

PRENATAL DEVELOPMENT OF CENTERS OF OSSIFICATION IN THE LIMB BONES OF CHABRO CHICKEN

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ABSTRACT

The present study was conducted on the bones of fore limb and hind limb of 63 apparently healthy embryos/fetuses of Chabro chicken irrespective of sex and aged from day one to 21 days of incubation, three chicks of each day to determine the day of appearance of center of ossification. The fertilized eggs of different incubation days were collected from the poultry farm of DUVASU, Mathura. The embryos/fetuses were harvested carefully and fixed in 10% Neutral Buffered Formalin. These embryos/fetuses were stained with Alizarin Red-S or Alcian blue Alizarin Red-S double staining methods to document the appearance of centers of ossification in different bones of forelimb and hind limb. The study revealed that the onset of ossification started simultaneously in the bones of fore limb and hind limb of embryos. On 9th day of incubation, the centers of ossification were observed in the clavicle, coracoid, scapula, humerus, radius, ulna and metacarpals of forelimb whereas, in hind limb the centers of ossification was observed for femur, tibia, fibula and all three metatarsal bones on the same day of incubation. On 17th day of incubation the clavicles of either side were completely ossified and fused distally to form bonyhypocondrium. In hind limb the centers of ossification for femur, tibia, fibula and all three metatarsal bones were first noticed on 9th day of incubation. Whereas, the centers of ossification for ilium and ischium were first observed on 13th day of incubation but for pubic bone it was observed on 14th day of incubation.

Keywords: Chabro chicken, Center of ossification, Fore limb, Hind limb

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Chabro chicken is a rural meat type bird and is a cross bred of Barred Plymouth Rock and Red Cornish birds. They are active, large in built, pugnacious in nature with contrast color. They are able to save themselves from their predators under free range and activeness and are adapted to all climatic zones of Indian subcontinent; hence they are recognized for backyard farming. They gain an average weight of 1.6- 1.7 Kg in 60 days (CPDO, 2017).

The skeleton of the fowl comprised of axial and appendicular components. The bones of appendicular skeleton include the humerus, radius, ulna and carpometacarpus in forelimb and femur, tibiotarsus and tarsometatarsus in hind limb. The forelimb of fowl is transformed into the wing. There are two major modes of bone formation viz; Intramembranous ossification and endochondral ossification. The bones of fore and hind limb are formed by the process of endochondral ossification where mesenchymal cells differentiate into cartilage, and this cartilage is later replaced by bone. Literatures are available on the development of appendicular skeleton in Kuttanad duck embryos (Firdous *et al.*, 2016), Japanese quail embryo (Nakane and Tsudzuki, 1999) and chick embryos (Sawad *et al.*, 2009).

A systematic study on the prenatal development of appendicular skeleton (fore limb and hind limb) of Chabro chicken has not been done so far. Hence the present study

has been conducted to document the appearance of centers of ossification in different bones of fore limb and hind limb of Chabro chicken.

MATERIALS AND METHODS

The study was conducted on the fore limb and hind limb of 63 apparently healthy embryos/fetuses of Chabro chicken irrespective of sex, aged from day one to 21 days of incubation, three chicks of each day. The fertilized eggs of different incubation days (0-21 days) were collected from the poultry farm of DUVASU, Mathura. The embryos/fetuses were harvested carefully and fixed in 10% neutral buffered formalin. The embryos/foetuses were stained with Alizarin Red-S or Alcian blue Alizarin Red-S double staining methods as practiced in the anatomy department of College of Veterinary Science, DUVASU, Mathura.

Procedure for Alizarin Red-S Staining: The fixed specimens were washed overnight in running tap water to remove the traces of fixative. The skin and the soft tissues as far as possible were removed from the fetuses. 3% potassium hydroxide (KOH) solution was prepared in distilled water. Fetuses were kept in this solution for 3-5 days for maceration under regular monitoring. The soft macerated tissues were removed gently and regularly. Then, 0.003% Alizarin Red-S solution in 1% potassium hydroxide was prepared in distilled water. The fetuses

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were kept in this solution for 3-4 days under regular monitoring for maceration and staining. Further, the clearing solution (Glycerine +1% KOH+ Benzene) was prepared and the well stained foetuses were kept in this solution for 2-4 days. Gradually the amount of glycerine was increased and after complete clearing of specimen, it was stored in 100% glycerine solution until examination. This technique was used to study the appearance of centres of ossification and extent of calcification, which was indicated by deep red coloration in the skeleton. The uncalcified areas and other soft tissue remained unstained.

