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Study of *ex-ovo* Embryonic Development of *Gallus gallus domesticus*

Estudio Inicial del Desarrollo Embrionario *ex-ovo* de *Gallus gallus domesticus*

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MACÍAS, M. O.; GUERRERO, B. A. L.; VALDIVIA, F. A. G.; QUEZADA, T. T.; AVELAR, G. F. J.; SOTO, P. M. M. & DE SANTIAGO, A. S. Study of *ex-ovo* embryonic development of *Gallus gallus domesticus*. *Int. J. Morphol., 41(2)*:668-674, 2023.

SUMMARY: The domestic chicken is a species of bird that has been extensively studied in regard to its biology and as a model organism for science. The reproduction of the species is by the laying of fertilized eggs, which in a period of 21 days will develop a chick inside. Several methods have been described to develop embryos *ex-ovo*, allowing the observation and manipulation of the organism. This work has the propose to standardize a method that allows the development of the embryos inside the artificial incubation system, which has a low cost and is easy to make. In this work, 100 chicken eggs were used to study the effects of humidity, mineral supplementation, and the preincubation time of the egg on the incubation *ex-ovo* of the embryos. Embryo development was documented through the different days. Pulverized eggshell was selected as an optimal source to provide calcium, magnesium, phosphorus, and other minerals to the developing embryo. By providing 900-1200 mg of pulverized eggshell, 40 mL of the 0.001 % solution of benzalkonium chloride, and a preincubation time of approximately 56 h, the embryos were able to develop until 19 days, and even though they did not reach hatching, the incubation conditions that allowed the survival and development of embryos until late stages were achieved. Thus, due to the conditions established for calcium, humidity and preincubation time, in the present work, the chicks reached 19 days of development.

KEY WORDS: Birds; Embryonic development; *ex-ovo* incubation; *Gallus gallus domesticus*; incubation conditions.

INTRODUCTION

Bird embryos have been used throughout time as an efficient biological model for the study of embryonic development in vertebrates, such as in genetic studies, due to their availability, low price, and easy manipulation. *Gallus gallus domesticus* is the most commonly used species due to its size and the accessibility of its embryo. The embryos are extracted in different developmental stages, and then samples, slides, and histological preparations can be made to study each stage (Vilches-Moure, 2019). Before the establishment of artificial cultures for the development of organisms by an *ex-ovo* technique, a common practice was the "window" aperture in the eggshell to facilitate the observation of the organism in development as well as to facilitate the introduction of genes and to perform other manipulations (Andacht *et al*., 2004). Perry (1988) reported a culture method for chick embryos using a surrogate eggshell as an incubation system for the *ex-ovo* culture of the fertilized egg; with this method, the hatching rate was approximately 7 %.

Ex-ovo embryonic development is the extraction of the embryo from the eggshell, which is placed in a medium that will provide the necessary conditions for its development, thereby helping the observation and manipulation of the organism (Kamihira *et al*., 2005). In the studies performed by Boone (1963), the embryos were incubated in 50 and 100 mL beakers, and the different materials that could provide better development for the organisms were examined. It was found that the use of 100 mL beakers favored better development than the use of 50 mL beakers, and the use of a plastic wrap halted the humidity loss and provided the right gas exchange. With these conditions, a development time of 8 days was achieved. Dunn (1974) described a technique in which the content of the

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bird egg is removed after 72 hours of incubation, which could be developed for extended periods of time until Day 15. The egg content is supported with the help of a plastic wrap over a plastic cup with the water solution to maintain the humidity of the embryo. Kamihira *et al*. (1998) reported an *ex-ovo* method for quail embryos using a plastic cup and plastic wrap; by this method, nearly 43 % of the embryos were developed and hatched with the supplementation of calcium lactate.

The supplementation of calcium to the *in vitro* developing embryos is essential for its development. The embryos that are developed naturally inside the eggshell obtain their calcium from the eggshell that protects them during embryo development until hatching (Kamihira *et al*., 2005).

