

## Development of the wing bones in quail's embryo; *Coturnix japonica*

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### Abstract

Quail is an essential model in avian research because of economic importance in poultry industries around the world. Furthermore, its use in the experimental embryology research field. Therefore, this study aimed to identify the onset of chondrification and ossification of the wing bones also to summarize the main histological sequences related to the formation of the humerus in Japanese quail. Six embryos were taken every 24 hrs from 3-16 days of incubation. Three embryos were prepared and stained with Alcian blue and Alizarin red for cartilage and bone, respectively. The other three embryos have been processed for histological examination. The macroscopical findings showed that the humerus, radius, ulna, coracoid, scapula, third, and fourth metacarpals were chondrified on 5<sup>th</sup> day. However, first signs of ossification were observed in the humerus, radius, and ulna on 8<sup>th</sup> day. While the minor digit remained none ossified at prehatching. The microscopical stages of developing humerus associated with the limb bud, apical ectodermal ridge formation, and chondrocyte differentiation on 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> day, respectively. The cartilage template of the humerus was established on 6<sup>th</sup> day. The diaphysis and epiphyses of the humerus were formed on 7<sup>th</sup> and 8<sup>th</sup> day, correspondingly. The periosteal-bone collar was formed on 8<sup>th</sup> day, and vascularization of chondroepiphysis has occurred on 9<sup>th</sup> day. There was a difference in the timing of chondrification and ossification in the forelimb skeleton and humerus developed by the endochondral mechanism. The obtained results should be considered in teratological and molecular studies in skeletogenesis.

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### Introduction

*Coturnix japonica* is one of the order Galliformes and the family Phasianidae. It is calm, adaptable, and economical to maintain (1,2). Japanese quail has assumed importance as an experimental model because of its distinct properties involving precocity of the species, fast and more productivity, early sexual maturity, short generation interval, and less feed and space requirements (3).

Osteogenesis occurs by two different mechanisms, intramembranous and endochondral ossification. Intramembranous ossification is a process responsible for the formation of the clavicle, and flat bones of the cranial vault. It is also a crucial process during the natural healing of bone

fractures. During intramembranous ossification, the mesenchymal cells directly differentiate into osteoblasts without the formation of the cartilage model (4,5). The long bones form and grow through endochondral ossification. Formation of the cartilage template is the initial stage of the endochondral mechanism, and it is made by aggregation of mesenchymal stem cells, follow they are transforming into chondrocytes. The cartilage model, once formed chondrocytes in the central region, undergoes more maturation to hypertrophic chondrocytes. These central hypertrophied chondrocytes undertake programmed cell death, and the matrix of the cartilage template is resorbed. The periosteal bud invaded the resorbed cartilage matrix at the center of the diaphysis and then invaded at both

epiphyses by osteogenic and hematopoietic precursor cells that make the primary and secondary centers of ossification, correspondingly. These centers of bone formation gradually invade the intact cartilage; eventually, the cartilage template entirely replaced by bone, and only the articular surface remained cartilaginous (6,7).

The prehatching development of the avian skeletal system has been commonly inspected. However, the histomorphological investigation on the prehatching development of the quail skeleton is limited. Therefore, this study aimed to determine the chondrification and ossification timing in the wing bones and to describe the histological stages of formation of the humerus in Japanese quail embryo.

## **Materials and methods**

### **Birds and housing**

A total of twenty healthy *Coturnix japonica* quail of both sexes aged three months were accommodated at the animal house, College of Veterinary Medicine, University of Sulaymani. The ratio of male to female was 1:3. The artificial photoperiod was 16 hrs, and the temperature maintained at 65-70°F. The collected eggs were stored at 15°C for one week.

### **Experimental design**

Eighty-four fertilized eggs weigh 10-12g, were incubated in the automatic egg turner incubator. The relative humidity and temperature of the egg incubator were adapted to 55-60%, and 37.5 ± 0.5°C respectively. Six embryos were taken at 24 hrs interval from 3-16 days of incubation. Three embryos were prepared for double staining, and the other three embryos processed for histological examination.

### **Preparation of embryo**

The eggs were cracked at the blunt end. The embryos removed from eggshells and separated from surrounding yolk and extraembryonic membranes. The internal organs were removed.

### **Double staining**

The prepared embryos stained with Alcian Blue 8GX (C.I.74240 Merk, Germany) and Alizarin Red-S9 (C.I.58005 Merk, Germany) for cartilage and bone, respectively. The double staining steps were carried out according to (8,9) with some modifications as follows; the skin and subcutaneous fat tissue have been removed carefully. This step was assisted by put the embryos in absolute ethanol or in hot water (50°C) for a few minutes. This step at the early stages of embryonic development (with aged between 3<sup>rd</sup>-5<sup>th</sup> day) did not require. The embryos with aged between 3<sup>rd</sup> - 9<sup>th</sup> day were fixed in absolute ethanol for 3 days, while embryos with aged between 10<sup>th</sup> -16<sup>th</sup> day were fixed for 7 days. Then, specimens were stained at 37- 40°C for 4 days in a staining solution of (0.3% (300 mg) Alcian blue in 70% ethanol (100

ml), 0.1% (100 mg) Alizarin red in 99% ethanol (100 ml), glacial acetic acid (100 ml), and 70% ethanol (1500 ml). The stained specimens were bathed for 2 hrs in changed water, and they macerated in an aqueous solution of potassium hydroxide as follows: embryos with aged between 3<sup>rd</sup> -5<sup>th</sup> day macerated in 0.5% KOH for 15 min, embryos with aged between 6<sup>th</sup> -8<sup>th</sup> day in 0.5% KOH for 30 min, embryos with aged between 9<sup>th</sup> -11<sup>th</sup> day in 1% KOH for 15 hrs, embryos with aged 12<sup>th</sup> -14<sup>th</sup> day in 1.5% KOH for 20 hrs, and embryos with aged 15<sup>th</sup> -16<sup>th</sup> day in 2% KOH for 24 hrs. Then, specimens cleared by an aqueous solution of gradually increasing concentration of glycerol 20%, 50%, and 80% for 3 days for each concentration. Lastly, embryos stored in a pure glycerol 100%. For permanent storage, kept embryos at 4°C, and few thymol crystals, have been added to inhibit fungus growth and putrefaction.

