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# Onset and Stages of Osteogenesis in the Rabbit (*Oryctolagus cuniculus*) using Diaphonisation

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## Abstract

**BACKGROUND:** Developmental anatomy is a prerequisite for a real understanding of gross anatomy and teratology. It is concerned with the sequential stages of embryonic and fetal development, beginning with fertilization. Moreover, it helps to describe developmental changes and abnormalities, heredity, sexing, and the appearance of vestigial structures. Powerful advancements in molecular genetic manipulation and assisted reproductive technologies are employed during embryo and fetal development, and these efforts have had profound impact on animal production worldwide.

**OBJECTIVES:** The present study was carried out to investigate the onset and stages of osteogenesis in rabbit (*Oryctolagus cuniculus*).

**METHODS:** Forty mature rabbits (33 does and 7 bucks) with a mean weight of 2 kg were included in this study. They mated twice at a ratio of three does to one buck on day zero (0). Mounting was carefully observed to ensure mating occurred and was confirmed by the physical examination of the vulva for swelling and redness, while pregnancy diagnosis was confirmed by ultrasonography and abdominal palpation. Embryos and fetuses were extracted via a ventral incision on the abdominal and uterine walls of one pregnant doe each day after euthanization with thiopental sodium at the dose of 31 mg/kg body weight intravenously. Diaphonisation was employed in studying osteogenesis.

**RESULTS:** Osteogenesis began at fetal day 21 (F21) using diaphonisation. In the skull, the bones of the splanchnocranium appeared before the neurocranium. The centrum and transverse processes were the first identifiable features of all different types of bone in the vertebral column to be ossified. The spinous process and the fusion of the sacrum and sternum were not detected throughout the gestation period. It was also observed that the epiphyseal and articular ends of none of the bones, neither in the fore nor hind limbs were ossified throughout gestation. Specifically, while the primary ossification centers of the middle phalanges were the last to appear in the manus and pes, all the carpals were absent, and only the calcaneous was evident in the tarsal bones.

**CONCLUSIONS:** We recorded the onset of osteogenesis and subsequent development throughout the gestation period of rabbits, which has added some valuable information to the documented literature on the bone development of this species.

**KEYWORDS:** Bones, Diaphonization, Ossification, Osteogenesis, Rabbit

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## Introduction

Rabbit (*Oryctolagus cuniculus*), commonly known as the European rabbit, is a representative of the leporidae family which constitutes a group of the order Lagomorpha along with the Hare and Pika (Fontanesi, 2021). It has been introduced on every continent except Antarctica and is known worldwide as a wild prey animal and a domesticated form of livestock as a pet adapting to variable environments, temperatures, and conditions (Dickman and Newsome, 2015). Its effect on cultures and ecologies in many areas of the world needs to be emphasized as it plays a part in food, clothing, companionship, and a source of artistic inspiration (Delibes and Delibes-Mateos, 2015). It has been widely kept as livestock since the ancient Rome era for its meat and is a lean source of high-quality protein and fur used in a broad range of coat colors, patterns, and lengths (Maertens and Coudert, 2006). It has also been and is continuously being utilized in laboratory works, such as the production of antibodies for vaccines, investigating human reproductive system toxicology, studying bronchial asthma, stroke prevention treatments, cystic fibrosis, diabetes, and cancer (Buseth and Saunders, 2015; Alhasson *et al*., 2022 ). With its reputation as a prolific breeder, selective breeding has generated a wide variety of rabbit breeds, with some strains being bred specifically as research subjects (Esteves *et al*., 2018).

Developmental anatomy is a prerequisite for a real understanding of gross anatomy and teratology (Smith *et al.,* 2020). It is concerned with the sequential stages of embryonic and fetal development, beginning with fertilization (McGeady *et al*., 2017). Moreover, developmental anatomy studies the growth and differentiation of an organism as it transforms from a single fertilized egg cell into a highly complex and independent living (Rao and Ramayya, 2013). It helps to describe developmental changes and abnormalities, heredity, sex determination, and the appearance of vestigial structures (Weiss and Refetoff, 2016). Powerful advancements in molecular genetic manipulation and assisted reproductive technologies are employed in the embryo and fetal development, and these efforts have had profound impacts on animal production worldwide (Hansen, 2020).