Procedure for Alizarin Red- S and Alcian Blue combined staining: First of all, there is overnight washing of specimen was done in running tap water. The specimen was placed in absolute ethanol for 7 days. Gently the soft tissue was removed and specimen was put in 0.01% Alcian blue solution for 2 days. Rehydration of specimen was done by placing them in descending grades of alcohol starting from 95% to 15% alcohol (2 hours in each solution). Washing of specimen was done in three changes of distilled water (1 hour in each). The specimen was kept in 2% potassium hydroxide (KOH) solution for 4-5 days for maceration. The specimen was then placed in 0.003% Alizarin Red- S solution in 1% KOH for 4-5 days. The specimen was transferred in clearing solution (Glycerine + KOH + Benzene). Gradually the concentration of Glycerine was increased up to 100% and the specimens were stored in this solution till examination. The bony (calcified) tissue stained red while the cartilages stained Blue. Other tissue remains unstained.

RESULTS AND DISCUSSION

Fore Limb: Results have been summarized in Table 1 and compared with standard information available in different birds' embryo. The limb buds for the fore limb appeared on 4th day of incubation in Chabro chick embryos, which further increased in size on 5th day of incubation. On 6th day of incubation, the differentiation of digits took place and the division between first and 2nd digits was remarkably visible. On 7th day of incubation, complete cartilaginous miniature of fore limb was formed, which was more appreciable on 8th day of incubation. Nakane and Tsuzuki (1999) and Nakamura *et al.* (2019) reported the cartilaginous forelimb bones of Japanese quail and Blue breasted quails, respectively on 6th day of incubation. On 9th day of incubation, the centers of ossification were observed in the clavicle, coracoid, scapula, humerus, radius, ulna and metacarpals of Chabro chick embryo (Fig. 1). Firdous *et al.* (2016) reported similar observations in 9 days old embryos of Kuttanad ducks while Onyeanusi *et al.* (2008) and Sawad *et al.*

(2009) in the chick embryos and the Guinea fowl embryos, respectively observed it on 10th day of incubation. In Japanese quail (Nakane and Tsuzuki; 1999) and also in the Blue breasted quails (Nakamura *et al.*, 2019) embryos the ossification of scapula and coracoid occurred on 8th and 9th days. Humerus, radius and ulna ossified on 7th day of incubation in both types of quails (Nakane and Tsuzuki, 1999; Nakamura *et al.*, 2019). The clavicle ossification occurred on 8th day in embryos of Japanese quail (Nakane and Tsuzuki; 1999). In grey breasted guinea fowl embryos, the ossification was delayed. The scapula and coracoids ossified on 13th day while the clavicle on 14th day. The long bones (humerus, radius and ulna) ossified a day earlier i.e., on 12th day. In clavicle of Chabro chick embryos, two centers of ossification were observed, one in the center of the shaft of the clavicle and the other in the ventral extremity i.e. hypocleidium (Fig. 2). The hypocleidium of either side of clavicle were not fused till 9th day of incubation. The periphery of scapula stained darker whereas, its central part took lighter color of Alizarin red-S stain, indicating more calcium deposition at the periphery. The ossification centers were observed in the middle of the shaft of coracoids, humerus, radius and ulna. On 10th day of incubation, the area of ossification gradually increased with the increase in the length and size of the bones and the intensity of staining is further increased. In the clavicles of either side the ossification reached up-to the proximal and distal ends of the bone but the proximal extremity near the shoulder joint as well as the distal extremity near the hypocleidium were not ossified completely. Therefore, the distal extremities of the clavicles of either side were not fused together up-to the 12th day of incubation. On 17th day of incubation, the clavicles of either side were completely ossified and fused distally to form the bony hypocleidium. The extremities of coracoid bones were still unossified (Fig. 3).