### **Observation and measurement of the embryo**

The stained specimens put in a glass or transparent plastic dish filled with 100% glycerol carefully to allow the specimen to settle down entirely. Observation of the skeletal elements was performed under a stereomicroscope (Optica, BG-Italy), and the embryos positioned laterally to expose the wing elements. The total length and the length of the ossified part of the humerus have been measured by using an ocular micrometer under dissecting microscope (x20) from 5-16 days of incubation. The number of cases was three embryos per day. Statistical analysis was performed by SPSS version 22 (SPSS Inc., USA). The data were expressed as mean ± SD (Standard Deviation). The calibration factor was (0.058) calculated as follows; 1 Stage division = 0.01 mm » 100 Stage divisions = 100 \* 0.01 = 1mm; Stage divisions / Ocular divisions = 1mm/17 = 0.058 mm.

### **Histological examination**

The prepared embryos were fixed in 10% neutral buffered formalin for 24 hrs. Tissues obtained for histological examination included the humerus, processed by routine histological processing method; sections (5 µm thickness) were taken and stained with hematoxylin and eosin, Alizarin red, modified von Kossa, and Masson's trichrome stains then examined by light microscope (Motic, China). The presented images were captured by using a digital camera (Am scope, China).

## **Results**

### **Macroscopical findings**

In the present study, the onset of chondrification and ossification of the forelimb elements of the Japanese quail embryo identified by using Alcian blue and Alizarin red, respectively. The cartilaginous parts stained blue with Alcian blue while the ossified regions were stained red with Alizarin red, as shown in (Figures 1 and 2). 3<sup>rd</sup> day of incubation: The limb buds were evident as slightly thickened ridges at

laterally about midway down of the body. 4<sup>th</sup> day of incubation: The limb buds more detectable and protruded from the embryo body. 5<sup>th</sup> day of incubation: The chondrification of the coracoid, scapula, humerus, radius, ulna, third, and fourth metacarpals were observed as slightly blue-stained. 6<sup>th</sup> day of incubation: The chondrification process followed by chondrification of the Os carpi radiale,

Os carpi ulnare, the second metacarpal and first phalanxes of the major and minor digits. 7<sup>th</sup> days of incubation: The second phalanx of the major digit was blue stained. 8<sup>th</sup> day of incubation: The humerus, ulna, and radius were started ossification. The ossified part was visualized as the small stained red center at the diaphysis. The first phalanx of the alular digit was blue stained.

