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Development of external morphological malformations induced by hyperthermia exposure during the blastula stage in an ex-ovo (shell-less) culture of *Gallus gallus domesticus* embryos

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ABSTRACT

The chicken is a valuable model to study embryonic development, genetic manipulation, regenerative medicine, tumorigenesis, vaccine development, toxicology, and teratology research. Different methods have been described for incubating chicken embryos outside the eggshell (ex-ovo or shell-less incubation). To date, no studies have focused on using ex-ovo incubation as a model to study the effects of hyperthermia as a teratogen. In this work, a total of 350 fertile chicken eggs were used to study the development of congenital malformations in an ex-ovo and in-ovo incubation system exposed to normal (37.5°C) and hyperthermia conditions (40°C). In the ex-ovo hyperthermia test, all ex-ovo embryos (n = 50) developed malformations; only 0.02 % reached 5 days of development. In the ex-ovo normal temperature group, none of the ex-ovo embryos developed malformations, and 48 % reached 21 days of development; there was no significant morphological difference between the ex-ovo normal temperature and the in-ovo normal temperature group. The time of exposure to hyperthermia conditions is crucial for the development of malformations, with the blastula stage (0 h) being the most susceptible. Blastula stage malformations, neural tube defects, microphthalmia, amelia, gastroschisis, caudal regression, and the development of twinning were identified. The ex-ovo incubation system does not increase the risk of the development of malformations, and it is a viable model for studying the effects of teratogens, as well as the morphology and physiology of the embryo.

Introduction

Throughout history, the chicken has been a valuable model to study embryonic development in vertebrates due to its availability, low price, and easy accessibility to the embryo (Mok et al., 2015; Vilches-Moure, 2019; Wittig and Münsterberg, 2016). The culture of chicken embryos has also been applied to embryo genetic manipulation, regenerative medicine, tumorigenesis, vaccine development, toxicology, and teratology research (Naik et al., 2018; Tahara et al., 2021). The field of teratology studies congenital anomalies or malformations and the external factors (biological agents, drugs, chemicals, radiation, temperature) that cause them, which are called teratogens (Vargesson and Fraga, 2017; Wachholz et al., 2021).

Several studies have shown the effects of temperature as a teratogen in the development of bird species (Noiva et al., 2014); the incubation temperature is a crucial determinant for successful incubation; chicken embryos require an incubation temperature range of 37.5°$C \pm 0.5$ for an optimal hatching rate (Tona et al., 2022). Research has shown that a 1°C change from the incubation temperature can significantly affect the organisms (French, 1994). Hyperthermia is an abnormally high body temperature caused by diverse sources, like an infection or exposure to a heat source (Edwards, 1986). Research has proven that hyperthermia is

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a teratogen causing severe malformations, embryonic death, and growth retardation in birds, amphibians, reptiles, fish, and mammals, including humans, however, there have not been enough studies and focus on the effects on humans (Haghighi et al., 2021; Krausova and Peterka, 2007; Narinç et al., 2016). The most frequent malformations after exposure to hyperthermic temperatures ≥40°C, are neural tube defects, anencephaly, microphthalmia, microcephaly, amelia, skeletal defects, and resorption of the embryo (Davey et al., 2018; Krausova and Peterka, 2007). Chicken embryos are classified as poikilothermic, meaning that their metabolism relies on the incubation temperature, which influences the use of egg nutrients and embryo development, making them more susceptible to the effects of hyperthermia (Yalcin et al., 2022).

The ex-ovo culture (shell-less) is an incubation technique that allows the development of avian embryos like chicken and quails (Kamihira et al., 1998) in an in vitro incubation system to allow the observation of embryogenesis (Dunn et al., 1981; Macias-Marin et al., 2023; Tahara and Obara, 2014). This incubation technique consists of removing the embryo, yolk sac, and albumen to be incubated outside the egg and discarding the eggshell and the membranes (Boone, 1963; Dunn, 2023; Tahara and Obara, 2014; Tahara et al., 2021). The authors Tahara and Obara (2014) developed a simple and inexpensive culture method with a high hatchability; the artificial vessel was made with an ordinary plastic cup (PET), a polymethyl pentene (PMP) plastic film as the incubation chamber and supplying different culture conditions such as calcium and water supplementation and oxygen aeration.

Previous studies required the ex-ovo incubation to have a period of pre-incubation at 37.5°C within the egg (in-ovo) for the first 55–70 h to allow a successful development of the embryo and incubation, thus preventing the incubation from the blastula stage in the ex-ovo incubation system, and early embryo development research (Auerbach et al., 1974; Dunn, 1981; Kamihira et al., 1998; Tahara and Obara, 2014; Tahara et al., 2021). Ono et al. (2005) developed blue-breasted quail from the blastoderm stage to hatch using a chicken surrogate eggshell. The author Dunn (2023) described a method for successful ex-ovo incubation of chicken embryos without a pre-incubation period in-ovo, using a disc of Milli-Wrap membrane to allow embryo development from the blastoderm stage until hatching.

To date, no studies have focused on using ex-ovo incubation as a model to study the effects of hyperthermia as a teratogen. This research aimed to assess the impact of hyperthermia exposure on embryos incubated in an ex-ovo system, determine if the ex-ovo incubation caused the development of malformations, and finally demonstrate the importance of the ex-ovo incubation system as a model to study teratogens and research the real-time development of malformed embryos.

Materials and methods

Chicken eggs

Fertile specific pathogen-free (SPF) Bovans White eggs (n = 350) were sourced from "Specific pathogen-free poultry" (‘‘ALPES’’, Sanfer, Mexico).

The temperature and mass of the eggs were measured before incubation. If the eggs were not incubated immediately, they were stored between 15.6 and 18.3°C to pause embryo development (diapause) until the incubation temperature was increased (Pokhrel et al., 2021).

The eggs were grouped in the ex-ovo (experimental group: hyperthermia and normal temperature) incubation tests and the in-ovo (control group: hyperthermia and normal temperature). This division aimed to determine whether the development of malformations could be correlated with the ex-ovo incubation system or the temperature (Table 1).

The in-ovo normal temperature eggs (n = 50) were placed in an automatic egg incubator (Harris Farms Nurture Right 360, Manna Pro®, Chesterfield, MO) set to a constant temperature of 37.5°C and a RH of 60 %. For the in-ovo hyperthermia group, the eggs (n = 50) were placed in the automatic egg incubator set to a constant temperature of 40°C and a RH of 60 %.

Table 1
Hyperthermia tests: embryo incubation groups.

Tests	In-ovo		Ex-ovo	
	Hyperthermia	Normal temperature	Hyperthermia	Normal temperature
Incubator	Nurture Right 360	Nurture Right 360	Labline 315	Labline 315
Number of eggs	50	50	50	50
Temperature	40°C	37.5°C	40°C	37.5°C
Relative Humidity	50–60 %	50–60 %	50–60 %	50–60 %
Egg turning	Automatic (90°/h)		Manual Rotation + Albumen Pipetting (every 4 h)	

The ex-ovo eggs were not pre-incubated before the extraction to allow an incubation outside the egg from the blastula stage (first day of incubation).