Bones are rigid organs that constitute a part of the endoskeleton of vertebrates (Laurin *et al*., 2021). They are living structures with blood vessels, lymphatic vessels, and nerves. Bones are subject to disease, can undergo repair, and adjust to stress-induced changes (Frandson *et al.,* 2010). The appearance of bone in the animal body requires a process called osteogenesis, which is the formation of true bone by the deposition of calcium salts in a matrix of osteoid tissue (Park *et al*., 2022). The process of osteogenesis has been studied mainly in birds using various techniques (Retnoaji *et al*., 2016). Diaphonisation, a technique of bone staining, has greatly enhanced the visualization of skeletal development in fish, frogs, turtles, birds, and small animals (Chitra and Sharon, 2020). In mammals, several researchers have stained cartilage and bone in rodent fetuses (McLeod 1980; Young *et al.*, 2000; Menegola *et al.,* 2002; Saralamoli *et al.,* 2018) to show the conditions of the skeleton in distinct embryonic stages and detect abnormalities in their development. Therefore, this study aimed to monitor the order of appearance and development of ossification centres in the prenatal rabbit.

## Materials and Methods

### Acquisition of Experimental Animals

Forty mature rabbits (33 does and 7 bucks) with a mean weight of 2 kg were utilized for this research. They were purchased and housed in a rabbit breeding house and were fed fiber-rich meals (tridax, carrots, starter's mash, and grower's mash) and *ad libitum* water prior to the study. The bucks and does were kept in different cages for three months to acclimatize before being paired at a ratio of three does to one buck on day zero (0) for mating. Mounting was carefully observed to ensure that mating occurred and was confirmed by the physical examination of the vulva for swelling and redness. Furthermore, pregnancy diagnosis was confirmed by ultrasonography and abdominal palpation. The bucks in each pairing were removed the next day. Each doe was then examined daily to further investigate osteogenesis.

### Extraction of Embryos and Fetuses

One pregnant doe was euthanized each day using thiopental sodium at the dose of 31 mg/kg body

weight intravenously (Mohammed *et al*., 2011). The does were placed on dorsal recumbency and dissected via a ventral incision on the abdominal wall starting from the umbilicus to a few inches above the vulva, exposing the uterus. Next, the uterus was incised to expose the embryos/fetuses to the placenta. The wall of the amniotic cavity was excised using small, pointed-end scissors to harvest the embryos/fetuses of an average litter size of four which were immediately preserved in 100% alcohol to harden the semi-solid content.

### Techniques for Studying Osteogenesis

Diaphonisation:

The embryos/fetus es were processed for staining after being removed from the uterus of the pregnant doe at each day of gestation. Afterwards, they were stained with Alizarin red and Alcian blue using the following modified staining protocol as described by Behringer *et al*., (2014).

Solutions prepared included:

- Alcian blue solution (0.03% Alcian blue powder+80% ethanol+20% glacial acetic acid)

For 200 mL=0.06 g of Alcian blue in 160 mL of 100% of ethanol and 40 mL of 100% glacial acetic acid.

- Alizarin red S solution (0.05% Alizarin red powder+1% KOH)

For 200 mL=10 mg (0.01 g) of Alizarin red+2 g KOH+200 mL $H_2O$

- 100% ethanol
- 100% acetone
- 1% KOH (10 g of KOH in 990 mL of $H_2O$)
- Glycerol

Staining procedure:

1) The embryo/fetus was placed in tap water for 1 h.
2) The embryo/fetus was then scalded in hot tap water (~65ºC) for 20-30 sec for the easier maceration of the tissue.

3) The embryo/fetus was skinned and eviscerated using forceps and was fixed in 95% ethyl alcohol (EtOH) overnight.

5) The fixed embryo/fetus was transferred to 100% acetone and was incubated overnight at room temperature.

6) It was stained for cartilage overnight at room temperature by placing in sufficient Alcian blue stain to cover the body.

7) Embryo/fetus was rinsed twice in 95% EtOH and was de-stained in 95% EtOH overnight at room temperature.

9) It was slightly cleared by placing in 1% KOH for 1 h and was counter-stained for bone in Alizarin Red stain for 4 h.

10) The embryo/fetus was cleared by placing in 1% KOH of decreasing strengths as 1% KOH for 2 days, 80:20 of 1% KOH to glycerol overnight at room temperature, 50:50 of 1% KOH to glycerol overnight at room temperature, and 20:80 of 1% KOH to glycerol overnight at room temperature. Next, the sample was stored indefinitely in 20:80 of 1% KOH to glycerol.

Afterwards, the stained and cleared embryo/fetus was photographed using a digital camera (Nikon COOLPIX A100) and the exposures printed. Stained bone deposits were noted and recorded as the primary centers of ossification in the fetus of the European rabbit. The chronological order of the emergence of these centers was also noted and recorded.

