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Avian incubation conditions: Role in embryo development, physiology and adaptation to the post-hatch environment

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Avian incubation conditions: Role in embryo development, physiology and adaptation to the post-hatch environment

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Editorial: Avian incubation conditions: Role in embryo development, physiology and adaptation to the post-hatch environment

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KEYWORDS

incubation conditions, development, embryo, metabolism, poultry

Editorial on the Research Topic
Avian incubation conditions: Role in embryo development, physiology and adaptation to the post-hatch environment

Introduction

This Research Topic on Avian Incubation Conditions is an inclusive treatise discussing the impact of several environmental factors during egg storage and in the incubators that may affect avian embryo and post-hatch development, physiology, metabolism, and growth. The objective of this project was to gather current relevant research on this topic, detect areas lacking knowledge or understanding, and propose methods of study to advance on embryology and avian incubation techniques. The impact of egg storage conditions, incubation temperature, hypoxia, moisture, and light, among other factors, were reviewed in this Research Topic. Embryo developmental and long-lasting effects of these factors were systematically revised. A total of 12 manuscripts were accepted for publication: 7 original research contributions; 3 reviews; one mini-review; and one paper related to methods.

Three papers presented recent findings related to egg storage effects on egg quality and embryo development. Egg storage is a common and necessary practice in poultry production to synchronize hatch, meet and regulate hatchling demands, and coordinate activities in the hatchery. However, prolonged storage and conditions during this period may significantly impact embryo survival, development, hatchability, and even life post-hatch. Minimizing these harmful effects has been a frequent Research Topic in avian incubation. Pokhrel et al. elucidated the molecular and cellular mechanisms involved in the better recovery of embryos during storage when eggs were exposed to 12°C rather than higher temperatures (18°C). These temperature-dependent mechanisms are related to the embryo's transition from blastulation to gastrulation.

Studying the sequential effects of egg storage on egg quality and embryo development on the same eggs is challenging since most methods to evaluate egg quality are destructive. Adriaensen et al. used non-invasive tools such as computed tomography and magnetic resonance imaging in a study to evaluate the effects of egg storage duration on egg quality and embryo

development. In this study, eggs were stored at 16°C and 80% relative humidity. These imaging technologies aided in visualizing and quantifying the negative impacts of egg storage on embryo development. Guinebretière et al. studied the effects of three storage temperature strategies (11.6°C or 18.3°C) together with pre-incubation in chicken eggs from young and old breeder flocks. The pre-incubation was applied on days 6 and 10 during the storage period of 14 days. Their results indicated that the low storage temperature (11.6°C) had similar results to warm egg storage (18.3°C) if pre-incubation is applied, and both treatments counterbalance the negative effects of prolonged egg storage on hatchability and chick quality regardless of breeder age; however, these authors did not detect differences among treatments on chicken live performance during rearing.

Original research and review manuscripts covered the impact of variation in temperature and other incubation factors on embryo development and long-lasting effects in the life post-hatch. Almeida et al. evaluated the effect of constant low (36°C), control (37.5°C), and high (39°C) machine temperature from day 13 onward on lipid metabolism, adipose tissue, and body composition. Their findings indicated that incubation temperature affects regional adiposity, lipid metabolism, and fat deposition in broilers.

Many studies in the past years have tried to determine the optimal embryo temperature during incubation, looking at diverse response parameters. Yalçin et al. reviewed current studies on this topic and the influence of temperature variation and light use during incubation. This review also included studies on cyclic temperature manipulations during critical periods of embryo development. Additionally, the review encompassed the potential impact of lighting on circadian rhythms vital for development and regulation related to improving the resistance of broilers to heat stress.

Tona et al. also reviewed the effects of incubation conditions such as temperature, relative humidity, turning, ventilation, *In ovo* feeding, and delay in feed access. These authors concluded that all these incubation factors might affect embryo parameters and post-hatch growth differentially according to exposure time and stage of development at the time of the stimulus in the incubator. The literature review presented by Wang et al. focused on the impact of temperature, humidity, oxygen density, ventilation, and lighting on the number, shape, and structure of embryo muscle fibers, with long-lasting effects on post-hatch muscle growth and meat quality. This paper also suggested future studies to evaluate the effects of incubation conditions on muscle cell regulation, proliferation, and meat quality looking for methods to improve the final poultry product.

Oxygen availability controls several mechanisms of development, tissue maturation, and cell metabolic regulation in avian embryos. The impact of hypoxia during incubation on the post-hatch performance of broilers subject to suboptimal environmental temperature was presented in original research conducted by Haron et al. Hypoxia for either 12 h (17% O_2) during days 16–18 of incubation or continuous hypoxia for 48 h from days 16–17 were compared with incubation under normoxic conditions (21% O_2). Hatchlings were raised under cold, hot, and diurnal cyclic temperatures. Broiler results, up to 42 days of age, indicated that hypoxia during that critical period of embryo development caused adaptive metabolic responses that improved thermoregulation, feed efficiency, and breast muscle growth. The authors proposed strategic hypoxia during incubation as a tool to adapt poultry to post-hatch suboptimal environmental conditions.

Relative humidity variability during incubation from 25% to 93% was evaluated by Branum et al. While hatchability, embryo dry body mass, and acid-base regulatory responses were not affected by this factor, incubator relative humidity caused significant differences in tissue water content and body mass at hatch. Embryo hydric balance can also be affected by egg treatments. Gregorich et al. induced moisture loss in leghorn and broiler eggs by drilling two 1.5 mm diameter holes in the eggshells. They observed the immunity cell response and transcription of interleukins in embryos and chicks facing a challenge with lipopolysaccharides. These researchers concluded that this double-hole treatment and faster moisture egg loss could reprogram embryo gene transcription to facilitate immunity cell survival and responses to an immunological challenge.

A mini-review by Sukparangsi et al. described the benefits of avian embryonic culture *In Ovo* and *In vitro* cell culture versus the traditional *Ex-Ovo* methods. However, the relationships among these methods are discussed. These methodologies can help better understand avian development mechanisms and unravel the transcriptional networks that regulate cell differentiation in embryos. In the same line of ideas related to methodologies of studying embryo development, Dave et al. proposed a novel methodology called the egg-in-cube system to study the avian blastoderm with intact tissue tensions on its native yolk. This technique could solve the common flaw of embryo culture techniques. This new methodology could allow researchers to explore fundamental questions in early embryogenesis on its native yolk.

In summary, the papers compiled on this Research Topic offer a complete overview of the impact of multiple factors that may affect various embryo developmental parameters during storage and incubation. It was evident in all papers that incubation conditions have consequences in the life post-hatch. Consequently, incubation conditions could be used to adapt the hatchlings to adverse environmental conditions or improve meat quality, immunity, and health. This Research Topic also includes reviews of methodologies to enhance the understanding of embryology and the impact of avian incubation factors.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Storage Temperature or Thermal Treatments During Long Egg Storage Duration Influences Hatching Performance and Chick Quality

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This study was designed to improve the hatching performance, chick robustness and poultry health in the event of long-term egg storage and suboptimal age of the reproductive flock. A total of 9,600 eggs from one young breeder flock (28 weeks of age, batch B) and 9,600 eggs from an older breeder flock (59 weeks of age, batch E) were used (ROSS 308). Each batch was separated into three sub-groups and stored for 14 days. The first sub-group of eggs (Cool, group C) was stored at 11.6°C. The second sub-group of eggs (Warm, group W) was stored at 18.3°C with two pre-incubation on days 6 and 10 of the storage period. The final sub-group of eggs (Control, group Ct) was stored at 18.3°C throughout the storage period. Eggs were similarly incubated and hatched birds were raised on the same experimental farm. In both batches, embryonic development was significantly more advanced in W eggs than in C and Ct eggs ($p < 0.01$). In both batches, C and W treatments decreased early embryonic mortality by more than 10% compared with Ct, decreased the proportion of late-hatched chicks and improved the percentage of first grade chicks: in batch E, 42% of Ct eggs were first grade chicks vs. 57% in group W and 59% in group C. Benefits were even higher in batch B, where only 60% of Ct eggs gave first grade chicks vs. 83% in others groups. The hatching rate was thus higher in groups C and W regardless of flock age: for batch B eggs, 85% hatched in W and 84% in C vs. 62% in Ct, while for batch E eggs, 59% hatched in W and 61% in C vs. 45% in Ct. Day-old Ct chicks from batch E were heavier than W and C ones, and heavier than W chicks from batch B ($p < 0.05$). Long-term parameters on farm were not significantly different between groups. Thermal treatments during the storage of eggs from both young and old breeder flocks counterbalance the negative effects of prolonged egg storage on hatching rate, without altering chicken performance during rearing.

Keywords: breeder flock age, storage conditions, embryonic development, embryo mortality, hatchability, chick quality, broiler performance, antioxidant status

INTRODUCTION

In order to coordinate hatchery activities and improve their flexibility to meet market fluctuations and demand, the duration of egg storage may vary in the broiler breeder industry. Hatcheries may have to extend this storage period up to 15 days to allow the incubation of thousands of eggs, and accordingly to obtain chickens of the same age in sufficient quantities. Hatcheries are also dependent on the availability of breeder flocks to meet demands for day-old chicks, with either young flocks (before 35 weeks of age) or older ones (over 55 weeks of age). However, neither the egg storage period nor the breeder flocks' age are always optimised in commercial situations, with potentially detrimental effects on egg quality, incubation efficiency, the quality of chicks and post-hatching performance.

Prolonged egg storage (7 days or more) has negative consequences on embryonic survival, the hatching rates of fertile eggs and chick quality (Lapão et al., 1999; Tona et al., 2003a,b; Fasenko, 2007; Hamidu et al., 2010). It can also have a negative effect on the long-term performance of chickens (body and carcass weight; Alsobayel and Al-Miman, 2010). Moreover, hatchability increases when breeder flocks are aged less than 40 weeks old (Abiola et al., 2008) and then decreases, in part due to increased total embryo mortality (Almeida et al., 2008) and lower fertility (Guise et al., 1998). Breeder flock age also affects egg weight (Tona et al., 2001) and therefore influences chick weight (Tanure et al., 2009) at hatching: the eggs are generally heavier if they come from an older flock than from younger flocks. The quality of chicks from a young flock appears to be better (Tona et al., 2004) and their mortality rate is lower (Peebles et al., 1999).

The duration of egg storage and the age of the breeder flock often interact (Nasri et al., 2020). The age of the flock is likely to play a major role in the embryo's ability to withstand prolonged storage periods; eggs from young breeder flocks can be stored longer without deterioration than eggs from older flocks (Reijrink et al., 2009).

Storage conditions can have a dramatic impact on hatchability (reviewed in Bergoug et al., 2013a). In commercial hatcheries, when eggs are stored for up to 1 week, they are generally kept at a constant temperature of 18°C–20°C and a relative humidity (RH) of 70–75% (Christensen et al., 2002). To help reduce the negative effects of prolonged storage, temperature and RH are empirically adjusted.

One solution is to decrease the storage temperature below physiological zero (Edwards, 1902 or embryonic diapause Fasenko, 2007), i.e. 20–21°C. Generally, the temperature in the storage room is decreased to 15–16°C as recommended by several authors (Christensen et al., 2002; Elibol et al., 2002). Under the influence of hypothermia, embryogenesis pauses, without any embryonic development: some cellular metabolic processes continue, but gross morphological (shape and structural) changes are stopped. Lowering temperature even more could also be beneficial. For instance, Pokhrel et al. (2018) compared egg storage for 7–28 days at either 18°C or 12°C with respect to hatchability and chick quality, and suggested lowering the storage temperature to 12°C for extended storage (beyond 7 days). Ruiz and Lunam (2002) also obtained better results for hatching rate and chick weight at hatching using eggs stored for 9–11 days at 10°C than at 16.5°C. However, according to Reijrink et al. (2008), this solution might not be enough for longer egg storage periods, since there is still an increase in the number of necrotic cellular indicators contributing to the early mortality of embryos. To date, very few publications about the impact of low temperature (under 12°C) on hatchability and chick quality are available.

Another solution is the pre-incubation of eggs, i.e. raising the temperature to 37°C for six continuous hours or several times during storage. Several authors have investigated this solution with respect to hatchability and chick quality (reviewed in Reijrink et al., 2008). The objective of this strategy is to synchronise the embryos to attain a more advanced stage of development that is less sensitive to prolonged storage (Pokhrel et al., 2017). The embryo germ cells could thus be in a better condition and the viscosity of the subgerminal liquid be maintained. However, the results obtained appear variable: hatching rate increased and embryonic mortality decreased when eggs were warmed for 6 h at 37°C (Silva et al., 2008), whereas another study (Fasenko et al., 2003) found adverse effects on the hatching rate by using this technique a few days after oviposition. Dymond et al. (2013) found better hatchability, lower embryo mortality and higher body weight over the 4 weeks post-hatching after several periods of pre-incubation of eggs every 4–5 days of storage, whereas Silva et al. (2008) advise against exposing eggs from old breeding flocks to a higher temperature (36°C) for extended periods of storage.

Pre-incubation appeared to be particularly important for embryos from young hens (Reijrink et al., 2009) but was not clearly demonstrated by Damaziak et al. (2018), who tested two pre-incubation periods at 5 and 10 days over 12 days of egg storage. It seems that the beneficial effect of pre-incubation depends on the interaction between several factors, such as the developmental stage of the embryo at oviposition, the length of storage, the length of the pre-incubation period and the number of times when it is applied (Reijrink et al., 2008). Finally, the relationship with the breeder flock's age is not fully clear as related studies are sometimes contradictory.

It has been well established that hatchability declines with storage duration and the breeder flock's age. However, the medium- and long-term consequences of cool or warm treatments prior to egg incubation have not been fully explored, especially those on animal health during rearing. The present research was designed to explore how to compensate for the negative impacts of long storage and the suboptimal age of breeder flocks in order to increase the hatching performance without deteriorating chick robustness, and improving poultry health throughout the rearing phase. The goal of this study was thus to measure the short-, medium- and long-term effects of different temperature conditions during storage when eggs, collected from young or old breeder flocks, are stored for 14 days. In one case, eggs were kept at low temperatures (11.6°C). In another, eggs were kept at 18.3°C with two successive periods of short pre-incubation thermal exposures. Egg quality (pH, weight loss,

embryo mortality, embryo staging and hatchability), chick quality (proportion of first grade, vitellus and body weight) and broilers' health and performance up to slaughter (mortality, body weight, breast yield and oxidative balance in the blood) were compared with eggs stored under conventional conditions (18.3°C, RH 69%).

MATERIALS AND METHODS

Animals and General Husbandry

Egg Origin

Two batches of 9,600 eggs from two separate ROSS 308 breeding flocks, one at the beginning and the other at the end of their laying period (B: 28 weeks of age, E: 59 weeks of age), were used (September 2018). All the eggs were purchased from the Perrot hatchery (Pommerit-Jaudy, France). They were then considered separately and designated as batches B and E.

Egg Storage

All the eggs from both batches were stored for 14 days at the ANSES Ploufragan experimental facilities (France) according to three modes, forming three groups of 3200 eggs: control group (Ct), cool group (C) and warm group (W). The average temperature of the Ct group was 18.3°C (18.3–18.4), with RH 69.4% (69.3–69.5) for the entire storage period. C eggs were stored in a cool room, the target being 11°C for the entire storage period. Actually, the average T° was 11.6°C (11.6–11.7) and RH 70.1% (69.6–70.5). Finally, W eggs were stored in the same room as Ct eggs, the difference being that they were warmed twice in incubators, on day 6 and day 10 after oviposition. During these two pre-incubations, the temperature reached a plateau of 34°C in 3.5 h, then remained at 34°C for 2 h before going back down under 26°C in 2 h. The total duration above 32°C was 4 h. The experiment workflow is summarised in **Figure 1**.

Incubation Period

After the 14 days of storage, all the eggs were incubated in the experimental hatchery using two incubators. One incubator was used for B eggs, another one for E eggs (Incubators/Hatchers, 9,600 egg capacity, Petersime®, Zulte, Belgium). The three groups were homogeneously distributed in each incubator. The incubation parameters (temperature, CO_2 and hygrometry) were in accordance with the manufacturer's recommendations.

Before incubation started, the eggs were pre-heated for different lengths of time depending on the group, slowly reaching 24°C to limit condensation on the eggshells, especially for C eggs. Thus, C eggs were pre-heated longer (12 h) than the other eggs (8 h). In addition, hatching time was supposed to be shorter for W eggs (Dymond et al., 2013). In order to have all the eggs hatching at the same time, the incubation of W eggs was therefore delayed by 8 h compared with Ct and C eggs.

In each group, eggs were candled after 18 days of incubation. Semi-transparent eggs were discarded. The others were put back into the incubators until hatching.

Hatching

At 21 days of incubation (523 h for Ct and C eggs; 515 h for W eggs), the results of incubation were divided into either unhatched products or hatched chicks.

Unhatched products were further divided into:

- Unhatched dead in shell embryos,
- Unhatched live in shell chicks at internal/external pipping, and
- Hatched dead chicks outside the shell.

Based on physical parameters, hatched chicks were blindly classified by trained staff as:

- First grade chicks: free of any visible abnormality, dry and clean, active and bright eyes (Tona et al., 2004).
- Second grade chicks: either inactive or with an abnormality or defect in the navel and legs/feet. They included in this category wet chicks, considered to be late-hatched as: these chicks were alive and outside of the eggshell, but although fully hatched their fuzz was still wet.

In each group, the first grade chicks were sexed (vent sexing).

Rearing Period

First grade chicks were placed directly next to the experimental farm's hatchery. One room housed B chicks, and another, E chicks. In both rooms, one side housed males and the other side housed females, which were separated by a central corridor. In each batch × sex block, the three groups were homogeneously distributed in each side, in pens with a density of 19 chicks/m^2. All the animals were reared under the same conditions: the rearing parameters (feeding, lighting programme, temperature, ventilation and vaccination) followed the classic standard commercial specifications (AVIAGEN, 2014). All animals had *ad libitum* access to water and feed. Animals were slaughtered in a commercial slaughterhouse at 35 days of age. Depending on the numbers of hatched chicks, 1,274–2,381 chickens were reared in each group. There were 3–6 pens of 170 animals per group × sex block.

Data Collection

Unless specified, all the following measurements were taken for each batch.

Egg Parameters During Storage

Before and after the storage period, 50 randomly selected eggs per group were weighed (0.1 g precision) to estimate weight loss, and 20 eggs were broken to measure egg white pH (HI 9321 microprocessor pH meter, HANNA Instruments, Lingo, France).

After 12 days of storage, 15 eggs per group were opened to determine the embryo staging according to Eyal-Giladi and Kochav classification (Eyal-Giladi and Kochav, 1976). Specific methods for embryo isolation and staging were described by Pokhrel et al. (2017) and Wade et al. (2014). Briefly, forceps were used to open the blunt end of the egg, exposing the

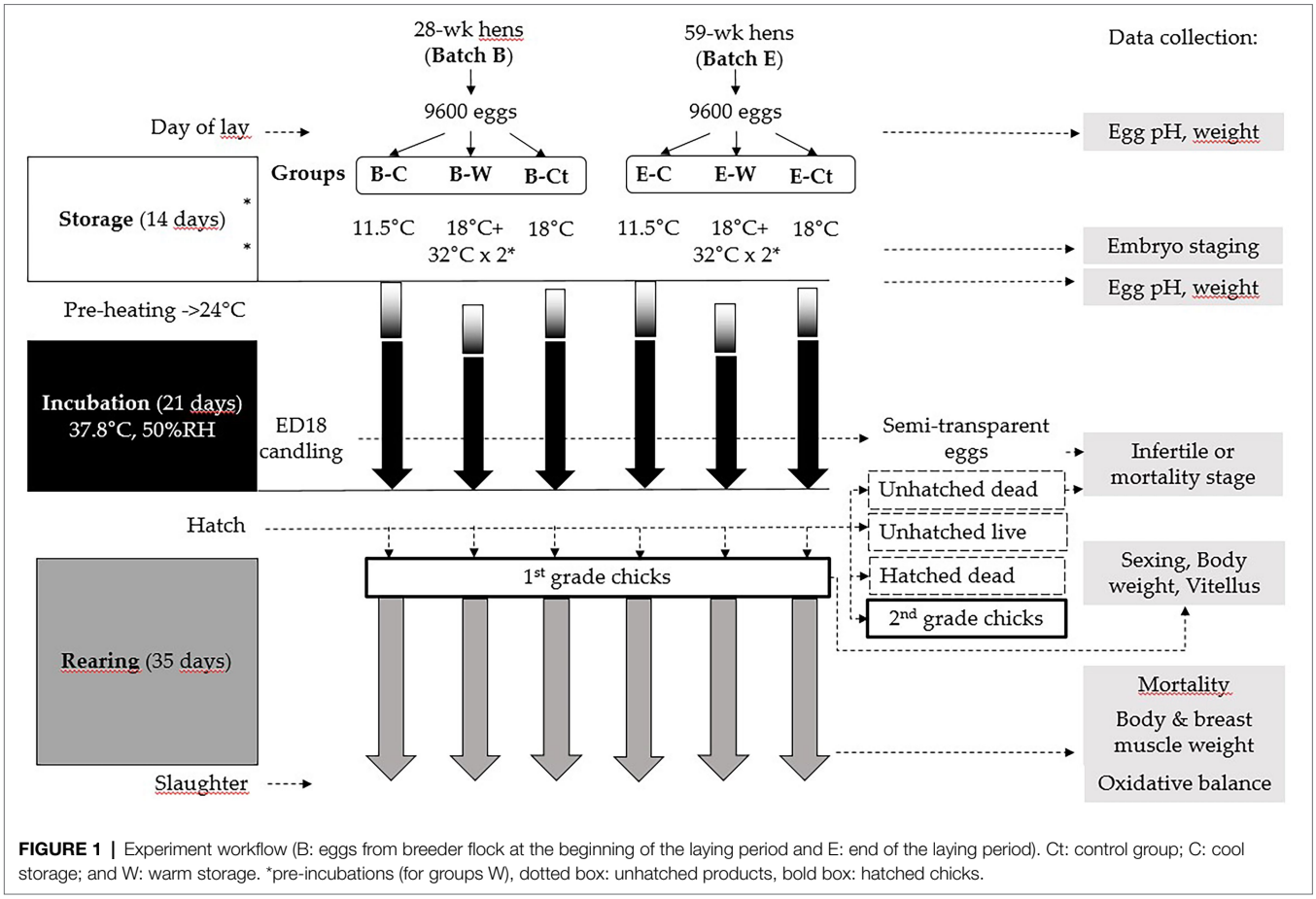

FIGURE 1 | Experiment workflow (B: eggs from breeder flock at the beginning of the laying period and E: end of the laying period). Ct: control group; C: cool storage; and W: warm storage. *pre-incubations (for groups W), dotted box: unhatched products, bold box: hatched chicks.

underlying air space. Albumen was removed from the yolk, and the embryo was removed from the egg by placing a filter paper disc containing a 1-cm hole in the centre, over the embryo. Dissection scissors were used to cut around the disc through the perivitelline layer. The disc containing the germinal disc embryo was removed and cleaned in PBS (pH 7.4) to remove any adherent yolk. The embryos were placed in Petri dishes and observed using a dissection microscope (Nikon SMZ1500, Melville, NY). Stage EGX was defined by completion of area pellucida formation (**Figure 2**). At this stage, polyingressing cells and cell clusters were visible on the ventral surface of the area pellucida. Notably, at stage EGX, no hypoblast cells in the posterior part of the embryo, adjacent to Koller's sickle, had formed. In the following stages, EGXI-XIII, the hypoblast formed in a posterior to anterior direction; at stage EGXI, the hypoblast covered one-third of the area pellucida; at stage EGXII, it covered two-thirds of the area pellucida; and by stage EGXIII, hypoblast formation was complete.