Preparation of the ex-ovo incubation system

The ex-ovo incubation systems were done following the method described by Tahara and Obara (2014) and with modifications described by Macias-Marin et al. (2023). The incubation system consisted of a polyethylene terephthalate (PET) plastic cup (Solo® TP12 Ultra Clear™ 12 oz, Mason, Michigan) filled with an aqueous solution (40 mL) of 0.01 % benzalkonium chloride (cat. no. 311040, Dermocleen®, degasa, Mexico), to maintain the internal RH. Above the solution, a chamber was placed to incubate the embryo, this chamber was made with a polymethyl pentene (PMP) plastic wrap (F.O.R. WRAP, Riken Technos, Japan), stretched into a concave shape. The incubation system was closed with the bottom of a plastic or glass Petri dish; once the system was completed (Fig. 1), it was sterilized in a UV-C light chamber (222 nm wavelength) for 1 h.

Ex-ovo embryo incubation

The extraction of the embryo was performed inside a laminar flow cabinet (Lab Companion model BC-11H, Jeio Tech, South Korea), which was sterilized by spraying 70 % ethanol, subsequently, the ex-ovo incubation systems were placed inside it to be sterilized once again by UV light exposure for 15 min. Once the sterilization cycle was completed, the eggs were prepared for extraction; first, the eggs were placed horizontally to allow the movement of the yolk sac to the top of the egg, after that the surface of the eggs was disinfected by using 70 % ethanol wipes, wiping down and drying the surface in one direction, in accordance to the protocols described by Tahara et al. (2021) and Dunn (2023). Once the eggs were dry, they were immediately cut around the middle section using a rotatory tool (Dremel® 7760, Dremel Tools, Mount Prospect, IL) equipped with a dental circular saw, after the cut was made, a sterile scalpel was used to cut the egg’s inner and outer membrane to help in the extraction, finally the eggs were opened inside the incubation chamber of the ex-ovo system, checking the presence and upper position of the blastoderm to determine a fertile egg. Finally, the system was closed with the Petri dish and placed inside the CO_2 incubator (Labline® model 315, Springfield, IL) coupled with an oxygen concentrator (CAIRE® Companion 5, CAIRE Inc., Ball Ground, GA) to provide 500 mL/h of O_2 to the embryos from day 14th until 21st, The O_2 levels were fixed at 21 % with a sensor (BH-90A Single Gas Detector, Henan Bosean Electronic CO., LTD, China). Another gas sensor (Aranet4 Home, SAF Tehnika JSC, Latvia) was placed inside the incubator to maintain the CO_2 levels around 1.0 % (measuring an average of 10013 ppm) during the initial incubation period (from day 1–10) and hatching (from day

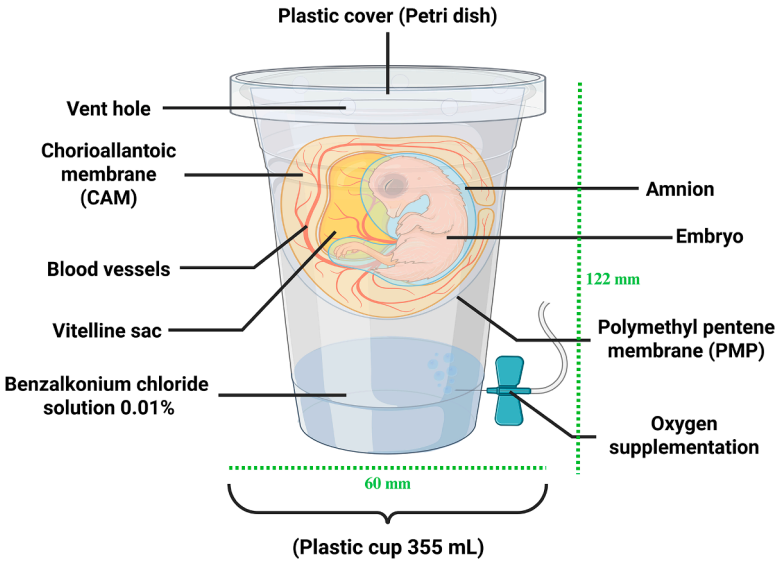

Fig. 1. Diagram of the ex-ovo incubation system.

18–21), in the middle incubation period (day 11–17) the CO_2 levels were decreased to 0.4 % as described by Tong et al. (2015) and Liu et al. (2022), to achieve a better incubation and hatching. For calcium supplementation, 250 mg of calcium carbonate (cat. no. 030- 00385, Fujifilm Wako Pure Chemical Corporation, Japan) was supplied directly over the chorioallantoic membrane (CAM) on the 10th day of incubation. Due to the inaccessibility of Milli-Wrap membrane (Dunn, 2023), a two-stage process was done to allow the development of the embryos from the blastula stage (Fig. 2): (1) Transfer of the egg's outer thin albumen to cover and humidify the blastoderm and yolk sac surface (until 6 d), (2) Manual rotation (360°) of the ex-ovo culture by tilting it at a 45° angle (until 18 d), ensuring a proper movement of the yolk sac to detach it from the walls of the incubation chamber. The temperatures were measured using a thermal camera (FLIR ONE® Edge Pro, Teledyne Technologies, Thousand Oaks, CA) and a digital thermometer/hygrometer (Digital Combo, Zoo Med Laboratories Inc., San Luis Obispo, CA) placed inside the incubator to get more accurate readings.

Hyperthermia test

In a previous study (Macias-Marin et al., 2023), we observed the development of embryos with malformations at an incubation temperature of 40°C and RH of 60 % (data not shown); for these tests, we continue with that temperature and RH set value. Experimental group to determine the effects of a hyperthermia incubation (Table 1): Ex-ovo hyperthermia (n = 50, 40°C, RH 60 %, Labline 315), Ex-ovo normal temperature (n = 50, 37.5°C, RH 60 %, Labline 315), In-ovo hyperthermia (n = 50, 37.5°C, RH 60 %, Nurture Right 360 Incubator), and In-ovo normal temperature (n = 50, 37.5°C, RH 60 %, Nurture Right 360 Incubator). The normal temperature group was developed to verify that the development of malformations is correlated to the incubation temperature and not the ex-ovo incubation system. To compare the development ex-ovo, the control group was prepared in-ovo to analyze the morphological differences in the incubation (Figs. 3A and 4).

Hyperthermia exposure time test

Test of experimental groups to determine the effects of hyperthermia incubation in an ex-ovo incubation during three different incubation times (Table 2): 0 h (n = 50, 40°C, RH 60 %, Labline 315), 24 h (n = 50, 40°C, RH 60 %, Labline 315), and 48 h (n = 50, 40°C, RH 60 %, Labline 315). These tests were developed to verify the sensitivity of the embryo in different stages to develop malformations (Fig. 3B and D).