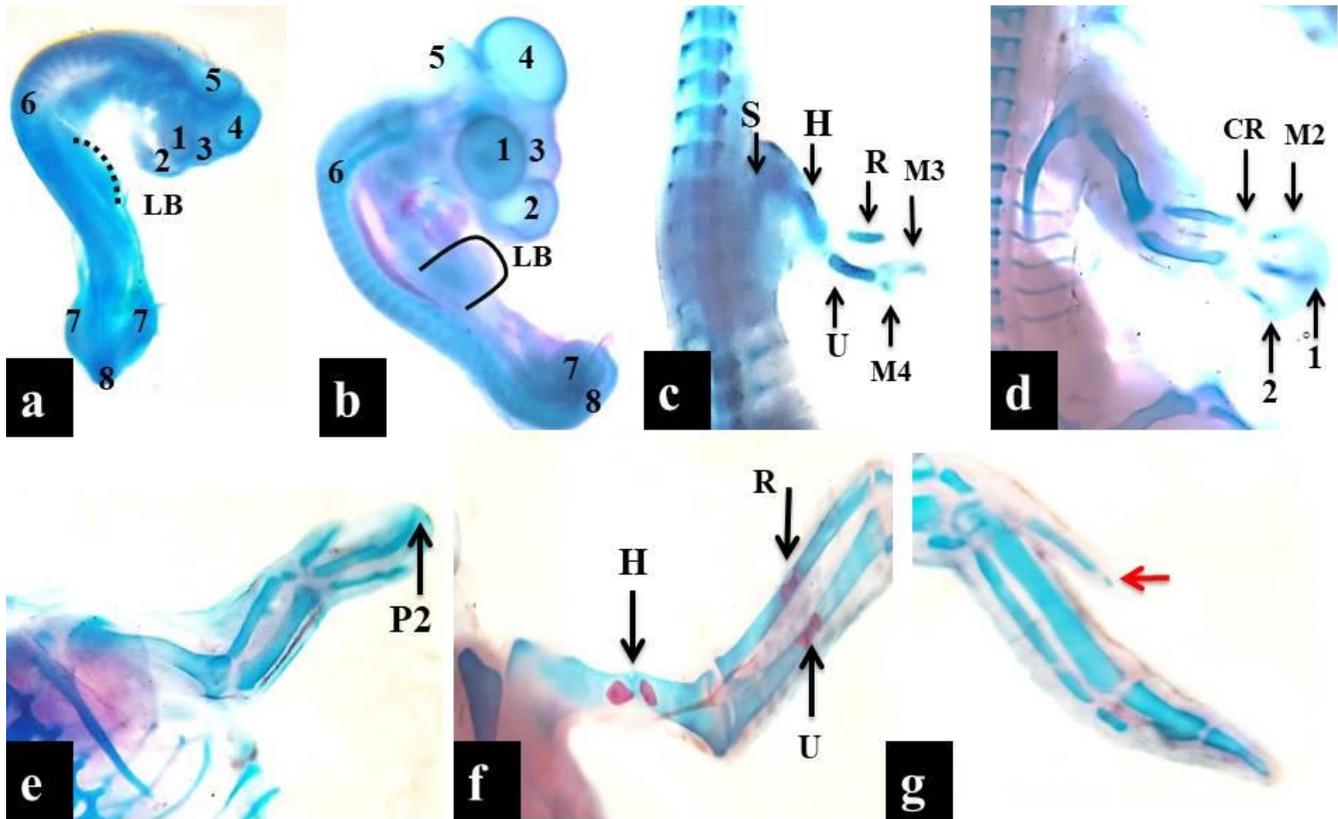


Figure 1: Japanese quail embryo: a. Small ridge of upper limb bud LB, (3<sup>rd</sup> day), b. Protruded limb bud LB, (4<sup>th</sup> day), in (a and b) 1. Eye, 2. Telencephalon, 3. Diencephalon, 4. Mesencephalon, 5. Metencephalon, 6. Spinal cord, 7. Lower limb bud, 8. Tail bud, c. Chondrification of scapula S, humerus H, radius R, ulna U, third metacarpal M3, fourth metacarpal M4 (5<sup>th</sup> day), d. Chondrification of Os carpi radiale CR, second metacarpal M2, the first phalanx of major digit 1, the first phalanx of minor digit 2 (6<sup>th</sup> day), e. Chondrification of the second phalanx of major digit P2 (7<sup>th</sup> day), f and g. Ossification center (black arrows) of the humerus H, radius R, and ulna U, chondrification of the first phalanx of the alular digit (red arrow), (8<sup>th</sup> day). (Double staining, x20).

9<sup>th</sup> day of incubation: The clavicles, coracoid, scapula, 3<sup>rd</sup>, and 4<sup>th</sup> metacarpals were ossified. 2<sup>nd</sup> phalanx of the alular digit was blue stained. The proximal extremity of the second metacarpal combined with the articular surface of the third metacarpal. 10<sup>th</sup> day of incubation: The carpi fused with metacarpals to form a carpometacarpal compound. The entire pectoral girdle and wing elements formed, and the ossification part extended through the extremities of the elements. 11<sup>th</sup> day of incubation: The first phalanx of the major digit was ossified and red-stained. 12<sup>th</sup> day of incubation: The second phalanx of the major digit and the

first phalanx of alular digits were turned red at the central portion. 13<sup>th</sup> day of incubation: The earlier ossified parts of the wing skeleton extended without any new ossification center. 14<sup>th</sup> day of incubation: The second phalanx of the alular digit was ossified. 15<sup>th</sup> - 16<sup>th</sup> day of incubation: Through 15<sup>th</sup> and 16<sup>th</sup> day of the prehatching period, no ossification was occurred in the first phalanx of the minor digit, extremities of the long bones, the distal end of the scapula, and carpal elements which were remained cartilaginous as indicated by blue stained. Finally, on 17 days, the chick of *Coturnix japonica* hatched.