The *ex-ovo* development method named ''chicks in cup'' allows the continuous observation of live bird embryos from the third day of the development until the eighteenth; by using this method, organogenesis, extra-embryonic membranes, and prehatching behavior can be studied (Fisher, 1993). In another work, Tahara & Obara (2014) reported a technique for *ex-ovo* embryonic development that allowed the hatching of chicks (57.1 %).

There have been different improvements in the *ex-ovo* development of embryos; however, most of these methods require the use of surrogate eggshells from larger bird species (Borwompinyo *et al*., 2005). On the other hand, Tahara *et al*. (2021) argue that the use of this method has disadvantages because of the preparation of the eggshells, inability to reuse them, and poor operability.

The standardization of a method that could allow *ex-ovo* development implies great importance because it poses a great utility for teaching purposes by allowing the observation of the different stages that comprise the embryonic development of a chordate (Kamihira *et al*. 2005). This method could also facilitate the creation of transgenic chickens and other genetic manipulations used in regenerative medicine, and it could support cell cultures by obtaining stem cells and the mass production of compounds in bioreactors (Tahara & Obara, 2014).

Table I. Quantity of minerals per milligram of pulverized eggshell.

Quantity of grounded eggshell (mg)	Quantity of minerals (mg)		
	Ca^{2+}	Mg^{2+}	P
300	97.0980	1.5383	0.3270
600	194.1960	3.0765	0.6540
900	291.2940	4.6148	0.9810
1200	388.3920	6.1530	1.3080
2500	809.1500	12.8188	2.7250
5000	1618.3000	25.6375	5.4500

The objective of this study was to establish the experimental conditions to improve the *ex-ovo* incubation and the conditions of *Gallus gallus domesticus* embryos.

MATERIAL AND METHOD

The current study was approved by the institutional ethics committee (CEADI-UAA). The *ex-ovo* development of *Gallus gallus domesticus* embryos was carried out with respect and care.

Procedure for obtaining and preparing embryos. Fertilized chicken eggs (n=100) were obtained from a farm. Once collected, they were placed inside a CO_2 incubator (Lab-Line Model 315) at a constant temperature of 37.5 °C and a relative humidity of 60 %. In the case that the eggs were not incubated immediately, they were stored between 15.6 and 18.3 °C so that the embryo would enter a diapause state and would resume its development when it was returned to the incubation temperature (Pokhrel *et al*., 2021).

Mineral supplement preparation. The chicken eggshell was selected as a source of Ca^{2+}, Mg^{2+}, P, and other essential minerals for the correct development of the embryos. After being obtained, they were washed thoroughly with distilled water to remove all residues of the egg white and to clean the surface. Then, they were dried in an oven at 90 °C for 30 minutes. The eggshell was pulverized with a mortar, and the various amounts to be tested were weighed on an analytical balance. Then, they were stored in centrifuge tubes with a screw cap to avoid contamination and facilitate manipulation. They were finally sterilized using an autoclave and UV light.

Quantitative analysis of the mineral composition of the supplement. To determine the contents of Ca^{2+}, Mg^{2+}, and P in the eggshells used as mineral supplements (Table I), the methods described in NMX-066 (1978) were used.

Preparation of the incubation system and placement of the embryo. The transfer and placement of the embryo was performed in an *ex-ovo* incubation system, following the method described by Tahara & Obara (2014). An artificial incubation system consisted of a plastic cup (PET plastic) with a capacity of 355 mL. Subsequently, a concave chamber was developed in which the embryo could be placed for its development. This chamber was designed using a Teflon plastic wrap (plastic film). This allows the gas exchange required for adequate oxygenation. At the cup's bottom, an aqueous solution of 0.001 % benzalkonium chloride (BKC) was placed to maintain the humidity in the cup, avoid embryo desiccation, and to keep the cup clean and sterile. Once the

incubation system was built, it was sealed with the top of a Petri dish to be sterilized in a UV light chamber for a period between 12 and 24 h. Finally, before proceeding with the embryo extraction, the pulverized eggshell for the mineral supplement test was added to 1 mL of distilled water to allow the gradual assimilation of the minerals by the embryo (Fig. 1).