On 21st day of incubation, the diaphysis of all the long bones were ossified. The centers of ossification for the extremities of long bones were not noticed even on 21st day. The centers of ossification for the carpals were not observed in prehatched Chabro chicken as has also been reported in chick embryos (Sawad *et al.*, 2009), Grey breasted guinea fowl (Salami *et al.*, 2012), Kuttanad duck (Firdous *et al.*, 2016), Blue breasted quail (Nakamura *et al.*, 2019) and Japanese quail (Nakane and Tsuzuki, 1999) embryos. The centers of ossification for the carpals were also not observed in prehatched chick embryo (Sawad *et al.*, 2009), Grey breasted guinea fowl (Salami *et al.*, 2012), Kuttanad duck (Firdous *et al.*, 2016), Blue breasted quail (Nakamura *et al.*, 2019) and Japanese quail (Nakane and Tsuzuki, 1999) embryos.

Hind limb: Results have been summarized in Table 2 and compared with literature in another avian embryo. On 4th day of incubation the limb buds appeared for the hind limb of embryos. It became more developed on 5th day of incubation. On 6th day of incubation, division between second and third digits was clearly discernible. On 7th day of incubation the cartilaginous miniature of hind limb was laid. Cartilagenous hind limb inlay have been reported in 6 days old embryos of Japanese and Blue breasted quail (Nakane and Tsudzuki, 1999) and (Nakamura *et al.*, 2019) embryo. On 9th day of incubation the centers of ossification were first noticed in the center of the diaphysis of femur, tibia and fibula as also reported in the embryos Guinea fowl (Onyeanusi *et al.*, 2008), Grey breasted Guinea fowl (Salami *et al.*, 2012), Kuttanad duck (Firdous *et al.*, 2016), Blue breasted quail (Nakamura *et al.*, 2019) and Japanese quail (Nakane and Tsudzuki, 1999). On 10th day of incubation the area of ossification gradually increased with the increase in the length of long bones. On 13th day of incubation the centers of ossification were seen in the ilium and ischium bones (Fig. 4). Further the center of pubic bone ossification appeared only on 14th day (Fig. 5). Sawad *et al.* (2009) recorded the ossification of all three bones of the pelvic girdle of chick embryos on 14th day. In Grey breasted Guinea fowl embryo ilium and ischium ossified on 13th day but pubis ossified on 19th day (Salami *et al.*, 2012). In Kuttanad duck (Firdous *et al.*, 2016), Blue breasted quail (Nakamura *et al.*, 2019) and Japanese quail (Nakane and Tsudzuki, 1999) embryos the ossification

started at an earlier date i.e. between 9-10 days. With further incubation, the area of ossification increased in the ilium, ischium and pubis bones (Fig. 5). On 17th day of incubation, the fibula was completely ossified.