Hatching Parameters

The number of semi-transparent eggs defined at 18 days of incubation was counted in each group and was related to the initial number of eggs in the incubator (Ni). The total number of unhatched eggs at 21 days of incubation was counted in each group and was related to Ni or to the number of eggs kept in the incubator after the candling process (Ns). Semi-transparent eggs (at 18 days of incubation) and unhatched eggs (at 21 days of incubation) were cracked open immediately after having been sorted and were visually analysed to determine infertility or embryo mortality staging:

- Stage 0: no visible embryo. The egg was considered as infertile or fertile but in which the embryo died before 4 days of incubation.
- Stage 1: embryo died before 4 days of incubation: the germinal disc was visible.
- Stage 2: embryo died between 4 and 6 days of incubation: presence of a blood network.
- Stage 3: embryo died between 7 and 17 days of incubation: presence of black eyes.
- Stage 4: embryo died after 18 days of incubation: growth of embryo nearly complete and yolk sac drawn into body cavity.

In each group, the number of eggs in each stage was related to Ni.

The number of dead chicks and the number of unhatched chicks were counted for each group and related to Ni or to Ns.

The number of first grade chicks and second grade chicks were counted for each group and related to Ni or to the total number of hatched chicks (Nh). This was also considered as the hatching rate. The number of wet chicks was counted for

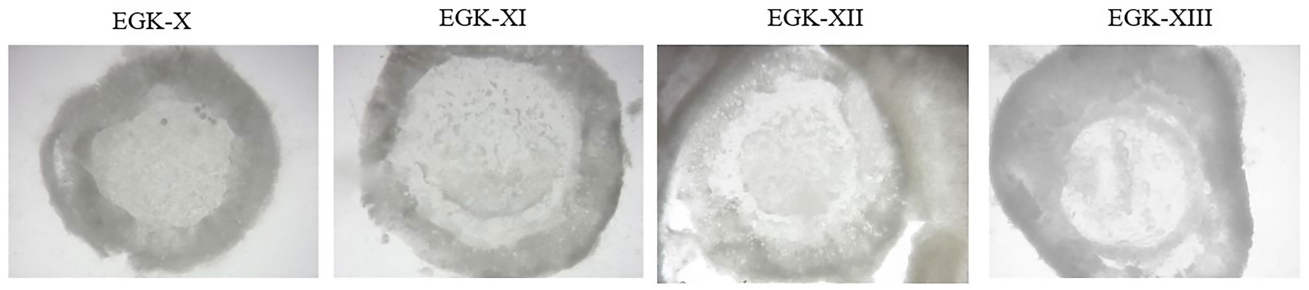

FIGURE 2 | Ventral view of the blastoderm from stages EGX to EGXIII—©ANSES.

each group and related to Ni or Nh. The total number of wet chicks plus unhatched chicks was counted for each group and related to Ni or to Ns.

Measurements on First Grade Chicks

The sex ratio of first grade chicks was determined in each group (number of females related to the number of first grade chicks). In each group, 100 chicks (50 males and 50 females) were weighed and 40 chicks (20 male and 20 females) were euthanised and their vitellus weighed, then related to the chick's total body weight.

Measurements on Broilers During Rearing

Mortality During the Rearing Period

During the rearing period, mortalities were recorded daily. For ethical reasons, birds that had severe health problems (i.e. fracture, blindness, severe lameness and malformation) were culled and recorded as dead. The cumulated mortality rates on days 5, 11, 14 and 35 were calculated as the cumulated number of dead animals to the initial number of animals on the farm.

Body Weight During the Rearing Period

In each group, 200 animals (100 males and 100 females) were weighed at 4, 11, 21, 28 and 32 days of age. Animals were randomly selected in each group. The individuals weighed in each group were thus potentially different or identical between each weighing session.

Breast Muscle Weight

In each group, 16 randomly selected broilers (eight males and eight females) were euthanised at 31 days (for E) or 32 days (for B). Their right muscle breast (pectoralis major) was weighed. The weight was multiplied by two and related to the bird's total body weight.

Oxidative Balance in the Blood of E Animals

Thermal treatments during egg storage could have an incidence on the antioxidative properties of chicks. These changes in oxidative balance may in turn have an impact on poultry health and even resistance to disease or other stresses. No research has been carried out in this area until now. An exploratory study was therefore performed to investigate the oxidative balance using several potential markers of oxidative stress. Markers of oxidation and antioxidant mechanisms were investigated in poultry from batch E at 7 and 31 days old. No poultry from batch B was included in this exploratory study. E birds were chosen based on the hypothesis that they would be more impacted by a long egg storage period.

At 7 and 31 days old, blood samples were taken from 16 broilers per E group (eight males and eight females). Blood was sampled from the occipital sinus. Whole blood was centrifuged at 3,000 *g* for 10 min, and plasma samples were stored at −80°C for further analysis of oxidative stress and antioxidant mechanisms. The thiobarbituric acid reactive substance (TBARS) index, commonly used to assess the susceptibility of tissues to peroxidation (Lin et al., 2004; Alnahhas et al., 2015), was measured in plasma samples according to Lynch and Frei (1993). The antioxidant status was assessed in plasma by measuring total antioxidant status (TAS; Pertusa et al., 2017) by using the Randox TAS colorimetric assay according to the supplier's recommendations and using an ARENA 20XT (Thermo Scientific) device. Plasma concentrations of glucose, uric acid (a marker of protein oxidation for energy purposes, but also a potent antioxidant) and triglycerides were measured using miniaturised commercial kits (BioMérieux, Craponne, France) and a clinical chemistry analyser (ARENA, Thermo Fisher Scientific, Courtaboeuf, France).

Statistical Methods

The statistical analysis was conducted using R software (R Core Team, 2020). The B and E batches are considered as two distinct batches, so they were analysed separately and they were not compared between themselves. Indeed, although conditions of storage were perfectly similar between the two batches, eggs came from different farms, different breeders flocks, which can strongly influence egg quality, egg fertility and thus hatching rate, and chick quality. Moreover, incubation and rearing were done in separate incubators and rearing rooms.

After checking the normality of the data by a Shapiro–Wilk test and the equality of variances by a Bartlett test, egg weight loss, pH and embryo staging after the 14-day storage period were examined by an analysis of variance, taking into account the group as explanatory variables, followed by a Student–Newman–Keuls test as *post-hoc* analysis. Chick weight, vitellus

weight, body weight during rearing and breast muscle weight were all examined using an analysis of variance taking into account the group and sex as explanatory variables and their interactions, followed by a Student–Newman–Keuls test.

The results of hatching parameters, embryo mortality stages in relation to Ni, Ns or Nh, sex ratio relative to first grade chicks and mortality rates on days 5, 11, 14 and 35 were analysed using Chi-square tests.

The results for oxidative balance in the blood were examined by an analysis of variance taking into account the age of the animals, the group and the sex as explanatory variables and their interactions, followed by a Student–Newman–Keuls test as *post-hoc* analysis.

For all analyses, differences were considered significant when $p < 0.05$.

Ethics Approval

All research was reviewed and approved by the Comité National de Réflexion Ethique sur l'Expérimentation Animale (National committee for ethics in animal testing) No. 016 at the French Ministry for Education, Higher Education and Research before data collection (number APAFIS#16128–2,018,071,314,183,633 v1). All efforts were made to minimise the number of animals used and their suffering.

RESULTS

Eggs

The results of egg weight loss, albumen pH and embryo staging after a 14-day storage period are shown in **Table 1**.

Before storage, pH values were 8.5 and 8.4 in B and E eggs, respectively. After 14 days of storage, in both B and E batches, the pH was higher for W eggs than for Ct eggs and was lower for C eggs than for Ct eggs.

Before storage, embryo staging was at 10.2 and 10.7 in B and E eggs, respectively. After 14 days of storage, in both batches B and E, the developmental stage of the embryo was higher in W than in Ct eggs, while Ct and C embryo stages were not significantly different.

Hatching Results

Table 2 summarises the hatching results per group in batches B and E, and the results of the statistical comparison of proportions relative to Ni.

For both B and E batches, the number of semi-transparent eggs (at ED18) relative to Ni was higher in Ct groups than in either C or W groups. They represented 25% of Ni eggs in the B-Ct group, whereas they represented less than 10% in the B-C and B-W groups. Semi-transparent eggs represented 43% of Ni eggs in the E-Ct group, whereas they were less than 33% in both E-C and E-W groups.

Similarly, for both B and E batches, there were more unhatched dead in shell (at E21) relative to Ni (or to Ns) in Ct groups than in C or W groups. Unhatched dead in shell represented 7.1% of Ni eggs in the E-Ct group, whereas they represented less than 5.5% in both E-C and E-W groups. In the B-W group, the number of unhatched dead in shell was even lower than in the C group: unhatched dead in shell represented 2.3% of Ni eggs in the B-W group, whereas it was 3.2% in group B-C and 5.5% in B-Ct.

TABLE 1 | Mean (SD) of weight loss (% of initial weight), albumen pH and embryo staging (EG&K classification) after 14 days of storage.

	B batch			
	C	**W**	**Ct**	**P Group**
Weight loss	1.0 (0.3)^{b}	1.9 (0.5)^{a}	1.7 (0.3)^{a}	<0.001
Albumen pH	8.7 (0.03)^{c}	8.9 (0.04)^{a}	8.8 (0.04)^{b}	<0.001
Embryo staging	10.0 (0.00)^{b}	12.4 (1.04)^{a}	10.1 (0.67)^{b}	<0.001
	E batch			
	C	**W**	**Ct**	**P Group**
Weight loss	1.1 (0.5)b	1.8 (0.4)^{a}	1.7 (0.3)^{a}	<0.001
Albumen pH	8.8 (0.04)^{c}	9.0 (0.04)^{a}	8.9 (0.05)^{b}	<0.001
Embryo staging	10.4 (0.70)^{b}	12.6 (1.09)^{a}	10.2 (0.63)^{b}	<0.001

Ct, control group; C, cool storage; and W, warm storage. B: eggs from breeder flocks at the beginning of the laying period; E: end of the laying period. n per group = 50 eggs (weight loss), 20 eggs (pH) and 15 eggs (embryo staging). p indicates the statistical difference between groups within batches. a, b and c: values not associated with common letters within batches are statistically different ($p < 0.05$).

The number of hatched dead chicks outside the shell was less than 1.6% of Ni in all groups, regardless of the batch (B or E) or the group (C, W or Ct).

The details of mortality stages in semi-transparent eggs and unhatched eggs are shown in **Figure 3**. Ct groups contained more stage 0 than other groups, in both B and E batches. They represented 18.8% of Ni eggs in the B-Ct group but less than 7% in the B-C and B-W groups. They represented 39.3% of Ni eggs in the E-Ct group but less than 29% in both the E-C and E-W groups.

Mortality stages 1, 2 and 3 decreased in the B-C and B-W groups in comparison with the B-Ct group. This was only the case for mortality stage 2 in batch E for the C group. The higher number of stage 0 in the Ct groups strongly affected the Ns that had lower values in the Ct group than in either the W or C groups in both B and E batches (**Table 2**).

For both B and E batches, Nh (or hatching rate) was lower in the Ct groups than in either C or W relative to Ni or Ns. The hatching rate increased from 62.4% of Ni in B-Ct to 85.4 and 84.0% in the B-W and B-C groups, respectively, and from 45.4% in E-Ct to 59.3 and 60.7% in the E-W and E-C groups, respectively.

For both B and E batches, the relative number of first grade chicks was lower in the Ct groups than in either C or W. In addition, the proportion of first grade chicks relative to Nh was even higher in the B-C group than in the B-W group: 96.1% of Nh in B-Ct were first grade, 97.5% in B-W and 98.5% in B-C, $p < 0.001$ for each pairwise comparison. Conversely, the proportion of second grade chicks relative to Nh was higher in B-Ct groups than in B-W groups, the latter being higher than in B-C groups (3.9, 2.5 and 1.5% in B-Ct,

TABLE 2 | Number of incubated eggs (Ni), semi-transparent eggs eliminated at ED18, eggs kept in the incubator after the candling process (Ns), unhatched products—detailed as unhatched dead in shell embryos, hatched dead chicks and unhatched live in shell chicks; number of hatched chicks (Nh)—detailed as first and second grade chicks including wet chicks.

		B batch				E batch			
		C	W	Ct	*p*	C	W	Ct	*p*
Incubated eggs (Ni)		3200	3200	3200	–	3200	3200	3200	–
Semi-transparent eggs		297[b]	285[b]	788[a]	<0.001	1024[b]	1036[b]	1384[a]	<0.001
Viable eggs (Ns)		2903[b]	2915[b]	2412[a]	<0.001	2176[b]	2164[b]	1816[a]	<0.001
Unhatched products	Unhatched dead	104[b]	74[c]	175[a]	<0.001	158[b]	173[b]	229[a]	<0.001
	Hatched dead	36	41	44	0.663	38	52	37	0.186
	Unhatched live	74[b]	67[b]	196[a]	<0.001	39[b]	41[b]	96[a]	<0.001
Hatchlings (Nh)		2689[b]	2733[b]	1997[a]	<0.001	1941[b]	1898[b]	1454[a]	<0.001
1st grade chicks	1st grade chicks	2649[b]	2666[b]	1919[a]	<0.001	1889[b]	1830[b]	1358[a]	<0.001
	males	*1350*	*1320*	*1086*	–	*929*	*932*	*677*	–
	females	*1299*	*1346*	*833*	–	*960*	*898*	*681*	–
2nd grade chicks	2nd grade chicks	40[b]	67[a]	78[a]	0.002	52[b]	68[b]	96[a]	<0.001
	wet chicks	15[b]	21[b]	60[a]	<0.001	21[b]	18[b]	36[a]	0.024

Ct, control group; C, cool storage; and W, warm storage. B: eggs from breeder flocks at the beginning of the laying period; E: end of the laying period. p indicates the statistical difference between groups within batches, comparing numbers relative to Ni. a, b: values not associated with common letters within batches are statistically different (p < 0.05).

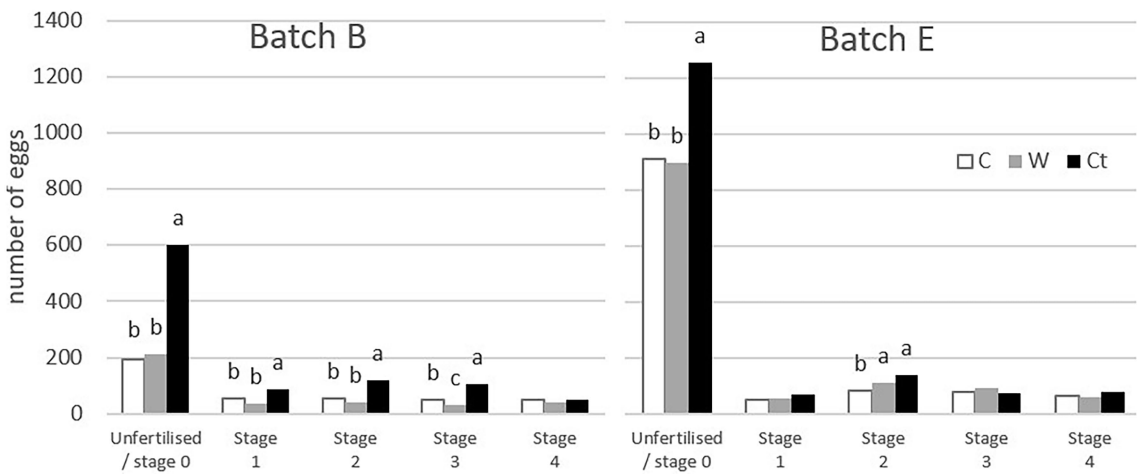

FIGURE 3 | Mortality stage of embryos from unhatched eggs (at ED21) and semi-transparent eggs (at ED18; B: eggs from breeder flock at the beginning of the laying period and E: end of the laying period). Ct: control group; C: cool storage; and W: warm storage. a, b and c: values not associated with common letters within stages are statistically different at threshold $p < 0.05$ [comparing numbers relative to the number of incubated eggs (Ni)].

B-W and B-C groups, respectively, $p < 0.001$ for each pairwise comparison).

In batch E, 93.4% of Nh in E-Ct were first grade, 96.4% in E-W and 97.3% in E-C groups (there was no significant difference between the E-W and E-C groups; E-Ct was lower than other groups, $p < 0.001$). Conversely, the proportion of second grade chicks relative to Nh was higher in the E-Ct groups than in E-C and E-W, without any significant difference between these two groups.

To sum up, 42.4% of group E-Ct eggs became first grade chicks vs. 57.2% in E-W and 59.0% in E-C. The benefits of cool or warm storage conditions were even higher in batch B where only 60.0% of eggs in group B-Ct were first grade chicks vs. 83.3% in B-W and 82.8% in B-C, respectively.

For both B and E batches, the relative number of wet chicks was higher in the Ct groups than the C or W groups. This was also the case for the number of unhatched chicks (higher in the Ct groups than in the C or W groups), which can also be considered as late-hatched chicks (live chicks but not fully hatched). These two categories taken together represented, respectively, 3.1, 3.0 and 10.6% of B-C, B-W and B-Ct eggs relative to Ns, while they represented 2.8, 2.7 and 7.3% of E-C, E-W and E-Ct Ns eggs, respectively.

First Grade Chicks

Sex Ratio

Females were fewer in the E-Ct group (43.4%, **Table 2**). This sex ratio was significantly different from that of groups E-W (50.5%, $p < 0.001$) and E-C (49.0%, $p < 0.001$). For batch E, there was no difference in the sex ratios of first grade chicks between groups (females represented 50.8, 49.1 and 50.1% of E-C, E-W and E-Ct chicks, respectively, $p = 0.562$).

Chick Weight and Vitellus Weight

Body weight and vitellus weight are shown in **Table 3**.

B-W chicks were lighter than B-Ct and B-C groups, with no difference between B-C and B-Ct chick weights. There was no difference in the vitellus/weight ratio between groups. E-W and E-C chicks were lighter than those from the E-Ct group, and both males and females had a relatively lighter vitellus.

In both B and E batches, females were heavier than males (36.7 vs. 35.8 g in batch B female and male chicks, respectively, and 46.2 vs. 45.2 g in batch E female and male chicks, respectively). In batch E chicks, females had a higher vitellus/weight ratio than males (9.8% vs. 8.4%).

Broilers During Rearing

Mortality During the Rearing Period

Mortality during rearing did not exceed 3% in any of the E batch groups. In batch B, mortality was less than 3% on the fifth day of age across all groups and increased to 4.2% by the end of the rearing period. No significant difference in mortality rates was observed among groups for both B and E broilers, or between males and females at any age.

Body Weight During the Rearing Period and Breast Muscle Weight

Table 4 shows the mean body weights during rearing and the relative breast muscle weight at the end of the rearing period for each group.

From 11 days of age onwards, males were heavier than females in all groups. No difference in body weight was observed among the groups, at any age except 4 days of age, when B-Ct chicks were heavier than both B-C and B-W chicks.

No difference in relative breast muscle weight was observed between groups ($p = 0.628$) and sexes ($p = 0.263$).