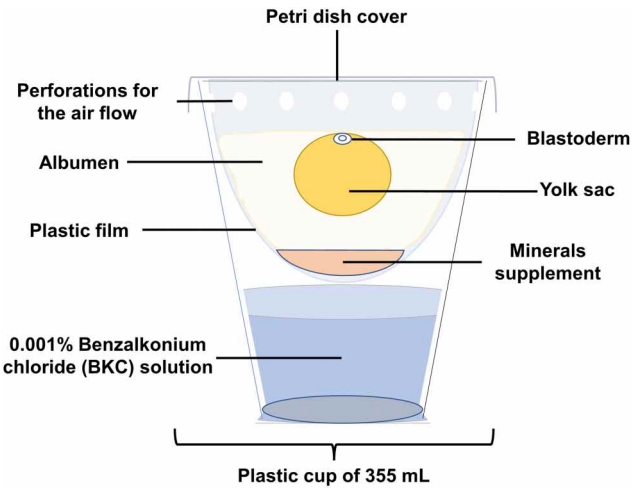

Fig. 1. Diagram of the *ex-ovo* incubation system.

Before extracting the embryos, the surface of the eggshell was sterilized with a sterile gauze pad impregnated with 70 % ethyl alcohol and cleaned in a single direction. Then, in a sterile space, the eggs were placed horizontally to allow the yolk sac to move to the top of the egg. After that, the eggs were quickly turned over 180°, and using a spatula, the eggs were cracked in the middle section to avoid embryo damage. The content of the eggs was placed in the incubation chamber of the *ex-ovo* incubation system and then closed with a Petri dish top. Finally, the incubation systems were placed inside the incubator to continue with embryonic development.

Three different tests were performed to evaluate the necessary conditions that allowed optimal development of the chick embryos:

Mineral supplement test (Ca^{2+}, Mg^{2+}, P). Experimental groups with five embryos each were implemented to test the effect of different amounts of pulverized eggshell as a mineral supplement (Table II): 300, 600, 900, 1200, 2500 and 5000 mg were tested. A volume of 40 mL of 0.001 % BKC solution and a preincubation time of 56 h were used.

Humidity test. Experimental groups with five embryos each were implemented to test the effect of the 0.001 % BKC solution volume (Table III): 0, 10, 20, 30, 40, 50, 60 and 70 mL. All the incubation systems were enriched with 900 mg of pulverized eggshells and a preincubation time of 56 h.

Preincubation time test. Experimental groups with ten embryos each were used to test the effect of preincubation time (Table IV): 32, 56 and 80 h were tested. After that, they were incubated at 37.5 °C with 900 mg of pulverized eggshell and 40 mL of 0.001 % BKC solution.

Table II. Average development time reached with different quantities of pulverized eggshell.

Incubation system number	Quantity of grounded eggshell (mg)	Days of development reached
1 - 5	300	5.2
6 - 10	600	8.8
11 - 15	900	16
16 - 20	1200	12.8
21 - 25	2500	2.2
26 - 30	5000	1.2

Table III. Average development time reached with the 0.001 % benzalkonium chloride solution treatment.

Incubation system number	Volume of 0.001% BKC solution (mL)	Days of development reached
1 – 5	0	1.5
6 - 10	10	2.3
11 – 15	20	5.3
16 – 20	30	10.5
21 – 25	40	16.5
26 - 30	50	11.8
31 – 35	60	4
36 - 40	70	1.8

Tissue processing and histological staining. Four embryos, corresponding to four different stages of development (52 h, 3 d, 4 d and 19 days), were fixed in Bouin solution (Ellenburg *et al*., 2020). The fixation time varied depending on the organism size to correctly fix the tissue. Afterward, the embryos were washed with water to remove excess Bouin fixative. The next steps regarding dehydration and clearing of the tissues were performed with the use of a Tissue Processor (Leica model TP1020). Then, paraffin infiltration was performed for sectioning. The slides were stained with hematoxylin-eosin, Masson's trichrome and Klüver-Barrera (Kluver & Barrera, 1953) to observe and compare the structures that are present during each different stage. An optical microscope (Nikon Eclipse NI-SS) was used.