## Results

According to the findings of diaphonization, as an anatomical technique, no osteogenesis was observed during the first and second trimesters of the gestation period in the rabbit. By the third trimester, many of the primary loci of ossification were observed in bony parts of the body. However, not all parts of the bones were formed. F21 presented the earliest evidence of bone formation (Figure 1). F22 to F28 had few new ossification centers and considerable growth of existing ossification centers.

### Skull

The ossification centers of the mandible, frontal, and nasal bones were first formed in F21 (Plate 1). The ossification loci of other bones, such as the parietal, occipital, sphenoid, ethmoid, pterygoid, malar, lacrimal, and temporal bones, appeared subsequently in F22 (Figure 2) and increased considerably from F23 to F28 (Plates 3-9). However, specific features of each skull bone could not be

identified. Points of individual bone attachments (sutures) were visible as clear spaces (Figure 7). On the dorsal midline, the parieto-frontal suture was incomplete, creating a diamond-shaped space (thereby exposing the brain) that decreased in size as the fetus grew (Figure 7).

## Vertebral Column

Ossification centers were first identified on the centrum and transverse processes of all different types of vertebral bones on day 21 and augmented considerably in later fetal days. The spinous processes and other specific features, such as cranial and caudal articular processes, as well as the mammillary and accessory processes of each vertebra, were not visible throughout the gestation period, thereby exposing the dorsal surface of the spinal cord. Each vertebra was uniformly separated from its preceding and succeeding adjacent vertebrae, which indicated the presence of cartilage at such spaces. Fusion was not observed at any point in parts of the vertebrae, such as the sacrum and coccygeal vertebrae (Figures 3-9).

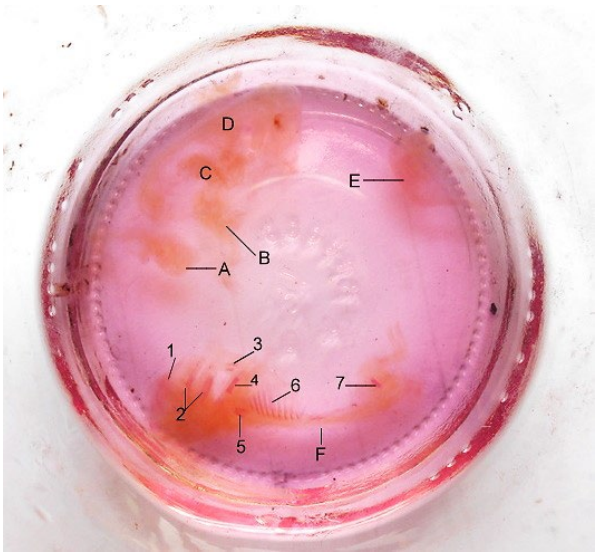



**Figure 1.** The appearance of diaphonised embryos and fetuses at days 16(B), 17(A), 18(C), 19(E), 20(D), and 21(F) of the gestation period in the rabbit shows ossification centers at day 21 only (Lateral view).

Ossification loci seen on the 1, Nasal bone; 2, Maxilla and Mandible; 3, Shaft of Ulna and radius; 4, Shaft of Humerus; 5, scapula; 6, shaft of ribs; 7, Shaft of Femur.