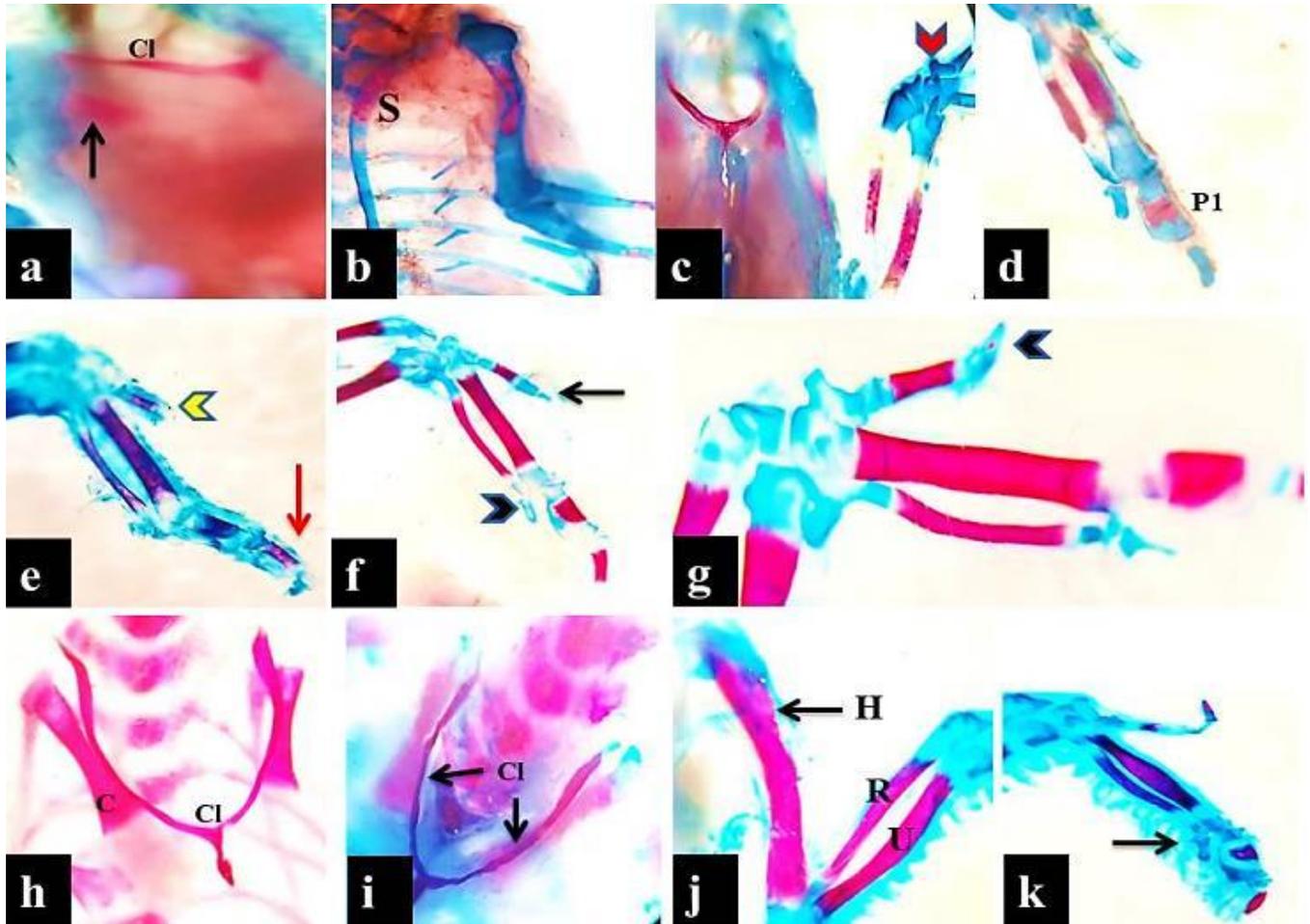


Figure 2: a and b. Chondrification of clavicles Cl, coracoid (black arrow), scapula S, (9<sup>th</sup> day), c. Carpometacarpal compound (red head arrow), (10<sup>th</sup> day), d. Ossification of the first phalanx of major digit P1, (11<sup>th</sup> day), e. Ossification of the first phalanx of alular digit (yellow head arrow) and the second phalanx of major digit (red arrow), (12<sup>th</sup> day), f. The second phalanx of alular digit (black arrow) and minor digit (black head arrow) remained non-ossified (13<sup>th</sup> day), g. Ossification of the second phalanx of alular digit (black head arrow) (14<sup>th</sup> day), h. Clavicles Cl, Coracoid C, (15<sup>th</sup> day), i. Clavicles Cl, j. Humerus H, Radius R, Ulna U, k. Only the minor digit remained cartilaginous (black arrow), i, j and k (16<sup>th</sup> day). (Double staining, x20). Note: In figures (d and e) the second phalanx of the alular digit lost during imaging.

The result of statistical analysis revealed that there was a significant increase at  $P < 0.05$  in the total length and length of the ossified part of the diaphysis of the developing humerus among studied days from 5<sup>th</sup> - 16<sup>th</sup> day of incubation, particularly there was a noticeable increase in the total length of the humerus on 8<sup>th</sup> day (Figure 3).

#### Microscopical findings

The histological examination of the embryo on 3<sup>rd</sup> day showed a lateral swelling from the body wall known as limb bud. The limb bud composed of undifferentiated mesenchymal cells surrounded by ectodermal cells layer (Figure 4a). The ectodermal cells layer increased in number and became thickening termed the apical ectodermal ridge (AER) on 4<sup>th</sup> day, which was covered the distal surface of the

limb bud (Figures 4b and c). On 5<sup>th</sup> day there was an aggregation of mesenchymal cells in the limb bud known as mesenchymal condensation. This condensation has appeared as a small round area consisted of undifferentiated mesenchymal cells. Some of the mesenchymal cells in the center of the condensation region, differentiated into round chondrocytes (Figure 4d), whereas mesenchymal cells at the periphery of the condensation synthesized the prospective perichondrium and demarcated the humerus from adjacent mesenchymal tissues. The cartilage template was formed on 6 days, and it consisted of small chondrocytes and surrounded by undeveloped perichondrium (Figure 4e).

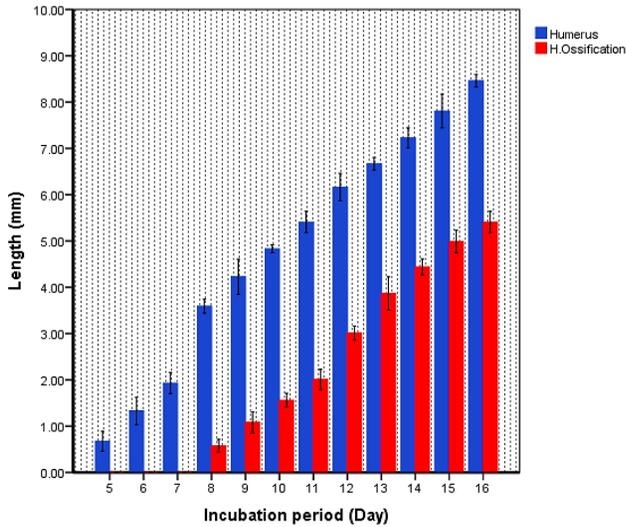


Figure 3: The graph showing the significant difference in the total length (blue color bars) and the length of the ossified part (red color bars) of the humerus for three embryos per day from 5-16 days of incubation, the data presented as (Mean  $\pm$  SD) at  $P < 0.05$ .