Data analysis. For the determination of the age of the organisms, the methodology proposed by Hamburger & Hamilton (1992) was used for the embryo development stages. This result was also corroborated by the time of incubation registered for each organism. Statistical analysis was performed by t test using GraphPad Prism 8.00 software (GraphPad Software, San Diego, California USA).

RESULTS

A total of 100 eggs were used, of which 18 were not fertilized or suffered damage during the extraction of the embryo. In the *ex-ovo* incubation, different development times were achieved, from 0 (Fig. 2A), 3 (Fig. 2B), and 4 (Fig. 2C) days of development to 19 days of development (Fig. 2F). It was also possible to observe the morphology and ethology of the organisms and their different stages.

When performing the quantitative analysis of the minerals present in the eggshell, an average of 323.66 mg Ca^{2+}/g eggshell, 5.1275 mg Mg^{2+}/g eggshell and 1.09 mg P/g eggshell was determined. In the mineral supplement test (Fig. 3A), the tests with 900 mg of pulverized eggshell reached the longest development time with an average of 16 days, followed by the test with 1200 mg with an average of 12.8 days. However, the statistical analysis (with a P value of 0.984 with a confidence level of 95 %) indicated that there was no significant difference between 900 and 1200 mg; rather, embryos that received these 2 levels of eggshell achieved the same development time.

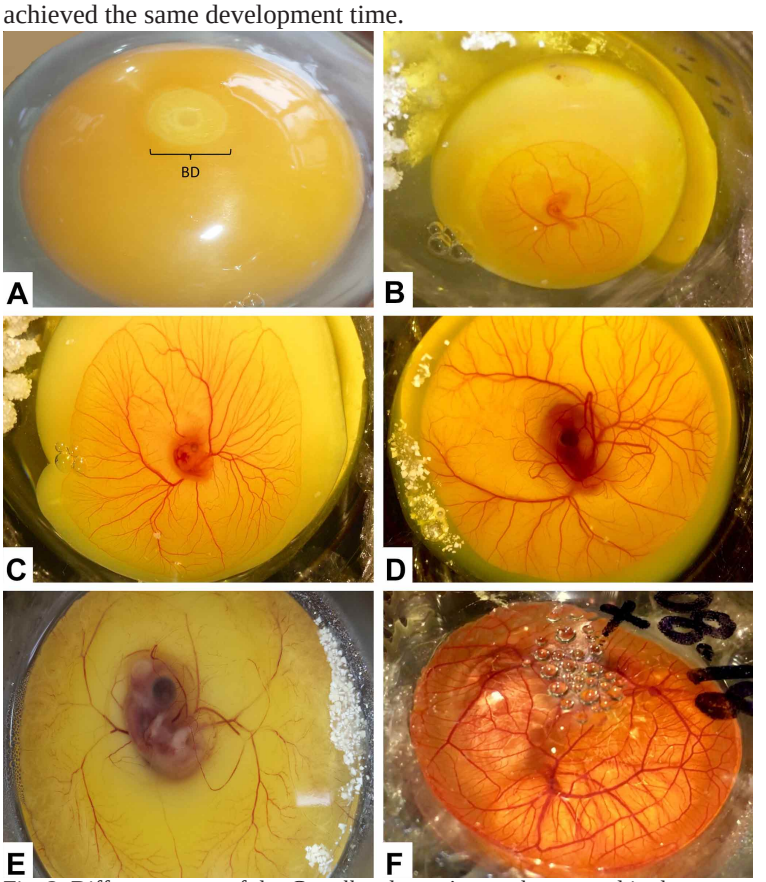

Fig. 2. Different stages of the *G. gallus domesticus* embryos used in the *ex-ovo* development: A) fertilized egg with blastoderm (BD), B) embryo of 3 days (Stage 18 HH), C) embryo of 4 days (Stage 23HH), D) embryo of 7 days (Stage 30 HH), E) embryo of 8 days (Stage 34 HH), F) embryo of 19 days (Stage 45 HH).