Euthanasia of the organisms

Following the strict national and international guidelines for the care of chicken embryos and fetuses, the protocol for euthanasia of embryos that had achieved 80 % incubation (17 d) was cooling at <4°C in a fridge for 4 h. For the remaining days (18–21 d), electroencephalogram (EEG) activity suggests the potential for pain perception in conscious embryos (AVMA, 2020), so the appropriate protocol was the use of an anesthetic overdose (intravenous dose on the CAM of 120 mg/ Kg of pentobarbital). To reduce the sacrifice of organisms, the organisms that reached the hatching period were examined, their mass and size measured and finally relocated to a farm to continue their growth.

Morphological analysis and malformations scoring

Once the euthanasia process was completed, an external morphological analysis and a visual scoring was performed on the organisms to determine the presence and identify the type of external malformation in the incubated embryos, using the in-ovo normal temperature embryos as control (Figs. 4 and 6), the data was corroborated with several articles that reported malformations on chicken embryos, and even research papers that reported malformations in other species that had not yet been described before for the chicken embryos (cojoined twins). To determine the age of the organisms, the results were corroborated with the times of incubation recorded, and the chicken development stages (HH) by Hamburger and Hamilton (1951) were used.

Tissue processing

The embryos were preserved by immersion in Bouin's fixative solution (HT10132-1 L, Sigma-Aldrich) for 24 h and stored in sample tubes.

Data analysis

Statistical analysis was performed using the t-test and one-way ANOVA using GraphPad Prism 8.00 software (GraphPad Software, San Diego, California, USA).

Results

The technique employed for the "turning'' of the ex-ovo incubation system (Fig. 2) allowed the development of embryos from the blastula stage, enabling ex-ovo incubation experiments without the in-ovo pre-

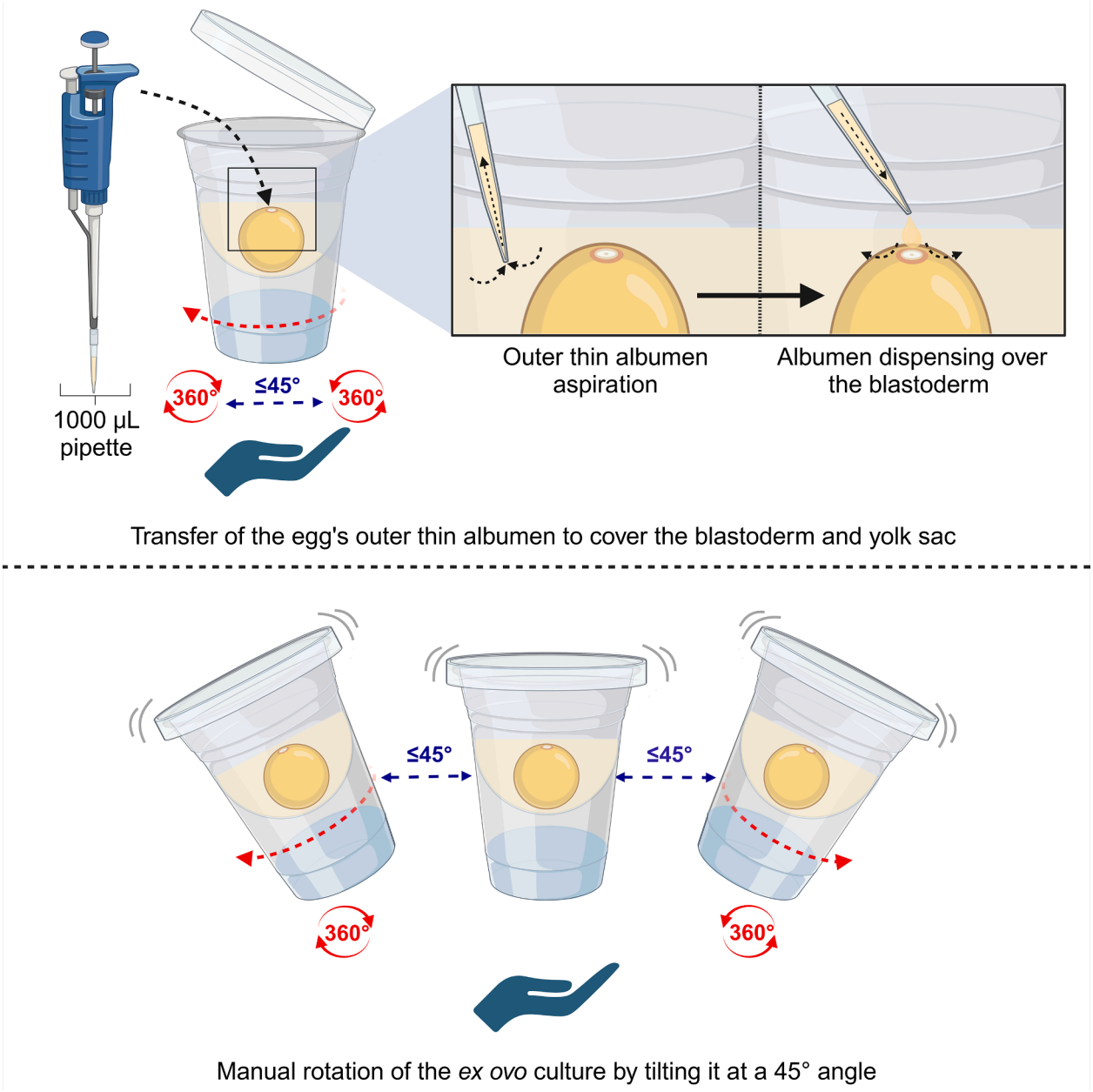

Fig. 2. Method for the rotation of the ex-ovo incubation system and humidification of the blastoderm to develop embryos without an in-ovo pre-incubation.

incubation.

A total of 150 eggs were used for the Hyperthermia test (Table 1), all the embryos (n = 50) of the ex-ovo hyperthermia group developed malformations, and only 0.02 % (1/50) reached 5 days of development (Fig. 3A), by comparison in the ex-ovo normal temperature group, none of the embryos developed malformations, and 48 % (24/50) reached 21 days of development. In the in-ovo normal temperature group, none of the embryos developed malformations, and there was a hatching of 96 % (48/50).

In the hyperthermia exposure time test, four groups of 50 eggs each were used (Table 2). The days of development reached by the embryos were measured (Fig. 3B), and there was a significant difference ($P \leq 0.05$) between the viability of the 0 – 48 h incubation times. However, when analyzing the malformations developed, there was a difference between the incubation times; by exposing the embryos during the blastula stage (0 h), all developed malformations; on the other hand, the frequency of malformations decreased by increasing the days of incubation (Fig. 4D).

A total of 169 embryos with malformations were identified (Figs. 5, 6 and Table 3): patterning defect (31.36 %), microcephaly (9.46 %), microphthalmia (26.62 %), heteropagus (0.59 %), cephalothoracopagus (0.59 %), amelia (11.24 %), encephalocele (4.73 %), ectopic heart (21.89 %), neural tube defects (28.40 %), caudal regression (7.69 %), polymelia (0.59 %), gastroschisis (12.42 %), anophthalmia (6.50 %), phocomelia (3.55 %) and hydrocephalus (8.28 %). The development of the embryos was observed and registered in vivo with the ex-ovo incubation system (Fig. 5). Necropsy analysis of the embryos helped with the identification of the malformations (Fig. 6).