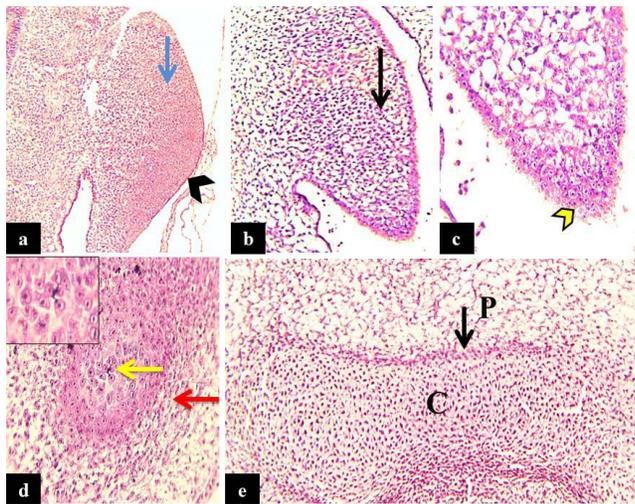


Figure 4: Microscopic sections of the embryo showed: a. Limb bud (blue arrow), ectoderm layer (black head arrow), (3<sup>rd</sup> day), b. Elongated limb bud (black arrow), c. Apical ectodermal ridge (yellow head arrow), (4<sup>th</sup> day), d. Round chondrocytes (yellow arrow) and specified by inset in the center of mesenchymal condensation, prospective perichondrium (red arrow), (5<sup>th</sup> day), e. Cartilage template C, prospective perichondrium P, (6<sup>th</sup> day). H&E stain, (a, b) x100, (c, d, e) x400.

The diaphysis of the cartilage template was established on 7<sup>th</sup> day, and surrounded by developed perichondrium, including an inner cellular layer and outer fibrous layer

(Figures 5a and b). The cartilage template attained the shape of the prospective humerus on 8<sup>th</sup> day; it consisted of mid-diaphysis and epiphyses at the ends (Figure 5c). Chondrocytes at the epiphyseal area were small rounded to oval shape and randomly distributed throughout the extracellular matrix. While chondrocytes in the center of the diaphysis were organized into a hypertrophic zone of large chondrocytes (Figure 5d), and on both peripheries of the hypertrophic zone there was a proliferative zone of flattened shape chondrocytes (Figure 5e).

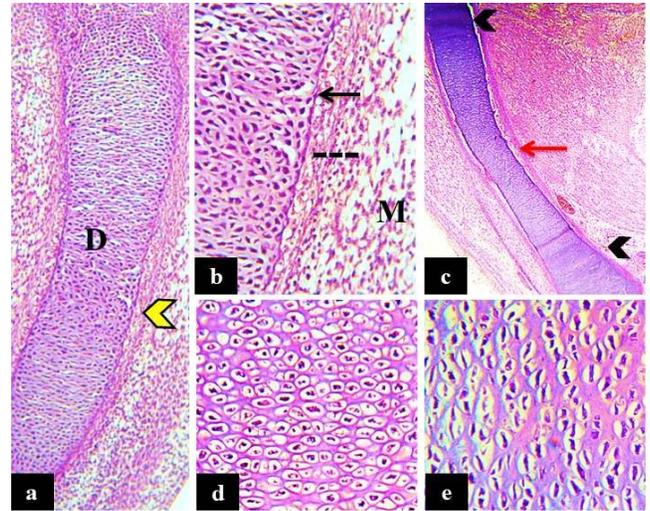


Figure 5: Microscopic sections of the humerus showed: a. The diaphysis D of the cartilage template, perichondrium (yellow head arrow), b. Inner cellular layer (black arrow) and outer fibrous layer (dash line) of the perichondrium, undifferentiated mesenchymal cells M, (7<sup>th</sup> day), c. Epiphyses (black head arrows), diaphysis (red arrow), d. Hypertrophied zone, e. Proliferative zone (8<sup>th</sup> day), H&E stain, (a) x100, (c) x40, (b, d, e) x400.

The differentiation of the inner cellular layer of perichondrium into osteoblasts occurred on 8<sup>th</sup> day. These osteoblasts deposited osteoid tissue as a bone collar around the mid-diaphysis at the site of the hypertrophied chondrocytes and perichondrium transformed into the periosteum (Figure 6a). Calcification of the bone collar was occurred on the same day as indicated by red and black-brown stained with Alizarin red and modified von Kossa stain, respectively (Figures 6b and c).

On 9<sup>th</sup> day, the second layer of bone collar deposited and accompanied by vascular and cellular penetration into hypertrophied chondrocytes on the periphery of the mid-diaphysis of the cartilage template (Figure 6d). Furthermore, vascularization of the chondroepiphysis was firstly observed. The proximal epiphysis of the humerus revealed a small, short unbranched canal introduced from the perichondrium known as epiphyseal cartilage canal on 9<sup>th</sup> day (Figure 6e).

The periosteal bud conveyed hemopoietic cells and osteoprogenitor cells into the space left after resorption of the cartilage matrix, and the medullary cavity formed on 10<sup>th</sup> day (Figure 6f). The periosteal bone collar more deposited by osteoblasts around mid-diaphysis and the thickness of it increased toward the center of the diaphysis on 11<sup>th</sup> day (Figure 6g). The endochondral ossification started when the osteoblasts used the cartilage matrix as a scaffold and began to secrete osteoid tissue and bone spicules formed. In turn, these spicules connected and bone trabeculae formed on 12<sup>th</sup> day (Figure 6h). Mature osteoblasts embedded in the matrix and transformed into osteocytes; thus, the primary woven bone formed on 13<sup>th</sup> day (Figure 6i).

Calcification of the trabecular bone has occurred on 14<sup>th</sup> day, as indicated by the positive reaction to von Kossa stain

(Figure 6j). On 15<sup>th</sup> day the endochondral bone trabeculae more synthesized till emerged with the periosteal bone collar and they were occupied all mid-diaphysis of the humerus and constituted the primary ossification center POC (Figure 6k). The bone trabeculae were intensively mineralized and deeply stained with Alizarin red stain (Figure 6l).

The osteoclasts digested the endochondral trabecular bone in resorption bay (Figure 7a) and bone marrow cavity within mid-diaphysis started to form on 16<sup>th</sup> day (Figures 7b and c). The histological section from the distal epiphysis of the developing humerus showed cartilage canals without the presence of the secondary ossification center (Figures 7d, e and f).