For the humidity test (Fig. 3B), forty eggs were incubated; however, seven eggs were not fertilized. From the five tests, 40 mL of a 0.001 % solution of BKC allowed the longest development time, with an average of 16.5 days.

For the preincubation test (Fig. 3C), thirty eggs were incubated, but only nineteen were fertilized or did not suffer damage during the extraction. The preincubation time under 32 hours gave an average of only 2 days of development; in contrast, a preincubation of 56 hours reached an average of 15 days of development, and by performing the 80 hours

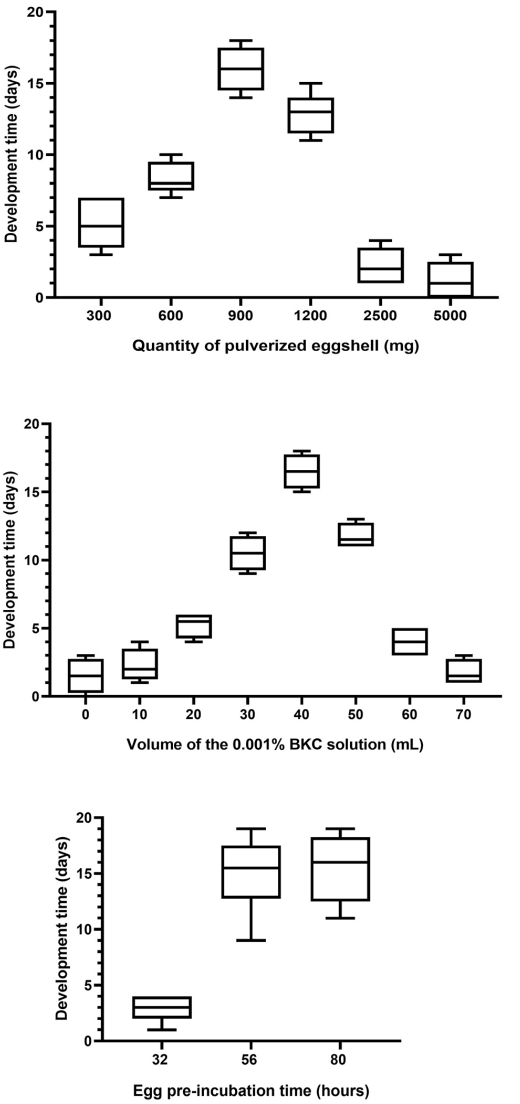

Fig. 3. Graphics of the *ex-ovo* incubation tests; A) embryo development time reached by the quantity of pulverized eggshell, B) embryo development time reached by the quantity of the 0.001 % BKC solution volume, C) embryo development time reached with the egg preincubation time. Made with GraphPad Prism 8 software.

preincubation time, the test reached an average of 15.5 days of development. In the statistical analysis of the 56 and 80 h periods, the results showed a P value of 0.7952 with a confidence level of 95 %; therefore, there was no statistically significant difference between the two periods. Through histological processing (Fig. 4), the anatomy of the embryos and the tissues during embryonic development could be compared (Fig. 5).