Markers of Oxidation and Antioxidant Mechanisms in Blood

Oxidative Stress and Antioxidant Markers in Plasma

Plasma TBARS results are reported in **Figure 4**.

There was no sex effect in this index, but age and group effects were observed, with no interaction between them. Indeed, the TBARS index decreased with age and was lower in the E-Ct group than in the E-C group, with the E-W group showing intermediate values.

The effect of age, group and sex on TAS was also studied (**Figure 5**). Only an effect of age was observed for this indicator of blood antioxidant defences.

Metabolite Concentrations in the Plasma

There was no significant difference between groups for the plasma concentrations of glucose, uric acid and triglycerides. However, we observed an effect of age (d31 > d7, $p = 0.011$ for TG and d31 < d7 for AU) and sex for TG (male > female; $p = 0.002$).

DISCUSSION

Whether eggs came from young or old breeder flocks, pre-incubations and cool treatment during egg storage both resulted in reduced embryo mortality, which led to a subsequent decrease in the number of unhatched eggs and unhatched chicks, consequently increasing the number of hatched chicks. Short-, medium- and long-term effects of pre-incubations and cool treatment are discussed hereinafter.

Short-Term Effects: Egg Weight Loss, Albumen pH, Embryo Development and Embryo Mortality

The detrimental effects of prolonged storage are mostly due to reduced albumen quality (Brake et al., 1997; Elibol and Brake, 2008) and the impairment of yolk membrane integrity, as the pH of the albumen increases with storage duration.

TABLE 3 | Mean (SD) of first grade chick weight (g; $n = 50$ chicks, by sex per group) and percentage of vitellus weight related to body weight ($n = 20$ chicks, by sex per group).

	B batch					
	C	**W**	**Ct**	**P Group**	**P Sex**	**P Group × Sex**
Chick weight	36.5 (2.9)[a]	35.6 (2.2)[b]	36.7 (2.5)[a]	0.0095	0.004	0.542
Vitellus %	8.0 (2.0)	7.5 (1.8)	8.3 (2.0)	0.169	0.847	0.626
	E batch					
	C	**W**	**Ct**	**P Group**	**P Sex**	**P Group × Sex**
Chick weight	45.0 (3.6)[b]	45.0 (3.6)[b]	47.0 (3.3)[a]	<0.001	0.037	0.096
Vitellus %	8.3 (3.4)[b]	8.5 (2.5)[b]	10.4 (4.0)[a]	0.011	0.019	0.656

Ct, control group; C, cool storage; and W, warm storage. B: chicks from breeder flocks at the beginning of the laying period; E: end of the laying period. p indicates the statistical difference between groups within batches; a, b and c: values not associated with common letters within batches are statistically different ($p < 0.05$).

TABLE 4 | Mean (SD) of body weight (BW, g) at 4, 11, 21, 28 and 32 days of age and relative breast muscle weight (pectoralis major PM, % in relation to body weight) at 31 (E) and 32 (B) days of age.

	B batch							
	C	**W**	**Ct**	**Male**	**Female**	**P Group**	**P Sex**	**P Group × Sex**
BW 4	72 (7)[b]	73 (6)[b]	74 (7)[a]	73 (6)	73 (7)	0.005	0.675	0.161
BW 11	281 (23)	281 (26)	280 (24)	285 (26)	276 (22)	0.801	<0.001	0.300
BW 21	884 (77)	891 (74)	897 (72)	929 (68)	852 (60)	0.251	<0.001	0.835
BW 28	1494 (161)	1513 (147)	1519 (159)	1618 (118)	1396 (95)	0.272	<0.001	0.358
BW 32	1925 (239)	1918 (183)	1923 (220)	2078 (153)	1765 (129)	0.940	<0.001	0.254
PM	18.4 (1.3)	18.6 (1.1)	18.0 (1.2)	18.4 (1.2)	18.4 (1.2)	0.287	0.946	0.636
	E batch							
	C	**W**	**Ct**	**Male**	**Female**	**P Group**	**P Sex**	**P Group × Sex**
BW 4	89 (7)	89 (7)	90 (8)	89 (7)	89 (7)	0.311	0.152	0.563
BW 11	315 (24)	316 (24)	315 (23)	322 (24)	310 (22)	0.983	<0.001	0.583
BW 21	962 (85)	956 (90)	965 (80)	1,022 (73)	915 (62)	0.244	<0.001	0.251
BW 28	1632 (182)	1610 (166)	1591 (166)	1759 (139)	1507 (106)	0.130	<0.001	0.244
BW 32	2037 (235)	1994 (247)	1987 (233)	2,198 (196)	1856 (140)	0.749	<0.001	0.951
PM	18.0 (1.2)	17.6 (1.2)	17.9 (1.1)	17.6 (1.4)	18.0 (1.0)	0.628	0.263	0.788

Ct: control group; C: cool storage; and W: warm storage. B: chickens from breeder flocks at the beginning of the laying period; E: end of the laying period. n, by sex per group = 100 animals (body weight), eight animals (PM). p indicates the statistical difference between groups within batches; a and b: values not associated with common letters within batches are statistically different ($p < 0.05$).

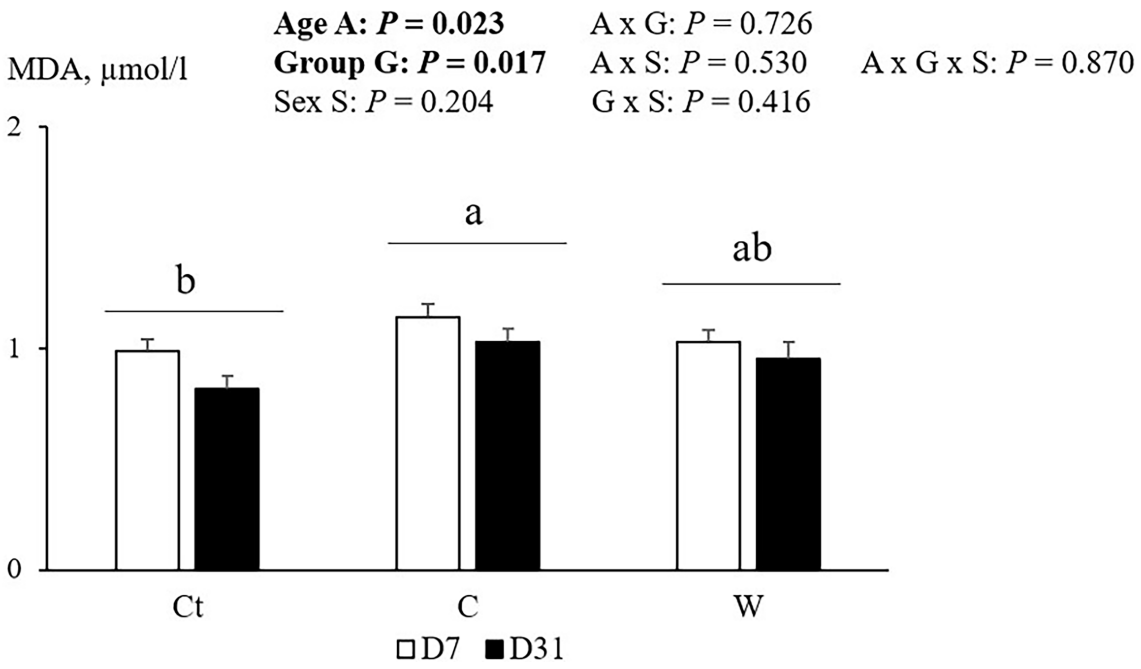

FIGURE 4 | TBARS plasma index [expressed as Malondialdehyde concentration (MDA)] for E chickens (from eggs of breeder flock at the end of the laying period) at 7 and 31 days, depending on egg storage conditions and age of broilers ($n = 8$ animals, by sex per group). Ct: control group; C: cool storage; and W: warm storage. a, b: values not associated with common letters are statistically different ($p < 0.05$).

Embryo viability depends on the total number of viable cells, the pH of the embryo's micro environment and the embryo's development stage (Reijrink et al., 2008).

In our study, storage at an average temperature of 11.6°C (vs. 18.3°C) appears to have limited the weight loss and increase in pH of eggs in group C compared to Ct. These results are in accordance with Hamidu et al. (2018), who showed that a lower storage temperature (18°C vs. 25°C) decreased egg weight loss, and Özlü et al. (2021), who observed a lower albumen pH with storage temperatures of 12 or 15°C rather than 18°C. Williams (1992) proposed that storage temperature should be maintained below 10°C in order to reduce the decline in

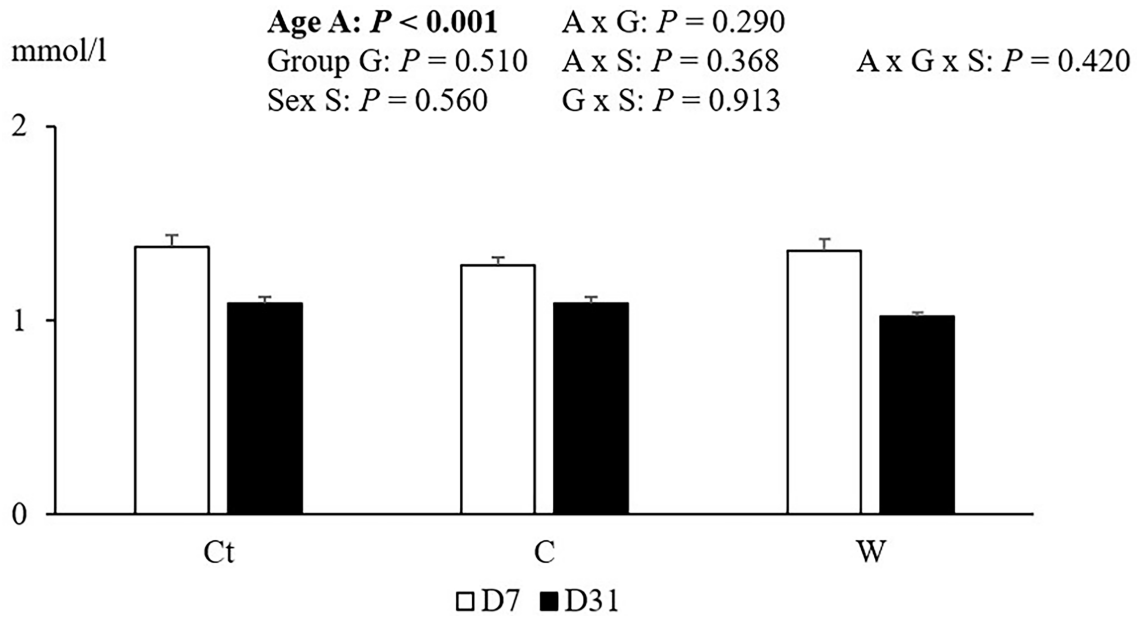

FIGURE 5 | Total antioxidant power (TAS) of plasma for E chickens (from eggs of breeder flock at the end of the laying period) at 7 and 31 days, depending on egg storage conditions and age of broilers ($n = 8$ animals, by sex per group). Ct: control group; C: cool storage; and W: warm storage.

albumen viscosity, but our study shows that an average of 11.6°C may be sufficient.

As expected, the pre-incubations conducted in our study on W eggs on days 6 and 10 after oviposition accelerated embryonic development from about stage EGX to XII, without however exceeding the critical stage of EGXIII (Reijrink et al., 2008). In contrast, embryo staging remained stable in other groups of eggs. A similar effect of pre-incubation on embryonic development was also reported in previous studies (Fasenko et al., 2001; Reijrink et al., 2009, 2010; Bakst et al., 2016; Özlü et al., 2021).

The number of eggs at stage 0 was high in Ct eggs: respectively, around 18.8 and 39.3% of eggs initially incubated in batches B and E. This result is in line with previous studies (reviewed in Bergoug et al., 2013a) showing that breeder flocks' age negatively impacts egg fertilisation, but also the embryo's ability to withstand prolonged storage periods in our study, pre-incubations and cool treatment decreased by more than 10% the proportion of eggs at stage 0 (in both B and E batches). This observation suggests that 10% of eggs discarded in Ct groups with the candling method could actually be considered as fertile eggs but with an embryo that died before 4 days of incubation. Thus, pre-incubations and cool treatment during storage preserved embryos from early mortality. Our results support previous studies (Pokhrel et al., 2018) suggesting that the first 3 days of incubation are critical for embryonic survival.

In Özlü et al. (2021), late embryo mortality decreased following pre-incubation treatments. In our study, middle and late embryo mortalities were generally much lower in all groups but were still impacted by pre-incubations and cool treatment in B eggs (until the 17th day of incubation). For E eggs, embryo mortality was probably less impacted at later stages because of the higher number of early mortalities (the weakest embryos were already dead at stage 0).

Regardless of the age of breeder flocks, there were no effects of pre-incubations and cool treatments on embryo mortality after 18 days of incubation, as there was no significant effect on embryo mortality at stage 4, nor on chick mortality at hatching.

Medium-Term Effects: Hatching Results, Proportion of First Grade Chicks, Late Hatching, Hatching Body Weight, Vitellus Weight and Sex Ratio

The beneficial effects of pre-incubations and cool treatment on embryo mortality helped improve hatching results, increasing the hatching rate by more than 21% for B eggs and more than 13% for E eggs.

The better hatchability observed in our study with cool treatment during egg storage—whether the eggs were from young or old breeder flocks—is in accordance with Pokhrel et al. (2018), who compared the effects of a temperature of 12°C vs. 18°C on eggs stored from 7 to 28 days (these eggs came from 32- to 63-week-old breeder flocks). They found lower rates of early apoptosis with a lower temperature and persistence of the embryonic cytoarchitectural properties.

Damaziak et al. (2018) also found better hatchability rates with pre-incubations, but more significantly in older groups, which is in contrast to other studies (Gucbilmez et al., 2013; Ebeid et al., 2017; Özlü et al., 2021) where the increase in hatchability of fertile eggs was higher in eggs from young flocks than in eggs from older flocks. Similarly, the positive

effect of pre-incubations on hatchability was more pronounced in our experiment for batch B (from 62% in Ct to 84% in W) than for batch E eggs (from 45% in Ct to 61% in W). This is probably due to the higher number of infertile eggs in older flocks that could not be changed whatever the storage conditions.

In Reijrink et al. (2009), pre-incubations had no effect on hatchability when the storage time was shorter than 7 days and were found to be both detrimental and beneficial when the storage period was prolonged. The reasons for this discrepancy were not clear. For instance, in Reijrink et al. (2010), the pre-incubation of eggs stored for 15 days had only a small effect on early embryonic mortality, and thus no effect on hatchability. In Fasenko et al. (2001), it improved hatchability from 72 to 82%. In both studies, the eggs were from breeder flocks whose hatchability is generally optimal [36 weeks old in Reijrink et al. (2010) and 32 in Fasenko et al. (2001)]. They both applied a single pre-incubation session at the beginning of storage. In our study, the better hatchability observed after several pre-incubations totalling 12 h during egg storage is in accordance with other studies such as Bakst et al. (2016) or Dymond et al. (2013), who found that pre-incubations restored hatchability to 84% by lowering both early and late embryo mortality. The total length of time of pre-incubation, whether applied at the beginning of the storage period or divided into various sessions throughout storage, thus appears to be of major importance.

Dymond et al. (2013) suggested that periodic pre-incubations on the blastoderm might reset the storage time perceived by the blastoderm and/or possibly provide an opportunity for blastodermal cells to perform basic cell functions and functions otherwise suppressed during egg storage. Regardless of the mechanisms involved, the beneficial effects of pre-incubations on hatchability appear to be due to the advancement of the developmental stage of the blastoderm. Fasenko et al. (2001) hypothesised that embryos between developmental stages EGXII and EGXIII are probably the most resistant to prolonged egg storage because they have already formed the hypoblast and are in a quiescent developmental stage.

Independently of the number of hatched chicks, the quality of hatched chicks—in terms of first/second grade ratio was improved in our study for both young and older breeder flocks after pre-incubations and cool treatment during egg storage. Within hatched chicks (Nh), cool treatment improved the proportion of first grade chicks even more than warm treatment, though significantly this only applies to batch B. However, although significant, the difference between the C and W groups was minor.

In several studies, chick quality was not affected by storage conditions. This was shown either in terms of the Pasgar score, body weight, chick length and yolk sac weight [in Pokhrel et al. (2018) comparing 12°C vs. 18°C storage conditions], or in terms of first/second grade ratio [in Özlü et al. (2021) comparing 12, 15 and 18°C storage temperatures, and in Damaziak et al. (2018) looking at pre-incubation effects], or in terms of chick lengths and yolk-free body mass [in Reijrink et al. (2009) applying pre-incubations]. However, all these studies found low percentages of second grade chicks (less than 1.5%), and chicks were of high quality in all storage conditions. This could explain the absence of an effect. Ebeid et al. (2017) found similar results to ours in that a pre-incubation period of 6 or 8 h improved the first grade proportion. This was not the case, however, for shorter pre-incubation times. This may partially explain the absence of an impact in other studies. Our results lead us to hypothesise that embryos resistant to long storage due to W or C treatments were of a higher quality and led to improved chick quality. They also suggest that as long as the embryo successfully recovers from diapause, the rest of its development continues normally. These findings are in agreement with Pokhrel et al. (2018), and with the fact that embryo mortality essentially occurs before the third day of incubation in Ct storage conditions.

While chicks are kept in the hatcher without water and feed, their body weight rapidly decreases due to dehydration and yolk use (Sklan et al., 2000; Bergoug et al., 2013b). Control (Ct) day-old chicks from batch B were heavier than W chicks and those from batch E were heavier than both W and C chicks. The proportional vitellus was also heavier in Ct chicks from batch E. This may be explained by the fact that Ct chicks hatched later: there were more wet chicks and unhatched chicks in Ct groups than in W or C groups in both B and E batches. In other words, pre-incubations (in batches B and E) and cool treatment (in batch E) foster precocious hatching. Many authors associate delayed hatching with egg storage. This delay could be explained by a delay in the initiation of embryogenesis after the start of incubation and/or by a decrease in the rate of embryonic development during incubation [reviewed by Nasri et al. (2020)]. The 8 h delay in the start of incubation of W eggs was efficient in our study, it being known that pre-incubation shortens the incubation period (Dymond et al., 2013). It also shows that the longer pre-warming (12 h) of C eggs to avoid condensation before the start of incubation was also efficient in shortening the incubation period or synchronising the hatching time (shorter hatching window). The later-hatched chicks in the Ct groups can be explained by a longer hatching window. On the contrary, W or C treatments made it possible to better synchronise hatching. Finally, W and C chicks had more time to resorb their vitellus into the abdominal cavity, thus making better use of energy resources for improved defences, increased robustness and limited pathogen entrance.

Long-Term Effects: Mortality During Rearing, Body Weight, Relative Breast Muscle Weight and Oxidative Balance

In batch B, Ct chicks were still heavier than the C and W groups at 4 days of age, but there was no difference among groups from batch E. However, although significant, the batch B differences were small (2 g in body weight). After 4 days of age, pre-incubations and cool treatment during egg storage had no further visible consequences on post-hatching performance, i.e. mortality during rearing, body weight and relative breast muscle weight at the end of the rearing period

(no interaction with sex). This is in accordance with Damaziak et al. (2018), who did not see any effect of pre-incubations on body weight until 42 days of life, but not with Dymond et al. (2013), who found a higher body weight following pre-incubations over the first 4 weeks post-hatching. However, in the latter study, mortality during the first week was not affected by pre-incubations.

The investigation of the oxidative balance conducted in poultry from batch E between 7 and 31 days of age was aimed to explore the metabolic effects of thermal treatments and on the redox balance during long-term effects. No effect of pre-incubations or cool treatment was shown on the plasma TAS. Although prestorage incubation has previously been shown to improve the antioxidative properties of chicks (Bakst et al., 2016; Ebeid et al., 2017), the present study did not reveal such an effect. Furthermore, the TBARS index—a potential marker of oxidative stress—was higher in C animals regardless of age. This raises the question of whether egg storage conditions perform a 'selection'. Indeed, our results could indicate that by limiting the number of dead embryos, the cool treatment keeps more embryos alive, which overall would have a slightly less favourable redox balance than Ct animals. However, in our study, this trait was independent of the quality of hatched chicks, which was better for chicks from the C group than from the Ct group. To determinate a potential short-term effect of thermal treatments, the investigation of the oxidative balance needs further research, earlier in the embryo development or on hatched chicks.

Finally, our study revealed a decrease in the plasma TBARS index with age. This finding is consistent with the results previously obtained by Lin et al. (2004) between 0 and 14 days of age. Our results show that the plasma TAS was similarly affected by age. A decrease in plasma antioxidant defences was also reported between 0 and 21 days of age by Balogh et al. (2001).

Sex Differences

The effect of thermal treatment during incubation on sex ratio was shown in other studies such as in Tzschentke and Halle (2009). In our study, the sex ratio of first grade chicks in the E batch was equally balanced and was not impacted by pre-incubations or cool treatment during egg storage. However, in the B batch, it appears that females were more sensitive to long storage because they represented only 43% of Ct first grade chicks, whereas the sex ratio at hatching was equally balanced when pre-incubations or cool treatment were applied. It is known that females hatch earlier than males (Van de Ven et al., 2011). The hatching window being longer in Ct—and leading to an interruption of the complete hatching process as hypothesised before—we should find more females than males in Ct, but the results for batch B showed the contrary. Mather and Laughlin (1976) reported that the difference in hatching time of both sexes disappears if the storage period is increased to 14 days. Wu et al. (2012) found that more female embryos died during incubation, especially in the early and middle stages. It is thus possible that embryo resistance and precocity in development are sex-dependent and should require further investigation. Indeed, the alteration in sex ratio induced by thermal stimulation could have a major impact on the commercial poultry industry, especially for layer hen producers, for whom male chicks have no commercial value and are culled upon hatching.