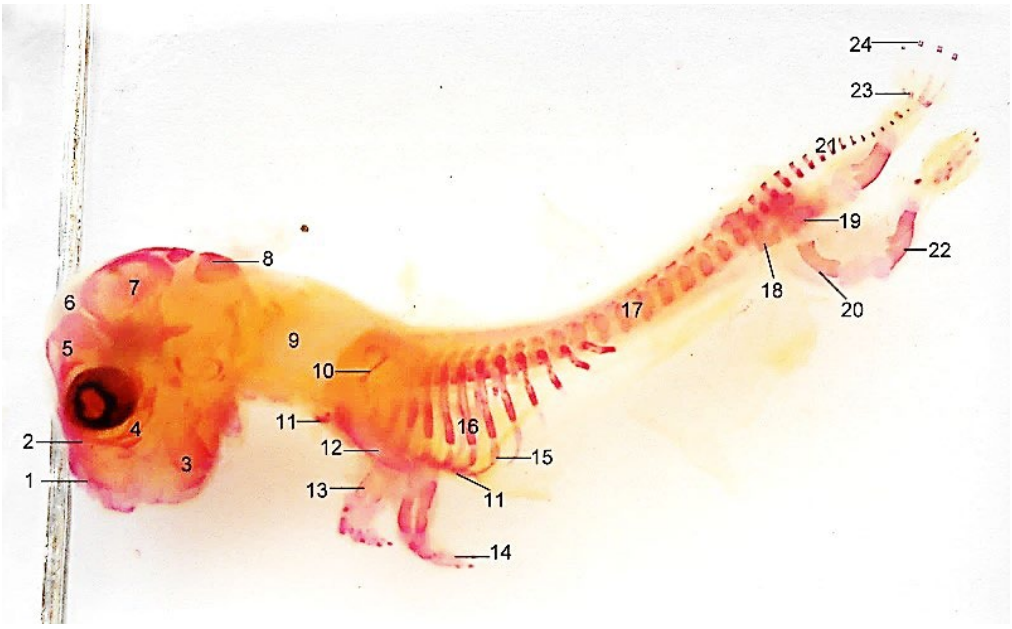



**Figure 2.** The appearance of diaphonised fetus at day 22 of gestation in the rabbit (*Oryctolagus cuniculus*) shows ossification centers on bones in different skeletal regions (Lateral view).

1, Nasal; 2, Maxilla; 3, Mandible; 4, Malar and lacrimal; 5, Frontal; 6, Parieto-frontal incisure; 7, Parietal; 8, Occipital; 9, Position of cervical vertebrae; 10, Scapula spine; 11, sternum; 12, Humerus; 13, Radis and Ulna; 14, Digits; 15, Coastal cartilage; 16, Sternal ribs; 17, Bodies and transverse processes of Vertebrae; 18, Ilium; 19, Ischium; 20, Femur; 21, Bodies of caudal vertebrae; 22, Tibia and Fibula; 23, Metatarsal; 24, Distal phalanx.

## Thorax

All types of ribs, including sternal, asternal, and floating, appeared simultaneously at the beginning of the third trimester. Ossification center was observed only on the shaft by day 21. Proximal (head, neck, and tubercle) and distal extremities of the ribs that form joints were not ossified. Bodies of sternebras uniformly spaced from each other appeared simultaneously from F22 with the growth of ossification centers as the fetus grows. Fusion of the sternum was not observed throughout this prenatal period.

## Forelimb

Osseous presentation of the forelimb was found simultaneously in the scapula, clavicle, humerus, radio-ulna, third phalanx, and metacarpals II, III, and IV by day 21. Loci of primary ossification were observed at the borders and spine of the scapula, shafts of the clavicle, humerus, and radio-ulna. Metacarpals II, III, and IV, and third phalanx were evident at day 22. Except for the scapula that presented distinguishing features, such as the spine, acromion, and supra and infraspinous fossa, those of the long bones were absent. Ossification loci of metacarpals I and V and proximal phalanx appeared in F26, while the middle phalanx was seen in F28. The ossification loci in the carpals and the proximal and distal extremities of long bones were not visible throughout the prenatal period.

## Hind Limb

The initial sequence of primary ossification in the hind limb was similar to that of the forelimb. Ossification occurred simultaneously in the pelvic bone, femur, tibia-fibula, tarsal, metatarsals II, III, and IV, and distal phalanx on day 22. Loci of ossification were noted at the shafts of the ilium, ischium, and long bones. Loci of the tarsal bone appeared as a single dot, which initially increased considerably with fetal growth. Its location suggested that it belonged to the calcaneus. Ossification loci of metatarsals I and V and proximal and middle phalanx appeared in F26. Loci of ossification in the pubis and proximal and distal extremities of long bones were not visible throughout this prenatal period.

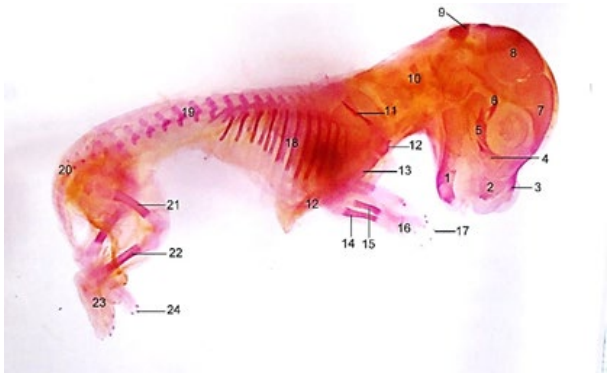


**Figure 3.** The appearance of diaphonised fetus at day 23 of the gestation period in the rabbit (*Oryctolagus cuniculus*) showing ossification centers on bones of different skeletal regions (Lateral view).