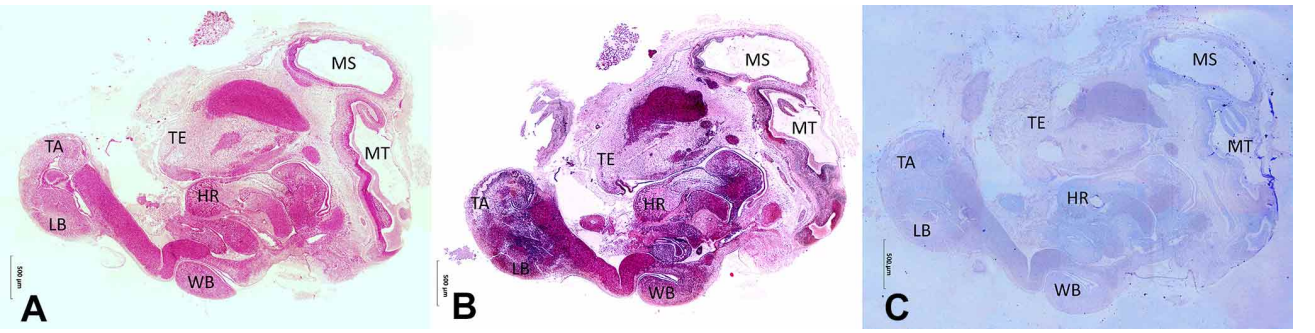

Fig. 4. Embryo (sagittal plane) with 3 days of development (Stage 18 HH). Three stains: A) hematoxylin-eosin, B) Mason's trichrome, C) Klüver-Barrera. Heart (HR), telencephalon (TE), mesencephalon (MS), metencephalon (MT), wing bud (WB), leg bud (LB) and tail (TA).