Whatever the group (C, W or Ct) or the batch (B or E), sex-related differences in chick/bird characteristics were observed. In both batches, day-old females were heavier than males with proportional vitellus also heavier in females (though this difference was significant only in E chicks). These differences were reversed from 11 days of age onwards. During rearing, males became heavier than females, as is commonly reported in various studies. Sexual dimorphism in breast yield is well documented in modern broilers (Zuidhof et al., 2014), but the number of birds used for breast muscle sampling was probably too low to reveal a statistical difference in breast muscle weight relative to body weight according to the sex.

CONCLUSION

Whether eggs came from young or old breeder flocks, pre-incubations and cool treatment during egg storage both resulted in beneficial short- and medium-term effects on hatching results compared with conventional storage conditions. However, we did not find any major long-term effects during rearing of pre-incubations or cool treatment.

Changing storage conditions to obtain better hatchability rates is only beneficial to the industry and poultry if chick quality remains high. The benefits of pre-incubations or cool treatment on the hatching rates were very high in the present study compared with the number of incubated eggs, an outcome that is economically and practically relevant for commercial hatcheries.

In light of these results, and taking into account other practical constraints in hatcheries, pre-incubation or cool storage is interesting options to reduce the negative effects of long-term storage. Pre-incubation accelerates the developmental stage of the embryo, whereas cool storage limits weight loss and pH increase, thus contributing to a higher egg quality (albumen and vitelline membrane). These strategies have no adverse effects on chick robustness or on the health and performance of poultry throughout the rearing phase, at least in the specific case of suboptimal breeder flock ages explored in this study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Comité National de Réflexion Ethique sur l'Expérimentation Animale (National committee for ethics in animal testing) No. 016 at the French Ministry for Education, Higher Education and

Research before data collection (number APAFIS#16128-2018071314183633 v1).

AUTHOR CONTRIBUTIONS

MG and JP designed the research studies. MG, JP, and RT carried out the experimental studies in collaboration with AK. AC designed and carried out the part on antioxidant mechanisms. PC, EC-A, and EC conducted the biochemical analyses. MG and AC analysed the data and performed the statistical analysis. MG, AC, and SR-G wrote the paper. All the authors read and approved the final manuscript.

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Effects of Induced Moisture Loss in Chicken Embryos at Embryonic Day 18 and Post-hatch Immune Response During *Salmonella enteritidis* Lipopolysaccharide Challenge in Broilers

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Two experiments were conducted to investigate the effects of induced moisture loss on embryonic development and the immune response following an inflammatory challenge immediately post-hatch. In Experiment I, fertile leghorn eggs (n = 100) and commercial broiler eggs (n = 300) were set at 37.5°C and moisture loss was induced in one-half of the Leghorn and broiler eggs by drilling two, 1.5 mm diameter holes. The Control eggs had 0 holes. At embryonic day (ED)18, layer and broiler eggs in the 2-holes treatment had a significant ($P < 0.01$) increase in moisture loss compared to the control treatment (10.1% vs. 8.2%). Similarly, at ED18, the broiler eggs with 2-holes had a significant increase ($P < 0.01$) in moisture loss compared with control eggs (9.9% vs. 8.4%). Thymocytes from both the leghorn (104%) and broiler (62%) embryos in the 2-holes treatment had significantly increased *in vitro* proliferation compared with the control embryos ($P \leq 0.05$). At ED18, layer and broiler embryos in the 2-holes treatment had an approximate twofold increase in the splenic $CD8^+/CD4^+$ ratio ($P \leq 0.05$) and $CD4^+CD25^+$ cells percentage in both the thymus and spleen ($P \leq 0.05$). At ED18, both layer and broiler embryos from the 2-holes treatment had a significant increase in splenic IL1-β, IL-6, IL-10, and TLR-4 mRNA transcription compared to the control group ($P \leq 0.05$). Experiment II was repeated with 300 fertile broiler eggs. On the day of hatch, chicks were randomly distributed into one of four treatments in a 2 (0, 2 holes) × 2 (0, 500 μg lipopolysaccharide, LPS) factorial arrangement of treatments. Chicks in the LPS groups were injected intraperitoneally with 500 μg/kg BW LPS. At 24 and 48 h post-hatch, chicks hatched from eggs with 2-holes and challenged with LPS had a significant increase ($P \leq 0.05$) in thymocyte proliferation at 24 h (42%) and 48 h (37%) when compared with chicks hatched from the control (0-hole; 0 μg LPS) treatment. Chicks hatched from the 2-holes treatment and challenged with the LPS had an approximately twofold higher splenic $CD8^+/CD4^+$ ratio and 1.5 fold increase in $CD4^+CD25^+$ percentage compared to control chicks ($P \leq 0.05$). In chicks hatched from

the 2-holes treatment, MUC2 mRNA transcription was comparable to control chicks at 24 and 48 h in response to the LPS challenge. Our data suggest that the 2-holes treatment reprograms gene transcription to facilitate cell survival via proliferation and differentiation during an LPS inflammatory challenge.

Keywords: chicken embryos, induced moisture loss, inflammatory challenge, $CD8^+/CD4^+$ ratio, Tregs percentage, cytokine response

INTRODUCTION

For successful hatching, developing avian embryos require an ideal temperature, turning, humidity, and ventilation (Romanoff, 1929). Avian eggs lose water during incubation and monitoring moisture loss during incubation is critical to achieving optimal hatchability and chick quality (Hoyt, 1979). Moisture loss via diffusion through eggshell pores occurs steadily during incubation and is greater than the metabolic water produced by embryonic oxidation of yolk lipids (Ar and Rahn, 1980). Incubator humidity is one of the important factors in controlling water loss during incubation (Landauer, 1967). The moisture loss percentage can range from 10 to 13% in multi-stage incubators and 9.5–12.5% of initial egg mass in single-stage incubators (Green, 2017). Experimentally, induced moisture loss (>20%) achieved by drilling holes in the eggshell over the airspace on an embryonic day (ED)1 caused osmotic stress due to early depletion of allantoic fluid (Davis, 1987).

Changes in the osmotic state can negatively influence an organism's function and the capacity of avian embryos to regulate hydration is critical for optimal hatchability (Moeller et al., 2013). Natural variation in the number of pores in an eggshell between individual eggs within a single population contributes to the variability in moisture loss during incubation and this variability can subsequently influence osmoregulation by the embryo (Tullett and Deeming, 1982). Molenaar et al. (2010) reported that chicks hatched from water-stressed embryos were smaller and lost more than 20% of their initial mass when compared with control chicks. Layer breeders were reported to have stronger eggshells than broiler breeders (Johansson et al., 1996), which may have contributed to the increased mortality observed in layers embryos compared to broilers at the end of incubation (Everaert et al., 2008). This difference between broilers and layer strains in eggshell thickness may affect both external pipping and also negatively influence pulmonary respiration by decreasing the relative weights of the lung (Molenaar et al., 2010).

Acellular structures like the egg yolk, vitelline membrane, egg white, and eggshell not only provide nutrients but also protect the chicken embryo against physical shock and microbial infection during development (Rehault-Godbert et al., 2011). Immune function is an important physiological process that can be compromised by any reduction in metabolic resources (Moeller et al., 2013). The production, maintenance, regulation, and physiological importance of the osmotic environment in and around the avian embryo have been well described (Romanoff, 1929; Romanoff and Romanoff, 1949; Adolph, 1967). However, there is not much information available to describe the role of the osmotic environment on immune cells during the latter part of incubation. The thymus and spleen have significantly increased osmolality than serum, suggesting that lymphocytes are exposed to a certain degree of physiologic osmotic stress (Go et al., 2004). Since lymphocytes have higher osmolality, it is not clear if immune system development would be affected by an increase in water loss during incubation. Several studies have investigated the effects of moisture loss on hatchability and embryonic development, but these studies, in general, did not differentiate between the effects of moisture loss or temperature on embryonic metabolism. Hence, we hypothesized that moisture loss during embryogenesis would negatively influence immune function. The objective of this study was to investigate the effects of induced moisture loss without changes in incubator temperature on embryonic development and the immune response following an inflammatory challenge immediately post-hatch.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at The Ohio State University approved all animal protocols (IACUC # 20140090).

Experiment I

This experiment studied the effect of induced moisture loss in broiler and layer strain eggs on embryos weight, dry weight, and proliferation efficiency of T-lymphocytes, $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ T-regulatory cells at ED18. Fertile leghorn eggs (n = 100) and commercial broiler eggs (n = 300; Orrville Chick hatchery, Orrville, OH, United States) were stored at 10°C and 65% relative humidity (RH) until use. All eggs were warmed to room temperature (20°C) for 12 h immediately prior to incubation. The layer and broiler eggs were stratified by weight to uniformly distribute egg weights across all the treatments prior to incubation. The eggs were set at 37.5°C and 65% RH in a single-stage incubator (Natureform, Jacksonville, FL, United States) at the OARDC Poultry Research Farm. Incubation temperature was regulated by thermistors connected to microprocessors with a temperature sensitivity of ±0.05°C. Humidity was controlled using humidity sensors. Infrared thermometers were placed in each incubator tray to monitor incubation temperature. Humidity levels and incubation temperature were logged daily.

Induced Water Loss

At ED8, all eggs were candled, and infertile eggs were removed. Fertile eggs were evenly distributed among six egg trays. Increased moisture loss in one-half of the Leghorn and broiler eggs was induced by drilling two, 1.5 mm diameter holes

approximately 1 cm apart into the airspace with a 16G needle (BD Biosciences, Franklin Lakes, NJ, United States). The egg surface was cleaned with 70% ethanol before the holes were drilled. The Control eggs had 0 holes. Trays with experimental eggs were randomly distributed within the setter and set at 37.5°C with 65% RH for the remainder of the incubation period.

Moisture Loss

On ED18, moisture loss was calculated by individually weighing leghorn and broiler eggs. Embryos were sacrificed at ED18 and dry mass was obtained by drying the yolk-free embryos (n = 25) in an oven at 60°C until there were no further decreases in weight. Spleen, and cecal tonsils from three embryos were pooled into one replicate sample (n = 6) in RNAlater® (Sigma-Aldrich, St. Louis, MO, United States) and stored at −70°C until further analysis. The trial was terminated at ED18.

$CD4^+$, $CD8^+$, and $CD4^+CD25^+$ T Regulatory Cell Percentage in the Spleen and Thymus

At ED18, the effect of moisture loss on $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ T-regulatory cells was determined by flow cytometry as described previously (Shanmugasundaram et al., 2015). The splenocytes and thymocytes were collected from 18 embryos per treatment group, with three individual samples pooled into one (n = 6). Single-cell suspensions from the spleen and thymus pools were enriched for lymphocytes by density centrifugation over Histopaque (1.077 g/ml, Sigma-Aldrich, St. Louis, MO, United States) for 15 min at 400 g. The cells were incubated with a 1:250 dilution of fluorescent-isothiocyanate conjugated mouse anti-chicken $CD4^+$ (Southern Biotech, Birmingham, AL, United States); 1:450 dilution of phycoerythrin-conjugated mouse anti-chicken $CD8^+$ (Southern Biotech, Birmingham, AL, United States), and 1:200 dilution of unlabeled mouse IgG for 15 min. The unbound antibodies were removed by centrifugation, and the percentages of $CD4^+$ and $CD8^+$ cells were analyzed using a flow cytometer (Guava EasyCyte, Millipore, Taunton, MA, United sates). For the T-regulatory cell percentages, cells (1×10^6) were incubated with 10 μg/ml of primary fluorescent linked mouse anti-chicken $CD25^+$, 1:250 dilution of fluorescent-isothiocyanate conjugated mouse anti-chicken $CD4^+$ (Southern Biotechnology Associates, Birmingham, AL, United States), and 1:200 dilution of unlabeled mouse IgG for 45 min. The unbound primary antibodies were removed by centrifugation. The percentage of $CD4^+CD25^+$ cells in the different organs was analyzed in a flow cytometer (Guava EasyCyte, Millipore, Taunton, MA, United sates) and expressed as a percentage of total $CD4^+$ cells.

Thymocyte Proliferation Efficiency

At ED18, the effect of moisture loss on the proliferation efficiency of thymocytes was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (Shanmugasundaram et al., 2019). Thymocytes were collected from 18 embryos per treatment group, with three samples pooled into one (n = 6). Thymocytes were collected by density centrifugation using Histopaque (1.077 g/ml, Sigma-Aldrich, St. Luis, MO, United States). Live cells were counted by trypan blue exclusion. Thymocytes (1×10^4 cells) were cultured in 200 μl of RPMI- 1640 media supplemented with 10% chicken serum and 1% penicillin, streptomycin. Cells were stimulated with 200 ng/ml phorbol 12-myristate 13-acetate (PMI), plus 50 ng/ml ionomycin (IM), in 96-well round-bottom plates for 72 h in a 5% CO_2 incubator at 37°C. Each sample was replicated three times. At 72 h of cell culture, 20 μl of 5 mg/ml MTT (Sigma Aldrich, St. Louis, MO, United States) solution was added to the cell culture and incubated for an additional 4 h. The supernatant was removed after centrifugation. The cells were suspended in 200 μl of isopropanol plus 10% dimethyl sulfoxide and 0.04N HCl for 1 h at room temperature. The concentration of MTT formazan formed in the 96-well plates was measured on an Epoch microplate spectrophotometer at 570 nm (BIOTek, VT, United States) and reported as the mean optical density. The thymocytes proliferation percentage was calculated as follows:

$$\text{Thymocyte proliferation percentage} = \frac{\text{Mean OD value of 2-hole treatment}}{\text{mean OD value of 0-hole treatment}} \times 100$$

Experiment II

This experiment studied the effect of induced moisture loss on the post-hatch immune response following a lipopolysaccharide (LPS) inflammatory challenge. The determined metrics included; body weight, splenic $CD8^+$: $CD4^+$ cell ratios, lymphocytes proliferation efficiency, the pro-inflammatory cytokine Interleukin (IL)1-β, IL-6, anti-inflammatory cytokine IL -10, and toll-like receptor-4 (TLR-4). The same incubation protocol described for Experiment I was subsequently repeated with 300 fertile broiler eggs (Orrville Hatchery, Orrville, OH, United States). On ED18, moisture loss was calculated as described in Experiment I and the eggs from each treatment were randomly distributed to six hatch baskets per treatment (n = 6), transferred to a hatcher, and incubated at 37.0°C and 70% relative humidity. Non-hatched eggs were opened at ED21, and the number of infertile eggs or dead embryos was determined. At the time of the hatch, chicks from each hatch basket were weighed individually.

Lipopolysaccharide Injection

On the day of hatch, chicks were randomly distributed among one of four treatments in a 2 (0 and 2-holes) × 2 (0 and 500 μg LPS) factorial setup. On d1 post-hatch, chicks in the LPS groups were injected intraperitoneally with 500 μg/kg BW LPS from *Salmonella enteritidis* (Sigma Aldrich, St. Louis, MO, United States) in 250 μl of PBS. Each treatment was replicated in six battery cages (n = 6) with ten chicks per replication. The chicks were housed in environmentally controlled rooms and had *ad libitum* access to water and a starter diet.

$CD4^+$, $CD8^+$, and Tregs Percentage in Thymus and Spleen

At 24 and 48 h post-LPS challenge, the effect of moisture loss on $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ T-regulatory cells

TABLE 1 | Primer sequences for genes under study.

Gene	Primer Sequence[1] (5′- 3′)	Annealing Temp (°C)	Product size (bp)	GenBank accession no.
IL10	F-CATGCTGCTGGGCCTGAA	57.5	94	AJ621254.1
	R-CGTCTCCTTGATCTGCTTGATG			
IL1-β	F-TCCTCCAGCCAGAAAGTGA	57.5	225	NM_204524.2
	R-CAGGCGGTAGAAGATGAAGC			
IL-6	F-ACAGCACAAAGCACCTGGCG	55	100	NM_204628.2
	R-TTGGCGAGGAGGGATTTCTGGG			
MUC-2	F-ACCAAGCAGAAAAGCTGGAA	61	257	XM_040673077.1
	R-CCTCCAGCCACCCAGTATAA			
TLR-4	F-ACCTACCCATCGGACACTTG	60	109	KP410249.1
	R-TGCCTGAGAGAGGTCAGGTT			
β-actin	F-ACCGGACTGTTACCAACACC	56	154	NM_205518.1
	R-GACTGCTGCTGACACCTTCA			
RPS13	F-CAAGAAGGCTGTTGCTGTTCG	55	169	NM_001001783.1
	R-GGCAGAAGCTGTCGATGATT			

[1]*F, forward; R, reverse.*

were determined by flow cytometry as described above in Experiment I.

Splenic and Cecal Tonsils IL1-β, IL-6, IL-10, TLR-4 and Jejunal Muc-2 Cytokine mRNA Transcription

At 24 and 48 h post-LPS injection, thymus, spleen, ∼1 cm jejunal tissue sampled proximal to Meckel's diverticulum, and cecal tonsils were collected from each treatment group in RNAlater® (Sigma-Aldrich, St. Louis, MO, United States). Samples from three chicks were pooled into one replicate sample and stored at –70°C until further analysis.

Total RNA was extracted from all experimental groups using the TRI reagent (Molecular Research Center, Cincinnati, OH, United States) following the manufacturer's instructions. RNA concentration and purity were determined by NanoDrop (Thermo Fisher Scientific, Waltham, MA, United States) using the 260/280 and 260/230 ratios. Two μg RNA was reverse transcribed into cDNA and analyzed for IL-1β, IL-6, IL-10, and TLR-4 expression by real-time PCR (iCycler, Bio-Rad, Hercules, CA, United States) using SYBR Green. Each well contained 10 μl SYBR Green PCR master mix, 7 μl RNAse-free water using IQ5 Cycler (iCycler, Bio-Rad, Hercules, CA, United States), 2 μl (∼600 ng/μl) cDNA, 0.5 μl forward primer (5 μM), and 0.5 μl reverse primer (5 μM). To perform real-time PCR, the following machine settings were used for all genes: an initial denaturation of 95°C for 10 min (1 cycle), followed by 95°C for 15 s, and 60°C for 45 s (40 cycles). The melting profile was determined by heating samples at 65°C for 30 s and then increasing the temperature at a linear rate of 10°C/s to 95°C while continuously monitoring fluorescence. The spleen mRNA analyzed for IL-1β, IL-6, IL-10, and TLR-4 were normalized with β-actin (primer sequences and annealing temperature found on **Table 1** (Shanmugasundaram and Selvaraj, 2011). The cecal tonsils mRNA analyzed for IL-1β, IL-6, IL-10, and TLR-4 were normalized with Ribosomal protein S13 (RPS13). The jejunal mRNA analyzed for Muc-2 was normalized with RPS13. The $2^{-\Delta\Delta Ct}$ method previously described (Livak and Schmittgen, 2001) using the Ct as the threshold cycle to calculate mRNA expression and the fold change calculated as $2^{(\text{Ct Sample} - \text{housekeeping})}/2^{(\text{Ct Reference} - \text{housekeeping})}$. The reference group was the non-challenged 0-hole control group.

Statistical Analysis

In Experiment I, data collected from multiple time points from the same eggs were analyzed using a paired student's *t*-test to determine the effects of 0- or 2- hole on moisture loss and a student's *t*-test to determine the effects of 0- or 2- hole on immune parameters. In Experiment II, a two-way ANOVA was performed using the fit model procedure (JMP, SAS Institute, Cary, NC, United States) to determine the effects of holes and LPS injection on measured parameters. Values reported are least-squares means ± SEM. Significance was established at $P \leq 0.05$ and Tukey's test was used to separate the means.

RESULTS

Experiment I

Moisture Loss at ED18

At ED18, the layer and broiler eggs in the 2-holes treatment had a significant ($P < 0.01$) increase in moisture loss compared to the control treatments, respectively (**Table 2**). At ED18, there were no significant treatment differences in yolk-free embryo dry weight in either the layer or broiler embryos (**Table 2**).

Thymocytes Proliferation

In Experiments I, inducing moisture loss had a significant effect on *in vitro* thymocyte proliferation. Thymocytes from both the layer (104%) and broiler (62%) embryos in the 2-holes treatment had significantly increased *in vitro* proliferation compared with the control embryos ($P \leq 0.05$; **Figure 1**).

TABLE 2 | Effects of 2-holes drilled on the eggshell into the airspace at 8 days of incubation on moisture loss.