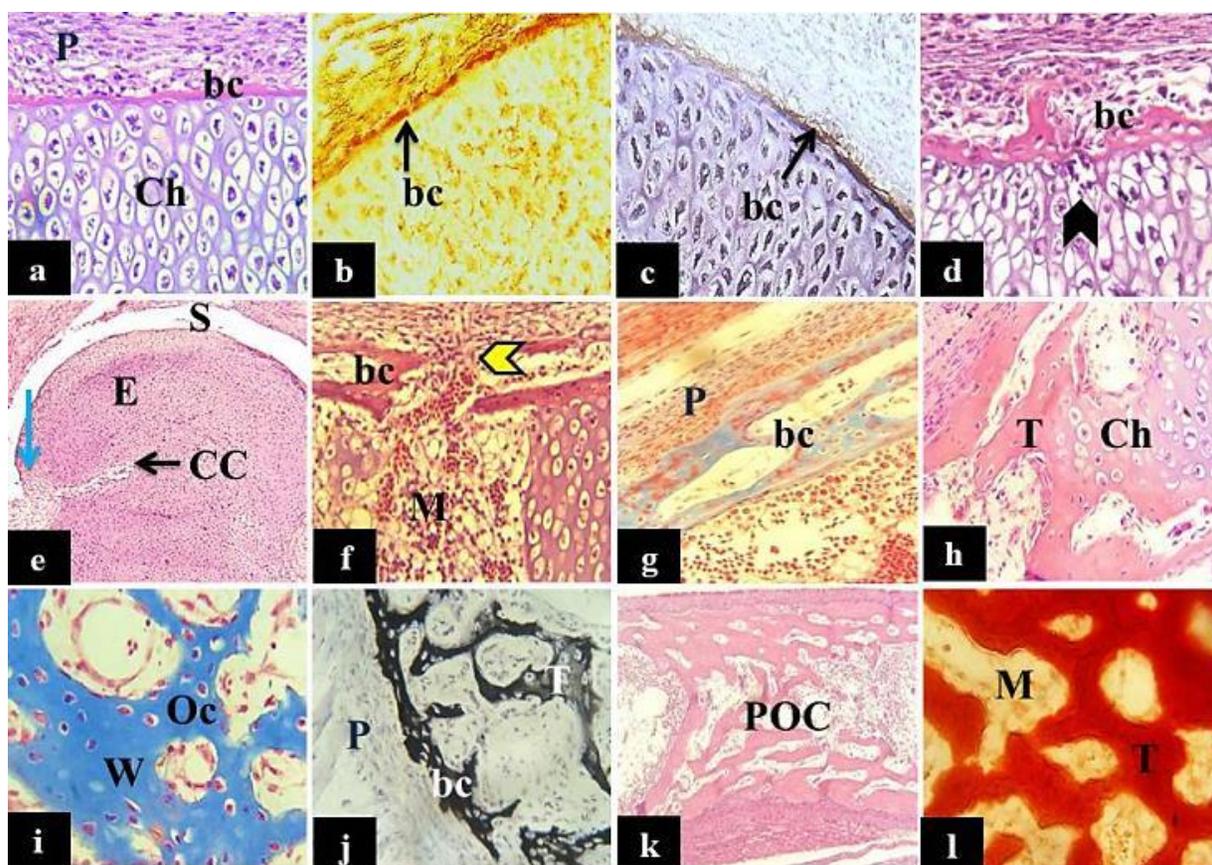


Figure 6: a. Formation of bone collar bc above hypertrophied chondrocytes Ch, periosteum P, b. The red color of calcium deposits of the bone collar bc with Alizarin red, c. Brown-black color of calcium deposits of the bone collar bc with modified von Kossa, (8<sup>th</sup> day), d. Periosteal bud invasion (black head arrow) from bone collar bc, e. Cartilage canal CC, proximal epiphysis E, perichondrium (blue arrow), shoulder joint S, (9<sup>th</sup> day), f. Medullary cavity M, cellular and vascular invasion as an irruption canal (yellow head arrow), bone collar bc, (10<sup>th</sup> day), g. Bone collar bc more deposited, and red color indicated the mineralized portion of the bone collar while blue color indicated collagen fiber of it, periosteum P (11<sup>th</sup> day), h. Chondrocytes Ch underwent endochondral ossification and they replaced by trabecular bone T (12<sup>th</sup> day), i. Primary woven W bone, embedded osteocytes Oc (13<sup>th</sup> day), j. Calcified trabecular bone T, bone collar bc, periosteum P (14<sup>th</sup> day), k. Primary ossification center POC, l. Mineralized trabecular bone T, medullary space M, (15<sup>th</sup> day). (a, d, e, f, h, k) H&E, (b, l) Alizarin red, (c, j) von Kossa, (g, i) Masson's Trichrome, (a, b, c, d, f, g, h, i, j, l) x400, (e, k) x100.