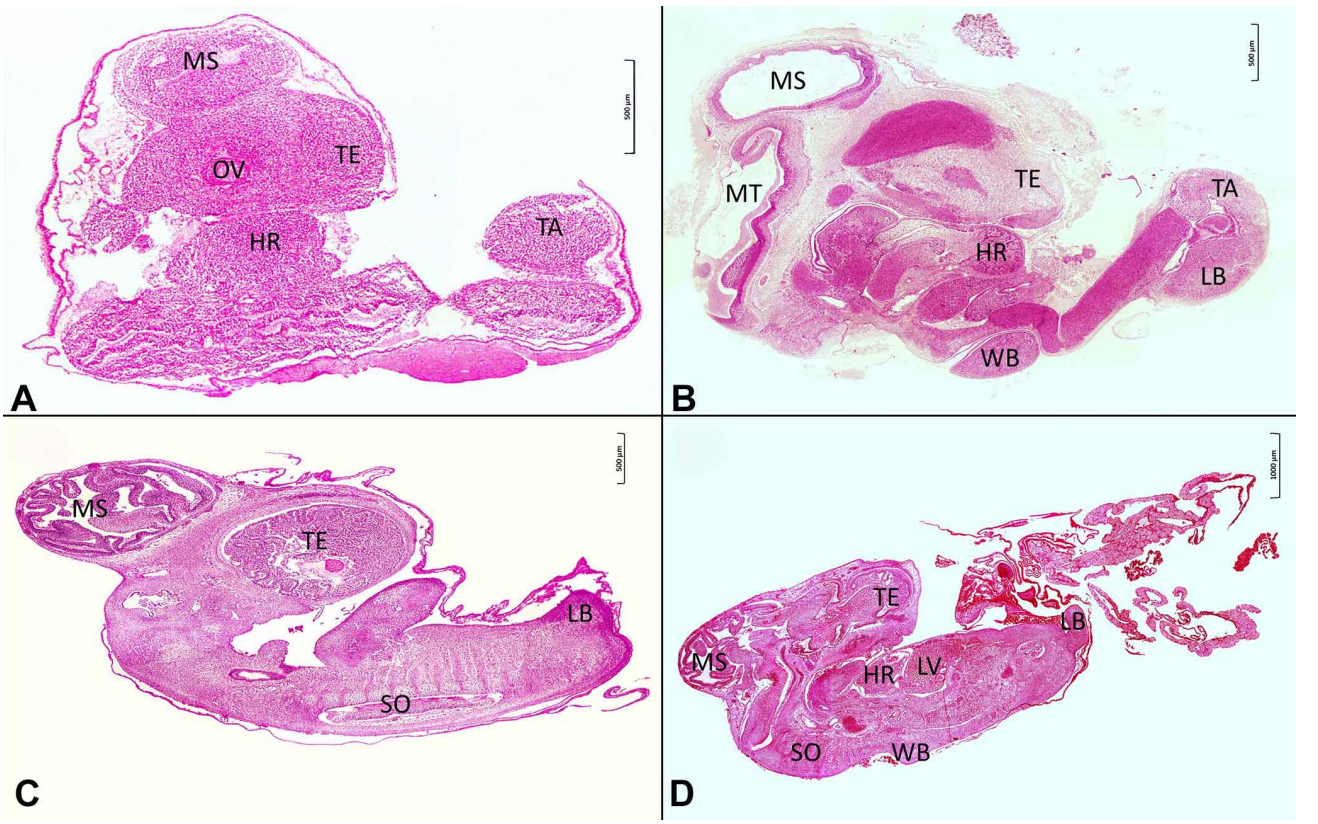

Fig. 5. Embryos (sagittal plane) with different days of development: A) 36 hours, B) 3 days, C) 4 days, D) 5 days. Heart (HR), telencephalon (TE), mesencephalon (MS), metencephalon (MT), wing bud (WB), leg bud (LB), tail (TA), optical vesicle (OV), somites (SO) and liver (LV).

DISCUSSION

Initially, the sterilization of the pulverized eggshell was performed inside a UV light chamber for one day; however, this resulted in contamination of the incubation systems, so it was decided to use an autoclave machine for 15 minutes at a temperature of 120 °C and a pressure of 15 psi. Once the process was performed, the tubes were stored inside the UV

light chamber until they were needed for the tests. In the subsequent tests, there were no contamination problems.

The absence of fertilized eggs complicated some tests because the embryos were extracted during the initial stages of development, so techniques such as egg candling (Adegbenjo *et al*., 2020) could not be used to determine fertilization. Another factor that affected the tests was damage to the yolk sac during the extraction, causing the death of the organism. In the articles that were reviewed, there was not a clear mention of the correct method for embryo extraction, so we tried different tools and found positive results with the use of a spatula. By performing the preincubation test (Table IV), a certain pattern was observed regarding the fragility of the yolk sac through the preincubation time. After 80 h of preincubation, despite the precautions taken, four out of ten eggs were broken. By examining the broken eggs, it was found that the consistency of the yolk sac was more fragile because of the effects of vascularization. After 56 h of preincubation, when the embryo was less developed, there were no effects present; similarly, preincubation for 32 h had no complications in the extraction. In this test, although 56 and 80 h were statistically equal, the higher risk of damage in the yolk sac of the egg preincubated at 80 h showed complications, and by using the 56 h period, the embryos could be easily extracted and reached a development time of 19 days. In their tests, Tahara & Obara (2014) reported that in a preincubation of less than 48 hours, a development of only 8 days could be reached, and with a period of 56 h, a development time of 17 days could be reached.

Pulverized eggshell, which was used by Kamihira *et al*. (1998) for the *ex-ovo* development of quail embryos, yielded positive results, but the embryos had a low hatchability. In our tests, the pulverized eggshell provided an effective mineral supplement for achieving late development stages. Tahara *et al.* (2021) used calcium carbonate directly in chorioallantoic membranes and reported the importance of the position of the mineral supplement for the correct absorption by the embryo. In the tests performed in the present study, the pulverized eggshell was placed near the yolk sac, so it is possible that not all the minerals were correctly absorbed. In the quantitative analysis of the minerals of the eggshell, Brun *et al*. (2013) reported a high content of calcium, 381 ± 89 mg Ca^{2+}/g eggshell, as well as 4 mg Mg/g eggshell and 1.05 mg P/g eggshell. These quantities were consistent with the values that were obtained in the analysis.

Table IV. Average development time reached using different times of preincubation.

Incubation system number	Egg pre-incubation time (hours)	Days of development reached
1 - 10	32	2.9
11 – 20	56	15
21 - 30	80	15.5

The 0.001 % BKC solution helped to maintain the normal levels of humidity needed for the organisms not to desiccate despite the fact that the incubation system was sealed. By not applying an appropriate amount of the BKC solution, the embryo could die due to excess humidity or a lack of humidity, as shown in Table III. A solution of 40 mL allowed a longer development time.