Treatment	Egg weight (g)			Moisture Loss (%) Dry mass (g)			
	D0	D8	D18	D 0–8	D 8–18	D 0–18	D 18
Layers							
0-hole	61.7	59.1	56.7	4.2^{b}	4.0^{b}	8.2^{b}	4.4
2-holes	61.6	58.4	55.4	5.01^{a}	5.01^{a}	10.1^{a}	3.9
P-value	0.91	0.65	0.4	0.01	0.01	0.01	0.22
SEM	0.6	0.6	0.6	0.1	0.1	0.1	0.3
Broilers							
0-hole	63	60.3	57.8	4.3	4.1^{b}	8.4^{b}	5.3
2-holes	63.3	60.5	57.2	4.4	5.05^{a}	9.9^{a}	4.8
P-value	0.58	0.67	0.27	0.43	0.001	0.001	0.79
SEM	0.2	0.3	0.4	0.1	0.1	0.1	0.1

Fertile layer eggs (n = 100) and broiler eggs (n = 300) were incubated at standard (37.5°C) from embryonic day (ED) 1–18. At ED8, half the eggs were incubated at 37.5°C, 2-holes were drilled on the eggshell into the airspace, and remained eggs served as control groups. The eggs were continued to be incubated at 37.5°C until ED18. Egg weights were measured before set in the incubator, D8, and D18. Moisture loss was calculated as a percentage from D8, D8–18, and D0–18. Dry mass of yolk-free embryos weight was calculated by drying the embryos in an oven at 60°C until there were no further decreases in weight. Means with no common superscripts differ significantly (P ≤ 0.05). n = 6.

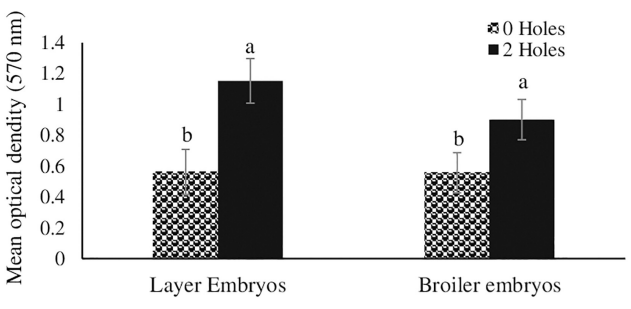

FIGURE 1 | Thymocyte proliferation efficiency in layer and broiler embryos at ED18 (Experiment I). Fertile layer and broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0-hole or 2-holes in the eggshell above the air cell and continued to incubate until ED18. At ED18, 1×10^5 thymocytes were stimulated *in vitro* with 200 ng/ml phorbol 12-myristate 13-acetate (PMI), plus 50 ng/ml ionomycin (IM). At 72 h of culture, thymocyte proliferation was analyzed by MTT assay. Means with no common superscripts differ significantly ($P \leq 0.05$; $n = 6$).

$CD8^+/CD4^+$ Ratio and $CD4^+CD25^+$ Cell Percentages in Thymus and Spleen at ED18

At ED18, layer and broiler embryos in the 2-holes treatment had an approximate twofold increase in the splenic $CD8^+/CD4^+$ ratio compared to the control treatment ($P \leq 0.05$; **Table 3**). The layer embryos from the 2-holes treatment had a significant increase in $CD4^+CD25^+$ cell percentages in both the thymus and spleen whereas the broiler embryos from 2-holes treatment only had a significant increase in $CD4^+CD25^+$ cell percentage in the spleen ($P \leq 0.05$; **Table 3**).

TABLE 3 | $CD8^+/CD4^+$ ratio and $CD4^+CD25^+$ cell percentages at ED18 in thymus and spleen of layer and broiler embryos (Experiment I).

	Treatment	Thymus		Spleen	
ED18	Holes	$CD8^+/CD4^+$	% Tregs	$CD8^+/CD4^+$	% Tregs
Layer embryos	0	1.4	2.4^{b}	5.2^{b}	3.5^{b}
	2	2.5	4.5^{a}	10.1^{a}	6.5^{a}
P-value		0.09	0.02	0.01	0.01
SEM		0.2	0.5	0.6	0.5
Broiler embryos	0	1.1	2.2	6.6^{b}	5.5^{b}
	2	2.3	3.4	9.6^{a}	8.1^{a}
P-value		0.14	0.07	0.05	0.01
SEM		0.2	0.3	0.6	0.5

Fertile broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0-hole or 2-holes in the eggshell above the air cell and continued to incubate until ED18. The embryos from each treatment group were transferred to the hatcher and maintained at 37.0°C and 70% relative humidity until hatch. At ED18 thymus, and spleen were collected from all the treatment groups and analyzed for $CD8^+/CD4^+$ ratio and $CD4^+/CD25^+$ by flow cytometry after staining with fluorescent-linked anti-chicken CD4, CD8, and CD25 antibodies. Means with no common superscripts differ significantly (P ≤ 0.05). n = 6.

Splenic and Cecal Tonsil IL1-β, IL6, IL-10, and TLR-4 mRNA Transcription at ED18

At ED18, layer and broiler embryos from 2-holes treatment both had a significant increase in splenic IL1-β, IL-6, IL-10, and TLR-4 mRNA transcription compared to that in the control group ($P \leq 0.05$; **Table 4**). Layer and broiler embryos in the 2-holes treatment had a 2.9 to 8.2 fold increase in transcription of the different splenic cytokine mRNAs studied compared to the control group. At ED18, layer and broiler embryos from the 2-holes treatment had a significant increase in cecal tonsil IL-6, IL-10, and TLR-4 mRNA transcription compared to the control group ($P \leq 0.05$).

Experiment II

Broiler Eggs Hatchability Percentage and Hatch Weight

In Experiment II, hatchability in the 0-hole and the 2-holes treatment was 88.9 and 74%, respectively. The average hatch weight of broiler chicks in the 2-holes treatment group was 42.6 g, while that in the control groups was 43.4 g. The chicks hatched from 2-holes treatment had a 1.9% decrease in body weight at the time of hatch than that in the control group.

$CD8^+/CD4^+$ Ratio and $CD4^+CD25^+$ Cell Percentages in Thymus and Spleen

There were significant interaction effects between hole number and LPS on the $CD8^+/CD4^+$ ratio at 24 h post-LPS challenge in both the thymus and spleen ($P \leq 0.05$; **Table 5**). Chicks hatched from the 2-holes treatment and challenged with LPS had an approximately two fold higher $CD8^+/CD4^+$ ratio compared to that in both the control group and 2-holes treatment with 0 μg LPS. Birds hatched

TABLE 4 | IL1-β, IL6, IL10, and TLR-4 mRNA expression at ED18 in spleen and cecal tonsils of layer and broiler embryos (Experiment I).

Spleen	0-hole-Control	2-holes-Control	*P-value*	SEM
Layer Embryos				
IL1-β	1.0[b]	4.6[a]	0.01	0.5
IL-6	1.0[b]	8.2[a]	0.01	0.3
IL10	1.0 [b]	5.1[a]	0.01	0.2
TLR-4	1.0 [b]	4.1[a]	0.01	0.3
Broiler Embryos				
IL1-β	1.0 [b]	2.9[a]	0.01	0.1
IL-6	1.0 [b]	4.3[a]	0.01	0.3
IL10	1.0 [b]	5.6[a]	0.01	0.4
TLR-4	1.0 [b]	4.6[a]	0.01	0.3
Cecal tonsils				
Layer Embryos				
IL1-β	1.0	2.6	0.07	0.2
IL-6	1.0[b]	14.5[a]	0.01	0.5
IL10	1.0[b]	5.3[a]	0.01	0.2
TLR-4	1.0[b]	3.1[a]	0.01	0.3
Broiler Embryos				
IL1-β	1.0	2.5	0.2	0.4
IL-6	1.0[b]	4.6[a]	0.01	0.3
IL10	1.0[b]	3.2[a]	0.01	0.2
TLR-4	1.0[b]	3.8[a]	0.01	0.2

Fertile broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0-hole or 2-holes in the eggshell above the air cell and continued to incubate until ED18. At ED18, spleen and cecal tonsils from both layer and broiler embryos were collected and analyzed for mRNA by real-time PCR analysis. The mRNA content of the spleen was normalized with β-Actin mRNA content, and the mRNA content of the cecal tonsil was normalized with RPS13 mRNA content. The fold change was calculated with the 0-hole control groups. Means with no common superscripts differ significantly ($P \leq 0.05$). n = 6.

from the 0-hole treatment and challenged with LPS had a significantly higher $CD8^+/CD4^+$ ratio compared to the control group.

There was also a significant interaction effect between hole number and LPS on the $CD8^+/CD4^+$ ratio at 48 h post-LPS challenge in the spleen ($P \leq 0.05$; **Table 4**). At 48 h post-LPS challenge, chicks hatched from the 0-hole treatment and challenged with LPS had a significantly higher splenic $CD8^+/CD4^+$ ratio than that in the control group, while birds hatched from 2-holes treatment and challenged with LPS had a comparable $CD8^+/CD4^+$ ratio to that in the control group ($P = 0.05$).

There was no significant interaction effect between hole and LPS on the thymic $CD8^+/CD4^+$ ratio. However, there was a significant main effect of LPS challenge on the thymic $CD8^+/CD4^+$ ratio at 48 h post-LPS challenge ($P < 0.05$). Birds injected with LPS had a significantly higher thymic $CD8^+/CD4^+$ ratio compared to the 0 μg LPS group.

There were significant interactions ($P \leq 0.05$) between the hole number and LPS challenge on $CD4^+CD25^+$ cell percentages in the thymus and spleen at both 24 and 48 h post-LPS challenge. Chicks hatched from 2-holes treatment and challenged with LPS had an approximately 1.5-fold increase in $CD4^+CD25^+$ percentage compared to that in the control group.

Thymocytes Proliferation at 24 and 48 h Post-lipopolysaccharide Injection

In broiler chicks, there was a significant interaction between 0 and 2-holes treatments and LPS on thymocyte proliferation at 24 and 48 h ($P < 0.05$) post-LPS challenge. At 24 and 48 h post-hatch, chicks hatched from 2-holes eggs and challenged with LPS had a significant increase in thymocyte proliferation ($P \leq 0.05$; **Figure 2**) at 24 h (42%) and 48 h (37%) when compared with control chicks (0-hole; 0 μg LPS), respectively.

Splenic IL1-β, IL-6, IL-10, and TLR-4 mRNA Transcription at 24 and 48 h Post-lipopolysaccharide Injection

At 24 h post-LPS challenge, there were significant interaction effects between the hole and LPS challenge on IL1-β and TLR-4 mRNA transcription in the spleen ($P < 0.05$; **Table 6**). At both 24 and 48 h post-LPS challenge, chicks hatched from the 0-hole treatment and challenged with LPS had significantly higher splenic IL1-β and TLR-4 mRNA transcription compared to control, non-challenge group; while chicks from the 2-holes treatment and with LPS challenge had comparable IL1-β and TLR-4 mRNA transcription levels to the control group ($P < 0.05$).

There was a significant main effect of LPS challenge on splenic IL-6 and IL-10 mRNA transcription at 24 h and on splenic IL-10 at 48 h post-LPS challenge ($P < 0.05$). Birds challenged with LPS had significantly higher IL-6 and IL-10 mRNA transcription compared to that in the 0 μg LPS group.

Cecal Tonsils IL1-β, IL-6, IL-10, and TLR-4 mRNA Transcription at 24 and 48 h Post-lipopolysaccharide Injection

There were significant interaction effects between the hole number and LPS challenge on IL1-β, IL-6, and TLR-4 mRNA transcription in the cecal tonsils at 24 h post-LPS challenge ($P < 0.05$; **Table 7**). At 24 h, chicks hatched from 0-hole treatment and challenged with LPS had significantly higher cecal tonsil IL1-β, IL-6, and TLR-4 mRNA transcription than control chicks, while those chicks in the 2-holes treatment and challenged with LPS had comparable IL1-β, IL-6, and TLR-4 mRNA transcription compared to the control group ($P < 0.05$).

There was a significant main effect of LPS challenge on IL-10 mRNA transcription at 24 h. Chicks challenged with LPS had significantly higher IL-10 mRNA transcription when compared with 0 μg LPS group.

At 48 h post-LPS challenge, there were significant interaction effects between hole number and LPS challenge on IL-10 mRNA transcription in the cecal tonsils ($P < 0.05$). Chicks hatched from 0-hole treatment and challenged with LPS had significantly higher cecal tonsil IL-10 mRNA transcription than that in the control chicks, while challenged chicks from the 2-holes

TABLE 5 | $CD8^+/CD4^+$ ratio and $CD4^+CD25^+$ cell percentages at 24 and 48 h post-LPS injection in thymus and spleen (Experiment II).

	0-hole-Control	2-holes-Control	0-hole-LPS challenge	2-holes-LPS challenge	Main effects		Interaction	SEM
					Hole P-value	LPS challenge *P-value*	Hole × LPS challenge *P-value*	
24 h								
Thymus								
CD8/CD4	2.5^{c}	2.8^{c}	4.0^{b}	5.7^{a}	0.01	0.04	0.01	0.2
Tregs	0.5^{c}	1.6^{b}	2.0^{b}	3.6^{a}	0.05	0.01	0.01	0.2
Spleen								
CD8/CD4	1.1^{c}	0.9^{c}	2.2^{b}	3.1^{a}	0.13	0.05	0.04	0.3
Tregs	3.1^{b}	3.5^{b}	4.0^{b}	5.0^{a}	0.26	0.07	0.05	0.3
48 h								
Thymus								
CD8/CD4	2.8	2.0	4.6	4.4	0.18	0.03	0.32	0.1
Tregs	2.2^{b}	2.8^{b}	3.7^{b}	5.8^{a}	0.34	0.05	0.05	0.1
Spleen								
CD8/CD4	1.6^{b}	1.6^{b}	4.1^{a}	2.5^{b}	0.48	0.05	0.05	0.3
Tregs	2.3^{c}	3.8^{bc}	4.3^{ab}	5.9^{a}	0.05	0.07	0.05	0.4

Fertile broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0-hole or 2-holes in the eggshell above the air cell and continued to incubate until ED18. The embryos from each treatment group were transferred to the hatcher and maintained at 37.0°C and 70% relative humidity until hatch. At D1, hatchlings from each treatment group were challenged intraperitoneally with either 0 or 500 μg lipopolysaccharide (LPS)/kg BW in a 2 (0-hole, 2-holes) × 2 (control, LPS challenge) factorial design. At 24 and 48 h post-LPS injection, thymus, and spleen from all the treatment groups were collected and analyzed for $CD4^+/CD8^+$ ratio and $CD4^+/CD25^+$ by flow cytometry after staining with fluorescent-linked anti-chicken CD4, CD8, and CD25 antibodies. Means with no common superscripts differ significantly ($P \leq 0.05$). n = 6.

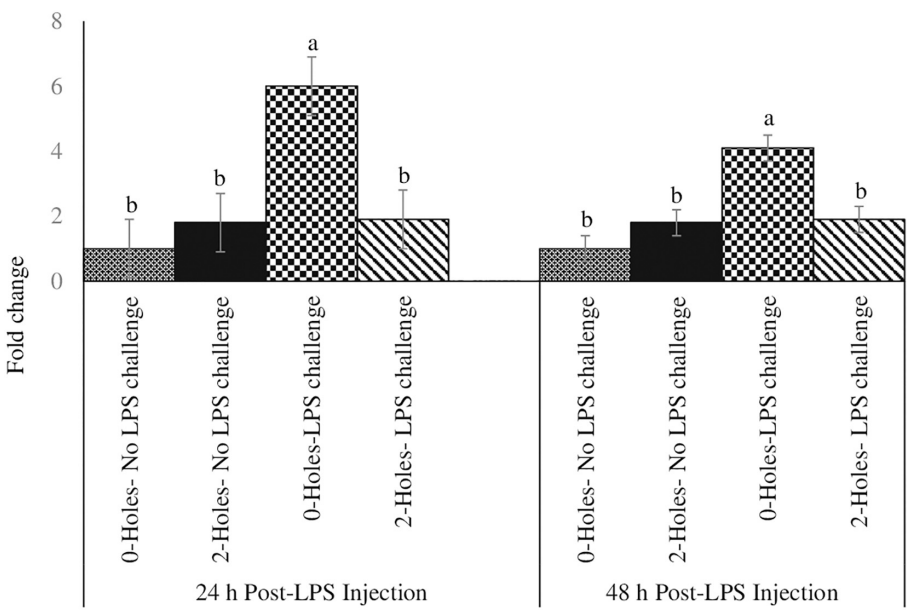

FIGURE 2 | Thymocyte proliferation efficiency at 24 and 48 h post-LPS injection (Experiment II). Fertile broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0-hole or 2-holes in the eggshell above the air cell and continued to incubate until ED18. The embryos from each treatment group were transferred to the hatcher and maintained at 37.0°C and 70% relative humidity until hatch. At D1, hatchlings from each treatment group were challenged intraperitoneally with either 0 or 500 μg lipopolysaccharide (LPS)/kg BW in a 2 (0-hole, 2-holes) × 2 (control, LPS challenge) factorial design. At 24 and 48 h post-LPS injection, 1×10^5 thymocytes were stimulated *in vitro* with 200 ng/ml phorbol 12-myristate 13-acetate (PMI), plus 50 ng/ml ionomycin (IM). At 72 h of culture, thymocyte proliferation was analyzed by MTT assay. Means with no common superscripts differ significantly ($P \leq 0.05$; $n = 6$). *P*-values: 24 h post- LPS challenge; holes × LPS challenge $P = 0.02$; Holes $P = 0.01$, LPS challenge $P = 0.05$. 48 h post- LPS challenge; holes × LPS challenge $P = 0.03$; Holes $P = 0.01$, LPS challenge $P = 0.05$.

TABLE 6 | Splenic IL1-β, IL-6, IL10, and TLR-4 mRNA expression at 24 and 48 h post-LPS injection (Experiment II).

Spleen	0-hole-Control	2-holes-Control	0-hole-LPS	2-holes-LPS	Main effects		Interaction	SEM
					Hole P-value	LPS challenge *P-value*	Hole × LPS challenge *P-value*	
24 h								
IL1-β	1.0^{b}	0.98^{b}	7.2^{a}	3.3^{b}	*0.07*	0.01	0.03	0.5
IL-6	1.0	1.4	5.6	2.6	*0.37*	0.05	0.24	0.7
IL10	1.0	2.8	2.1	5.2	*0.01*	0.03	0.37	0.4
TLR-4	1.0^{b}	1.7^{b}	5.6^{a}	1.8^{b}	*0.07*	0.01	0.01	0.4
48 h								
IL1-β	1.0^{b}	1.1^{b}	3.8^{a}	1.5^{b}	*0.02*	0.01	0.01	0.2
IL-6	1.0	2.4	1.8	2.2	*0.16*	0.63	0.41	0.3
IL10	1.0	1.9	4.1	2.8	*0.81*	0.03	0.25	0.5
TLR-4	1.0^{b}	2.1^{b}	3.2^{a}	1.7^{b}	*0.78*	0.23	0.05	0.4

Fertile broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0–holes or 2-holes in the eggshell above the air cell and continued to incubate until ED18. The embryos from each treatment group were transferred to the hatcher and maintained at 37.0°C and 70% relative humidity until hatch. At D1, hatchlings from each treatment group were challenged intraperitoneally with either 0 or 500 μg lipopolysaccharide (LPS)/kg BW in a 2 (0-hole, 2- holes) × 2 (control, LPS challenge) factorial design. At 24 h, 48 h post-LPS injection, spleen from all the treatment groups were collected and analyzed for mRNA by real-time PCR analysis. The mRNA content was corrected for reference gene β-Actin mRNA content and normalized to the mRNA content of the 0-hole-control groups. Means with no common superscripts differ significantly ($P \leq 0.05$). n = 6.

TABLE 7 | Cecal tonsils IL1-β, IL-6, and IL-10 mRNA expression at 24 and 48 h post-LPS injection (Experiment II).

Cecal tonsils	0-hole Control	2-holes Control	0-hole- LPS	2-holes-LPS	Main effects		Interaction	SEM
					Hole P-value	LPS challenge *P-value*	Hole × LPS challenge *P-value*	
24h								
IL1-β	1.0^{b}	1.1^{b}	11.8^{a}	2.3^{b}	*0.01*	0.01	0.01	0.6
IL6	1.0^{b}	1.8^{b}	3.9^{a}	1.9^{b}	*0.36*	0.02	0.03	0.3
IL10	1.0	1.4	2.6	4.7	*0.09*	0.01	0.26	0.4
TLR4	1.0^{b}	1.6^{b}	4.1^{a}	2.1^{b}	*0.20*	0.01	0.01	0.3
48 h								
IL1-β	1.0	1.1	1.0	1.5	*0.76*	0.07	0.64	0.2
IL6	1.0	1.2	2.4	1.5	*0.53*	0.17	0.28	0.2
IL10	1.0^{b}	1.8^{b}	5.7^{a}	2.8^{b}	*0.14*	0.01	0.01	0.4
TLR4	1.0	1.5	2.5	2.2	*0.83*	0.10	0.50	0.3

Fertile broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0-hole or 2-holes in the eggshell above the air cell and continued to incubate until ED18. The embryos from each treatment group were transferred to the hatcher and maintained at 37.0°C and 70% relative humidity until hatch. At D1, hatchlings from each treatment group were challenged intraperitoneally with either 0 or 500 μg lipopolysaccharide (LPS)/kg BW in a 2 (0-hole, 2 holes) × 2 (control, LPS challenge) factorial design. At 24 and 48 h post-LPS injection, cecal tonsils from all the treatment groups were collected and mRNA was quantified by real-time PCR analysis. The mRNA content was corrected for reference gene RPS13 mRNA content and normalized to the mRNA content of the 0-hole-control group. Means with no common superscripts differ significantly ($P \leq 0.05$). n = 6.

treatment and had comparable IL-10 mRNA transcription to the control group ($P = 0.05$).