1, Mandible; 2, Maxilla; 3, Nasal; 4, Lacrimal; 5, Malar; 6, Temporal; 7, Frontal; 8, Parietal; 9, Occipital; 10; Cervical vertebrae; 11, Scapula spine; 12, Sternum; 13, Humerus; 14, Radius; 15, Ulna; 16, Digits; 17, Distal phalanx; 18, Sternal and floating ribs; 19, Bodies and transverse processes of vertebrae; 20, Bodies of Caudal vertebrae; 21, Femur; 22, Tibia; 23; Metatarsal; 24, Distal phalanx.

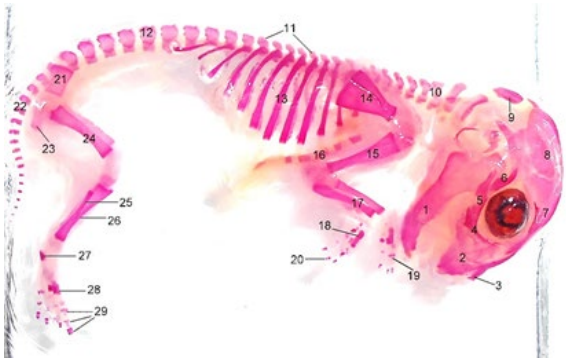


**Figure 4.** The appearance of diaphonised fetus at day 24 of the gestation period in the rabbit (*Oryctolagus cuniculus*) showing ossification centers on bones of different skeletal regions (Lateral view).

1, Mandible; 2, Maxilla and Premaxilla; 3, Nasal; 4, Lacrimal; 5, Malar; 6, Frontal; 7, Temporal; 8, Parietal; 10, Occipital; 11, Cervical vertebrae; 12, Scapula; 13, Humerus; 14, Ulna; 15, Radius; 16, Metacarpal; 17, Distal phalanx of fore limb; 18, Ribs; 19, Body of vertebrae; 20, Transverse process of vertebrae; 21, Ilium; 22, Ischium; 23, Femur; 24, Tibia; 25, Fibula; 26, Distal phalanx of hind limb.

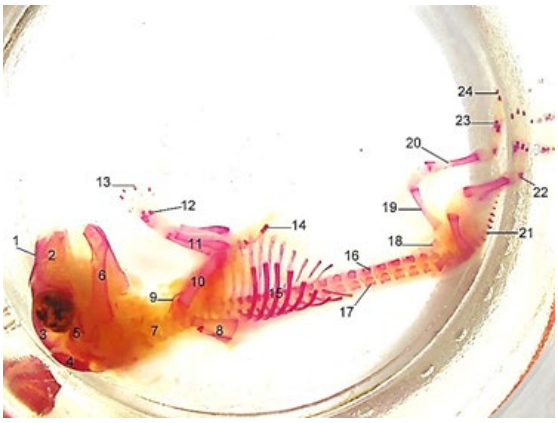


**Figure 5.** The appearance of diaphonised fetus at day 25 of the gestation period in the rabbit (*Oryctolagus cuniculus*) showing ossification centers on bones of different skeletal regions (Lateral view).
1, Nasal; 2, Maxilla and Premaxilla; 3, Frontal; 4, Parietal; 5, Temporal; 6, Mandible; 7, Cervical vertebrae; 8, Scapula; 9, Clavicle; 10, Humerus; 11, Ulna and Radius; 12, Metacarpal; 13, Distal phalanx of forelimb; 14, Sternum; 15, Sternal and floating ribs; 16, Bodies of vertebrae; 17, Transverse process of vertebrae; 18, Ilium; 19, Femur; 20, Tibia and Fibula; 21, Caudal vertebrae; 22, Calcaneus; 23, Metartarsal; 24, Distal phalanx of hind limb.