Discussion

The development of a method that allowed the ex-ovo incubation of the embryos from the blastula stage enabled studies that required that development period, like the study of the effects of hyperthermia as a teratogen. Previous studies show that a method for the ex-ovo incubation of the blastoderm was not viable; authors reported major difficulties

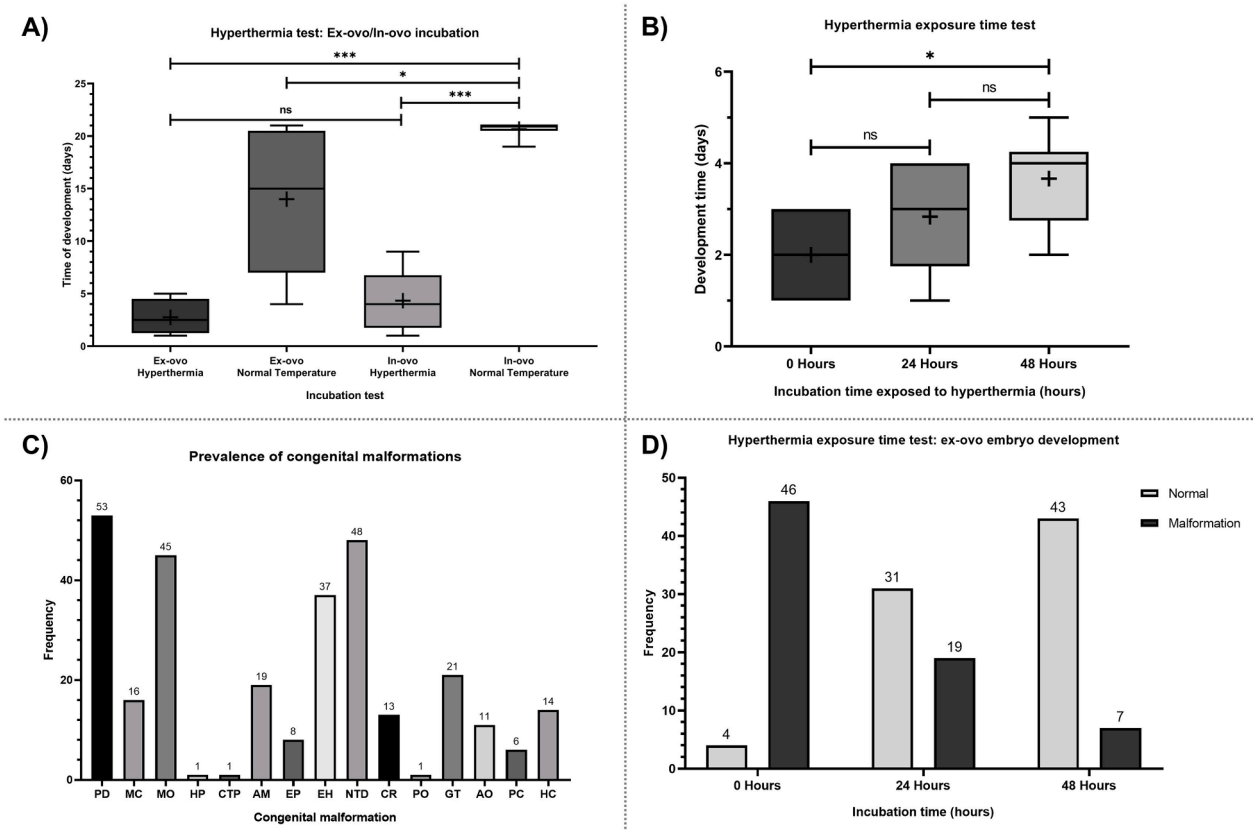

Fig. 3. Graphs of the ex-ovo incubation tests: (A) Hyperthermia test on the ex-ovo incubation, there is a significant difference ($P \leq 0.001$) between the days of development reached by the in-ovo normal temperature (21 d), the ex-ovo hyperthermia (5 d), and the in-ovo hyperthermia group (9 d). (B) Ex-ovo hyperthermia exposure time test, the 0 h and 24 h are non-significant ($P > 0.05$), the 24 h and 48 h exposure times are non-significant ($P > 0.05$), there is a significant difference $P \leq 0.05$ between the 0 h and 48 h tests. (C) Prevalence of the malformations on the ex-ovo incubation (PD: patterning defect, MC: microcephaly, MO: microphthalmia, HP: heteropagus twin, CTP: cephalothoracopagus twin, AM: amelia, EP: encephalocele, EH: ectopic heart, NTD: neural tube defect, CR: caudal regression, PO: polymelia, GT: gastroschisis, AO: anophtalmia, PC: phocomelia, HC: hydrocephalus), (D) Ex-ovo embryo development under a hyperthermia exposure time test (46/50 embryos developed malformations during the first 24 h, after the first day only 19/50, and finally after the second day only 7/50).

in maintaining the humidity of the blastoderm and yolk sac surface because the yolk sac tends to float to the surface of the incubation vessel above the surface level of the albumen, causing it to dry out. During the in-ovo incubation, the blastoderm requires the humidity, nutrients, and ions present in the albumen to secrete fluid downward from the surface albumen into the underlying yolk sac and embryo, allowing the metabolic processes the developing embryo requires (Dunn, 2023; New, 1956). Perry (1988) reported success in preventing the drying out of the blastoderm by bathing the yolk sac with the egg's albumen, thus allowing the development of the embryos, however, the incubation technique used a surrogate eggshell, not in an ex-ovo system. In the ex-ovo incubation systems, this technique of bathing the yolk sac with albumen proved ineffective in developing the embryos from the blastula stage until Dunn (2023) reported successful ex-ovo incubation from the blastula stage using a disc of Milli-Wrap membrane to maintain humidity and allow gas exchange.

Here was used a modified method of bathing the yolk sac described by Perry (1988) to develop the embryo by pipetting albumen to cover and hydrate the blastoderm and yolk sac in combination with a rotation of the incubation system (Fig. 2); we achieved the development of the embryos from the blastula stage, reaching 21 days of development although none of the ex-ovo chicks hatch. The principal factor for the successful incubation was the frequency of the pipetting of albumen and rotation of the vessel (every 4 h), guaranteeing the proper humidification of the blastoderm and yolk sac surface, thus far allowing an incubation without alterations or malformations (Fig. 4B) compared with the control group (Fig. 4A–C). However, it must be noted that this procedure was entirely manual, which takes more time to maintain each embryo and must be repeated every 4 h, thus reducing the number of embryos that could be incubated without compromising the optimal incubation care. An automated procedure that ensures the ex-ovo development from the blastula stage, like the one described by Dunn (2023) and Silver (1960), must be considered to allow a standardized high-throughput incubation.

