Study of Ossification Stages in Quail Embryo (*Coturnex japonica*)

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Abstract

A morphogenesis study has been made for the skeleton formation of the quail (*Coturnex japonica*) which included the analytical study to make the skeletal elements and the transitional changes that happens in the two processes of cartilage formation (Chondrofication) and bones (Ossification) during the grow of the different natural phases for the diverse bones of the body.

A quail was gathered of about (100) birds and brought up at home. Then the fertilized egg was gathered from those birds in about (7-9) days, then those egg were incubated by an Ukranian electronic incubator in a about (37,5-38 C^0) and 70% of temperature and humidity respectively. The embryos were used starting from day 3 of incubation to day 16 of incubation , which is the hatching day.

Embryos were extracted from the egg starting from day 3 of incubation, then they were treated with Alcian blue pigment that is related to paint the (non ossified) cartilaginous parts of bones, and Alizarin Red – S pigment that is related to paint the ossified parts of the bones as an indicator for calcium in order to recognize the ossified parts from those that didn't ossified for each bone of the skeletal system of the quail.The method that used to treat the embryos with the two pigments differed with the age progress of the embryo , also it's concentration increased with the progress of the embryos age due to the formation of the organs, skin and muscles as well as the strengthening of the cartilage and it's transformation into solid bones day after day.

The successive phases for the growth of the different bones were traced with complete concentration on the evolution of the cartilage (Chondrofication) and it's transitional transformation into bone (Ossification) for each bone of the vertebral skeleton , thorax gage , and the fore and hind limbs bones.

The comparison of the transitional natural changes that happen in both processes chondrofication and ossification during the growth of the embryo with the un natural disorder events helps in the explanation of the effect of different factors on the growth including the genetic factors and the mutations that happens during ossification and the effect of hormones on the formation of bones and it's growth in the diverse stages, these effects are studied now in a special field called (Teratological studies).

Introduction

Embryology defined as the study of morphogenesis of an individual life from the fertilization to the sexual maturation, including growth, differentiation and ways of controlling the activities of fetal configuration like regeneration, wound healing and hormonal control (2 and 7).

The quail is a small migratory bird , domesticated for two purposes, to produce egg and meat. And it's called in our Arabian countries in different names like(السمان والسلوى) .The most common name is (السلوى). It's characterized by it's very tasty taste, and it's one of the most important investable birds and became the focus of attention of researchers and investors. It's a domesticated bird despite being a migritory bird, where it's migrate to Iraq during autumn. (1, 2, 16, 17 and 21)

The quail is characterzed by being small in size, skinny bird, having no tail and it is very different wild birds and able to adapt.Females are characterized than males by it's light-colored feathers with the present of black dots in throat area and upper chest area.Males being gray colored with brown neck area and it has the cloacal gland above the anal whole secrete white liquid (foamy substance) help during the process of sexual intercourse (4).

Ossification is the process of creating bone , that is of transforming cartilage (or fibrous tissue) into bone .The animal skeleton initially consists largely of cartilage which is relatively soft and gradually transformed into hard bone during infant and child development .

There are two types of ossification

1- Intramembranous Ossification

Bone develops within stromal connective tissue that is characterized by mesenchymal stem cells, connected by thin cell processes, lying in a matrix of haphazardly arranged collagenous fibrils. Immediately before ossification commences two changes are observed; the mesenchymal stem cells proliferate and start to differentiate, finally forming osteoblasts, and the intercellular matrix becomes more dense and homogeneous.

These changes alone are sufficient to induce a suitable environment for early calcification to commence, and the mineral content of the matrix increases rapidly. The osteoblasts augment the process by producing more matrix that is calcified, and some of these cells will become trapped in the tissue and will transform into osteocytes. Until the bone has reached the final size, a layer of osteoblasts remain on the periosteal surface. The same process occurs for flat bones and on the periosteal surface of the diaphyses of long bones. (18).

2- Endochondral Ossification .(Interacartilagenous).