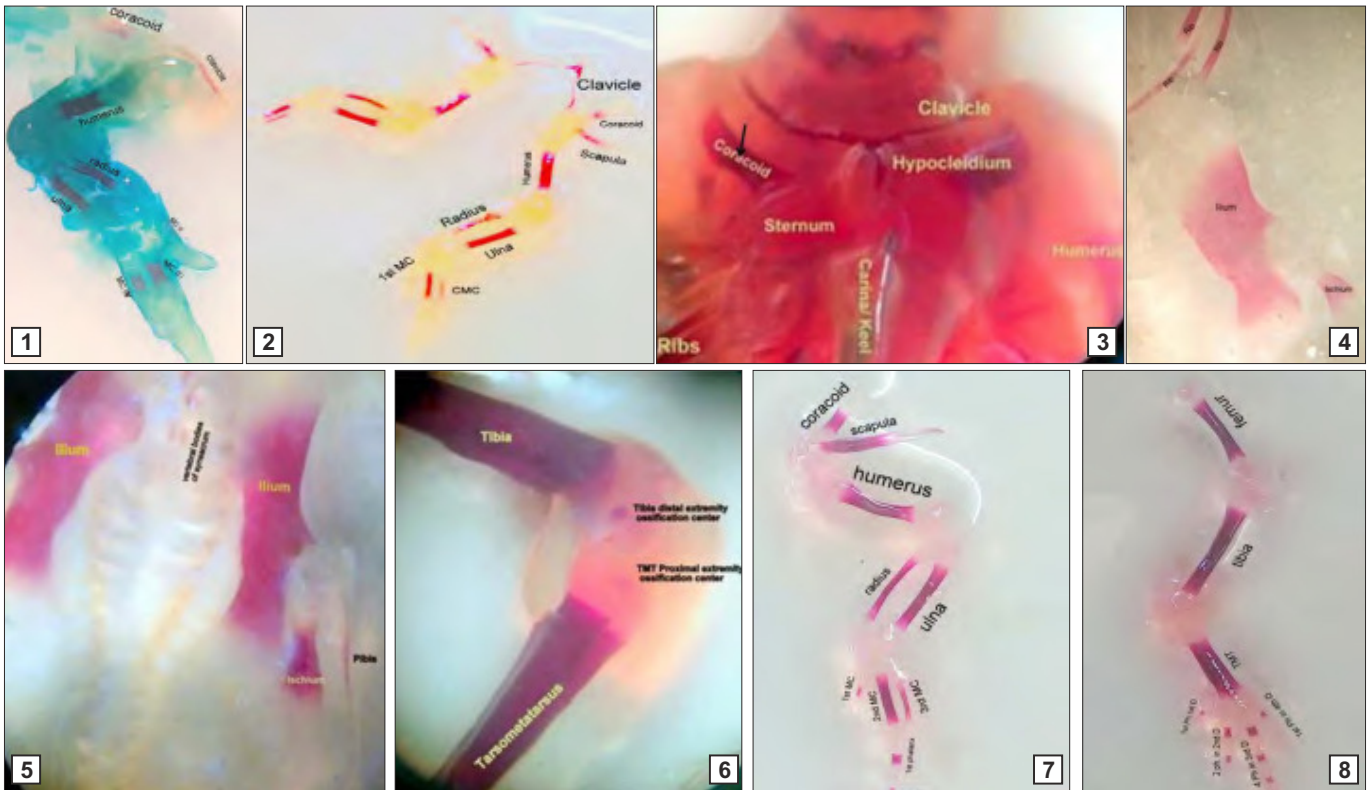
On 20th day of incubation the diaphysis of long bones was completely ossified and the centers of ossification for the distal extremity of tibia was seen (Fig. 6). However, the center of ossification for the extremities of femur and proximal extremity of tibia were not noticed till hatching (21st day of incubation). This goes in conformity with the study of Sawad *et al.* (2009), Salami *et al.* (2012), Firdous *et al.* (2016), Nakamura *et al.* (2019), Nakane and Tsudzuki (1999) in the embryo of Chicken, Grey breasted guinea fowl, Kuttanad duck, Blue breasted quail and Japanese quail embryos, respectively. The center of ossification for tarsals and patella was also not observed in prehatched Chabro chick embryos. This view finds support with the observations of Salami *et al.* (2012), Nakamura *et al.* (2018), Nakane and Tsudzuki (1999) and Firdous *et al.* (2016) in different avian embryos.

Manus and Pes: The three metacarpal (II, III and IV) bones were observed on 9th day of incubation in chabro chick embryos. They were in contact with each other proximally but distally the second metacarpal (first digit) was free, and other two were joined together at both the extremities. The ossification centers were observed only in the middle of the shaft of III and IV metacarpals (Fig. 1). The metacarpals ossification showed species variations. It

Table 1. Age (days of incubation of egg) of appearance of centers of ossification in the bones of fore limb of avian embryos

