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J Vet Adv 2016, 6(8): 1301-1309

DOI: 10.5455/jva.20160916045153
Prehatch Developmental Ossification Sequences of the Appendicular and Axial Skeleton in Kuttanad Duck Embryos (*Anas Platyrhynchos Domesticus*)

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Abstract

The processes of ossification sequences are poorly investigated for birds in general, even for domestic and experimental species and when it comes to the waterfowl it is almost negligible. Such sequences constitute a rich source of data on character evolution, and may even provide phylogenetic information. A prehatch developmental study on ossification sequences of axial and appendicular skeletal system in Kuttanad duck embryos was undertaken using 39 viable embryos. From day three to 7th day of incubation no ossification densities were seen both by alizarin red staining and computerized radiography. The first indication of ossification as small ossification centers in skull bones, clavicle, scapula, humerus, radius and ulna in forelimb and ilium, pubis femur and fibula in hind limb were observed on 9th day of incubation. The calcification of the body of the ribs started at the 11th day of incubation towards the proximal extremity. On day 13th the ossification process of vertebrae started from cervical end. The variation in appearance of the ossification centres in different bones at different stages of incubation period suggests relative importance of phylogeny to the sequences.

**Keywords:** Alizarin red, duck, ossification, radiography.
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Introduction

It is poorly understood or still in infancy, what factors influence ossification sequences and what the relative importance of phylogeny is to the sequences. Among domestic birds Galliformes constitute a good model to examine these variables. Waterfowl comes second to the Galliformes and will substitute the domestic fowl in many ways as an experimental valuable model for studying the vertebrate skeletal defects. These birds are osteologically conservative, have precocial young, but have a broad spectrum of body sizes and incubation periods. Birds are said to show the least embryonic variation of all groups of vertebrates (Kerr, 1919; Richardson et al., 1997). It has been argued that specific differences between birds arise largely through modifications at later stages of development (Ricklefs and Starck, 1998).

Patterns of early embryonic development have traditionally been viewed as invariant within vertebrate taxa. The passive component of avian skeletal system i.e. bone development and health is an important subject in avian research, because of its significance in the poultry industry. Many of the pathological skeletal deformities are still common place and do not appear to be linked to defined causes e.g. Varus and valgus deformation of the long bones are expressive examples. Although there are dissimilarities between human and avian bone development, the avian is considered a valuable model for human skeletal defects (Cook, 2001). Even then a lot of work has been done on the developmental aspects of the skeletal system in birds; research on skeletal system development waterfowl is scanty. So the present work was taken with the aim of investigating the developmental way of the passive part of the skeletal system that will add the knowledge in the anatomical literature and will help the allied subjects on comparative basis.

Materials and Methods

While there is a large body of data dealing with developmental pattern of skeletal system in other birds at later stages of development, i.e., after hatching or birth, data about developmental timing during embryonic stages are scarce in all birds in general and that of waterfowl in particular. In this regard a prehatch study was taken in Kuttanad duck embryos—a variety of waterfowl. The material for this study was formed by 39 viable embryos collected from 3rd to 28th day of incubation procured from the University Poultry and Duck Farm, Mannuthy, Thrissur Kerala India. The number of viable embryos collected in a given period of incubation is given in table 1.

| Incubation period (days) | 3rd | 5th | 7th | 9th | 11th | 13th | 15th | 17th | 19th | 21st | 23rd | 25th | 27th |
|-------------------------|-----|-----|-----|-----|------|------|------|------|------|------|------|------|------|
| Number of embryos collected | 3   | 3   | 3   | 3   | 3    | 3    | 3    | 3    | 3    | 3    | 3    | 3    | 3    |

Techniques to Access the Sequence of Ossification

Toluidine Blue-Alizarin Red S Staining of Cartilage and Bone Protocol (Alphonse, 1965)

Cartilage and bone were differentiated in whole-mount preparations with toluidine blue-alizarin red S staining after formalin, acetic acid and alcohol (FAA) fixation. Specimens were fixed in FAA solution having the ratio of three components as 1:1:8 for approximately 40 minutes. Then they were stained in 0.06 per cent toluidine blue made in 70 per cent ethyl alcohol for 48 hours at room temperature. 20 volumes of stain solution to the estimated volume of the specimen were used. Soft tissues were destained in 35 per cent ethyl alcohol for 20 hours; 5 per cent for 28 hours and 70 per cent for 8 hours respectively.