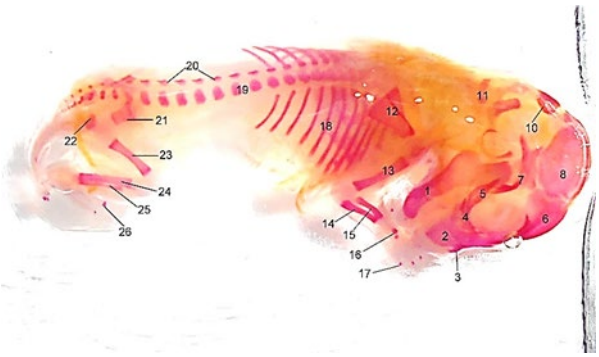
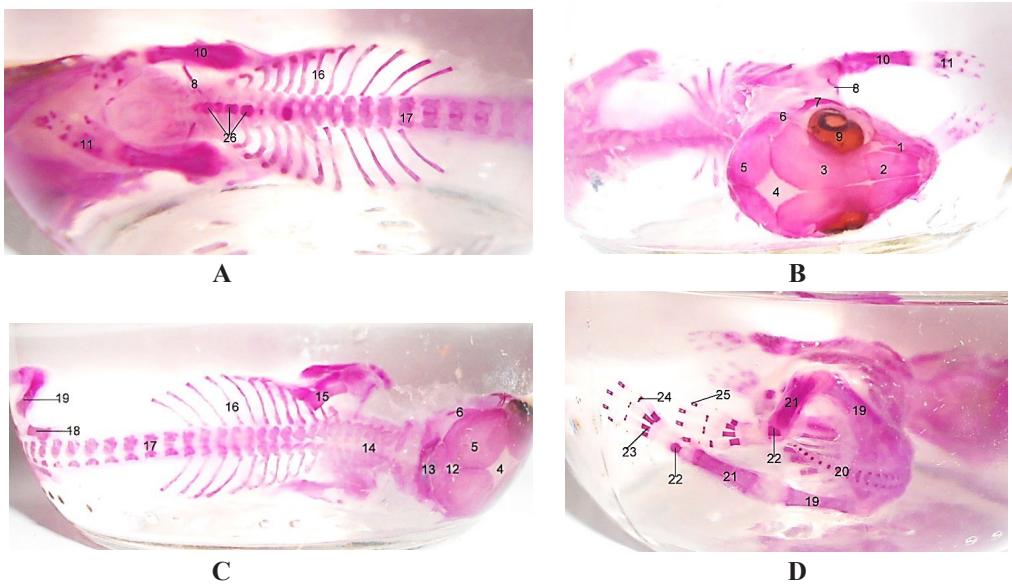


**Figure 6.** The appearance of diaphonised fetus at day 26 of the gestation period in the rabbit (*Oryctolagus cuniculus*) showing ossification centers on bones of different skeletal regions (Lateral view).
1, Mandible; 2, Maxilla and Premaxilla; 3, Nasal; 4, Lacrimal; 5, Malar; 6, Temporal; 7, Frontal; 8, Parietal; 9, Occipital; 10, Cervical vertebrae; 11, Thoracic vertebrae; 12, Lumbar vertebrae; 13, Sternal and floating ribs; 14, Scapula; 15, Humerus; 16, Sternum; 17, Ulna and Radius; 18, Metatarsal; 19, Proximal phalanx of forelimb; 20, Distal phalanx of forelimb; 21, Ilium; 22, Caudal vertebrae; 23, Ischuim; 24, Femur; 25, Fibula; 26, Tibia; 27, Calcaneus; 28, Metatarsal; 29, proximal and distal phalanx of hind limb.



**A** **B**

**C** **D**

**Figure 7.** The appearance of diaphonised fetus at day 27 of the gestation in the rabbit (*Oryctolagus cuniculus*) showing ossification centers on bones of different skeletal regions, Ventral (A), Rostral (B), Dorsal (C), and Caudal (D) views.

1, Maxilla; 2, Nasal; 3, Frontal; 4, Diamond-shaped Parieto-frontal incisure; 5, Parietal; 6, Temporal; 7, Malar; 8, Clavicle; 9, Orbital space with developing eye; 10, Ulna and Radius; 11, Forelimb digits; 12, Parieto-occipital incisure; 13, Occipital; 14, Cervical vertebrae; 15, Scapula; 16, Ribs; 17, Lumbar vertebrae; 18, Ilium; 19, Femur; 20, Caudal vertebrae; 21, Tibia and Fibula; 22, Calcaneus; 23, Metatarsal II-V; 24, Proximal phalanx of hind limb; 25, Distal phalanx of Hind limb; 26, Sternabrae.

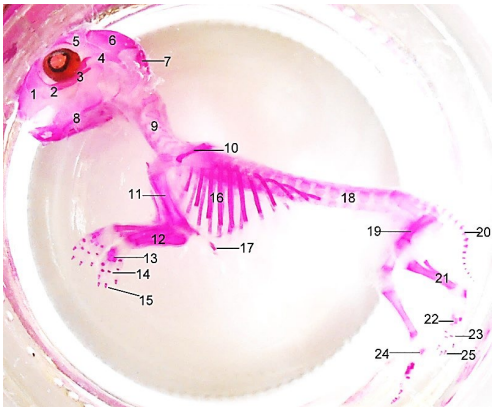



**Figure 8.** The appearance of diaphonised fetus at day 27 of the gestation in the rabbit (*Oryctolagus cuniculus*) showing ossification centers on bones of different skeletal regions (Lateral view).
1, Maxilla; 2, Lacrimal; 3, Malar; 4, Temporal; 5, Frontal; 6, Parietal; 7, Occipital; 8, Mandible; 9, Cervical vertebrae; 10, Scapula; 11, Humerus; 12, Ulna and Radius; 13, Metacarpal; 14, Proximal phalanx of fore limb; 15, Distal phalanx of fore limb; 16, Ribs; 17, Xyphoid process of Sternum; 18, Lumbar vertebrae; 19, Femur; 20, Caudal vertebrae; 21, Tibia and fibula; 22, Metatarsal; 23, Proximal phalanx of hind limb; 24, Calcaneus; 25, Distal phalanx of Hind limb.