Endochondral ossification occurs where bones elongate at a growth plate. This plate is arbitrarily divided into specific regions for descriptive purposes. At the epiphyseal front there is a layer of hyaline cartilage formed by cartilage cells, some of which may be embedded in matrix.The older cartilage cells begin to multiply and form into columns separated by wide parallel bands of interstitial substance. The cells are separated from each other by a thin capsule of matrix. These cells hypertrophy and incorporate stores of glycogen. Providing there are adequate concentrations of minerals available, the intercellular matrix then starts to

calcify, particularly between adjacent columns of cells. This zone forms a provisional structural framework between the growth plate and the cancellous bone of the metaphysis. Loops of blood vessels then invade the connective tissue and penetrate into the vertical columns. The interstitial tissue is removed, leaving calcified vertical columns of matrix known as the primary spongiosa. This primary spongiosa is considered to be the necessary scaffolding upon which the bone matrix can be deposited. In this way the newly formed endochondral bone mirrors the cartilage model which it has replaced. The key feature of this hypothesis is that the cartilage model forms first and the bone is laid down onto that model. As bone matrix is laid down upon the primary spongiosa they are transformed into secondary spongiosa, a more permanent set of trabeculae. These will be modified by the joint action of osteoblasts and osteoclasts to form the thickened adult trabeculae, which are clearly visible upon gross examination of the cut surface of bone. The pattern of mineralization at the growth plate can be clearly demonstrated by autoradiography and is of some interest. (9) showed that soon after calcium 45 (^{45}Ca) administration heavy deposits of radioactive ion are seen in the growth plate and adjacent trabecular bone of the metaphysis. Thirty days after (^{45}Ca) administration, the radioactive content of the plate is relatively low and concentration in the trabecular bone is less than on day one. By 60 days, osteoclastic activity has removed and remodeled almost all the newly formed bone and the level of radioactivity observed is low in all areas. Once an animal achieves skeletal maturity, bone stops growing in length and there is no further new formation of bone. The skeleton continues to be modeled and remodeled but the rate of change is considerably less than during the growth phase. Radioactive calcium introduced into bone at this stage may take years to be resorbed and removed. This underlines concerns about the hazards from certain radionuclides, for example strontium 90 (^{90}Sr) or strontium 89 (^{89}Sr), which have been shown to accumulate selectively in the skeleton (13).

Materials and methods

Experimental design

Fertilized eggs ,weighing (8-14) grams obtained from *Coturnix japonica* where stored at 15 ^{o}C. They where incubated in an Ukrainian manufactured incubator ,after adjustment of the temperature and humidity at 37.5 - 38 ^{o}C. and 70% respectively .With continous turning of eggs three times daily ,except last three days of hatching period wich is 16-17 days in *Coturnix japonica* .Then at the day 3 of incubation four embryos will be prepared daily throughout the whole 16 – 17 days to the double staining technique.

Skeletons of the embryos were stained with Alcian blue and Alizarin red - S for cartilage and ossified bones , respectively (14).The modified procedure of (15) , (20) , and (19) was used in the current study.

Observation of the skeletons (3-7 day embryos) was performed under a dissecting microscope paying attention to the timing of chondrofication and calcification. Observation of the skeletons (8-16 day embryos) was performed under a digital camera , since the size of the embryos became bigger than the eye pieces and the objective lenses of the dissecting microscope , and can't display the whole size of the embryo. Chondrofication was confirmed by blue color stained with Alcian blue and calcification by red color stained with alizarin red-S.

Results and discussion

The developmental features of the whole skeleton of 3-16 day embryos were described during the course of incubation . For convenience of description , the skeleton was divided into five parts , that is vertebrae, ribs, sternum, forelimb , and hind limb, on and after 5 days of incubation . The forelimb and hindlimb include the pectoral girdle and pelvic girdle , respectively. For (3 – 7) day embryos were observed and examined through dissecting microscope , but for (8 – 16) day embryos were observed and examined through digital camera.

At day 6 of incubation.

The length of the humerus (LH)= (3.7 +_ 0.25 mm) . The humerus , radius , and ulna are now recognizable well and they were stained blue at this stage, also the first phalanges of the third and fourth digits were stained blue.

The length of the femur (LF)= (4.3 +_ 0.25 mm).The femur, tibia , and fibula are now stained blue and well recognized. The first phalanx of the second digit, the first and second phalanges of the third and fourth digits were stained blue also.