One of the major advantages of an ex-ovo incubation from the blastula stage without a pre-incubation period is the ability to do research during these crucial stages of development and facilitate the extraction of the embryo for the ex-ovo incubation, as mentioned by Macias-Marin et al. (2023) during the in-ovo incubation as time progresses the yolk sac becomes more delicate and easy to break during the extraction, in contrast with the yolk sac during the initial stages of development which is more durable and resistant, thus improving the yield of ex-ovo incubation cultures that can be made.

One factor that could increase the risk of development of malformations is the time in which the eggs are incubated after being laid, as mentioned by Janikovičová et al. (2019), when time passes by and the eggs are not incubated promptly but stored (15-18°C, 75 % RH), there is an increase of malformations and mortality in the embryos. To reduce this risk, all the eggs were incubated on the same day the package of eggs arrived at the laboratory (approximately 2 days since the eggs were laid).

In the hyperthermia test, four incubation groups were analyzed; there was a significant difference between the time of development reached by the normal temperature ex-ovo incubation group and the

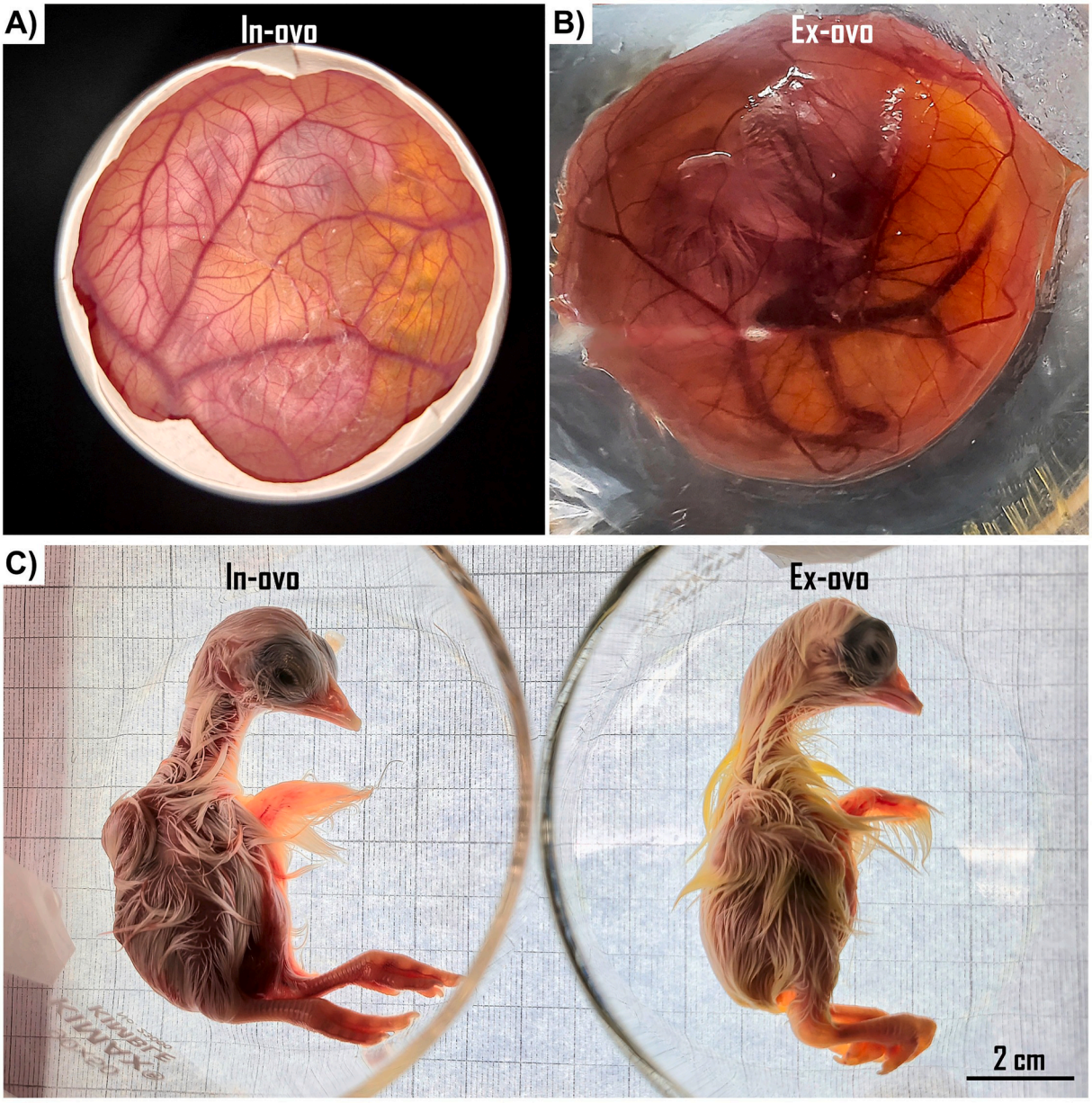

Fig. 4. Morphological comparison of the chick development (21 d, HH46) under normal incubation temperature (37.5°C): (A) Control (egg windowing), (B) Ex-ovo normal temperature, (C) Comparison of the in-ovo and ex-ovo chicks.

Table 2
Hyperthermia exposure time tests.

Tests	Exposure time			
	0 h	24 h	48 h	
Number of eggs	50	50	50	
Temperature	40°C	40°C	40°C	
Relative Humidity	50–60 %	50–60 %	50–60 %	
Egg turning	Manual Rotation + Albumen Pipetting (every 4 h)			

hyperthermia group. We observed the development of all the embryos (50/50) from the blastula stage for the normal temperature group (Fig. 3A); for the hyperthermia group, the embryos developed past the first day of incubation; however, they had a high rate of mortality (50 % in day 2), and only reaching up to day 5 of incubation. There was a significant difference between the time of development reached by the ex-ovo normal temperature incubation group and the ex-ovo hyperthermia group ($P \leq 0.001$), and only the hyperthermia group embryos developed malformations. By comparing the two hyperthermia groups (ex-ovo and in-ovo), the results of the days of incubation reached were non-significant ($P > 0.05$); however, in the in-ovo incubation, three embryos did not show any external malformation, and one reached 9 days of development (Fig. 6V), without any major malformations besides a slight gastroschisis. This result could be attributed to the thermal properties of the eggshell compared to the plastic cup, or the presence of components in the egg's membranes that help in the development of the embryo; however, more research is needed to determine a difference in the incubation techniques, and also the use of biochemical or molecular procedures to identify the presence of non-morphological abnormalities.