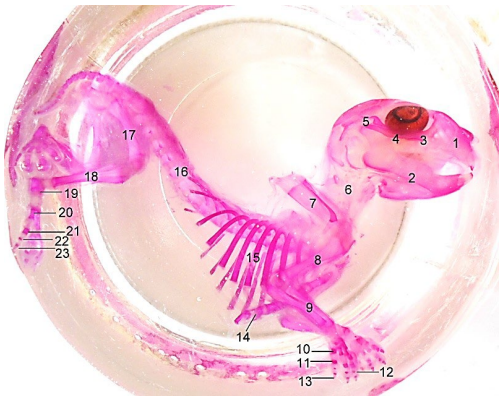



**Figure 9.** The appearance of diaphonised fetus at day 28 of the gestation period in the rabbit (*Oryctolagus cuniculus*) showing ossification centers on bones of different skeletal regions, lateral view.
1, Maxilla; 2, mandible; 3, Lacrimal; 4, Malar; 5, Temporal; 6, Cervical vertebrae; 7, scapula; 8, humerus; 9, Ulna and Radius; 10, Metacarpal; 11, Proximal phalanx of forelimb; 12, Middle phalanx of forelimb; 13, Distal phalanx of forelimb; 14, Sternum; 15, Ribs; 16, Lumbar vertebra; 17, Femur; 18, Tibia and Fibula; 19, Calcaneus; 20, Metatarsal; 21, Proximal phalanx of hind limb; 22, Middle Phalanx of Hiond limb; 23, Distal phalanx of Hind limb.

## Discussion

Many of the primary loci of ossification were observed in the bony parts of the body during the third trimester. However, some parts of the bones were not formed. The earliest evidence of bone formation was recorded using diaphonisation on F21. This finding further buttresses the point documented by (Hill, 2020), who reported that the skeletal development of rabbits has three stages, namely the notochordal, cartilaginous, and bone development stages. It further reveals that each of these stages is not a further development of the preceding stage but an independent one, which displaces its predecessor. Therefore, the cartilaginous skeleton does not arise from the notochord but outside this and is independent of it. It gradually displaces and obliterates the notochord to be displaced by the bony skeleton.

The loci of ossification in the skull were found on day 21. This finding agreed with the results of Mohammed and Saleh (2012), who performed a morpho-histological study on the supraoccipital bone development of domestic rabbit fetus es. They reported that the primary ossification centres of supraoccipital appeared first on day 22 of gestation. The bones of splanchnocranium (mandible, frontal, and nasal) appeared before the neurocranial bones. Although they are both formed by intramembranous ossification, skull bones originate from different sources. In general, the front of the head is derived from the neural crest, while the back of the head is derived from a combination of neural crest cells and head mesoderm (Hytell *et al.,* 2010).

The mentioned results infer that the neural crest cells, which form the facial skeleton of a vertebrate, move at a faster rate to the rostral region of the embryo when forming the skull before the caudal region. The rate and direction of the movement of neural crest cells strongly influence the shape of facial skeleton (Hytell *et al.,* 2010). The inability to identify specific features, such as the processes and foramina of these bones during the skull development, may be because they appear post-partum and after further development. The diamond-shaped incomplete space noted on the dorsal surface between the parietal and frontal bone gave room for further growth and development of the skull. Its closure,

along with other sutures post-natally, influences the final shape of the skull.

Ossification centers were first identified on the centrum and transverse processes of all different types of vertebral bones, namely cervical, thoracic, lumbar, sacral, and coccygeal bones and increased considerably in later fetal days. This finding agrees with the reports of Hytell *et al.* (2010), Rao and Ramayya (2013), and Mcgeady *et al.* (2017) on the general formation of the vertebral column due to resegmentation, a process where densely packed caudal half of one sclerotome joins with the loosely packed cranial half of the next to form the centrum (body) of a vertebra and subsequently the transverse processes and neural arches. The appearance of separate centers for the centrum and arch of the vertebral bones observed in the current study is in line with reports in domestic fowl (Rumpler, 1962; Hogg, 1980). In addition, our results showed that the spinous processes and other specific features of each vertebra were not visible throughout the gestation period, thereby, exposing the dorsal surface of the spinal cord. This was also reported in altricial domestic animals, such as cat and dog, where these ossification centres do not fuse dorsally before birth. Secondary ossification centres appear during postnatal development in the periphery of the body to form the epiphyses and distal parts of the transverse processes (Mcgeady *et al.,* 2017). The lack of the fusion of the sacrum and sternum suggested that fusion only took place via further postnatal ossification in this species, which obliterated the cartilages to form the common unpaired bodies.

