STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 69, 1, 2024 (p. 143-154)
doi:10.24193/subbbiol.2024.1.07

Adaptation of the diaphonization protocol and the highlight of some significant structures development in the chicken embryo (*Gallus gallus*) skeleton

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Article history: Received 14 February 2024; Revised 19 April 2024; Accepted 10 June 2024; Available online 30 June 2024.

Abstract. Diaphonization is a technique used in developmental biology, anatomy, and comparative morphology to visualize and study the internal structures of small organisms. In this study, we used diaphonization to visualize the development of chicken embryos (*Gallus gallus*). Diaphonization was performed on chicken eggs at different stages of development, from 10 to 13 days of incubation, and the resulting specimens were analyzed using microscopy. The results suggest that for embryos older than 14 days, a longer storage time in 1% KOH is recommended (approximately 30% longer incubation time compared to the original protocol). In the case of more developed chicken embryos, it is also recommended to carry out evisceration. These results provide insight into the early stages of avian development and may have applications in the fields of developmental biology and anatomy.

Keywords: diaphonization protocol, chicken embryo development, skeletal visualization, developmental biology, avian skeletal adaptation.

Introduction

Diaphonization, also known as "clearing and staining", is a staining technique used to prepare wet specimens, typically animal embryos or small vertebrates (Liutenko *et al.*, 2023). The process involves making the tissues of the specimen transparent while staining cartilage and bone to make them more visible (Chitra and Sharon, 2020; Vovk *et al.*,2022).

In the past, this technique has been used primarily for anatomical studies, scientific research and educational purposes. It allows for a detailed examination of internal structures, particularly skeletal and soft tissues, aiding in the understanding of anatomical features, their development, and variations among different species (Atanasoff *et al.*, 2018). The resulting specimens can be further used for scientific purposes or as an educational tool in universities or public museums (Liutenko *et al.*, 2023). Moreover, by making internal structures visible, diaphonization aids in the comparison of anatomical features between different species, contributing to the understanding of evolutionary relationships and adaptations (Khan *et al.*, 2015; Tsandev *et al.*, 2020).

While newer imaging technologies have advanced in recent years, providing non-destructive ways to visualize internal structures, diaphonization continues to be used due to its ability to provide detailed, three-dimensional insights into anatomical structures, especially bones and cartilage, which some imaging technologies like X-ray microtomography or magnetic resonance microscopy may not capture as comprehensively. Additionally, this technique is crucial nowadays in the field of developmental biology because it allows for the visualization of embryos and their developmental stages in intricate detail (Stern, 2022). This leads to researchers being able to study how skeletal elements and organs form and change during development, identify abnormalities or variations in development and understand evolutionary relationships and adaptations across different taxa (Atanasoff *et al.*, 2018).

The diaphonization protocol used on rat embryos (see materials and methods section) was tested on chicken embryos and optimized in order to obtain viable wet specimens. The modifications were mandatory since the chicken embryo is significantly larger than the rat embryo after twelve days of incubation (Hamburger and Hamilton, 1951). The specific stage of embryonic development is an important element in adapting the diaphonization protocol. Late-stage embryos, from the 15th day of incubation, have some internal organs, such as air sacs and guts, much more developed than mid-stage embryos, from the 10th day of incubation, which need to be taken into consideration when using this technique (Chatterjee, 2015). While rat embryos follow mammalian developmental patterns, chicken embryos show avian developmental characteristics such as feathering (Sullivan *et al.*, 2017). Those feathers are a hindrance to the staining process, making them also a thing to consider when working with chicken embryos (Gofur, 2020).

In this context the main objectives of this study were to optimize the diaphonization protocol, making it viable for chicken embryos; and to highlight skeletal structures in upper limb, lower limb and tail.

