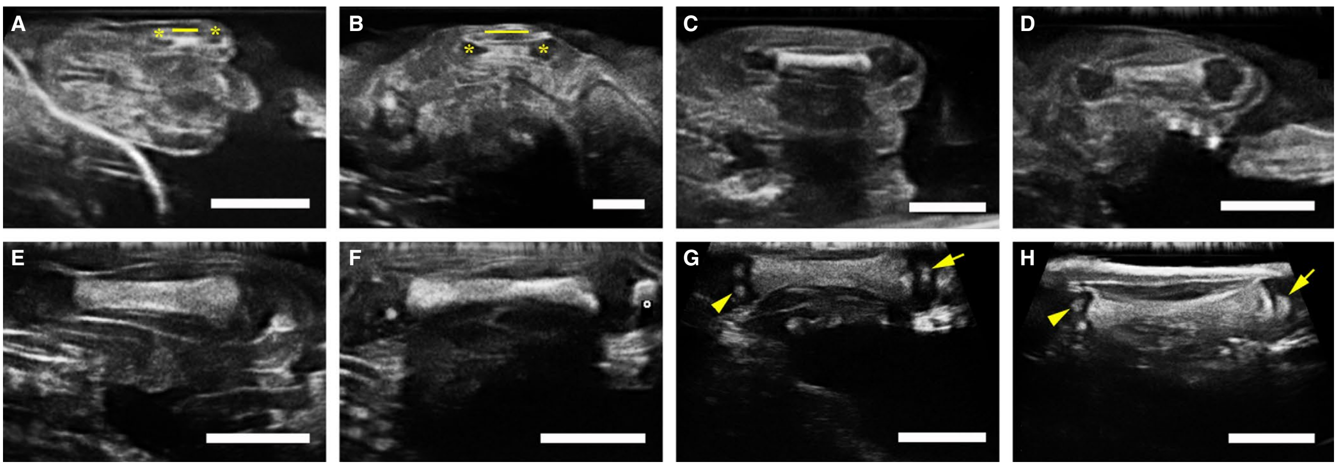

FIGURE 10 Ultrasound images of the humerus development in the lowland paca (*Cuniculus paca*): (A) fetus with total dorsal length (TDL) 7.8 cm (scale bar: 6 mm), (B) fetus with TDL 9.6 cm (scale bar: 5 mm), (C) fetus with TDL 12.8 cm (scale bar: 8 mm), (D) fetus with TDL 14 cm (scale bar: 10 mm), (E) fetus with TDL 19.6 cm (scale bar: 9 mm), (F) fetus with TDL 22.4 cm (scale bar: 11 mm), (G) fetus with TDL 27.1 cm (scale bar: 13 mm), and (H) fetus with TDL 35.9 cm (scale bar: 12 mm). Mineralized diaphysis (solid line), epiphysis (*), proximal epiphyseal center (arrowhead), distal epiphyseal center (arrow).

of the tibia (96 days, 64.4% GP), and the patella (116 days, 77.8%). This result contrasts with findings in the laboratory mouse, in which the development of the epiphyseal centers of the femur and tibia occurs in the first postnatal week, and while the development of the patella occurs in the second postnatal week (Patton and Kaufman, 1995;). In domestic carnivores, the distal epiphyses of the femur and proximal epiphyses of the tibia arise during the third postnatal week, and the patella is observed to be mineralized at the ninth postnatal week (Dyce et al. 2010).