The in-ovo normal temperature (control group) allowed for the best incubation of embryos; there was a significant difference ($P \leq 0.05$) between the in-ovo normal temperature and ex-ovo normal temperature groups, however by doing a morphological analysis in vivo by employing the windowing technique (Andacht et al., 2004) for the

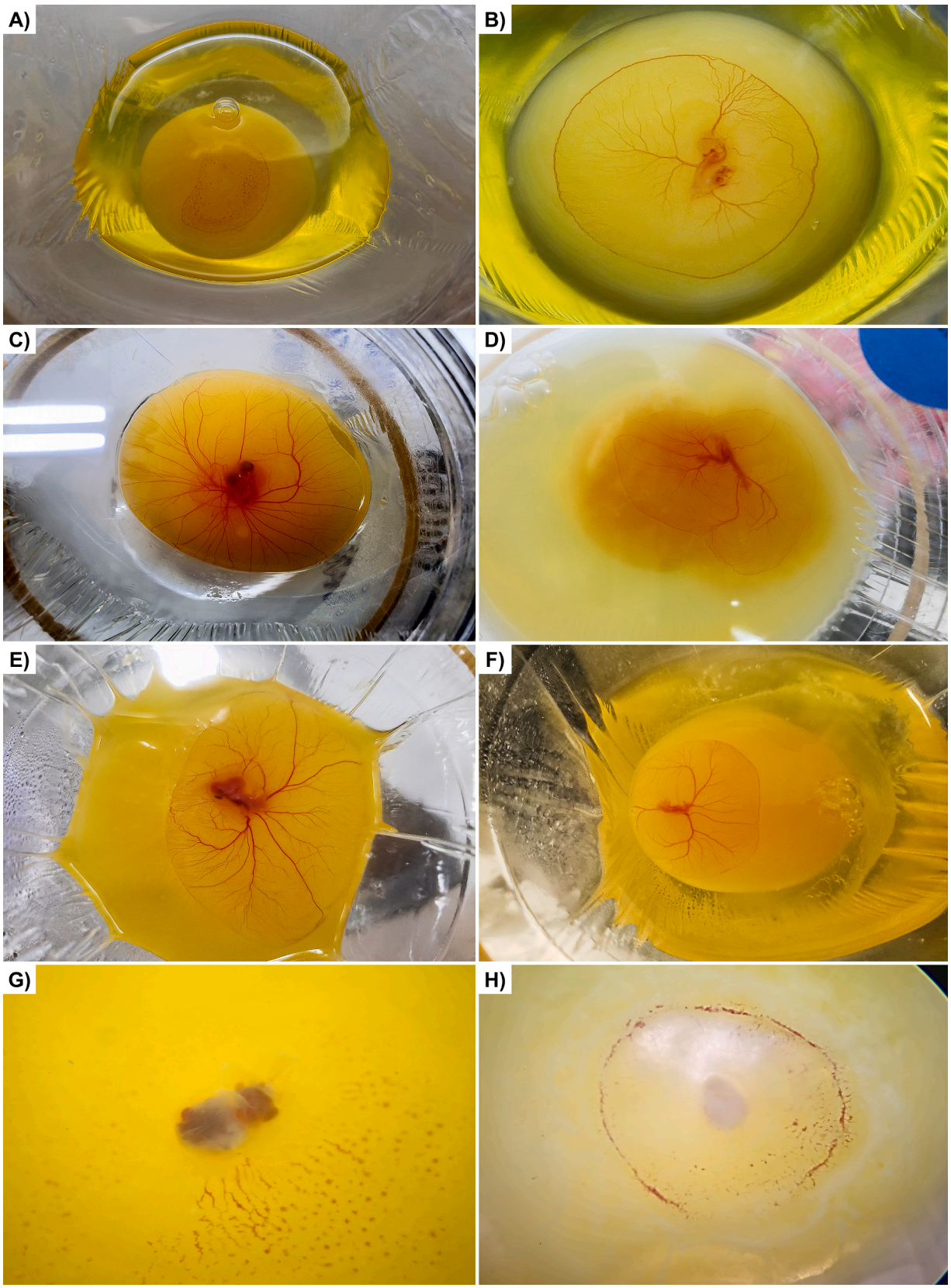

Fig. 5. Comparison of malformations in ex-ovo incubated embryos under hyperthermia conditions: (A) Patterning defect embryo (2 d, HH13), (B) Heteropagus twin embryo (3 d, HH23, an alteration of the left-right asymmetry can be observed), (C) Embryo with midbrain malformation and hypoplasia of the legs (4 d, HH24), D) Cephalothoracopagus twin embryo (3 d, HH21), (E) Embryo with midbrain malformation and microphthalmia (4 d, HH25), (F) Embryo with midbrain malformation and amelia (3 d, HH19), (G) Embryo with a patterning defect (2 d, HH13), (H) Embryo with neural tube malformation (36 h, HH10).