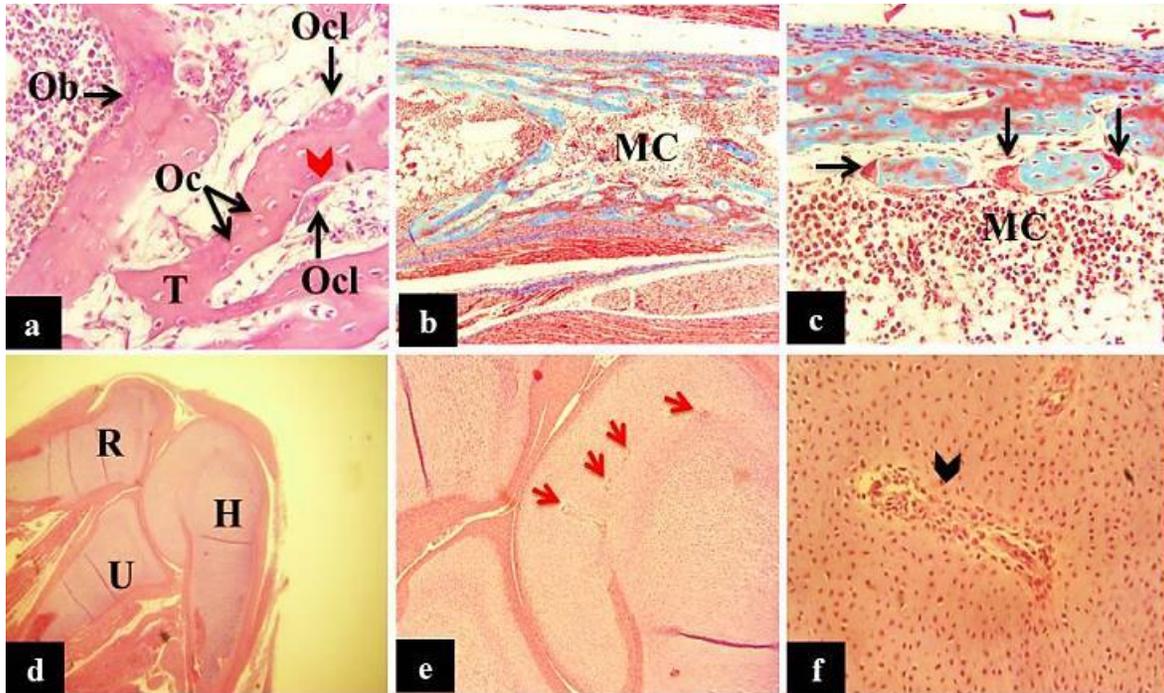


Figure 7: a. The multi-nucleated osteoclasts Ocl in resorption bay (red head arrow) digested bone trabeculae T, entrapped osteocytes Oc, osteoblasts Ob on the free surface of bone trabeculae, b. Bone marrow cavity MC, c. Small bone trabeculae at mid- diaphysis digesting by osteoclasts (black arrows), bone marrow cavity MC, d. Humerus H, radius R, and ulna U at the elbow joint, e. Numerous small and short cartilage canals (red arrows) within epiphysis of the humerus H without secondary ossification center, f. Higher magnification from section (e) showed cartilage canal (arrow head) with definitive outline and contain loose connective tissue within chondroepiphysis, 16<sup>th</sup> day, (a, d, e, f) H&E stain, (b, c) Masson's Trichrome, (a, c, f) x400, (b, e) x100, (d) x40.

## Discussion

Quail is not only an agricultural bird but also is one of the ideal vertebrate models used to study a variety of investigations (10). More recently, in third countries, this species has been housed as an alternative source of animal protein (11,12). There is much study regarding the prehatching skeletal development of Galliformes, and many authors did research concerning quail such as (13-15). The purpose of this study was to determine the onset of chondrification and ossification of the forelimb bones in the Japanese quail embryo.

This study on 3<sup>rd</sup> day showed that forelimb region has appeared as a lateral ridge known as limb bud. The first occurrence of chondrification was observed in the scapula, coracoid, humerus, radius, ulna, second, and third metacarpal bones on 5<sup>th</sup> day. In a previous study (14) in Japanese quail, the chondrification of the humerus, radius, and ulna recorded much earlier on 4<sup>th</sup> day. More recently (13) in Chinese quail, the chondrification of the humerus documented on 4<sup>th</sup> day, and chondrification of the scapula, coracoid, radius, ulna, second, and third metacarpal bones

recorded on 5<sup>th</sup> day. The first sign of ossification was observed at the center of the diaphysis of the humerus, ulna, and radius on 8<sup>th</sup> day. The ossified part appeared as a red-stained center. In the past, the early ossification center of these bones on 7<sup>th</sup> day stated by (14). On the other side (15) reported a remarkable earlier of the ossification of these bones on 6<sup>th</sup> day. Statistically, the data showed that the length of chondrified and ossified parts of the humerus significantly increased particularly; there was a remarkable increase in the length of the cartilage part of the humerus on day 8.

The thin U shaped clavicles were ossified directly by intramembranous mechanism on 9 day, whereas (15) and (14) reported the intramembranous ossification of clavicles on 7<sup>th</sup> and 8<sup>th</sup> day, respectively. The attained result showed that the first phalanx of minor digit remained cartilaginous at prehatching in contrast (15) documented the ossification of this phalanx on 14<sup>th</sup> day and (14) on 16<sup>th</sup> day. In Chinese quail, the ossification of this digit stated on 14<sup>th</sup> day (13).

In the current study, variations in the developmental timing of the wing skeleton with other studies have been stated. Variations in these results are probably related to strain, health, age, nutrition, management, and photoperiod

of breeder flock. Size, weight, quality, standardized collection, transportation, and storage of eggs, factors associated with the incubation procedure such as temperature, relative humidity, the time taken for the embryo to warm to the incubation temperature, and turning per day could be considered as factors that contributed these differences.

In the mammals and birds, most bones are made by endochondral ossification, in which an essential cartilage template is formed and gradually replaced by bones (7). Microscopically on 3<sup>rd</sup> day the limb bud composed of undifferentiated mesenchymal cells surrounded externally by ectoderm layer. On 4 day, the ectodermal cells became thick, and the apical ectodermal ridge was established (AER). The (AER), allowing the limb bud to grow by preserves the underlying mesenchymal cells in a proliferative state (16). On 5<sup>th</sup> day, a group of cells in the mid-point of the mesenchymal condensation area differentiated into chondrocytes. Transcription factor Sox-9 is considered as a major regulator of mesenchymal cell aggregation and differentiation of chondrocyte (17).