(14), said that the pubis , ischium , first metatarsus , first phalanx of the second digit , first and second phalanges of the third digit , and the first and second phalanges of the fourth digit were stained blue. Also said that the proximal ends of the second to fifth metatarsi fused with the nodule of the fused distal tarsi.

At day 10 of incubation.

Forelimb, LH= (8.4 +_ 0.15mm). PH= (50 +_ 3%).The first and the second phalanx of the third digit turned red at the central portion. (6) said that the chondrotic drafts of carpal elements including carpiradiale and carpiulnare, and 1st phalanx at 3rd digit of the forelimb were stained blue. Likewise the pelvic girdle elements including the ilium , ischium, and pubis, in addition to chondrotic drafts of the first metatarsal and first phalanx of the second ,third , and fourth digits were stained blue. That is approximately parallel to that of turkey except pelvic girdle and digits occurred at day 11 of incubation (5).

Hindlimb, LF= (9.7 +_ 0.15mm). PF= (58 +_ 2.5%).The pubis , first phalanx of the first digit, second phalanx of the second digit, and the third phalanx of the third digit were stained red at the central portion of each bone. That is near to (14) observations , but they added , a cartilaginous process extended toward the distal direction from the back portion of the proximal region of the tarsometatarsus.

At day 12 of incubation.

Forelimb, LH= (9.4 +_ 0.2mm). PH= (70 +_ 2.5%).The first and the second phalanges of the second digit partly turned red. While (14) observed only elongation in the fore limbs at this day of incubation. (6) observed the first red turning of the humerus at this stage, which occurred later in relation to the femur was typically parallel to accumulating data in turkey embryo (5). But he disagreed with them in the timing, which occurred in the fore and hind limb at 12 and 13 days of incubation , respectively. They speculate this slight delay as an indicator for the difference of the precocity degree.

The lifestyle and feeding may plays an important role in the sequences of the skeletal developmental differentiation between species (10).

Hindlimb, LF= (10.8 +_ 0.2 mm). PF= (80 +_ 3%).The red (ossified) regions of the third phalanx of the second digit, and the fourth phalanx of the third digit still expanding.

At day 14 of incubation.

Forelimb,LH= (10.8 +_ 0.2 mm). PH = (85 +_ 2%). The first and second digits are still ossified at the central portion and the red (ossified region) still expanding. The coracoid partly turned red. (14) said that the forelimb only elongated at this day of incubation without mention any other changes. (6) observed at this day of incubation in geese , the coracoid elements of the pectoral girdle at it's mid – diaphyseal portion turned red. For comparison , this element turned red in chick at stage (7.5-8) days (11), and 12 days (8), and in the turkey at 14^{th} day of incubation (5).

Theese variations of time of onset of ossification of this bone may related directly to the degree of precocity (12).

Hindlimb, LF= (12.9 +_ 0.25 mm). PF= (88 +_ 1.5%).The femur , tibia, and fibula completely turned red. The 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} phalanges of the hindlimb digits partly turned red . Ilium turned red, but ischium and pubis still partly ossified.

At day 16 of incubation. (Fig.5).

Forelimb, LH = (14.8 +_ 0.4 mm). PH = (95 +_ 2%). Elongated and enlarged significantly. That was typically similar to (14).

Hindlimb, LF = (17.3 +_ 0.5 mm). PF = (95 +_ 0.25%). Elongated and enlarged significantly.

In geese and at this day of incubation the phalangeal elements including the 1^{st} phalanx of the 2^{nd} digit of the forelimb , in addition to the 1^{st} phalanx of the 1^{st}, 2^{nd} , and 4^{th} digits and 2^{nd} phalanx of the 3^{rd} digit showed appearance of ossification centers (6).