The specimens were counterstained in a freshly prepared 1 per cent aqueous solution of KOH to which was added 2-3 drops of 0.1 per cent alizarin red S per 100 ml of solution. The specimens were transferred into the fresh 1 per cent KOH-alizarin mixture daily for 3 days, or until the bones had reached the desired intensity of red and soft tissues. The specimens were rinsed in water, placed in a 1:1 mixture of glycerol and ethyl alcohol for 1-2 hours and then transferred into fresh glycerol-alcohol for final clearing and storage.
Radiography
Radiographs of the skeletal system were taken at different ages in Kuttanad duck for arriving at the extent of ossification and development of various components of this system. Radiographs were taken by using computerized digital radiography equipment. The facility was provided by Department of Veterinary Surgery and Radiology, College of Veterinary and Animal sciences Mannuthy.

Stereozoom Microscopy
In order to view the embryos the stereozoom microscope was used. This facility was provided by department of veterinary physiology and department of veterinary parasitology.

Results and Discussion
A detailed description of skeletogenesis and ossification sequence for the chicken and Japanese quail has been provided by many researchers (Rogulska 1962; Schumacher and Wolff 1966, Hogg 1980, Starck 1989; Nakane and Tsudzuki 1999). The ossification sequence of the Kuttanad duck waterfowl variety has not previously been described. This study besides describing the process of ossification in Kuttanad duck may also reveal the relative importance of egg size and incubation period to ossification sequence in this species. The relative sequence of ossification is the same whether enzymatic clearing and staining or histological sections are used, and so studies using different methodologies are also comparable (Clark and Smith 1993). In this regard a prehatch developmental study of ossification sequences of axial and appendicular skeletal system in Kuttanad duck embryos was undertaken using 39 viable embryos. From day three to 7th day of incubation no ossification centers were seen. Both alizarin red staining and computerized radiography cannot detect any ossification densities (Fig. 1).

![Fig. 1: 5th day Kuttanad duck embryo without any signs of ossification.](image)

The degree of ossification during different stages of incubation period in Kuttanad duck was efficiently visualized by computerized radiography in the present study (Fig. 2, 3, 4). For convenience of description, the skeleton was divided into four parts, viz., skull, vertebrae, ribs and sternum, forelimb and hind limb.
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Fig. 2: Digital radiograph of the Kuttanad duck embryo.

Fig. 3: Digital radiograph of Kuttanad duck embryo’s showing ossification centres in different bones.

Fig. 4: Digital radiograph of 25th day old Kuttanad duck embryo showing ossification centres.
Skull
In earlier stages of the embryonic life in Kuttanad ducks most elements of the skull were still separated. By 27 days of incubation day facial bones were largely fused but the skull was still opened dorsally. The bones of the skull from 3rd day of incubation till 7th day did not stain with alizarin red. The first indication of ossification in skull bones as a small ossification centers were observed in squamosal bone on 9th day of incubation (Fig. 5 and 6).

![Fig. 5: First indication of ossification (→) in skull at 9th day of incubation.](image1)

![Fig. 6: Ossification centres in skull bones at 11th day of incubation in Kuttanad duck.](image2)

The process of ossification was followed by palatine, pterygoid, prefrontal, and the bones forming the beak such as maxilla, jugal, quadratojugal, splenial, angular, supraangular and premaxilla. The frontal and nasal bones ossified lastly and by 13th day all skull bones appeared and were maximally ossified (Fig. 7). In case of quail as reported by Jollie (1957) the skull bones appeared on 11th day of incubation. The red regions of each skull bone were enlarged with the advancement of incubation period.
Forelimb and Hind Limb

The long bones in the post cranial part of the skeleton in the Kuttanad duck were the first to show the process of mineralization. The front and hind limbs showed endochondral calcification, the ossification process starts at the center of the diaphysis and extends towards the epiphysis. On 9th day of incubation small ossification centres were observed in clavicle, scapula, humerus, radius and ulna in forelimb and ilium, pubis femur and fibula in hind limb (Fig. 8).