Materials and methods

The diaphonization protocol was completed two times. For each attempt, 20 chicken eggs of farm origin were incubated at 37°C in a manual, still-air mini-incubator, and turned daily. The eggs were evenly taken out of incubation after 10, 11, 12 and 13 days, following this, the protocol was applied. The experiment was performed in duplicate during a two month period. During the second experiment, the eggs were removed from the incubator two days earlier compared to the first batch. Details of the experimental design are presented in Table 1.

Table 1. Steps of the diaphonization protocol performed on chicken (*Gallus gallus*) embryos.

Day	Number of eggs sampled	Steps	Number of viable embryos
1	20	Start of incubation	-
2-11		Manual egg turning	
12	3	Embryo extraction, washing in tap water and fixation in 35mL ethanol 96%	3
13	6	Embryo extraction, washing in tap water and fixation in 35mL ethanol 96%	5
14	11	Embryo extraction, washing in tap water and fixation in 35mL ethanol 96%	3
	11 embryos were used for the rest of the experiment		
15	Staining with Alcian blue solution for 24 hours		
16	Washing twice in 35mL ethanol 96% for 5 minutes each and store in 35mL ethanol 96% for 24 hours		
17	Clarification with 35 mL KOH solution (1%) for 6 hours Staining with Alizarin red solution for 3 hours Clarification with 35 mL KOH solution (2%) for 2 days		
18	Washing in 35 mL of 80/20 solution made of KOH (2%) and glycerin for 5 hours		
19	Washing in 35 mL of 60/40 solution made of KOH (2%) and glycerin overnight		
20	Storage for unlimited time in 35 mL of 20/80 solution made of KOH (2%) and glycerin		

The experimental design was adapted after the diaphonization protocol for rat embryos (provided courtesy of Prof. Dr. Iacob Checiu, not published):

1) Fixation using 35mL of 96% ethanol for 3-5 days;
2) Staining for 24 hours. using Alcian blue (final concentration 0.15 mg/mL) dissolved in a solution consisting of 80 mL of 96% ethanol and 20 mL of glacial acetic acid;
3) Washing twice using 35mL of 96% ethanol for 5 minutes each;
4) Storage of the resulting embryos for 24 hours. in 35 mL of 96% ethanol;
5) Carification using 35 mL of KOH solution (1%) for 2-6 hours;
6) Staining for 1-3 hours. using Alizarin red (final concentration 0.05 mg/mL) dissolved in 1L of KOH solution (2%);
7) Clarification using 35mL of KOH solution (2%) for 4 hours. to 3 days;
8) Washing the embryos twice after clarification with 35 mL of KOH(2%)/ glycerin solution with a ratio of 4:1 and 3:2 for 24 hours each.
9) Storage the embryos for unlimited time in 35 mL of 1:4 solution made of KOH (2%) and glycerin.

Results and discussions

The protocol was successfully implemented for chicken embryos between 10 and 13 days of incubation. As embryos approach the late-stage of development, their bones and cartilages are much more matured and well-defined, making the 12th and 13th day embryos the most successful in terms of staining from our batch (Figure 1).

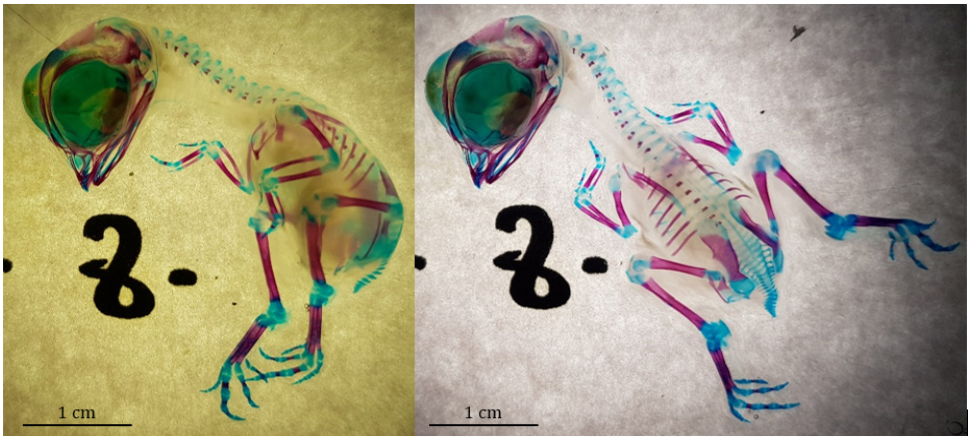

Figure 1. Chicken (*Gallus gallus*) embryos from the 12th day of incubation.