Ossification centers of all ribs appeared simultaneously on the shaft at the start of the third trimester, while proximal (head, neck, and tubercle) and distal extremities of the ribs that form joints were not ossified. This is mainly due to endochondral ossification, which starts in the shaft. This finding differed from a report on the mice by Sawad and Al-Asadi (2006) that revealed ossification on day 16 at the proximal extremity while the distal part remained cartilaginous. However, in avian species (Fell, 1925; Fujioka, 1955; Hogg, 1980), separate ossification centers were developed for each vertebral and sternal segment of the ribs. These differences may be attributed to species and gestation period differences.

Similar to bone development in most documented mammals and aves, primary ossification centers arise in the middle of the diaphyseal cartilage of long rabbit bones. This indicated that limb bone development is by mesenchymal cell condensation into forms that approximate the various bones of the limbs (Hytell *et al.,* 2010). These mesenchymal templates are replaced by cartilaginous models, which subsequently undergo endochondral ossification and form the bones of the limb (Sadler, 2012).

The presence of ossification loci at the borders and spine of the scapula on day 21 and the ossification of distinctive features, such as acromion and fossae, demonstrated in the present study differ slightly from reports on the laboratory mouse by Patton and Kaufman (1994). These researchers observed the primary center of the scapula on day 15 in the blade and spine. Its borders were only ossified post-natally. However, the current study was consistent with Elgendy *et al.* (2018), who reported the appearance of the primary ossification center within the scapula on day 21. Two primary ossification centers were found within the center of the scapular blade and in the scapular spine, and ossification extended bi-directionally on both sides.

We observed the appearance and subsequent growth of the primary ossification center along the shaft of the forelimb bones without the ossification of proximal and distal extremities and carpals throughout the prenatal life. The latter finding agrees with bone developmental studies on laboratory mice (Sawad and Al-Asadi, 2006), domestic fowl (Sawad *et al.,* 2009), and Guinea fowl (Salami *et al.,* 2012). Patton and Kaufman (1994) reported that carpals ossify on day 7 postnatal in the mouse. Primary loci of ossification in the forelimb and hind limb were observed on day 21 of the current study, which is to some extent in line with the research of Ahmed *et al.* (2015) on the histological sequences of long bone development in New Zealand rabbits. They reported the establishment of the medullary cavity by day 21 with no sign of calcification in the bone collar and a primary ossification center within diaphysis on day 24 of embryonic development. The appearance of the ossification loci of metacarpals and metatarsals II, III, and IV two days earlier than metatarsals I and V suggested that length may be a determining factor

in ossification because the former are usually longer than the latter. Ossification loci of the distal or third phalanges appeared before the proximal and middle phalanges. This finding may be attributed to the subsequent development of claws, which are endoskeletons on the distal phalanges.

In the present study, the pubis was the only bone of the ossa coxarum that was not ossified during gestation. Moreover, ossification loci for the tarsus appeared as a single dot initially, followed by a considerable increase with fetal growth. The location suggested that it belonged to the calcaneus (fibular tarsal bone). This result agreed with and differed from reports on the tarsal and pubic bones of the laboratory mouse (Patton and Kaufman 1994). They indicated that pubis appeared later than ischium and ilium during gestation, while only talus and calcaneus ossified during prenatal gestation.

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## Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Kenechukwu Tobechukwu ONWUAMA, James Oliver NZALAK, Tavershima DZENDA, Joseph HAMBOLU, and Sulaiman Olawoye SALAMI.The first draft of the manuscript was written by Kenechukwu Tobechukwu ONWUAMA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Ethical Statement

The procurement of specimen carcass and experiments conformed to the European Convention for the protection of Vertebrate animals used for scientific purposes (Council of Europe No. 123, Strasbourg 1985). The experimental protocols described were approved by the Ethics review committee for Animal experimentation of Ahmadu Bello University, Zaria, Nigeria with approval number ABUCAUC/2020/33.

## Conflict of Interest

The authors declare they have no conflict of interest associated with this work.

## Data Availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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