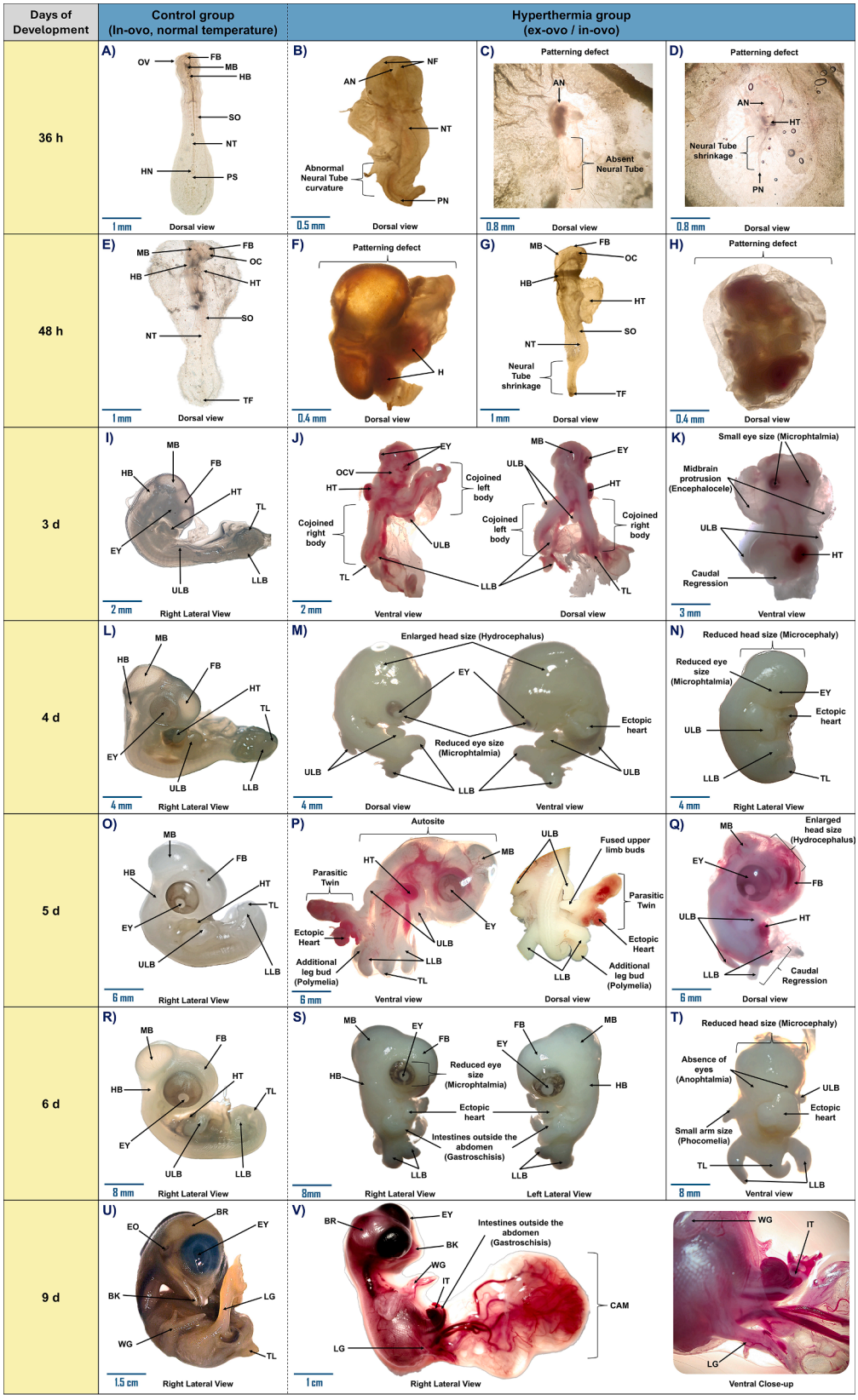

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Fig. 6. Anatomical comparison of embryos incubated under hyperthermia conditions that developed malformations: (A) Control embryo (36 h, HH26), (B) Ex-ovo embryo with a neural tube defect (36 h, HH26), (C) Ex-ovo embryo with a neural tube defect (36 h, HH26), (D) In-ovo embryo with a neural tube defect (36 h, HH26), (E) Control embryo (48 h, HH13), (F) Ex-ovo embryo with a patterning defect (48 h, HH13), (G) Ex-ovo embryo with a neural tube defect (48 h, HH13), (H) In-ovo embryo with a patterning defect (48 h, HH13), (I) Control embryo (3 d, HH18), (J) Ex-ovo cephalothoracopagus embryo (3 d, HH18), (K) Ex-ovo embryo with multiple malformations (3 d, HH18), (L) Control embryo (4 d, HH23), (M) Ex-ovo embryo with multiple malformations (4 d, HH23), (N) In-ovo embryo with multiple malformations (4 d, HH23), (O) Control embryo (5 d, HH26), (P) Ex-ovo heteropagus embryo (5 d, HH26), (Q) Ex-ovo embryo with multiple malformations (5 d, HH26), (R) Control embryo (6 d, HH28), (S) Ex-ovo embryo with multiple malformations (6 d, HH28), (T) In-ovo embryo with multiple malformations (6 d, HH28), (U) Control embryo (9 d, HH35), (V) In-ovo embryo with gastroschisis (9 d, HH35). Abbreviations: AN (Anterior neuropore), BK (Beak), BR (Brain), CAM (Chorioallantoic membrane) EY (Eye), FB (Forebrain), HT (Heart), HB (Hindbrain), HN (Hensen's node), IT (Intestines), LG (Leg), LLB (Lower limb bud), MB (Midbrain), NF (Neural folds), NT (Neural tube), OC (Optic cup), OCV (Oral cavity), OV (Optic vesicle), PN (Posterior neuropore), PS (Primitive streak), SO (Somites), TL (Tail), TF (Tail fold), ULB (Upper limb bud), WG (Wing).

Table 3
Types and frequency of morphological malformations in the hyperthermia ex-ovo/in-ovo embryos.

Malformation	Description	Frequency	
		(No.)	(%)
Anophthalmia	Absence of one or both eyes	11	6.51
Amelia	Absence of one or more limbs	19	11.24
Caudal Regression	Abnormal development of the lower part of the body (sacrum and lumbar spine)	13	7.69
Cephalothoracopagus	Imperfect fusion of the head and chest of cojoined twins, with separated columns, limbs, and pelvis	1	0.59
Ectopic heart	Abnormal location of the heart, either partially or completely outside of the thorax	37	21.89
Encephalocele	Protrusion of the brain	8	4.73
Gastroschisis	A congenital defect in which the intestines extend outside of the abdomen	21	12.43
Heteropagus	Asymmetrical cojoined twinning, in which a "parasitic'' twin is dependent on the main twin (autosite)	1	0.59
Hydrocephalus	Accumulation of fluid in the brain, enlarging the head	14	8.28
Microcephaly	Abnormally small head and brain size	16	9.47
Microphthalmia	A disorder in which one or both eyes are abnormally small	45	26.63
Neural Tube Defect	Category of neurological disorders related to malformations of the spinal cord	48	28.40
Patterning defect	Disruption of embryonic development, preventing the proper formation of body structures and organs	53	31.36
Phocomelia	Abnormally short length of the limbs	6	3.55
Polymelia	Presence of accessory limbs attached to various body regions	1	0.59

control group eggs, the development of the embryos was optimal (Fig. 4A–B), in the necropsy of 21-day old chickens (Fig. 4C), there were no significant differences in the external morphology of the control and the ex-ovo normal temperature embryos. Although there were no external malformations or differences in the anatomy of the normal temperature group, it must be noted that past studies have identified alterations in the organisms incubated by the ex-ovo technique, causing alterations in the ossification (Jourdeuil et al., 2015).

In the hyperthermia exposure time tests, there was a non-significant difference in the days of development reached between the 0 – 24 h and 24-48 h, but there was a significant difference ($P \leq 0.05$) between the 0 – 48 h; however, there was an interesting pattern proving the critical factor that is the exposure time to hyperthermia (Fig. 3B), the embryo is more susceptible during the first stages of development (Janikovičová et al., 2019; Krausova and Peterka, 2007), causing an increase in the development of malformations, but by increasing the days of development in which the embryos would be exposed to the hyperthermia, the malformation rate decreased (Fig. 6D). Hyperthermia disrupts the normal cell development in the embryo, particularly during the critical initial stages, through mechanisms like protein denaturation, apoptosis, and impairing cell migration (Buckiová et al., 1998; Edwards et al., 1997). One of the main problems of hyperthermia is that it causes a high rate of early embryo mortality (Fig. 3A)

As the mortality rate increased, so did the malformations (Fig. 3C); the most frequent malformation was at the blastula stage (Fig. 6C, E, F, H). Initially, these malformations were classified as early mortality for the embryo due to early development of the circulatory system blood islands (Fig. 5A, G, H); however, upon closer inspection with the stereoscopic microscope, the embryo was visualized, and the presence of malformations was discovered, in some embryos the development stopped during neurulation (Fig. 6B), and in other cases, the underdeveloped embryo appeared to be dead; however, the heart was still beating (Fig. 6F).

Other external malformations were identified, such as microphthalmia, neural tube defects, gastroschisis, and ectopic heart, which were present in conjunction with other malformations as shown in Fig. 6K the embryo presents the three anomalies of the development and only reached 3 days of development (HH18). These results are consistent with the reports of various in-ovo hyperthermia research studies; the authors Krausova and Peterka (2007) reported a high frequency of microphthalmia (7.3 %), although, in our research, we had a higher frequency (26.62 %). Various authors also reported gastroschisis (Krausova and Peterka, 2007; Peterka et al., 1996), with a frequency of 7 %; however, in our research, we also report a higher frequency (12.42 %).