Highlighted structures

The skeleton of birds is particularly adapted to flight. Both limbs and girdles have been modified during evolution to make this type of movement more effective (Chatterjee, 2015; Gofur, 2020). These changes can be observed during the development stages of the embryos (Hamburger and Hamilton, 1951, Namba *et al.*, 2010), the most important and significant ones, captured by us are described below.

Upper limb. In the upper limb of the 10th day chicken embryo (Figure 2A) the staining observed is predominantly Alcian blue, with little to no red, due to the coloring agent's action on the polysaccharides, specifically on the glycosaminoglycans present in cartilages (Liutenko *et al.*, 2023). It allows for the differentiation of the structures constituting the wing, such as the humerus, radius, ulna, carpals, metacarpals and phalanges. Embryos from the 11th day of incubation (Figure 2B) present a more intense Alizarin red staining compared to the embryos on the 10th day. The phalanges are more clearly defined, and due to the staining, we can observe the metacarpals, which will later fuse. Fingers 3 and 4 display Alizarin red staining, while finger 2 only shows Alcian blue staining. This finger displays

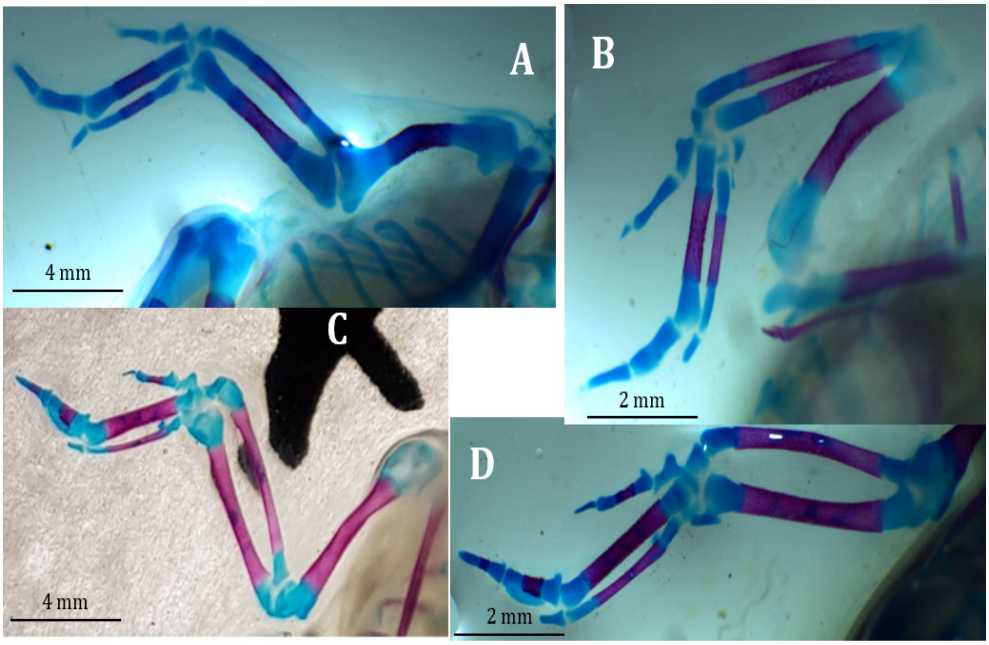

Figure 2. Upper limb of the chicken embryo from the 10th (A), 11th (B), 12th (C) and 13th (D) day of incubation.