On 6<sup>th</sup> day, the cartilage template was formed; this cartilage template is growth by two main mechanisms; interstitial growth which is responsible for elongation of cartilage template by the mitotic activity of chondrocytes, while appositional growth lead to the increasing thickness of template by addition of cartilage matrix by chondroblasts in the inner cellular layer of the perichondrium (18). The chondrocytes within the diaphysis were arranged into central hypertrophic and peripheral proliferative zones on day 8. Collagen type X is the most specific marker of hypertrophic chondrocytes (19). Indian hedgehog (Ihh) is a major factor which regulates bone development expressed by pre-hypertrophic chondrocytes (chondrocytes leaving the proliferative zone) that keeps chondrocyte in proliferation state and inhibits hypertrophy of chondrocyte. The chondrocyte proliferation is reliant on the Ihh induction by expression of the parathyroid hormone-related peptide (PTHrP) (20). PTHrP is expressed by chondroblasts and early proliferating chondrocyte. In addition, Ihh in endochondral bone formation plays a direct role in enhancing osteoblast differentiation in the perichondrium, also affects vascular and cellular invasion into cartilage template which, occurs at the next stage of bone formation (7). Runx2, which is expressed highly by mature and hypertrophic chondrocytes induces chondrocyte hypertrophy and succeeding bone formation in cartilage (21). The hypertrophic chondrocytes undergo physiological cell death or survive and dedifferentiated into osteoblasts (19).

In the present study, cells in the inner layer of the perichondrium differentiated into osteoblasts, and they formed a thin layer of bone around the mid-diaphysis of the cartilage model named periosteal collar bone on 8<sup>th</sup> day. This bone collar considered the first hallmark of endochondral ossification in long bones (22). Positive reaction to Alizarin

red and von Kossa satin (Figures 6b and c) on 8<sup>th</sup> day confirmed that the red-color of double staining in the mid-diaphysis of the humerus (Figure 1f), it means the onset of ossification. On day 9, the second layer of bone collar deposited by osteoblasts. Concomitantly periosteal bud invasion of the cartilage template started. These findings substantially were comparable with (23). In the current study, resorption of the cartilage matrix began when a small cavity became evident and occupied by undifferentiated cells originated from the periosteum. In mammals, hyaline cartilage is first calcified and then resorbed, but in the embryos of birds, cartilage resorption is independent of calcification (20). The cartilage canal penetrated the proximal epiphysis of the humerus on 9<sup>th</sup> day. The cartilage canal is present in human, mammalian, and some avian species. It has similar structural characteristics among vertebrates. In the chicken femur, cartilage canals contain arterioles, venules, capillaries, and mesenchymal cells, which are embedded in the canal matrix. In the bird, they form at prehatching; however, in murine, the cartilage canals are found after birth (24). The cartilage canals are significant for the nourishment of the developing cartilage and removal of waste materials. In addition, they are vital for the creation of the secondary ossification center, normal epiphyseal development and establishment of the growth plate (25).

## Conclusion

There are differences in the timing of bone development among quails. In Japanese quail, the chondrification and ossification for the first time found on 5<sup>th</sup> and 8<sup>th</sup> day of incubation, respectively. Intramembranous ossification of the furcula observed on 9<sup>th</sup> day. The carpal bones and minor digit were remained none ossified at the prehatching period. The humerus as long bone developed by endochondral ossification and cartilage template was formed on 6<sup>th</sup> day. The cartilage canals penetrated the epiphyses without the formation of a secondary ossification center at prehatching.

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## Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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## تطور عظام الجناح في جنين السمان الياباني *Coturnix japonica*

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### الخلاصة

السمان هو نموذج أساسي في أبحاث الطيور بسبب الأهمية الاقتصادية في صناعة الدواجن في جميع أنحاء العالم. وفضلاً على ذلك، استخدامه في مجال بحوث الأجنة التجريبية. لذا هدفت الدراسة إلى التعرف على بداية التغضرف والتعظم لعظام الجناح بالإضافة إلى تلخيص التطورات النسيجية الرئيسية المتعلقة بتكوين عظم العضد في السمان الياباني. تم أخذ ستة أجنة كل ٢٤ ساعة من ٣ إلى ١٦ يوماً من الحضانة. تم تحضير وتلوين ثلاث أجنة باللون الأزرق والأليزارين بالأحمر للغضاريف والعظام على التوالي وتم تحضير الأجنة الثلاثة المتبقية للفحص النسيجي. أظهرت نتائج الفحص المجهرى أن عظم العضد والكعبرة والزند والغرابي والكتف وعظم المشط الثالث والرابع التي بدأت بالتغضرف في اليوم الخامس. وعلى الرغم من ذلك، لوحظت علامات التعظم الأولى في العضد والكعبرة والزند في اليوم الثامن. بينما بقي الأصبع الصغير غير متعظم حتى قبل التفقيس. أظهرت المراحل المجهرية لعظم العضد النامي المرتبط ببرعم الأطراف، وتكون الأديم الظاهر القمي والتمايز الغضروفي في اليوم الثالث والرابع والخامس على التوالي. تم إنشاء قالب الغضروف في اليوم السادس. تشكلت الغشاء والصفي من قالب الغضروف في اليوم السابع والثامن، تكون طوق السمحاق العظمي في اليوم الثامن، بدأ التجهيز الدموي في الغضروف في اليوم التاسع. لوحظ اختلافاً في توقيت ظهور التغضرف والتعظم في الهيكل العظمي للقائمة الأمامية، وكان تطور عظم العضد بطريقة التعظم داخل الغضروف. يجب أن تؤخذ النتائج التي تم الحصول عليها من الدراسة الحالية في الدراسات المسحية والجزيئية في تكوين الهيكل العظمي.