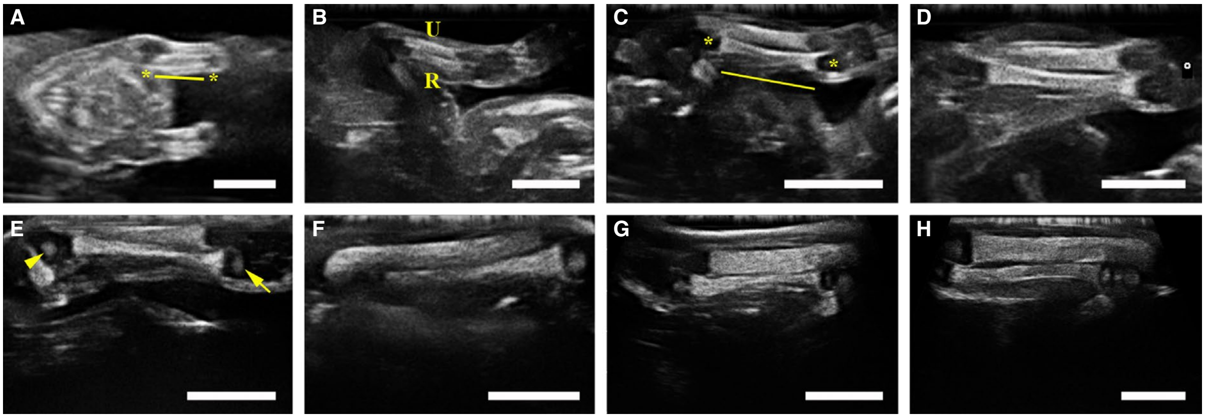

FIGURE 11 Ultrasound images of the radius development in the lowland paca (*Cuniculus paca*): (A) fetus with total dorsal length (TDL) 8 cm (scale bar: 3 mm), (B) fetus with TDL 12.2 cm (scale bar: 6 mm), (C) fetus with TDL 16.5 cm (scale bar: 12 mm), (D) fetus with TDL 18 cm (scale bar: 11 mm), (E) fetus with TDL 22.2 cm (scale bar: 11 mm), (F) fetus with TDL 25.9 cm (scale bar: 11 mm), (G) fetus with TDL 27.1 cm (scale bar: 11 mm), and (H) fetus with TDL 34.7 cm (scale bar: 9 mm). Mineralized diaphysis (solid line), epiphysis (*), proximal epiphyseal center (arrowhead), distal epiphyseal center (arrow), radius (R), ulna (U).

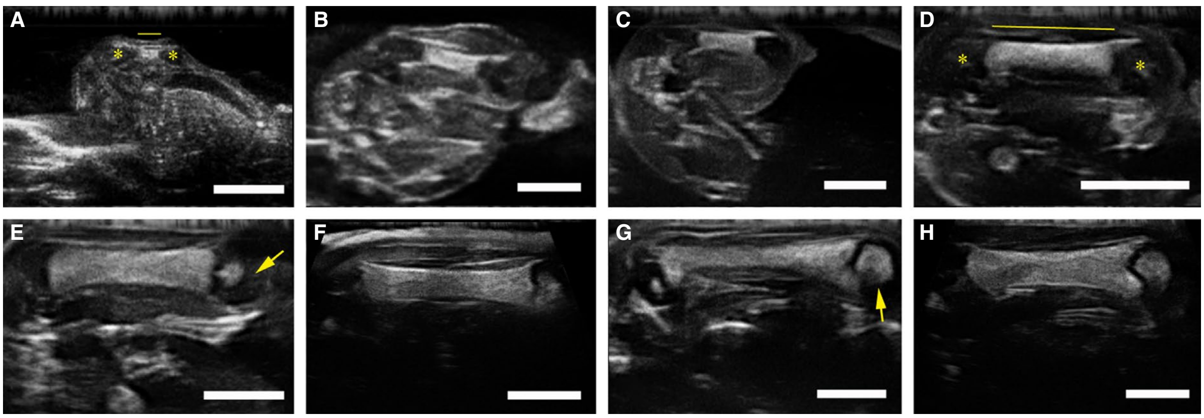

FIGURE 12 Ultrasound images of the femur development in fetuses of lowland paca (*Cuniculus paca*): (A) 6.9 cm total dorsal length (TDL; scale bar: 5 mm), (B) 10.4 cm TDL (scale bar: 5 mm), (C) 14 cm TDL (scale bar: 9 mm), (D) 16.5 cm TDL (scale bar: 10 mm), (E) 20.4 cm TDL (scale bar: 10 mm), (F) 22.2 cm TDL (scale bar: 10 mm), (G) 27.1 cm TDL (scale bar: 10 mm), and (H) 35.9 cm TDL (scale bar: 13 mm). Mineralized diaphysis (solid line), epiphysis (*), distal epiphyseal center (arrow).