Alizarin red staining for the first time in the specimens from the 12th day (Figure 2C), those also having the radius, ulna, and the metacarpal of finger 3 wider compared to the previous embryos. On day 13 (Figure 2D), the carpal and wrist bones are clearly visible. Additionally, the dilation of the phalange in finger 3 increases while the metacarpal of finger 4 becomes thinner.

Table 2. Summary of observations for the upper limb of chicken embryos.

Day of incubation	Observations
10th day of incubation	The structures constituting the upper limb, such as the humerus, radius, ulna, carpal bones, metacarpal bones, and phalanges, are differentiated. The predominant staining is Alcian blue.
11th day of incubation	Alizarin red staining appears in the humerus, radius, ulna, carpal bones, metacarpal bones, and phalanges, except for the phalanges of finger 2.
12th day of incubation	The more intense Alizarin red staining is due to osteogenesis, which also appears in the phalanges of finger 2. There is a dilation observed in the phalanx of finger 3. The radius, ulna, and metacarpal of finger 3 are wider.
13th day of incubation	The carpal and the wrist bones are visible. The dilation of the phalanx in finger 3 increases, while the metacarpal bone of finger 4 becomes thinner.

Lower limb. On the 10th day of incubation we observe the femur, tibia, and fibula very clearly (Figure 3). Between the tibia and the phalanges, which only have an Alcian blue staining, are the three bones that, through fusion, will form the tarsometatarsal bone. On the 11th day of incubation, the phalanges are more developed, the fibula differentiates more slowly compared to the tibia, and the Alizarin red staining is more pronounced due to the presence of osteocytes containing calcium. The embryo on the 12th day of incubation shows Alizarin red staining on the phalanges, except for the posteriorly positioned digit 1, which changes its shape by narrowing the areas where osteocytes are present. The Alcian blue staining is only found at the knee and heel levels, otherwise, Alizarin red is predominant. On the 13th day of incubation, osteocytes are present even in digit 1, evidenced by the Alizarin red staining. The three bones forming the tarsometatarsal are close but not yet fused. The claw-like shape of the distal phalanges in all digits is very distinct (Table 3).

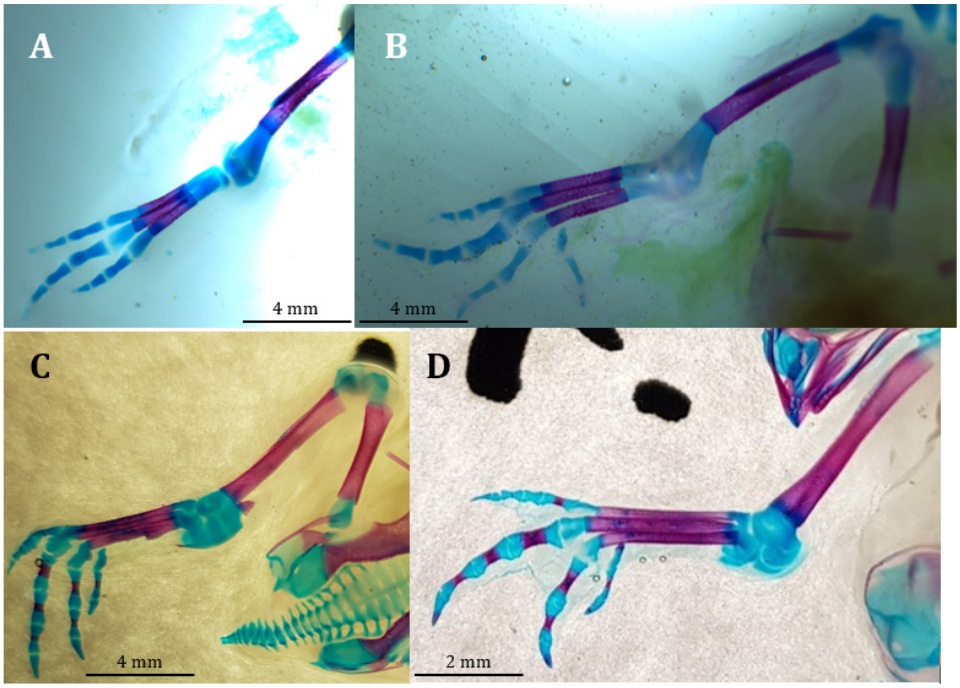

Figure 3. Lower limb of the chicken embryo from the 10th (A), 11th (B), 12th (C) and 13th (D) day of incubation.