Jejunal Muc-2 Gene Expression at 24 and 48 h Post-lipopolysaccharide Challenge

There were significant interaction effects between the hole number and LPS challenge on Muc-2 mRNA transcription in the jejunum ($P < 0.05$; **Figure 3**). At 24 and 48 h post-LPS challenge, chicks hatched from 0-hole treatment and challenged with LPS had significantly higher Muc-2 mRNA transcription than that in the control group, while chicks from the 2-holes treatment and challenged with LPS had comparable Muc-2 mRNA transcription compared with control chicks.

DISCUSSION

Osmoregulation is the maintenance of the proper concentrations of water and solutes. Any changes in the appropriate ratios are generally balanced by an equal and opposite loss or gain of water (Molnar and Gair, 2013). In biological systems, fluid compartments between and within the cells are separated by semipermeable membranes, and the ability to regulate the intracellular and extracellular solute microenvironments is critical in maintaining cellular homeostasis (Shoval and Alon, 2010). The developing avian embryo contains rudimentary tissues to maintain osmoregulation and relatively very little is known about the consequences of excess water loss on

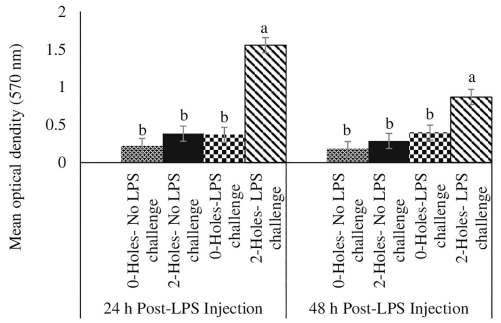

FIGURE 3 | Jejunal Muc2 gene expression at 24 and 48 h post-LPS injection (Experiment II). Fertile broiler eggs were incubated at standard (37.5°C) incubation temperature from embryonic day (ED) 1–8. At ED8, the eggs incubated at 37.5°C were drilled either 0-hole or 2-holes in the eggshell above the air cell and continued to incubate until ED18. The embryos from each treatment group were transferred to the hatcher and maintained at 37.0°C and 70% relative humidity until hatch. At D1, hatchlings from each treatment group were challenged intraperitoneally with either 0 or 500 μg lipopolysaccharide (LPS)/kg BW in a 2 (0-hole, 2-holes) × 2 (control, LPS challenge) factorial design. At 24 and 48 h post-LPS injection, jejunal tissue from all the treatment groups was collected and analyzed for MUC2 mRNA by real-time PCR analysis. The mRNA content was normalized to the mRNA content of housekeeping gene RPS13 and data presented as a fold change compared to the mRNA content of the no hole -control group. Means with no common superscripts differ significantly ($P \leq 0.05$). $n = 6$. P-values: 24 h post- LPS challenge; holes × LPS challenge $P = 0.02$; Holes $P = 0.11$, LPS challenge $P = 0.11$. 48 h post- LPS challenge; holes × LPS challenge $P = 0.01$; Holes $P = 0.03$, LPS challenge $P = 0.01$.

osmoregulatory mechanisms and the development of the avian embryonic immune system. Hence, the goal of this study was to identify the effect of an induced moisture loss on selected immune responses of broiler and layer embryos at ED18 and the acute inflammatory response in broiler chicks to an LPS challenge on the day of hatch.

In Experiment I, the average weight of eggs from a layer strain was 61 g while the broiler hatching eggs' average was 63 g. The eggs in both treatment groups lost 10% moisture at ED18. The loss of water occurs via diffusion through pores in the shell and, is an important regulatory process during incubation (Paganelli, 1980). Moisture loss includes both the water content of the egg along with metabolic water produced from the oxidation of lipids, particularly during the later stages of embryonic development (Ar and Rahn, 1980). At ED18, moisture loss was approximately 2% greater in the 2-holes treatment (2-holes, 10.1%; 0-hole, 8.20%; $P < 0.01$, **Table 2**) in layer embryos; and 1.5% (2-holes, 9.9%; 0-hole, 8.4%; $P < 0.01$) in broiler embryos. Similar results were previously observed (Davis et al., 1988; Buhr, 1995) for avian embryos incubated at 69% RH (7.6% moisture loss). In chicken eggs, hatching success is normally achieved if eggs lose approximately 7 g of water, which would represent 12% for 60 g of the initial egg mass. A major increase or decrease in total water loss during the 21-day incubation period may decrease hatching success (Barott, 1937; Landauer, 1967; Lundy, 1969). Commercial hatcheries maintain RH at 55% during the first 15 days of incubation with an increase to 60% RH after 15 days to maintain optimal hatchability (Cartwright, 2000). An increase in RH will decrease the moisture loss from the egg whereas a decrease in RH will increase water evaporation from the egg (Davis et al., 1988; Hamdy et al., 1991). In this study, the eggs were incubated at relatively higher RH (65%) with a constant incubator set temperature (37.5°C) throughout the experiment. When eggs were incubated at a constant temperature, decreasing humidity caused increased the incubation time and decreased the chick weight (Romanoff, 1929; Townsley, 1931). Eggs were incubated at a higher RH of 65% to minimize excessive water loss and subsequent embryonic mortality (Bruzual et al., 2000). The small increase in the moisture loss in 2-holes treatment could also be a result of water recycling via osmoregulation in the chorioallantoic membrane to minimize dehydration of embryos (Hoyt, 1979; Simkiss, 1980). This moisture loss did not affect the dry mass of the yolk-free embryos suggesting that induced moisture loss occurred in extra-embryonic membranes, but not from embryonic body tissues (Van der Pol et al., 2013). The increased moisture loss observed in this study at ED18 decreased the hatchability percentage and this is consistent with the earlier studies (Romanoff, 1929; Davis et al., 1988).

Though previous studies have examined the effect of dehydration on different physiological systems during incubation (Davis et al., 1988), not much information is available on the effects of embryonic water loss and the development of the avian embryonic immune system. To the best of our knowledge, we are the first group to demonstrate that induced moisture loss altered the immune indices of the embryos. In Experiment I, thymocytes, stimulated with PMA + Ionomycin, had increased proliferation in the 2-holes treatment. The chorioallantoic membrane is associated with the active transport of Na + ions from the allantoic fluid into the blood, calcium transport from the eggshell (Stewart and Terepka, 1969; Hoyt, 1979; Graves et al., 1986; Gabrielli and Accili, 2010). The increase in moisture loss likely induced a hyperosmotic environment and altered the Na^+, K^+, and Ca^{2+} ion transporter channels in T cells and subsequently stimulated T cell receptors to increase thymocyte proliferation (Feske et al., 2012). The induced moisture loss during embryogenesis could have activated the immune response thereby reducing BW by the time of hatch (1.9%), suggesting a physiologic trade-off between immune response versus BW.

The induced moisture loss also significantly increased the percentage of T-regulatory cells in the thymus and spleen and the splenic $CD4^+/CD8^+$ ratio at ED 18 compared with the control treatment. The activation and clonal expansion of $CD4^+$ T cells is a tightly regulated process that stimulates the proliferation of resting $CD4^+$ T cells and is critical for the activation of immune responses (Li et al., 2014). The $CD4^+CD25^+$ T regulatory cells (Tregs) are a unique subset of T-cells that regulate immune response and establish peripheral tolerance. The data in Experiment I suggested that induced moisture loss likely induced some degree of osmotic stress. Induced water losses ≥ 20% depletes allantoic fluid as resulted in prolonged osmotic stress in chicken embryos (Davis et al., 1988). Osmotic stress has been reported to alter the homeostasis of T cells (Brocker et al., 2012). At ED18, induced moisture loss increased the splenic Tregs percentage and upregulated IL-10 mRNA transcription in spleen and cecal tonsils, suggesting that the spleen and cecal tonsils were sensitive to the induced osmotic changes at ED18.

Moreover, avian embryos most likely sensed the extracellular hyperosmolarity at the cell membrane (Lunn and Rozengurt, 2004) and subsequently increased the transcription of splenic IL-6, TLR-4, and IL-10 in both layers and broiler embryos at ED18. Induced moisture loss altered the function of the immune system by increasing the proliferation of T cells and modulating the production of IL1-β, IL-6, and IL-10 (Coimbra et al., 1995). These changes in cytokine expression are most likely due to the activation of the conserved adaptive mechanisms in response to osmotic imbalances during embryonic development (Hubert et al., 2004). Taken together, the observed increase in the $CD8^+/CD4^+$ ratio and Tregs in embryos with induced moisture loss is likely a compensatory mechanism to facilitate immune and inflammatory responses for possible pathogen infections.

To determine if the induced moisture loss could play a role in the protection of newly hatched chicks to an inflammatory challenge, broiler chicks, hatched from control and induced moisture loss eggs, were injected with LPS. Moisture loss during incubation is a major factor that dysregulates immune response and increases mortality post-hatch (Yassin et al., 2009). Cells have developed several adaptive response mechanisms, by secreting cytokines to counter osmotic stress and restore osmotic equilibrium (Brocker et al., 2012). Chicks hatched from eggs with 2-holes and challenged with LPS had a significant increase in thymocyte proliferation compared to the control group is consistent with Galindo-Villegas et al. (2016) who reported that newly hatched germ-free (GF) zebrafish sense the hyperosmolarity of the aquatic environment and mount a protective adaptive immune response.

Osmotic stress can also activate the intracellular MAP kinase pathway and a subsequent non-specific inflammatory response via the release of selected cytokines (Uhlik et al., 2003). Studies with osmotic stress reported that human aortic endothelial cells and peripheral blood mononuclear cells secrete IL-1β (Shapiro and Dinarello, 1997; Fernandes et al., 2009) and rat peritoneal macrophages secrete IL-6 (Wade, 2002). Exposure to osmotic stress reprograms the inflammatory responses to a subsequent LPS challenge resulting in decreased pro-inflammatory cytokines in mice (Pimentel et al., 2019). In our study, the mRNA transcription of pro-inflammatory genes IL-1β and IL-6 was reduced but the transcription of the anti-inflammatory cytokine IL-10 was not altered in the spleen at either 24 or 48 h post-LPS challenge. The data reported herein suggest that induced moisture loss during embryogenesis causes LPS tolerance via inhibition of TLR-4 signaling at 24 h post-LPS challenge. An earlier study identified similar LPS tolerance through inhibition of TLR signaling (Foster et al., 2007). Further, comparable IL-10 production in the spleen in response to the LPS challenge indicates that induced moisture loss during embryogenesis may precondition the chicks toward LPS tolerance (Melo et al., 2010; Pimentel et al., 2019).

Among mucin isoforms, Muc-2 is the predominant isoform in the chick intestine, and Muc-2 transcription can be modulated by local inflammatory activity (Oh et al., 2019). The LPS challenge in the chicks hatched from the 2-holes treatment did not influence Muc-2 mRNA transcription at either 24 and 48 h compared to the control groups. This is most likely due to the induced moisture loss was not sufficient enough to activate NF-κB which is necessary for the induction of Muc-2 expression and the subsequent modulation of the gut mucosal immune response. On the other hand, the LPS challenge did increase Muc-2 transcription in the chicks hatched from 0-hole treatment. Earlier reports identified that LPS induced overexpression of Muc-2 in biliary epithelial cells and chicken jejunum through TLR-4 signaling (Zen et al., 2002; Oh et al., 2019).

CONCLUSION

The induced moisture loss was sufficient to decrease BW at hatch by 2%, increase thymocyte proliferation at ED18, 24 and 48 h post-LPS challenge, and decrease pro-inflammatory cytokines transcription without altering IL-10. Our data suggest that inducing moisture loss reprograms gene transcription in embryos to enhance cell survival via proliferation. Our study also suggests that osmoregulation is critical for regulating the immune system. Induced moisture loss could regulate specific gene patterns depending on the severity of osmotic stress. In future studies, the determination of osmoregulatory transcription factor NFAT5 transcription might help us to better understand how osmotic stress reprograms gene expression during embryogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at The Ohio State University approved all animal protocols.

AUTHOR CONTRIBUTIONS

JG: methodology, writing-review, and editing. MSL: conceptualization, funding acquisition, project administration, writing-review and editing. RS: conceptualization, data curation, formal analysis, investigation, methodology, validation, writing – original draft, writing – review and editing. All authors: contributed to the article and approved the submitted version.

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METHODS
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A Novel Egg-In-Cube System Enables Long-Term Culture and Dynamic Imaging of Early Embryonic Development

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The avian egg is a closed system that protects the growing embryo from external factors but prevents direct observation of embryo development. Various culture systems exist in the literature to study the development of the embryo for short periods of incubation (from 12 h up to a maximum of 60 h of egg incubation). A common flaw to these culture techniques is the inability to culture the unincubated avian blastoderm with intact tissue tensions on its native yolk. The goal of this work is to create a unique novel egg-in-cube system that can be used for long-term quail embryo culture initiated from its unincubated blastoderm stage. The egg-in-cube acts as an artificial transparent eggshell system that holds the growing embryo, making it amenable to microscopy. With the egg-in-cube system, quail embryos can be grown up to 9 days from the unincubated blastoderm (incubated in air, 20.9% O_2), which improves to 15 days on switching to a hyperoxic environment of 60% O_2. Using transgenic fluorescent quail embryos in the egg-in-cube system, cell movements in the unincubated blastoderm are imaged dynamically using inverted confocal microscopy, which has been challenging to achieve with other culture systems. Apart from these observations, several other imaging applications of the system are described in this work using transgenic fluorescent quail embryos with upright confocal or epifluorescence microscopy. To demonstrate the usefulness of the egg-in-cube system in perturbation experiments, the quail neural tube is electroporated with fluorescent mRNA "in cubo", followed by the incubation of the electroporated embryo and microscopy of the electroporated region with the embryo in the cube. The egg-in-cube culture system in combination with the "in cubo" electroporation and dynamic imaging capabilities described here will enable researchers to investigate several fundamental questions in early embryogenesis with the avian (quail) embryo on its native yolk.

Keywords: embryo development, egg-in-cube, avian embryo culture, imaging, long term culture, quail (*Coturnix japonica*)

INTRODUCTION

The main advantage of avian embryos for their use in developmental studies is easy accessibility and the ability to culture the embryos inside the eggshell (in ovo) or on artificial substrates (ex ovo). These embryo culture techniques made it possible for biologists to first describe the normal development of the chicken embryo in detail.

The technique of isolating and culturing embryos on a semi-solid substrate was first started by Waddington (Waddington, 1932) and further improved by Spratt (Spratt, 1947) in a seminal study of amniote form and function. The embryo isolation and ex ovo culture technique developed by New (New, 1955) provided a straightforward technique to culture, manipulate, and observe early avian embryogenesis from primitive streak stages up to Hamburger and Hamilton (HH) stage 12–13 (Hamburger and Hamilton staging system, Hamburger & Hamilton, 1951). Several groups modified New's culture by placing the isolated embryo between glass/metal rings to primarily extend the period of development observable from primitive streak stages up to HH17 (Gallera and Nicolet, 1961; Jacob, (1971); Jaffee, 1974; Seidl, 1977; Stern and Ireland, 1981). The "Cornish pastry" culture method (Connolly et al., 1995) and its modification MC culture (Nagai et al., 2011) were also derived from New's culture by adapting to a novel "fold and culture" method in liquid media made from a mixture of albumen and saline. Both these culture methods also worked to achieve better vasculature development up to stage HH18.

The Early Chick (EC) culture method (Chapman et al., 2001) improved upon New's culture technique by making it quicker and easier to culture chick embryos with more normal tissue tension. The EC culture system enables embryos to be cultured on either dorsal or ventral surfaces from stages HH3-15. However, the use of EC culture in culturing pre-primitive streak embryos remains limited due to low embryo survival rates at those early stages (Chapman et al., 2001). EC culture is still widely used to isolate and culture embryos between late HH3 and HH15, after which some neural and heart morphogenesis defects are often observed (Chapman et al., 2001). One advantage of EC culture over modified New's culture would be the ease of adapting the culture system to high-resolution microscopy. To enable the culture of pre-gastrulating embryos, New's culture was modified to isolate and culture pre-primitive streak embryos as early as EGK XII (Voiculescu et al., 2007) [EGK staging system (Eyal-Giladi & Kochav, 1976)]. This work also describes the first few time-lapse imaging experiments to understand the development of the chick embryo in the pre-gastrulation stages. This modified technique enabled isolation of pre-primitive streak embryos but unfortunately did not overcome the laborious nature of the culture technique inherent to the New's culture system.

Several whole-yolk culture systems have been developed (Auerbach et al., 1974; Tufan et al., 2004; Borwompinyo et al., 2005; Yalcin et al., 2010) to address the shortcomings of the various ex ovo culture methods. In these culture systems, the embryo remains on top of the intact yolk, and all the egg contents with the albumen are transferred to another container for incubation. The containers varied from a turkey or chicken surrogate eggshell (Nirasawa et al., 1992; Borwompinyo et al., 2005), a Petri dish (Auerbach et al., 1974), or cling wrap hammocks (Tufan et al., 2004; Yalcin et al., 2010; Schomann et al., 2013; Tahara and Obara, 2014) for culture. Some systems also include sequential culture in multiple containers involving a plastic cup (0–24 h), quail eggshell (24–76 h), and then in a chicken eggshell (until hatching) with a supply of chicken thin albumen for optimal growth (Ono et al., 1994). Embryos in these whole-yolk culture systems have increased accessibility and can be grown until hatching. Most of these techniques are very useful for studying the development of embryos after the vascular system is established while studying early morphogenesis and gastrulation remained challenging to achieve. While some culture systems enabled access to the early development of the quail embryo (Ono et al., 1994), they were unsuitable for direct observation/microscopy due to culture in a non-transparent eggshell after 24 h of embryo growth.

There is a growing need for a culture system that can enable microscopy of early embryos on their native yolk to mimic natural conditions and maintain proper tissue tension when imaging embryo development. One of the first in ovo imaging studies involved tracing the migration of hindbrain neural crest cells using dye injections, keeping the embryo on its yolk in its native egg environment (Kulesa et al., 2000; Kulesa & Fraser, 2000; Kulesa et al., 2010). These studies used a Teflon membrane on the eggshell window to provide optical transparency and maintain humidity around the embryo during imaging. A complication with this in ovo approach is, that the embryo can drift away out of focus due to its natural 3D expansion on the yolk. This lateral movement is not optimal for long-term time-lapse imaging. An ideal imaging and culture system should address concerns for both ex ovo and in ovo systems described above.

We recently established a generalized design method of the artificial egg-in-cube system, focused on optimizing the oxygen permeability through the cube surfaces and culture conditions for the chick embryo starting at day 3 (E3) until day 7 (E7) of development (Huang et al., 2015). We demonstrate normal embryo development in the cube identical to an eggshell with an added advantage of accessibility to the embryo and its vasculature. We standardized the thickness of the Polydimethylsiloxane (PDMS) membrane (the membrane forms the cube's sides) to enable higher oxygen diffusion yet provide flexibility enough to insert surgical instruments through the sides of the cube for embryo access.

In the work presented here, we customize the egg-in-cube system to the smaller quail egg, coupling it with fluorescent transgenic quail lines to demonstrate the power of the egg-in-cube system to enable long-term culture and high-resolution imaging of avian embryos from egg-laying stages (EGK-X).

MATERIALS AND METHODS

Fabrication of the Cube and Sample Preparation

The fabrication process of the cubic eggshell and the Polydimethylsiloxane (PDMS) membranes are done as in

(Huang et al., 2015) with minor modifications (**Supplementary Figure S1**). Briefly, PDMS membranes (Sylgard 184 Silicone Elastomer Base and curing agent, Dow, # 2646340) were fabricated and cut into the dimensions of the shorter cube (L*B*H: 24*24*18 mm). These membranes were attached to five sides of the cube frame using liquid PDMS as glue and cured on a hot plate (80°C for 20 min) with its top surface kept unwrapped. The cube was tested for leakage and then sterilized with distilled water and 70% ethanol. The contents of a fertilized quail egg are transferred to the cube. The cube is sealed with a high transparency oxygen permeable membrane (High sensitivity stretch membrane, YSI/Xylem Inc., #098095) or a rectangular piece of sterile & clean cling wrap stretched taut on the open surface and held in place with an elastic rubber band. (**Supplementary Figure S1**).

Detailed Design for the on-Stage Incubator

We designed a customized box incubator to house the cube that can be connected to a power supply. This box incubator is equipped with a cylindrical holder to house the cube encapsulated by two heaters made from carbon fibers. A USB carbon heater was modified using heat-shrinkable tubes to make the tubular coil structure. One coil was placed at the center of the holder (lengthwise) on its outer rim. The other was attached to the lid of the box to ensure heating at the level of the embryo. The resistance value (heating capacity) of the two heating coils was calibrated and adjusted by changing the length of the heating coils to obtain the same temperature output from both coils. After assembling the heating coils onto the compact incubator, 5 V DC voltage was applied to the heater coils. By using a thermal imaging camera, the heating output of the coils was adjusted to 37–38°C by changing the DC voltage applied. A small plastic water reservoir fit with an electric fan was used to maintain optimal humidity in the incubator system. For the testing and calibration of the environmental conditions in the incubator, a precise micro-TEMP/RH sensor (Sensirion SHT35 flexible, SysCom Corp.) was used to check the incubation conditions before the experiment. A portable temperature and humidity sensor (EEEKit LCD digital thermometer/hygrometer, supplier: Amazon, #B07KBW4W12) was then used to monitor the real-time temperature and humidity fluctuations in the incubator environment if any. The output terminals for this sensor were extended with electrical wires to measure the local environment very close to the embryo in the cube. This customized incubator setup is highly useful for imaging on microscopes not equipped with on-stage incubators (**Supplementary Figure S2A**).