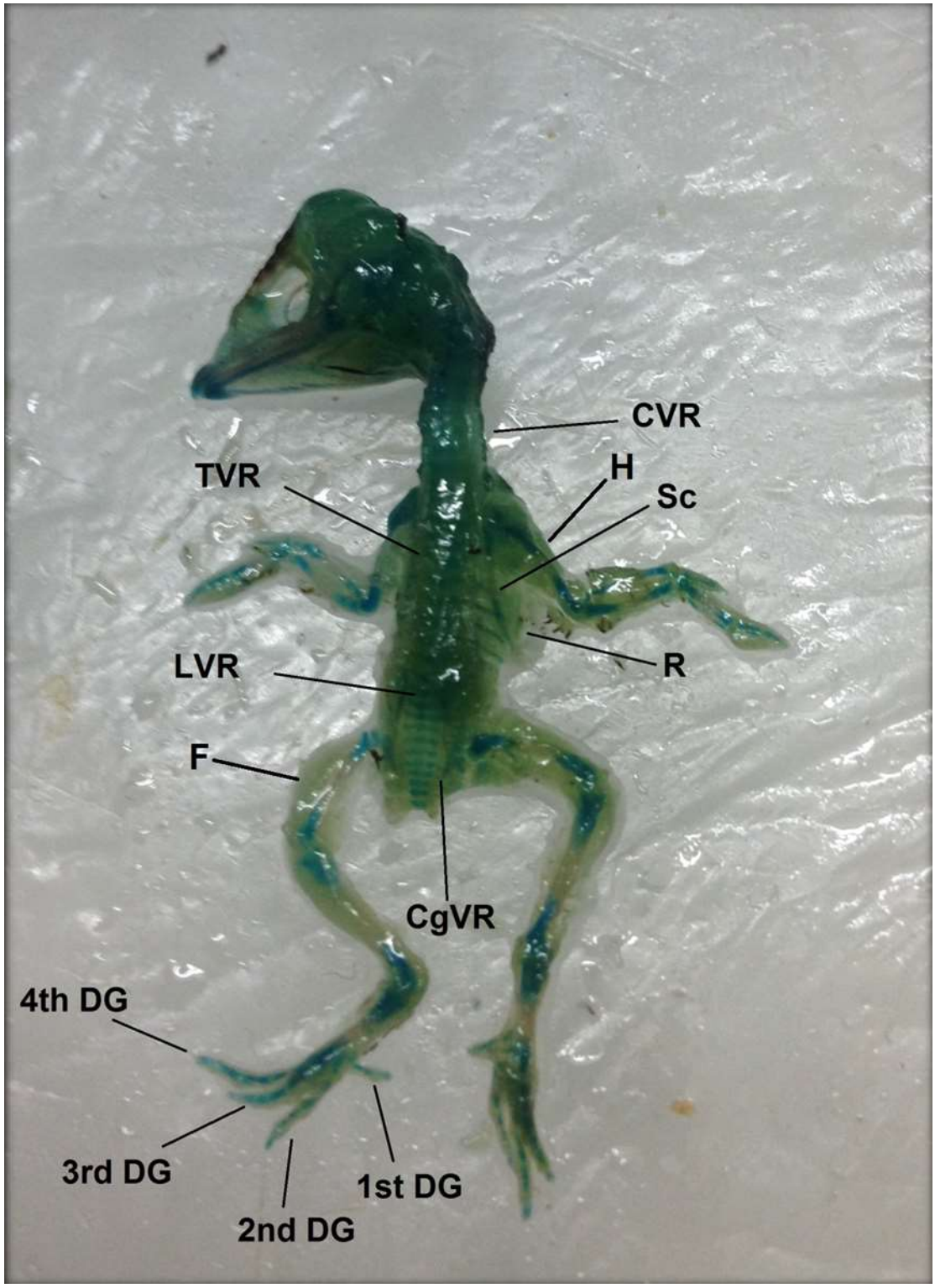

Fig (1). Whole mount of 9 days of incubation embryo, Cervical vertebral region (CVR), Thoracic vertebral region (TVR); Lumbosacral vertebral region (LuVR); Coccygeal vertebral region (CgVR); Scapula (Sc);Humerus (H); Ribs (R); Femur (F); Digits (1st, 2nd, 3rd, and 4th DGs) .