Avian Species Name of bones	Chabro Chick embryo (present observation)	Chick Embryo I	Grey Breasted G. Fowl-II	Guinea fowl-III	Kuttanad duck-IV	Blue breasted quail V	Japanese quail VI
Scapula	9 th DOI	10 th DOI	13 th DOI	10 th DOI	9 th DOI	8 th DOI	8 th DOI
Coracoids	9 th DOI	NO	13 th DOI	10 th DOI	NO	9 th DOI	9 th DOI
Clavicle	9 th DOI	10 th DOI	14 th DOI	10 th DOI	9 th DOI	7 th DOI	8 th DOI
Humerus	9 th DOI	10 th DOI	12 th DOI	10 th DOI	9 th DOI	7 th DOI	7 th DOI
Radius	9 th DOI	10 th DOI	12 th DOI	10 th DOI	9 th DOI	7 th DOI	7 th DOI
Ulna	9 th DOI	10 th DOI	12 th DOI	10 th DOI	9 th DOI	7 th DOI	7 th DOI
Carpals	NO	NO	NO	NO	NO	NO	NO
Metacarpals (II, III, IV)	9 th DOI	NO	12 th DOI	11 th DOI	13 th DOI	MC II 7; MC III 8	NO
1 st digit	↓	NO	NO	15 th DOI	15 th DOI	NO	NO
I phalanx	18 th DOI	NO	NO	NO	NO	10 th DOI	NO
II phalanx	NO	NO	19 th DOI	NO	NO	14 th DOI	NO
2 nd digit	↓	NO	NO	15 th DOI	15 th DOI	NO	NO
I phalanx	11 th DOI	NO	12 th DOI	NO	NO	10 th DOI	9 th DOI
II phalanx	13 th DOI	NO	14 th DOI	NO	NO	10 th DOI	10 th DOI
3 rd digit	NO	NO	NO	↓	15 th DOI	↓	NO
I phalanx	NO	NO	NO	15 th DOI	NO	14 th DOI	10 th DOI
II phalanx	NO	NO	NO	NO	NO	NO	10 th DOI
Total days of incubation period	21	21	26-28	26-28	28	16	18

Abbreviations and explanation: DOI- Day of incubation; NO- not observed I-(Sawad *et al.*, 2009); II- (Salami *et al.*, 2012); III- (Onyeanusi *et al.*, 2008); IV- (Firdous *et al.*, 2016); V- (Nakamura *et al.*, 2019); VI- (Nakane & Tsudzuki 1999), Arrow indicating the appearance of phalanx in digits



Figs. 1 to 8. (1) Photograph of forelimb of Chabro chick embryo on 9th day of incubation showing the centers of ossification in clavicle, coracoid, humerus, radius, ulna and metacarpals. (Alizarin Red- S and Alcian Blue combined staining); (2) Photograph of forelimb of Chabro chick embryo on 12th day of incubation showing the centers of ossification in clavicle, coracoid, scapula, humerus, radius, ulna and metacarpals. (Alizarin Red-S Staining); (3) Photograph of Chabro chick embryo on 17th day of incubation (ventral view) showing the ossification in the clavicle, coracoids (extremities of coracoids are not ossified). (Alizarin Red-S Staining); (4) Photograph of Chabro chick embryo on 13th day of incubation showing centers of ossification in ilium and ischium bones. (Alizarin Red-S Staining); (5) Photograph of Chabro chick embryo on 14th day of incubation showing center of ossification in pubis. Progressive ossification is seen in the femur, tibia and metatarsal bones. (Alizarin Red-S Staining); (6) Photograph of prehatch chick on 20th day of incubation showing centers of ossification for the distal extremity of tibia and proximal extremity of metatarsal bone. (Alizarin Red-S Staining); (7) Photograph of forelimb of Chabro chick embryo on 13th day of incubation showing the centers of ossification in the 1st and 2nd phalanx of large metacarpal bone along with progressive ossification in other bones. (Alizarin Red-S Staining); (8) Photograph of Chabro chick embryo on 13th day of incubation showing centers of ossification in 1st phalanx of 1st digit, 1st and 2nd phalanx of 2nd digit, 1st, 2nd and 3rd phalanges of 3rd digit and 1st phalanx of 4th digit bones. Progressive ossification was also seen in the femur, tibia and metatarsal bones. (Alizarin Red-S Staining)

was observed by Nakamura *et al.* (2019) earlier (7-8 days) in the Blue breasted quails but later (11 days) in Guinea fowl (Onyeanus *et al.*, 2008) and Grey breasted Guinea fowl on 12th day (Salami *et al.*, 2012). The metacarpals of Kuttanad ducks ossified even much later on 13th day (Firdous *et al.*, 2016). In the Chabro chick embryos the center of ossification was observed in the first phalanx of large digits of forelimb (wing) on 11th day of incubation. On 13th day of incubation the centers of ossification were observed in the 1st and 2nd phalanx of large metacarpal bone (Fig. 7). Beyond 13th day of incubation the area of ossification increased with increase in the size of bones.