Table 3. Summary of observations for the lower limb of chicken embryos.

Day of incubation	Observations
10th day of incubation	The femur, tibia, fibula, and the bones that will form the tarsometatarsal exhibit Alizarin red staining in addition to Alcian blue staining. The phalanges only have blue alcian staining.
11th day of incubation	The phalanges are more developed and the fibula differentiates more slowly compared to the tibia. The Alizarin red staining is more pronounced.
12th day of incubation	All phalanges, except those of digit 1, show Alizarin red staining, and the areas where it appears are narrower. Alcian blue staining is present around the phalanges only at the knee and heel level.
13th day of incubation	The phalanges of digit 1 exhibit Alizarin red staining; the three bones forming the tarsometatarsal are close but not fused. The distal phalanges of all digits have a claw-like shape.

Tail and pygostyle. The vertebrae that will contribute to the formation of the tail are spaced and decrease in length up to the last coccygeal vertebrae in the embryo on the 10th day of incubation (Figure 4). The structure is entirely Alcian blue. Condensed areas of Alcian blue staining are observed on the sides of the vertebrae in the embryo on the 11th day of incubation, and the last vertebrae are getting closer to each other. The vertebra from the embryo in the 12th day of incubation are larger and closer to each other in order to form the pygostyle. The last ones are going to partake in the process of fusion. In the 13th day of incubation, the developing pygostyle is very well observed. All vertebrae are closer and interconnected, and the coccygeal ones are partially fused or in the process of fusion. Alizarin red staining is absent in this case as well (Table 4).

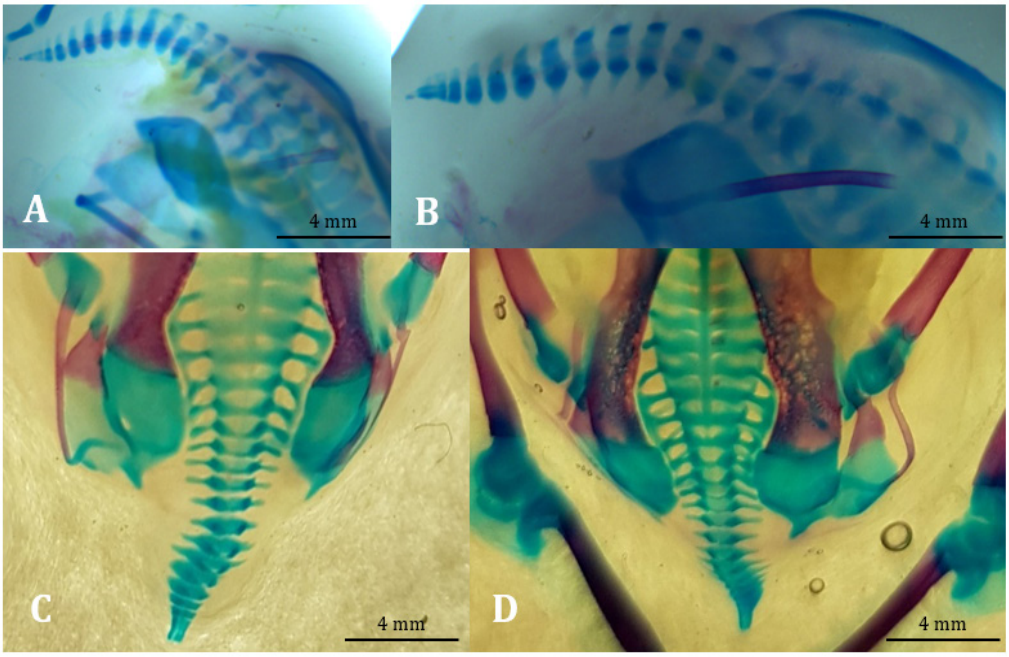

Figure 4. Tail of the chicken embryo from the 10th (A), 11th (B), 12th (C) and 13th (D) day of incubation.