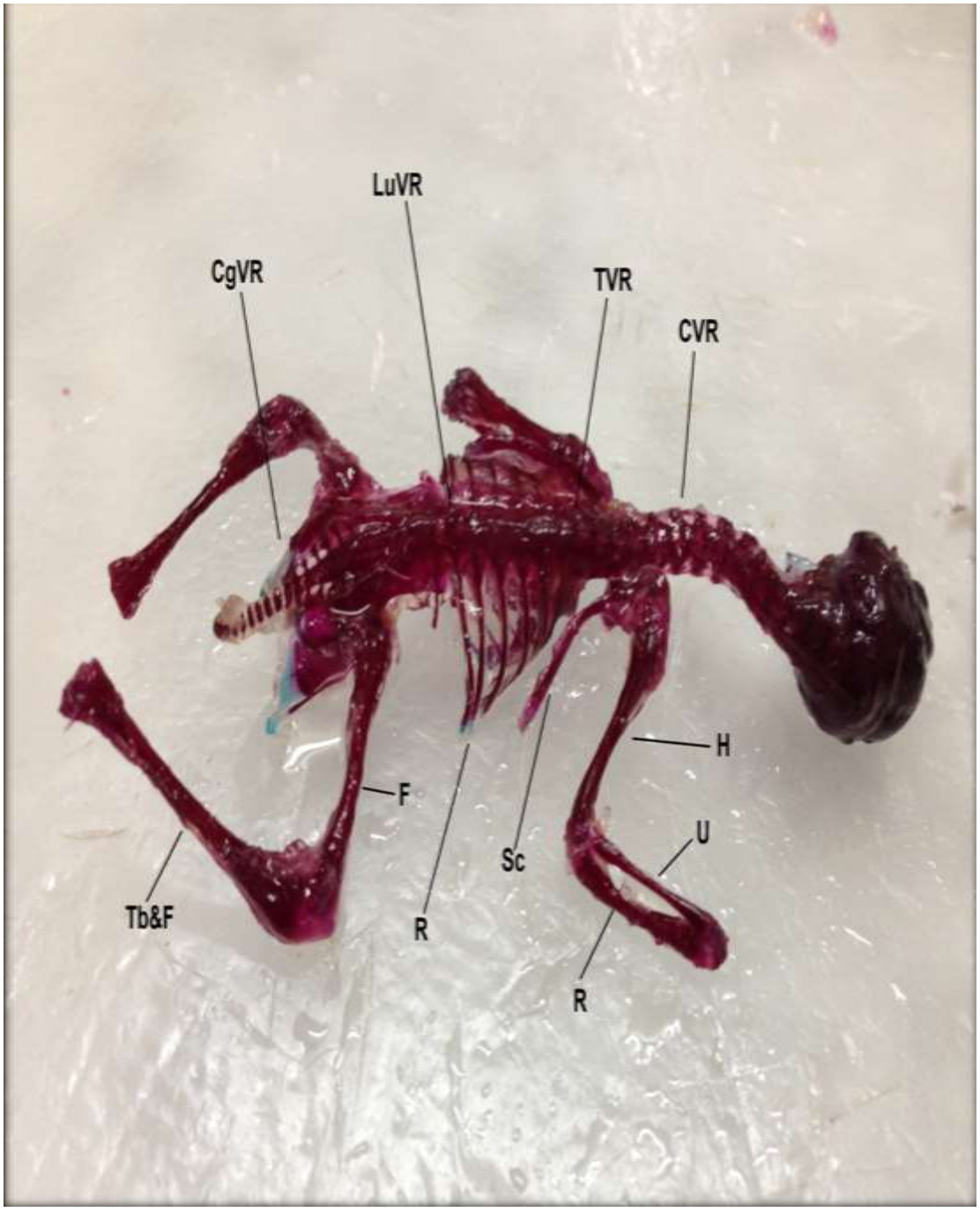

Fig (2). Whole mount of 16 days of incubation embryo, cervical vertebral region (CVR); Thoracic vertebral region (TVR); Lumbosacral vertebral region (LuVR); Coccygeal vertebral region (CgVR); Humerus (H); Radius and Ulna (R&U); Scapula (Sc); R (Ribs); Femur (F); Tibia and Fibula (Tb&F).