On 9th day of incubation the centers of ossification were first noticed in the center of the diaphysis of three metatarsal bones. Thus the three distinct metatarsals were clearly evident on 9th day of incubation. On 10th day of incubation the area of ossification gradually increased with the increase in the length of long bones. On the 10th

day of incubation the center of ossification was seen in the first phalanx of second and third digits of embryos. The three metatarsals came close to each other but still remained separated. Further the centers of ossification were evident in the 1st phalanx of 1st digit, 1st and 2nd phalanges of 2nd digit, 1st, 2nd and 3rd phalanges of 3rd digit and 1st phalanx of 4th digit also on the 13th day of incubation (Fig. 8). The third and fourth metatarsal bones fused together but the 5th metatarsal lay still separated. In 14 days old chick embryo the shafts of all the three metatarsals came close to each other and started fusing together. However, at the extremities they still lay separated from each other. On 15th day of incubation all the four phalanges of the third digit were showing ossification centers including the claws. Beyond 15th days of incubation, the area of ossification increased along with the increase in the length of long bones. The centers of ossification for the distal extremity of metatarsals were seen on 20th day of

Table 2. Age (days of incubation of egg) of appearance of centers of ossification in the bones of hind limb of avian embryos

Avian Species Name of bones	Chabro Chick embryo (present observation	Chick Embryo I	Grey Breasted G. Fowl-II	Guinea fowl-III	Kuttanad duck-IV	Blue breasted quail V	Japanese quail VI
Ilium	13 th DOI	14 th DOI	13 th DOI	10 th DOI	9 th DOI	9 th DOI	9 th DOI
Ischium	13 th DOI	14 th DOI	13 th DOI	12 th DOI	9 th DOI	10 th DOI	9 th DOI
Pubis	14 th DOI	14 th DOI	19 th DOI	10 th DOI	9 th DOI	9 th DOI	9 th DOI
Femur	9 th DOI	5 th DOI	12 th DOI	10 th DOI	7 th DOI	7 th DOI	7 th DOI
Tibia	9 th DOI	10 th DOI	12 th DOI	NO	7 th DOI	7 th DOI	7 th DOI
Fibula	9 th DOI	10 th DOI	12 th DOI	10 th DOI	7 th DOI	7 th DOI	7 th DOI
Tarsals	NO	NO	NO	NO	NO	NO	NO
Metatarsal	9 th DOI	NO	MT III & IV 12 I & II MT 14	NO	13 th DOI	7 th DOI	11 th DOI
1 st digit	↓	NO	↓	15 th DOI	13 th DOI	↓	↓
I phalanx	13 th DOI	NO	14 th DOI	NO	NO	7 th DOI	11 th DOI
II phalanx	NO	NO	14 th DOI	NO	NO	7 th DOI	9 th DOI
2 nd digit	↓	NO	↓	15 th DOI	13 th DOI	↓	↓
I phalanx	10 th DOI	NO	14 th DOI	NO	NO	9 th DOI	9 th DOI
II phalanx	13 th DOI	NO	14 th DOI	NO	NO	7 th DOI	NO
III phalanx	—	NO	NO	NO	NO	7 th DOI	11 th DOI
3 rd digit	↓	NO	↓	↓	↓	↓	↓
I phalanx	10 th DOI	NO	14 th DOI	15 th -16 th DOI	15 th DOI	9 th DOI	9 th DOI
II phalanx	13 th DOI	NO	14 th DOI	15 th -16 th DOI	13 th DOI	10 th DOI	9 th DOI
III phalanx	13 th DOI	NO	14 th DOI	15 th -16 th DOI	15 th DOI	7 th DOI	NO
IV phalanx	15 th DOI	NO	14 th DOI	NO	NO	7 th DOI	11 th DOI
4 th digit	↓	NO	↓	NO	NO	↓	↓
I phalanx	13 th DOI	NO	14 th DOI	NO	NO	10 th DOI	9 th DOI
II phalanx	18 th DOI	NO	14 th DOI	NO	NO	11 th DOI	9 th DOI
III phalanx	18 th DOI	NO	14 th DOI	NO	NO	7 th DOI	11 th DOI
IV phalanx	18 th DOI	NO	14 th DOI	NO	NO	7 th DOI	11 th DOI
V phalanx	18 th DOI	NO	16 th DOI	NO	NO	14 th DOI	11 th DOI
Total days of incubation period	21	21	26-28	26-28	28	16	18