These findings underscore the intricate process of skeletal development in chicken embryos, offering a detailed timeline of bone and cartilage formation. This knowledge is pivotal for developmental biology, providing a framework

for studying skeletal abnormalities and evolutionary biology. One example of evolutionary study is the tarsometatarsal bone, which in the chicken embryo is made up of three bones unfused, meaning that it has the structure closely related to that of reptiles, such as *Archaeopteryx*.

Table 4. Summary of observations for the tail of chicken embryos.

Day of incubation	Observations
10th day of incubation	The staining is entirely Alcian blue. The vertebrae are consistently spaced and naturally decrease in length.
11th day of incubation	Distinct areas of condensed Alcian blue staining are observed on the sides of the vertebrae. The last vertebrae are getting closer to each other.
12th day of incubation	The proximity of the vertebrae intensifies. They are wider and narrower, and the last ones are in the process of fusion.
13th day of incubation	The developing pygostyle can be observed. All vertebrae are close and interconnected, and the coccygeal ones are partially fused or in the process of fusion.

Protocol improvements

The improvements made to the diaphonization protocol are targeted towards embryos with darker skin pigmentation or those that have surpassed 14 days of incubation and are more developed. Since the staining shows lower efficiency and clarity in these cases, we recommend a longer storage in 1% KOH, approximately 30% more than the initially mentioned time. The clarification stage being extended by almost two hours allows the KOH to chemically break down more soft tissue. Another suggestion would be to add an evisceration step before fixation, ensuring that the stains in bones and cartilages are more distinct and clear.

Such improvements can be applied for staining other organisms as well, but further research needs to be done. The size and complexity of organs or specific features, such as feathers in our case, contribute to the list of factors needed to be taken into account for a successful staining.

Conclusions

The protocol was successfully applied for chicken embryos up to the 13th day of incubation, those being also the best specimens obtained. Due to the reduced efficiency and clarity of staining the embryos older than 14 days of incubation or with darker skin pigmentation, we propose the following modifications to the protocol: extending the clarification step and performing an evisceration. Firstly, by extending the clarification stage by 30% beyond the initial time, we allow the 1% KOH to chemically decompose more soft tissue. Secondly, through evisceration, we remove internal organs and facilitate the staining of the structures of interest.

Highlighted structures that are closely linked to phylogenetic evolution are the upper limbs, the lower limbs, and the tail and pygostyle. In embryos from days 10-13 of incubation, the upper limb undergoes significant changes at the wing tip level. Alizarin red staining appears later in the wing tip compared to the humerus, radius, or ulna. Digit 4's metacarpal thins to fuse with digit 3's metacarpal, which dilates at the penultimate phalanx. Digit 2 remains less developed, maintaining a consistent size. The lower limb shows notable changes at the terminal segment, where phalanges enlarge, thin medially, and take on a claw-like shape. The three bones forming the tarsometatarsal bone remain unfused, a trait seen in reptiles, particularly *Archaeopteryx*. In the tail, caudal vertebrae converge, with the last four fusing to form the pygostyle, and the beginning of the fusion of the first caudal vertebrae is also clearly observed to form the sacral bone. This region is entirely stained with Alcian blue, with no Alizarin red staining observed.

Further exploration with this staining technique will most likely lead to advances in the quality of the protocol for a broader range or organisms. With this development, more progress can be made in the field of developmental biology and anatomy by using diaphonization for comparative analysis between different species, embryonic studies or even for educational purposes in universities of public museums.

Acknowledgements. We thank Dr. Dorina Podar and Dr. Horia Banciu for the support given with reagent acquisition for the laboratory protocol.

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