Conclusion

The appearance of cartilage was late in the fore and hind limbs skeletal system, next after the vertebral skeletal system and before the ribs and sternal system. Where the long bones of the limbs showed chondrofication at day 4 of incubation. But the onset of ossification was earliest among the other systems (Vertebrae , ribs and sternum), where most of the fore and hind limbs proximal bones had an ossified regions at day 8 of incubation, Chondrofication and calcification progressed from proximal to distal bones. In all long bones, Calcification first occurred at the medial region and then progressed to the proximal and distal regions. The patella appeared at day 9 of incubation and remained cartilaginous at hatching.

Recommendations

1- It is important to complete a post hatching study on the non-ossified cartilaginous elements , in order to determine the time of calcification of these elements.
2- Studying the fine structure of the beginning of cartilaginous cell by using EM.
3- Using specific techniques, chemicals , and materials to make a teratological studies of some skeletal elements of the Japanese quail , correlated with the ossification.
4- Looking at the skeletal developmental disorders , as compared with the events takes place during the normal developmental morphogenesis of skeletal elements of this bird. In this study May resulting in studies can help to avoid like this embryonic disorders.
5- Study the hormonal interference , particularly , parathyroid hormones accompanied with the skeletal development of this bird.
6- Studying the genetic material that affect the normal developmental morphogenesis of the skeletal system of this bird. (Gene Expression).

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دراسة مراحل ألتعظم في جنين طائر ألسمان (ألسلوى)

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الخلاصة

اجريت دراسة النشأة المظهرية لتكوين الهيكل العظمي في طائر السلوى , (*Coturnex japonica*) والتي تضمنت الدراسة التشريحية لتخلق العناصر العظمية والتغيرات الانتقالية التي تطرأ في عمليتي تكوين الغضاريف (التغضرف) والعظام (التعظم) خلال نمو الانماط الطبيعية المتنوعة لعظام الجسم المختلفة., تم جمع طائر السلوى بعدد (١٠٠) طائر وتمت تربيتها منزليا . ثم تم جمع البيض المخصب من هذه الطيور بفترة ٧–٩ ايام ثم تمت عملية حضن هذا البيض المخصب بواسطة حاضنة كهربائية اوكرانية الصنع بدرجة حرارة ٣٧،٥ – ٣٨ درجة مئوية ونسبة رطوبة بلغت ٧٠%. استخدمت الاجنة ابتداءا من عمر ٣ ايام ولغاية ١٦ يوم حضانة (يوم الفقس).حيث تم استخراج الاجنة من البيض ابتداءا من عمر ٣ ايام , ثم تمت معاملتها بصبغتي (Alcian Blue) الخاصة بصبغ الاجزاء الغضروفية غير المتعظمة للعظام و صبغة (Alizarin Red – S) الخاصة بصبغ الاجزاء المتعظمة " ككاشف عن الكالسيوم". للتمييز بين الاجزاء المتعظمة عن الاخرى غير المتعظمة لكل عظم من عظام الهيكل الغضروفي لطائر السلوى .طريقة معاملة الاجنة بالصبغتين اختلفت مع تقدم عمر الجنين واختلاف المراحل العمرية له, وكذلك تراكيز هذه الصبغات ايضا ازدادات مع تقدم عمر الجنين نظرا لتكون الاحشاء والجلد والعضلات وتصلب الغضاريف وتحولها الى عظام صلبة يوما بعد يوم .تمت المتابعة المستمرة للمراحل المتعاقبة لنمو العظام المختلفة مع التركيز التام على نشوء الغضروف (التغضرف Chondrofication) وتحوله الانتقالي الى عظم (التعظم Ossification) لكل عظم من عظام الهيكل الفقري والقفص الصدري والاطراف الامامية والخلفية.

ان مقارنة التغيرات الطبيعية الانتقالية التي تطرأ في عمليتي تكوين الغضاريف (التغضرف) والعظام (التعظم) خلال نمو الجنين مع الاحداث المشوهة غير الطبيعية, تساهم في التفسير عن تأثير العوامل المختلفة على النمو ومن ضمنها العوامل الوراثية والطفرات التي تحدث احيانا خلال عملية تكوين العظام. وتأثير المواد المختلفة ومن ضمنها الهرمونات على نشوء وتكوين العظام ونموها في المراحل المختلفة.أن هذه التأثيرات تدرس حاليا في مجال خاص سمي (Teratological Studies).