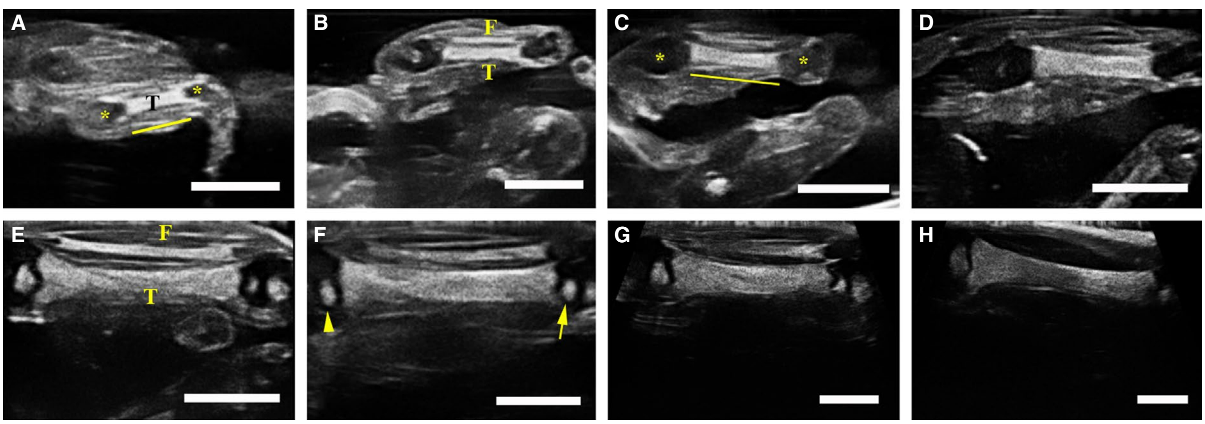

FIGURE 13 Ultrasound images of the tibia development in the lowland paca (*Cuniculus paca*): (A) fetus with total dorsal length (TDL) 8.6 cm (scale bar: 6 mm), (B) fetus with TDL 12.8 cm (scale bar: 8 mm), (C) fetus with TDL 14 cm (scale bar: 12 mm), (D) fetus with TDL 18.8 cm (scale bar: 12 mm), (E) fetus with TDL 22.2 cm (scale bar: 10 mm), (F) fetus with TDL 24.5 cm (scale bar: 11 mm), (G) fetus with TDL 27.1 cm (scale bar: 10 mm), and (H) fetus with TDL 35.9 cm (scale bar: 12 mm). Mineralized diaphysis (solid line), epiphysis (*), proximal epiphyseal center (arrowhead), distal epiphyseal center (arrow), tibia (T), fibula (F).

Regarding the bones of the forepaw and hindpaw, the latter had a more accelerated gestational growth than the former, reaching a greater length at the end of gestation. The lowland paca has seven bones in the carpus and eight in the tarsus (Oliveira et al. 2007). At 144 days (96.6% GP) in this species all the carpal and tarsal bones and secondary centers in the forepaw and the hindpaw, respectively, were mineralized. In contrast, altricial species, such as the marmoset, have incomplete hand and foot mineralization (Phillips, 1976). The paws are important structures for the postnatal capacity to swim, and the presence of long hindpaws (Samuels and Van Valkenburgh, 2008) and long calcaneal tubers is especially important (Carrizo et al. 2014). Similarly, pacas build burrows close to water bodies as a protection strategy (Beisiegel, 2006; Aquino et al. 2012; Figueroa-de-León et al. 2016; Leuchtenberger et al. 2017), from where they emerge and jump into the streams and rivers when threatened by predators (El Bizri et al. 2016).

At 143 days (96% GP), all primary and most secondary ossification centers of the lowland paca were mineralized. Furthermore, at 138 days (93% GP), this species had fully developed external characteristics, including complete covering pelage and open eyelids, which are important for independent temperature control, predator detection, and food recognition, and claws for handling seeds and digging burrows (El Bizri et al. 2017). In contrast, in altricial species, such as the marmoset, laboratory mouse, and domestic carnivores, most secondary ossification centers appear mineralized from 1–2 weeks after birth onwards (Patton and Kaufman, 1995; Dyce et al. 2010).

In the paca, all primary and most secondary ossification centers are mineralized in the final third of gestation. The early intrauterine development of the skeletal system in the paca may be related to the natural habits of this mammal during its postnatal life (El Bizri et al. 2017). This early development represents an important advantage for neonates, who can independently feed and escape from predators, by making burrows and swimming (Smythe, 1978; Aquino et al. 2009). The development of the skeletal and nervous systems is responsible for movement coordination, and it determines the animal's early motor capacity during the first hours after birth (Rengifo et al. 1996). While precocial species have mineralized patella and carpal/tarsal bones before birth and can walk and run shortly after birth, the mineralization of these components in altricial animals occurs after birth, resulting in a less developed postural capacity (Patton and Kaufman, 1995; Muir, 2000).

Between the offspring's second and third weeks of life, the female paca begins the weaning period and remains separate from the offspring for longer periods (Rengifo et al. 1996). The pups begin to ingest solids 21 days after birth and are weaned at approximately 42 days (Collet, 1981). Thus, the high energy level that is spent during pregnancy is compensated for by the shorter maternal time of breastfeeding and care of the offspring (Martin et al. 2005), allowing the mother to quickly recover her body condition for the next reproductive event (El Bizri et al. 2017).

The results of the present study contribute to a better understanding of the chronological events regarding the appearance and growth of the axial and appendicular skeletal structures in lowland pacas. This knowledge may be useful for gestational management and monitoring, serving as parameters for comparisons between precocial and altricial mammals and their life history strategies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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