Temperature is an incubation factor that impacts the development of embryos. As mentioned by Vilches-Moure (2019), chicken embryos are poikilothermic during their early stages of life, so they require an external heat source for correct development. The optimal incubation temperature for chicks is 37.5 °C, as it helps in the metabolism of the embryo (Noiva *et al*., 2014). In the experiments, the use of a digital thermometer helped to accurately determine the temperature necessary in the incubation chamber for appropriate development. Another important factor for the correct incubation of the chicks involves the use of oxygen; however, for this study, there was no oxygen employed in the methods that would allow the correct supplementation to the embryos. In the work done by Tahara & Obara (2014), it was reported that 14 eggs were supplied with oxygen, but only 8 (57.1 %) survived, thanks to this method, and the ones that did not have oxygen supplementation could not develop further. These results could explain why there were complications in the development of chicks and why none could hatch, but more studies that could establish the optimal incubation conditions are needed.

In the different tests, it could be seen how while the development time was passing by, the survivability of the incubated embryos decreased, as was reported by Andacht *et al.* (2004). By performing an *ex-ovo* incubation of the embryos, the hatchability tended to be reduced; however, by supplying the correct conditions, the development time achieved could be improved, as reported by Tahara & Obara (2014).

CONCLUSION

To be able to carry out the *ex-ovo* embryonic development of *Gallus gallus domesticus*, it is necessary to provide adequate conditions for its development, appropriate mineral supplements, oxygen, humidity, and temperature. However, the correct procedure for the creation of the incubation system and the periods of incubation of the embryo should also be considered to achieve a successful

extraction, which will allow a longer development time to be reached. Despite that in all experiments done, none of the incubated organisms reached the end of their development, and significant progress was made in the standardization of a method that allows *ex-ovo* embryonic development until 19 days. Additionally, this versatile study of *ex-ovo* development has provided methods for different preparation techniques with the purpose of evaluating the effects of these different incubation procedures on the tissues of the developing organisms.

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RESUMEN: El pollo doméstico es una especie de ave que ha sido ampliamente estudiada en cuanto a su biología y como organismo modelo para la ciencia. La reproducción de la especie es por la puesta de huevos fecundados, que en un período de 21 días desarrollarán un polluelo en su interior. Se han descrito varios métodos para desarrollar embriones *ex-ovo*, permitiendo la observación y manipulación del organismo. Este trabajo tuvo como objetivo estandarizar un método que permita el desarrollo de los embriones dentro del sistema de incubación artificial, el cual tiene un bajo costo y es fácil de realizar. En este trabajo se utilizaron 100 huevos de gallina para estudiar los efectos de la humedad, la suplementación mineral y el tiempo de preincubación del huevo sobre la incubación *ex-ovo* de los embriones. El desarrollo embrionario se documentó a través de los diferentes días. Se seleccionó la cáscara de huevo pulverizada como una fuente óptima para proporcionar calcio, magnesio, fósforo y otros minerales al embrión en desarrollo. Al suministrar 900-1200 mg de cáscara de huevo pulverizada, 40 mL de la solución de cloruro de benzalconio al 0.001 % y un tiempo de preincubación de aproximadamente 56 h, los embriones lograron desarrollarse hasta los 19 días, y aunque no llegaron a eclosionar, los embriones lograron desarrollarse hasta los 19 días. Se lograron condiciones de incubación que permitieron la supervivencia y desarrollo de los embriones hasta etapas tardías. Así, debido a las condiciones establecidas de calcio, humedad y tiempo de preincubación, en el presente trabajo los pollitos alcanzaron los 19 días de desarrollo.

PALABRAS CLAVE: Aves; Desarrollo embrionario; Incubación ex ovo; *Gallus gallus domesticus*; Condiciones de incubación.

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