We reported the development of two rare twinning malformations incubated ex-ovo under a period of hyperthermia during the blastula stage (0 h): a heteropagus parasitic twin (Figs. 5B and 6P) and a cephalothoracopagus twin (Fig. 5D and D). We observed the development of these two cases since the blastula stage, in which we initially did not notice anything abnormal in the blastoderm, yolk sac, or albumen, however as the development time increased, the malformations were observed and described in vivo (Figs. 5B and 6J), thanks to the capability to observe the development of the embryo in the ex-ovo system.

A heteropagus is the development of asymmetric conjoined twins in which the parasitic twin's tissues depend on the main organism called the "autosite'' (Sharma et al., 2010; Zhu et al., 2023). The heteropagus parasitic twin (Fig. 6P) reached 5 days of development; the autosite presented its normal morphology; however, in the ventral abdominal region (epigastric), it presented the parasitic twin, as mentioned by Sharma et al. (2010), the parasitic twin was completely dependent on the circulatory system of the main twin, each twin had its own functioning heart but shared the dorsal aorta. There are no previous reports of this chicken malformation.

Cephalothoracopagus embryos are conjoined twins who share parts or are fused in their heads, necks, and bodies (Bates and Dodd, 1999; Maurer et al., 2012). The cephalothoracopagus twin (Fig. 6J) reached 3 days of development; the twins shared the same head, neck, and heart but were separated into two bodies with their respective limb buds, somites, and tails. Maurer et al. (2012) reported a case of chicken cephalothoracopagus twins and hypothesized that since the abnormal development started until the neurulation, the embryo should be considered as a single embryo instead of conjoined twins; in our tests, we could support this theory by the description of only one blastoderm, that due to the effects of the hyperthermia caused the alteration during the

development.

Jin et al. (2024) described fission as a model to support the development of monozygotic twins, which we can support with our results by reporting only one blastoderm and yolk sac present in each egg (heteropagus and cephalothoracopagus), and not the partial fusion of two zygotes. The alterations that cause the fission process (Anca et al., 2015) could be related to the effects of hyperthermia, which causes multiple alterations (apoptosis, protein denaturation, alterations in cell migration), during the development of the organisms due to the heat stress (Edwards et al., 1997; Janikovičová et al., 2019; Noiva et al., 2014).

The effects of hyperthermia as a teratogen seem to be limited to the initial stages of development; however, during the rest of the development and post-hatching, it increases the morbidity and mortality of the organisms (Haghighi et al., 2021; Krausova and Peterka, 2007; Narinç et al., 2016). In the case of avian species there is no known risk of developing malformations due to infections during pregnancy like in humans (Sass et al., 2017), due to the type of incubation and embryonic development, however, in recent years, the environmental temperature has proven to be a major healthcare concern for most species. Aas the global temperature rises each year (Lindsey and Dahlman, 2024; NCEI, 2023), longer and more extreme heatwaves have been reported (US EPA, 2024) causing frequent periods of hyperthermia, which could increase the risk of development of malformations and mortality in humans and animals (livestock and wildlife), causing major economic losses.

The ex-ovo incubation allowed us to observe the embryonic development directly (morphology and ethology) and, as seen in the tests, the development of malformations; these advantages help us to do more research in the fields of toxicology and teratology using the standardized ex-ovo incubation system (Dunn, 2023; Macias-Marin et al., 2023; Tahara et al., 2014, 2021). A hyperthermia incubation temperature range test and an exposure time (short-term and long-term) test during the blastula stage should be considered for further research to help us understand the exact temperature and time threshold required to promote the development of malformations.

We reported the development of malformations starting from an incubation temperature of 40°C. Krausova and Peterka (2007), reported that temperatures from 42 to 44°C induced a higher rate of malformations and embryo toxicity, this temperature difference could be attributed to insulation properties of the materials (eggshell and plastic cup), which could have different heat exchange values, making the embryos that are incubated ex-ovo more susceptible to the changes on the incubation temperature. Further research should study the thermal properties of the incubation vessel materials (plastic cup) to help determine if the vessel increases or decreases the internal temperature of the embryo, to achieve an optimal incubation temperature.

The research focused solely on the external morphological malformations, which were abundant in the tests done; however, molecular and biochemical methods should be included in future research to identify and determine the malformations present in the embryos, like: immunohistochemistry, quantitative PCR, and fluorescence in situ hybridization (FISH). Genetic and metabolic alterations can be studied using these methods of identification (Boije et al., 2012), which would be ignored by only doing a morphological analysis. As we limited the research to the external morphology, internal abnormalities are highly likely to be present, like congenital heart defects (CHD), which are common (Houyel and Meilhac, 2021; Tikkanen and Heinonen, 1991) and can be identified with histological and biochemical procedures. An analysis of the expression of heat shock response (Sakatani et al., 2012) should be done in the embryos that developed malformations and in the embryos that did not develop severe external morphological malformations (Fig. 6), to determine if those embryos had a higher resistance to the effects of the hyperthermia and could adapt to the incubation condition. Most of the hyperthermia malformations identified (microphthalmia, amelia, twinning, etc.) originate from neural tube alterations (Moradi et al., 2017); such hyperthermia defects can be identified with RT-PCR by an induced upregulation of connexin-43 (CX43, GJA1) in the neural tube (Zhang et al., 2012). Connexins are proteins that construct gap junctions between cells and have an important role in regulating cellular differentiation and proliferation during embryonic development (Liu et al., 2006; Zhang et al., 2012), the overexpression of these proteins disrupts the gap junction channels, causing neural tube defects.

In conclusion, the ex-ovo incubation system does not cause the development of malformations; hyperthermia incubation increases the risk. Hyperthermia (40°C) is a teratogen that causes a variety of malformations in chicken embryos: blastula stage malformations, neural tube defects, microphthalmia, amelia, and the development of parasitic twins. The exposure to 40°C during the blastula stage of incubation (0 h) developed malformations in all the embryos; however, this rate decreased as the incubation days continued. The use of the albumen pipetting technique, in addition to the manual rotation of the ex-ovo culture (every 4 h), allows to reactivate the embryo development from the blastula stage by humidifying the blastoderm and yolk sac, therefore allowing the development of a standardized technique for the ex-ovo incubation from the blastula stage to hatching. The ex-ovo incubation system is a viable model for studying the effects of teratogens on the development of chicken embryos, allowing real-time observation and manipulation of the embryo from the blastula stage until the last day of incubation (21 d). More research is needed to understand the whole effects of temperature as a teratogen in human and animal healthcare.

Ethics approval

The current study was reviewed and approved by the Autonomous University of Aguascalientes Institutional Ethics Committee for Animal Experimentation (Permit Number of Protocol: CEADI-UAA-09-2022). The experiments were performed in strict accordance with national and international (Norma Oficial Mexicana: NOM-062-ZOO-1999, 2001; American Veterinary Medical Association, 2020; National Research Council, 2011) guidelines for the use, care, and euthanasia of research animals.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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