Detailed Design for a Custom Incubator for Long Term Embryo Imaging

The bigger custom incubator is modified from a Tupperware container with carbon fiber-based heaters to provide a constant source of heat to maintain the temperature at 37–38°C. A plastic grating platform keeps the cubes at a height above the small water reservoir. A small fan is used to maintain uniform humidity in the environment. In addition to a precise temperature/humidity sensor with a feedback controller connected to a laptop PC, this incubator was also fitted with a portable temperature/humidity sensor to check the conditions visually. Water was added to the container every 24 h using a small port on the side of the incubator without disturbing the cubes.

Transgenic Quail

The [Tg(hUbC:H2B-cerFP-2A-Dendra2)] quail line (source: Lansford lab, CHLA) ubiquitously co-expresses histone 2B-ceruleanFP (H2B-cerFP) and Dendra2 (Huss & Lansford, 2017). Dendra2 is a photoconvertible green fluorescent protein that converts to its red form after exposure to near-UV light (Gurskaya et al., 2006). The [Tg(hUbC: Membrane-eGFP)] quail line [Kindly provided by Dr. Jerome Gros (Pasteur Institute, Paris, France)] labels the plasma membrane of all cells (Saadaoui et al., 2020). The [Tg(PGK1:H2B-mCherry)] quail line ubiquitously expresses histone 2B-mCherryFP (H2B-mCherryFP) (Huss et al., 2015a). All animal procedures were carried out following approved guidelines from the Children's Hospital Los Angeles and the University of Southern California Institutional Animal Care and Use Committees.

Microscopy and Image Analysis

For the upright confocal imaging modality, time-lapse images were acquired on a Zeiss 780 LSM upright confocal microscope (ZLSM780u) using the W Plan-Apochromat 20x/1.0NA DIC (UV) VIS-NIR M27 75 mm objective or with an upright Olympus MVX10 epifluorescence stereomicroscope with an MVPLAPO x1/0.25NA objective coupled with an Olympus XM10 camera controlled by the Olympus CellSens dimension software (Olympus, RRID: SCR_016238). Imaging on an inverted modality was performed on the Zeiss 780 LSM inverted confocal microscope (ZLSM80i) with ×5/0.16NA, ×10/0.45NA M27, or ×20/0.8NA M27 Plan-Apochromat objectives. Time-lapse images were also acquired using brightfield imaging from an Android Motorola E5 phone camera (original specifications: 13 MP, f/2.0, 1/3.1″, 1.12 μm, PDAF, JPEG resolution: 4368 × 2912). The camera was programmed using a "debug mode" to communicate with a PC laptop. This programming modified the camera resolution to acquire JPEG images at 960 × 720 pixels at 0.7 MP. Images were acquired at regular intervals and stored on the laptop. Images and time-lapse files were processed using the Zeiss Zen (black) software (Zeiss, RRID: SCR_018163), NIH ImageJ (PMID 22743772, NIH, RRID: SCR_003070), and Imaris 9.5 (Bitplane, RRID: SCR_007370).

Embryonic Staging

Quail embryos were staged based on previously established criteria (Hamburger & Hamilton, 1951; Eyal-Giladi and Kochav, 1976; Ainsworth et al., 2010) with additional detailed descriptions of primitive streak morphology and staging from (Streit & Stern, 2008).

Time-Lapse Analysis of Embryo Growth in the Cube

The contents of several fertilized wild-type quail eggs were transferred to cubes and set into a customized box incubator

(**Supplementary Figures S2B, C**) for imaging along with intact eggs as controls. A small LED light source was mounted vertically at a suitable distance to provide a constant source of white light on the cubes for bright field imaging. An Android Motorola E5 phone camera was programmed to automatically capture an image at 5-minute intervals. The acquired JPEG images were stored in a computer attached to the phone. Imaging was continued uninterrupted until 96 h of incubation. At 96 h, cubes with normal embryo morphology were repositioned under the camera, and other cubes were discarded. Imaging was continued on these selected embryos until they deteriorated. The acquired JPEGs were converted into TIFF files using ImageJ 1.53. Results obtained from this methodology are described as a part of **Section 3.1**.

Testing the Effect of 60% O_2 on Embryo Growth in the Cube

The contents of several fertilized wild-type quail eggs were transferred to cubes and incubated along with intact eggs as controls in a forced air incubator set at 37°C for 3 days. On day 3, some of the cubes with living embryos were then transferred to a hyperoxia chamber that maintained a constant environment of 60% oxygen (O_2) along with a set of intact eggs as controls. Embryos in cubes incubated in the forced air incubator and at 60% O_2 were allowed to incubate and checked every 18–24 h for viability. Non-viable embryos were removed from the cube along with corresponding egg controls and fixed with cold 4% formaldehyde in PBS for 48 h at 4°C. These fixed embryos were then washed in PBS. For staging of these fixed embryos, the length of the 3rd toe and the beak length were measured to stage them according to the Hamburger and Hamilton system (Hamburger & Hamilton, 1951; Ainsworth et al., 2010). The measurement was performed by acquiring images of the 3rd toe and beak placed alongside a plastic ruler on the upright Olympus MVX10 epifluorescence stereomicroscope with an MVPLAPO ×1/0.25NA objective coupled with an Olympus XM10 camera controlled by the Olympus CellSens dimension software.

Bead Injections Into Embryos

The empty customized box incubator (shown in **Supplementary Figures S2A, A'**) is pre-equilibrated to 37°C by placing the box in the egg incubator before working with the eggs for 1 h. Wild-type quail embryos were incubated at 37°C in a humidified incubator for 72 h to reach stage E3 (HH18). Contents of these fertilized eggs were gently transferred to several cubes and screened for normal morphology and appropriate staging of embryos was performed. Fluorescent microspheres (1 µm in diameter, Crimson Ex/Em:625/645) (FluoSpheres, ThermoFisher Scientific, #F8816) were diluted 1:1000 into sterile PBS and microinjected using a pulled glass needle into the left lateral vitelline vein along the direction of blood flow. These injected "in cubo" embryos were then transferred back to the 37°C incubator to recover for 30 min. The best injections are first screened on the upright Olympus MVX10 epifluorescence stereomicroscope with an MVPLAPO ×1/0.25NA objective and then transferred to the box incubator on stage and used for dynamic imaging.

Electroporation of the Quail Embryo "in Cubo"

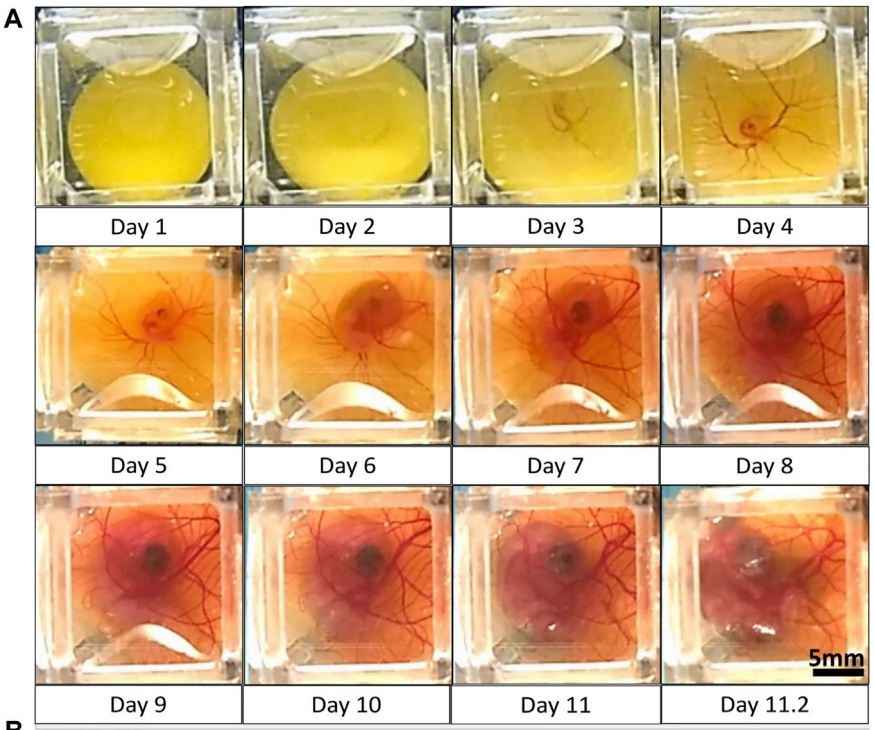
Multiple [Tg(PGK1.H2B.mCherry)] embryos were transferred to cubes and electroporated with 1 µL of 0.5 µg/µL mRNA, *in vitro* transcribed from the pCS2.membrane.eGFP plasmid (pCS2.membrane.eGFP was a kind gift from Dr. Le Trinh) targeting the midbrain-hindbrain and neural tube regions. Cubes with the electroporated embryos were incubated for 2 h at 37°C in the humidified incubator. After 2 h, electroporated embryos were screened for GFP expression under the upright Olympus MVX10 epifluorescence stereomicroscope and then transferred to the Zeiss LSM inverted confocal microscope stage for imaging. Electroporation conditions: 25 V 50 ms ON/100 ms OFF for 3–5 pulses (Nakamura and Funahshi, 2001).

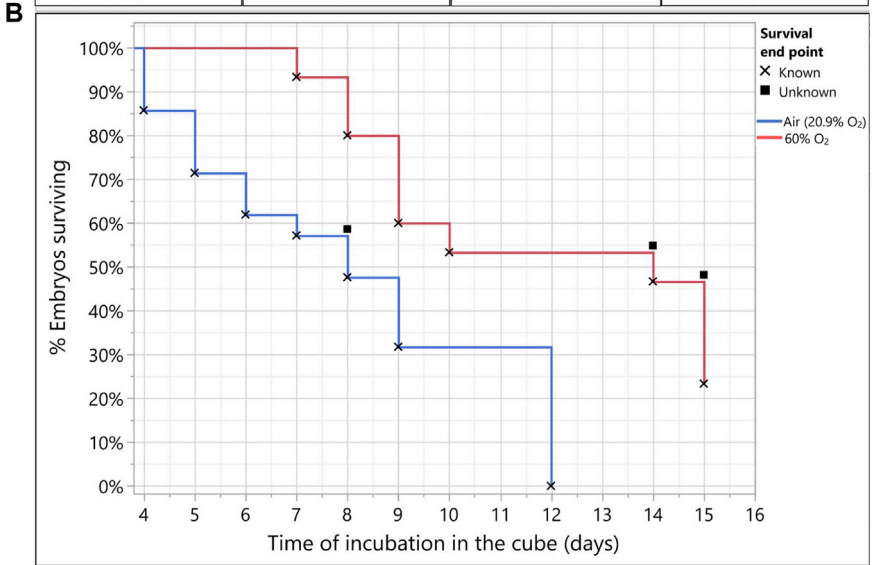
RESULTS

Long Term Culture of the Quail Embryo in the Cube

The egg-in-cube system developed in our previous work was built to hold the contents of chicken eggs (Huang et al., 2015). To adapt it to the quail eggs, we reduced the Polycarbonate (Takiron Co., Ltd, Cat # PCP1609A) cube frame dimensions to a smaller size (L×W×H: 24 × 24 × 18 mm). Our first aim was to characterize the egg-in-cube system and test it for the long-term culture of the quail embryo starting from the unincubated EGK-X stage. Embryo development was much slower, and its morphology was irregular in cubes where the embryo was in close contact with the membrane surface (**Supplementary Figure S3**). We found that for long-term culture (a few days) in the cube starting from EGK-X, it was essential to have an air gap of ~5 mm between the surface of the high transparency membrane (top of the cube) and the embryo. Imaging the long-term development (several days) of the embryo on a high resolution upright confocal microscope with the air gap would be restricted to low magnification long working distance objectives like the ×5 and ×10 objectives. Although this air gap did not hamper imaging on upright fluorescent stereoscopes because of their long-distance working objectives.

To test the effect that the revised culture conditions had on the long-term survival of quail embryos in the cube system, we transferred the contents of several EGK-X (unincubated) wild type quail eggs to cubes, introduced an air gap, and set them in a customized box incubator for imaging (See **Supplementary Figures S2B, C**). **Figure 1A** shows representative time frames taken from the time-lapse movies of an embryo over 11.25 days (11 days and 4 h) of incubation starting from day 0 (EGK-X, unincubated eggs). The embryo goes through early expansion in the first 2 days, and vasculature develops by the end of day 2 (see **Supplementary Video S1**). Through days 3–4 of incubation, the blastoderm edges cover the top of the visible yolk, the embryo grows bigger, curves into a C-shaped structure, and now is evident in the brightfield images with its heart beating. By the start of day 5, the allantois is visibly expanding with its vasculature beginning to envelop the embryo. Pigmentation in the eyes is visible; its size increases along with the differentiation of the limbs (~Day 6).





	4	5	6	7	8	9	10	11	12	13	14	15	Days of incubation in cube
Air (20.9% O_2)	21	18	15	13	12	3	3	3	2				Number of embryos at risk
60% O_2	15	15	15	15	14	12	9	9	9	9	8	4	

FIGURE 1 | Hyperoxia (60% O_2) improves embryo survival for long-term culture in the egg-in-cube system. **(A)** Captured frames from live imaging of an EGK-X wild-type quail embryo. The images shown here are acquired using an Android Motorola E5 phone camera taken from the dorsal aspect of the embryo in the cube at 5 min intervals. Images were acquired from EGK-X (E0) to day 4 of development, the lighting and cube were readjusted, and imaging was continued until the embryo started to deteriorate by day 11.25. Raw JPEG files were acquired from the phone and processed into TIFF files using ImageJ. Time frames at approximately 24 h intervals were isolated from these TIFFs to make a representative figure here. The original video file can be seen in **Supplementary Video S1**. Scale bar = 5 mm. **(B)** Kaplan Meier survival curve plotted between embryos incubated in Air (20.9% O_2, blue line, $n = 21$ embryos) and 60% O_2 (red line, $n = 15$ embryos). Percent embryos survival plotted on the Y-axis vs. Time of incubation in the cube (in days) on the X-Axis. X denotes the death of embryos and filled squares denote the survival endpoint of embryos is unknown/embryos were censored from analysis. Mantel-Haenszel Log-rank test, $\chi^2 1 = 7.16$, $p < 0.0074$.

The head and thorax of the embryo increase in size as the shape of the yolk becomes less spherical due to yolk absorption and metabolism by the yolk sac (Day 7). The chorioallantoic membrane (CAM) vasculature develops further into a dense network of capillaries and vessels on the walls of the cube. The embryo goes through normal development until 11.25 days of incubation; after which its yolk contracts, the vascular system begin to malfunction, and imaging is stopped. Once the imaging was stopped, the embryo was isolated out of the cube for staging. We observed the formation of eyelids, but the embryo had an absence of brown and black pigmentation on its back. Using the presence of these features, we assigned the embryo to a developmental stage of HH35 (~E8). Thus, the growth of the embryo in the cube was slightly retarded than its expected age at 11 days of incubation.

Embryos cultured in the egg-in-cube system have variable survival times when incubated from EGK-X. In several trials of embryo incubation in the cube, around 23% of embryos died on day 3 of incubation and 34% of embryos survived until 8 days of incubation. The peak in death rate in the initial 3–4 days of incubation has been observed before in literature (Payne, 1919; Riddle, 1930). Embryo death in the first 4 days can occur due to intrinsic factors like gross structural abnormalities and congenital malformations (Byerly 1930), abnormal accumulation of CO_2 during carbohydrate metabolism, and accumulation of lactic acid (Bohr and Hasselbalch, 1903; Tomita, 1921). One of the important extrinsic factors could be inadequate oxygen supply to the early developing embryo (Riddle, 1930) as it lays down the vascular bed as seen in **Figure 1A** (Day 4 of incubation). To test this hypothesis, we decided to investigate if supplying a higher amount of oxygen to the embryo from E3 onwards would increase the time of embryo development that can be achieved in the cube and improve the early survival rates.

For this study, several embryos were first transferred to cubes at EGK-X and incubated in a forced air incubator at 37°C for 3 days ($n = 36$ embryos). Some of the cubes ($n = 15$ embryos) with living embryos were then transferred to a hyperoxia chamber maintaining a constant oxygen supply of 60% O_2 (Stock and Metcalfe, 1984) at 37°C along with matched in ovo controls for long-term incubation. Kaplan-Meier survival curves were plotted for % Percent embryo survival (Y-axis) vs. Time of embryo incubation in the cube (days, X-Axis) to analyze if embryos survived longer due to the added oxygen in their environment. The survival curves (**Figure 1B**) show that embryos incubated in cubes at 60% O_2 ($n = 15$ embryos) had significantly better odds of survival (Mantel-Haenszel Log-rank test, $\chi^2 1 = 7.16$, $p < 0.0074$) as compared to those incubated in the air ($n = 21$ embryos, 20.9% O_2). The median survival time for embryos incubated in cubes at 60% O_2 (14 days) is also much higher than those incubated in the air (20.9% O_2, 8 days). Representative images of embryos incubated at 60% O_2 vs. those incubated in the air (20.9% O_2) are shown in **Supplementary Figures S6A, B** along with the way they were staged.

Thus, with the addition of a hyperoxic environment (60% O_2), we improved the survival and growth of quail embryos from a median survival time of 8–9 days up to 14 days of incubation. Cultures of all living embryos in the egg-in-cube were terminated at a maximum incubation of 15 days according to the Children's Hospital Los Angeles (Protocol # 351-16) Institutional Animal Care and Use Committee protocol.

Imaging Tissue Movements Occurring in a Pre-gastrulation Embryo Using an Upright Fluorescent Stereoscope

To demonstrate the capabilities of embryo culture and dynamic imaging using the cube system on an upright fluorescent stereoscope, we used an unincubated EGK-X [Tg(hUbCp.membrane.EGFP)] (Saadaoui et al., 2020) embryo mounted in the cube for imaging. We captured the development of the embryo through the first 10 h of incubation (**Supplementary Video S2**). The embryo starts expanding slowly through the first 4 h of incubation with polonaise-like movements (Gräper, 1929) in the epiblast accompanied by the classical anterior hypoblast movements (Stern, 1990; Lawson & Schoenwolf, 2003). Both these movements aid in the formation and elongation of the primitive streak towards the anterior end of the embryo, which increases the length of the embryo along the A-P axis as the movie ends at 9 h 50 min. **Figure 2** shown here includes representative images from the time-lapse movie highlighting the morphological changes described above.

Blood Flow Dynamics in an HH15 (E2.5) Wild Type Embryo Injected With Fluorescent Microspheres

Fluorescent microsphere injections have been widely used in mammals to understand myocardial infarctions and regional blood flow measurements (Glenny et al., 1993; Deveci & Egginton, 1999; Goyal et al., 2013). This technique has been extended to chick embryos for the measurement of cardiac output in E10 chick embryos in normal and hypoxic conditions (Mulder et al., 1997; Mulder et al., 1998). Here, we microinjected fluorescent crimson microspheres into the blood circulation of an E3 wild-type quail embryo and imaged the movement of microspheres in smaller capillaries in the extra-embryonic vasculature.

Within 30 min of injection, fluorescent beads are distributed throughout the embryonic and extra-embryonic circulation. **Figure 3A** (**Supplementary Video S3A**) shows the beads circulating in the extra-embryonic blood vessels on the left side of the A-P axis of the embryo. Panels in **Figure 3B** shows a zoomed-in view of a region of interest from **Figure 3A** with small capillaries. As a proof of concept for studying regional blood flow using the cube, a cluster of beads (circled in white, **Figure 3B**) was tracked through the 30 frames in the time-lapse (4 representative frames at 50, 450, 900, and 1500 ms shown in **Figure 3B**, **Supplementary Video S3B**). **Figure 3C** shows the representative initial and final positions of the fluorescent bead tracked in **Figure 3B**. The average velocity of beads in circulation was found to be 0.55 ± 0.14 mm/s (values are mean ± SEM, $n = 6$ beads tracked).