Abbreviations and explanation: DOI- Day of incubation; NO- not observed

I- (Sawad *et al.*, 2009); II- (Salami *et al.*, 2012); III- (Onyeausi *et al.*, 2008); IV- (Firdous *et al.*, 2016); V- (Nakamura *et al.*, 2019); VI- (Nakane & Tsudzuki 1999), Arrow indicating the appearance of phalanx in digits

incubation. However, the center of ossification for proximal extremity of metatarsal was not noticed till hatching (21st day of incubation).

The study revealed that the earliest onset of center of ossification in manus and pes of fore and hind limbs occurred simultaneously on 9th day of incubation in metacarpal and metatarsal followed by I phalanx of 2nd digit (11th day) in forelimb and I phalanx of 2nd and 3rd digits (10th day) in hind limb of Chabro chicken. The ossification centers appeared in I phalanx of 2nd digit and I phalanx of 1st digit of fore limb in Chabro chicken on 13th and 18th days of incubation, respectively. Similar trend of appearance of ossification centers in phalanges of digits of forelimb was reported by Salami *et al.* (2012) in Grey breasted Guinea fowl embryos. Onyeausi *et al.* (2008) in

Guinea fowl and Firdous *et al.* (2016) in Kuttanad duck embryos observed the appearance of centers of ossification for 1st, 2nd and 3rd digits on 15th day of incubation. Nakamura *et al.* (2019) reported the appearance of center of ossification in I phalanx of 1st digit and I and II phalanges of 2nd digit on 10th day of incubation whereas in the II phalanx of 1st digit and I phalanx of 3rd digit it appeared on 14th day of incubation in Blue breasted quail embryos.

Further the ossification (calcification) in the phalanges of hind limb of Chabro chicken started one day earlier (10th day) than those of the fore limb (11th day). Nakamura *et al.* (2019) in Blue breasted quail also reported the much earlier onset of ossification in the phalanx of hind limb than in the phalanx of fore limb, however, Nakane and Tsudzuki (1999) in Japanese quail embryos observed their

appearance on the same day (9th day). But Salami *et al.* (2012) observed an earlier onset of ossification in the phalanges of fore limb than the hind limb in Grey breasted Guinea fowl embryos. Although the commencement of ossification was noticed earlier in the phalanges of hind limb of Chabro chick embryos, the completion of ossification in all the phalanges of all digits occurred on the same day (18th day of incubation) in the fore and hind limbs both. Similar trend of onset and completion of ossification in the phalanges of digits of fore and hind limbs was observed by Nakamura *et al.* (2019) in Blue breasted quail embryos. However, in Grey breasted Guinea fowl embryo (Salami *et al.*, 2012) the ossification of phalanges of digits of hind limb completed earlier (16th day of incubation) than that of the fore limb (19th day of incubation). On contrary Nakane and Tsudzuki (1999) observed an earlier completion of ossification in the phalanges of fore limb (10th day) than the hind limb (11th day).

Thus the onset of ossification started almost simultaneously in the bones of fore limb and hind limb of Chabro chick embryos and took nearly same time period for the completion of ossification in all the bones of fore and hind limbs. Similar observations were recorded by Salami *et al.* (2012), Firdous *et al.* (2016) and Nakamura *et al.* (2018) in Grey breasted guinea fowl, Kuttanad duck and blue breasted quail embryos, respectively. However, in Japanese quail (Nakane and Tsudzuki, 1999) and Guinea fowl (Onyeausi *et al.*, 2008) embryos, the ossification of different bones of fore limb completed at an earlier date

than the bones of hind limb.

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