The wing ossified earlier as compared to the foot. Daniel et al., (2003) reported the same in hind limb of chicks but in disagreement to Janina et al., (2003) in mice. The ossification process of the forelimbs is in agreement to the studies of Hill (2000) in fore limbs of pigs and Saunder (1998) in chicks. By day eleven of incubation the size of the ossification centers got increased. On day 13th of incubation new ossification centres were observed on radius, ulna, carpometacarpal joint, ulnar carpel, metatarsus, tibiotarsus, first phalanx of digit ii and second phalanx of digit iii. Day fifteen marked the ossification centres on digit ii, iii, iv of forelimb and additional ossification centres were observed in 1st, 2nd, 3rd phalanx of digit iii and 4th phalanx of digit ii of pelvic limb. Between days 17-19 of incubation
there were prominent and clear increase the ossification centres in both the limbs (Fig. 9). Right and left clavicles showed the process of ossification on day 9th but were not fused. Both clavicles were joined on 23rd day of incubation but not properly fused. On 28th day of developmental process the growth plates of long bones were still open. Gambia (1990) reported in chicken that the clavicles appeared on day eight of incubation and got fused on day 16.

Vertebrae

On day 13th the ossification process of vertebrae started from cervical end (Fig. 10). By this day the vertebral arches of the first three vertebrae showed ossification centers and on day 15th fourth was also involved in the process. On 17th day the vertebral arches of all the cervical, thoracic, lumbar and sacral vertebrae showed ossification (Fig. 11). Andreo et al., (1998) reported in mice the vertebrae ossified at the 16 day but Fernando et al., (2000) reported the process of vertebral ossification on day 15th of incubation. The ossification of the cervical vertebrae body in chick embryo started at 12 -13th day of incubation while as sacral vertebrae get ossified on day 19th of incubation (Shapiro., 1992). On day 21st of incubation ossification was seen on pygostyle.
**Ribs and Sternum**

The calcification of the body of the ribs started at the 11th day of incubation towards the proximal extremity (Fig. 12). In both vertebral and sternal ribs; ossification first occurred at the middle region and then progressed towards the proximal and distal regions. As the ossification extended in both ways an area towards the vertebra remained cartilaginous till hatching Sawad et al., (2009) said that in Gallus the primary calcification of the rib body at the proximal extremity at the 10 day of incubation.

The uncinate process of the rib first appeared as cartilage then it was calcified. In case of chicken as reported by Hamburger and Hamilton (1951) the uncinate process calcifies directly. In Kuttanad ducks the keel bone began to ossify firstly towards the caudal side and then cranially. Nakane and Tsudzuki (1999) reported in quail that sternum ossification occurred in the laterocaudal process at 14 days of incubation, and in the laterocranial process at 15 days incubation.

**Conclusion**

In the present study, embryonic ossification sequences of skeletal system in Kuttanad duck were revealed. In this paper it is presumed that the stages
of ossification of various elements of the body presented will be useful as a normal control in the fields of general embryology, developmental engineering, and teratological studies. The difference in the sequence of ossification of various skeletal elements in Kuttanad duck may be due to the appearance of ossification centres at different stages of incubation. Besides there was also some difference on comparative basis possible be due to the difference in the incubation period among different birds.

Acknowledgement

The authors highly acknowledge the Dean of faculty for providing the necessary facilities to carry this work. The authors are grateful to department of veterinary surgery and radiology for providing the facilities of radiography.

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