Fluorescent microbeads have been used by other groups in quail embryos for studying blood flow dynamics, values for blood

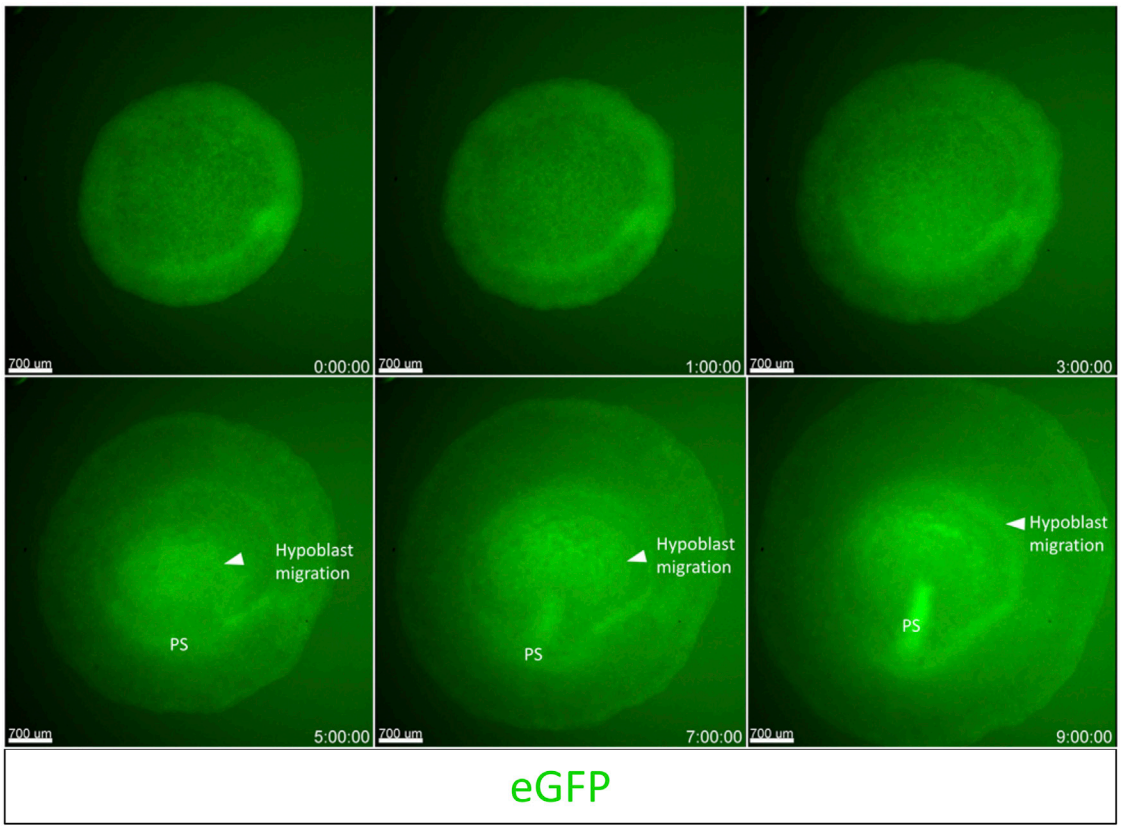


FIGURE 2 | Imaging gastrulation using an upright fluorescent stereoscope. Captured frames from live imaging of an EGK-X [Tg (hUbC.membrane.GFP)] quail embryo. The images shown here are acquired using the upright Olympus stereomicroscope taken from the dorsal aspect of the embryo in the cube at 10 min intervals. Images were acquired from EGK-X (E0) to HH3+ (9.8 h of incubation). Time frames at 1–2 h intervals were isolated from the original TIFF file. The original video file can be seen as **Supplementary Video S2** (EGK-X to HH3+). Scale bar = 700 µm.

flow velocity reported by these groups varied significantly in extra-embryonic vasculature regions imaged across different embryos at HH13 (0.3–2 mm/s) depending on vessel diameters and proximity to major blood vessels (Ghaffari et al., 2015). These studies employ an elaborate setup to keep the embryo growing atop its yolk in a Petri dish covered with albumen. The egg-in-cube system will make it much easier to microinject, track, and quantify the velocity of beads in circulation using potentially all possible types of imaging modalities.

Imaging Cardiac Neural Crest Cell Migration Using Photoconversion of the Dendra2 Protein Using Transgenic Embryos in the Cube

Cardiac neural crest (CNC) cells are pluripotent stem cells that give rise to the pharyngeal arch arteries and cardiac outflow tract (Le Lièvre & Le Douarin, 1975; Kirby et al., 1983). They arise from the dorsal neural tube region between the otic vesicle and the 3rd somite at HH11 and migrate laterally towards the ventral surface of the embryo until at HH17 when they start to appear in the precardiac wall, in regions circumscribing the pharyngeal ectomesenchyme and the anterior cardinal vein (Kirby et al., 1985; Hutson & Kirby, 2007). Confocal imaging of neural crest cell migration was achieved in ovo by (Kulesa et al., 2000; Kulesa & Fraser, 2000). This in ovo imaging technique enabled a long time of observation and had the advantage of keeping the embryo with its proper orientations and tissue tension intact.

To demonstrate the use of the egg-in-cube system for imaging cardiac neural crest migration in embryos atop yolk, we photoconverted Dendra2 expressing cells in the neural tube between the otic placode and the 3rd somite of an HH10 [Tg(hUbC:H2B-Cerulean-2A-Dendra2)] quail embryo mounted in the cube using the 405 nm laser. The 780LSM upright confocal microscope was used to photoconvert and image the migration of cardiac neural crest cells over time. Images were acquired before and after photoconversion to confirm the extent of the Dendra2 photoconversion in the region of interest. Representative images in (**Figure 4A**) show the photoconverted region of interest. Photoconverted cardiac neural crest cells start migrating out of the neural tube and are positioned laterally closer to the somites at 7 h into the time-lapse (**Figure 4B**), **Supplementary Video S4**). The orthogonal slice view aids in the visualization of the neural crest migration and calculating the distance covered by the photoconverted CNC cells on either side of the neural tube (**Supplementary Figures S4A, B**). When we quantified the number of photoconverted cells at t

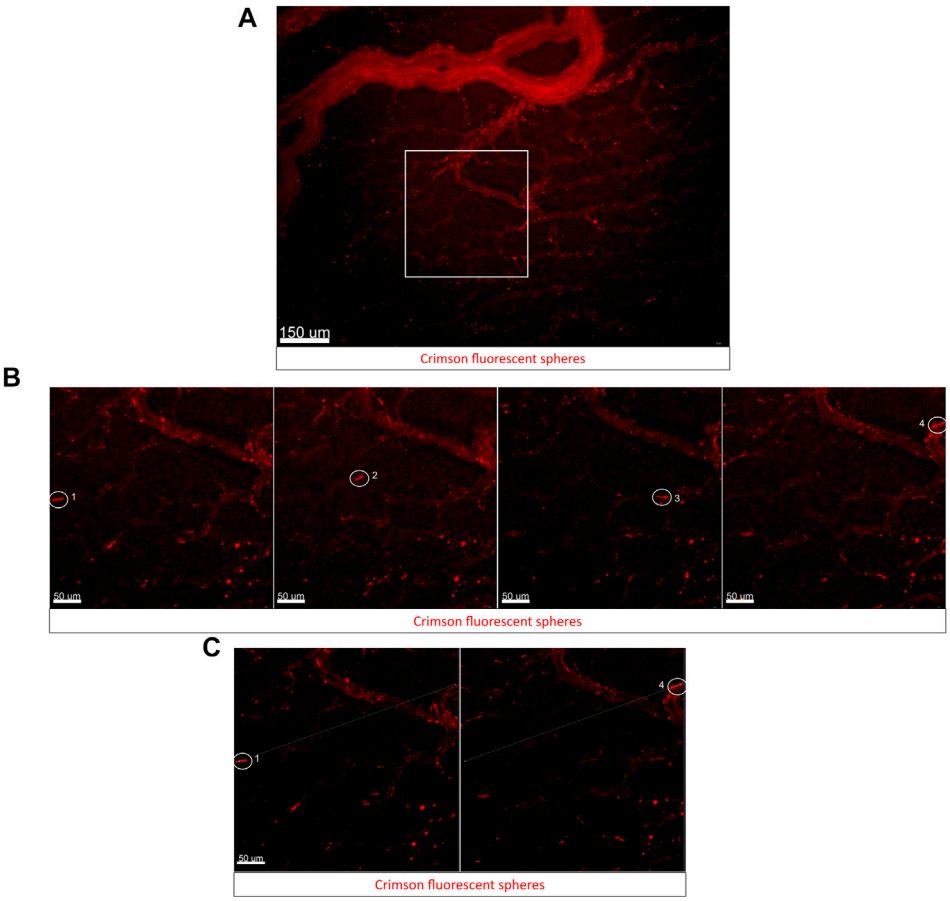


FIGURE 3 | Using fluorescent bead injections to study blood flow dynamics in embryos using the cube on an upright fluorescent stereoscope. **(A)** Captured frame from live imaging of an E3 wild-type quail embryo in the cube. The embryo was microinjected with a small bolus of fluorescent microspheres/beads in the lateral left vitelline vein, the cube was covered with the high transparency membrane and mounted into the custom incubator for imaging. The images shown here are acquired using the upright Olympus stereomicroscope taken from the dorsal aspect of the embryo in the cube at 50 ms intervals. Panel **(A)** shows the representative region of interest (ROI) used for bead tracking analysis. **(B)** Captured frames from the ROI (white square) from **(A)**. The encircled bead moves through the small capillary and is tracked through its path in the vessel here. Representative images from four time frames 50 ms, 450 ms, 900 ms, and 1500 ms from the start of the time-lapse show the bead moving through the vessel (labeled 1–4 in order of frame sequence). **(C)** The first frame and last frame are shown in **(B)** and are used for tracking the displacement of the microsphere through the circulation (Measurement tool in Imaris). The original video file can be seen as **Supplementary Video S3A** [original time-lapse of region shown in Panel 3A] and **Supplementary Video S3B** [Zoomed in time-lapse of region shown in Panel 3B].

= 0 h and t = 7 h, the number of cells had increased 2.5 fold (334 cells in the neural tube at t = 0 h, 890 cells total in the neural tube + CNCs at t = 7 h, **Supplementary Figure S4C**). This increase in number represents a faithful transmission of photoconverted Dendra2 fluorescent protein through successive cell divisions between cells in the neural tube marking a subset of migrating CNCs. The egg-in-cube system enables us to image the migration of cardiac neural crest cells and it potentially can be used for *in vivo* manipulations of signaling pathways targeting this migratory behavior to further understand the role of these cells in embryo development.

Imaging Cell Movements in the Early Blastoderm

We were also curious about how embryo development proceeds if we flipped the egg-in-cube so that cell movements could be imaged on inverted microscopes. In the case of an inverted confocal microscope, the gap between the embryo and the transparency membrane shrinks to the presence of a thin layer of albumen (~100 μm) between the embryo and the membrane surface. When we tested the growth of the embryo in inverted cubes (n = 3 embryos in cubes) and incubated for 48 h, embryos developed marginally slower (by 3–4 h as compared to the expected stage), probably due to an unnatural yolk weight on the developing embryo (data not shown here). However, these embryos were morphologically normal for the period of development observed. In contrast, embryos from egg incubated controls developed normally.

Considering our observations from the embryo growth experiments above, we demonstrate the confocal time-lapse imaging of the first 24 h of avian embryo development using an EGK-X [Tg(hUbC.membrane.EGFP)] quail embryo in the egg-in-cube system. The embryo in the cube is inverted and placed in the on-

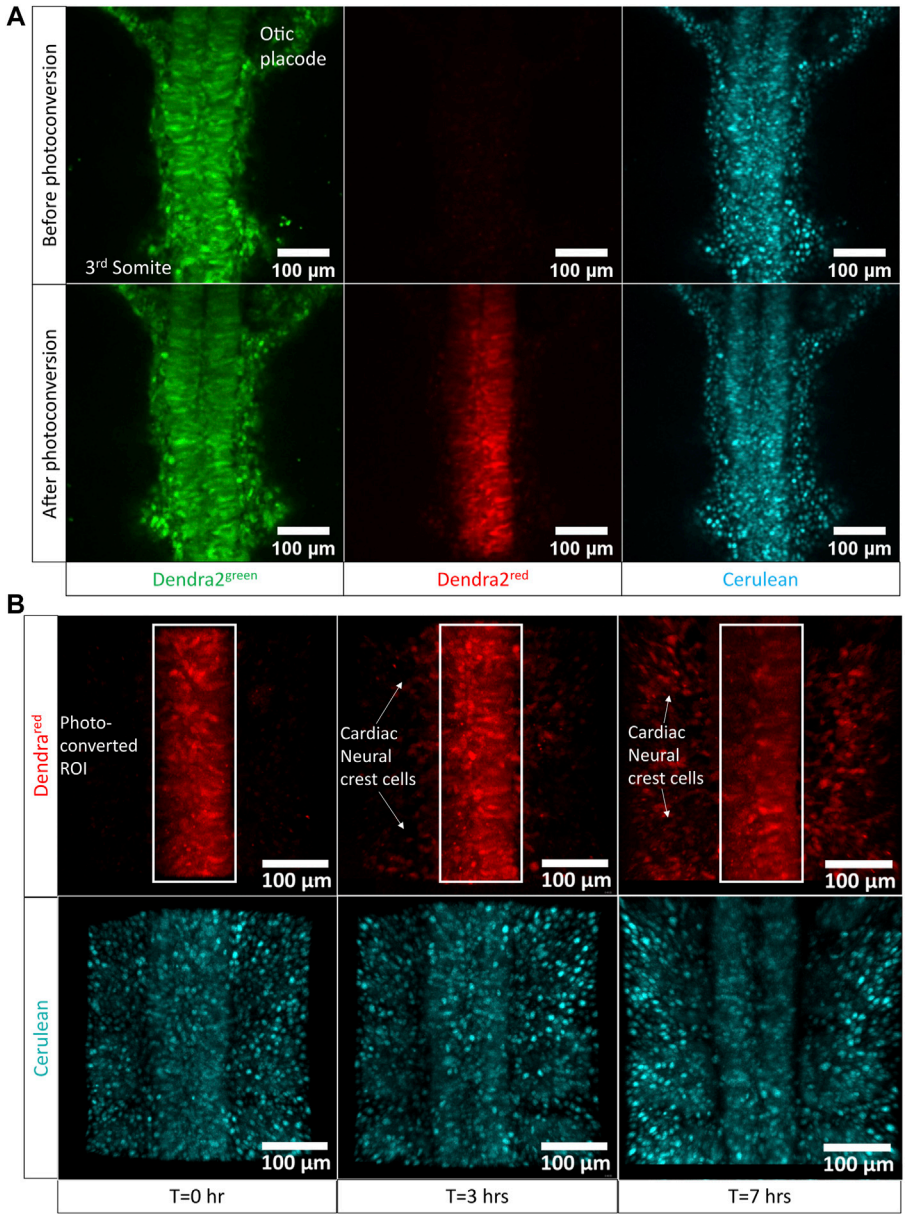


FIGURE 4 | Cardiac neural crest cell migration imaged by photoconversion of an HH10 [Tg(hUbC:H2B-Cerulean-2A-Dendra2)] embryo using the egg-in-cube system. **(A)** Confocal images of the native green form of Dendra2 (referred to as Dendra2^{green} here), photoconverted red form of Dendra2, and Cerulean channels in an HH10 [Tg(hUbC:H2B-Cerulean-2A-Dendra2)] embryo before and after photoconversion (PC). The neural tube cells between the otic placode and the 3rd somite expressing the Dendra2^{green} are photoconverted using the 405 nm UV laser to induce the activated red form of Dendra2 (referred to as Dendra2^{red} here) expression. The Dendra2 green fluorescence intensity decreases with its photoconversion into its red form whereas the fluorescent intensity of the Cerulean channel remains unchanged. **(B)** Confocal images of the photoconverted red form of Dendra and Cerulean channels at different time points were sampled from the time-lapse data at 0 h, 3 h, and 7 h acquired using the upright confocal microscope. Photoconverted cardiac neural crest cells migrate laterally out of the neural tube towards the somites. The original time-lapse files can be seen in **Supplementary Video S5**.

stage incubator of a Zeiss LSM780 inverted confocal microscope for dynamic imaging. Cells in the lateral posterior edges of the EGK-X embryo start to move towards the midline to initiate the polonaise-like movements in the epiblast at 30 min (**Figure 5**, **Supplementary Video S5A**). These movements give rise to the prospective primitive streak as a small triangular structure at 9 h (HH2) and elongate to half its final length by 12 h into the time-lapse (~HH3+). During these early events, the blastoderm expands not only in the X-Y dimension but also elongates in the Z dimension along its natural crescent-shaped curvature on the yolk, which also becomes apparent (**Figure 5B**, **Supplementary Video S5B**). We used the orthogonal section view in Imaris to quantify the change in the length and curvature of the blastoderm in the first ~10 h of development. The blastoderm varies from being a flat disc-like shape at EGK-X: 3.3 mm

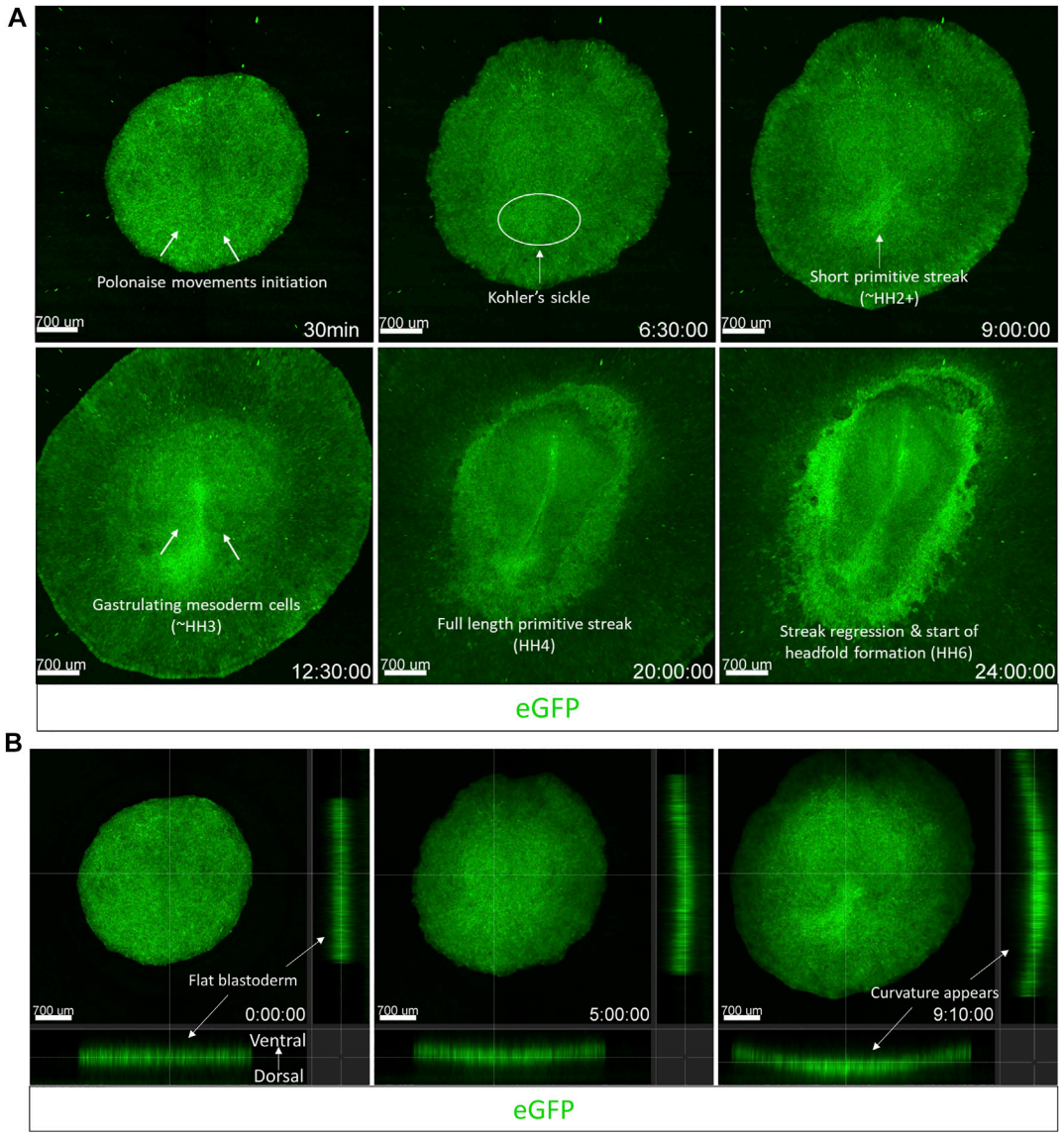


FIGURE 5 | The egg-in-cube system enables dynamic imaging of early embryonic development. Captured frames from live confocal microscope imaging of an EGK-X [Tg(hUbC.membrane.EGFP)] quail embryo. The 5x images, with ×0.6 optical zoom, are maximum intensity projections of 11 optical slices (100 µm each) taken at 10 min intervals taken from the dorsal aspect of the embryo on the inverted confocal microscope. **(A)** Frames are shown at different time points highlighting key features of early avian development through the first 24 h of development. **(B)** Frames shown at different time points highlight the expansion of the blastodisc in 3D following its natural curvature on the yolk. Time-lapse images were acquired using the Zen 2011 (black) software, the acquisition was halted momentarily to readjust the focus and resumed several times. Acquired images were converted to a maximum intensity projection and stitched together in time to present the development as a continuous time-lapse. Images shown in **(B)** were taken from the first 9.1 h of development in 4D and presented as an orthogonal slice view using Imaris. The video files can be seen in **Supplementary Video S5A** (Panel 5A) and **Supplementary Video S5B** (Panel 5B).

length to a curved structure with an arc length of 6.5 mm at HH2+ (**Supplementary Figure S5A**). As gastrulation continues, prospective mesoderm cells can be seen arising in the form of mesodermal wings from either side of the elongating primitive streak (visible by 12.5 h HH3). By 20 h (HH4), the mesodermal wings have reached the anterior halves on both sides of the germinal crescent as the streak reaches its full length. Hensen's node becomes apparent by 22 h, and the streak begins to regress as the head fold begins to appear by 24 h (HH6). The embryo gets brighter with development due to the accumulation of EGFP in the cell membranes. Using the egg-in-cube system, we report the imaging of the first 24 h of avian embryo development starting from EGK-X.

Imaging the Migration of the Hypoblast Using Dendra2 Photoconversion

To image the anterior migration of the hypoblast, we used stage HH2 (10 h of incubation) embryos from the [Tg(hUbC:H2B-

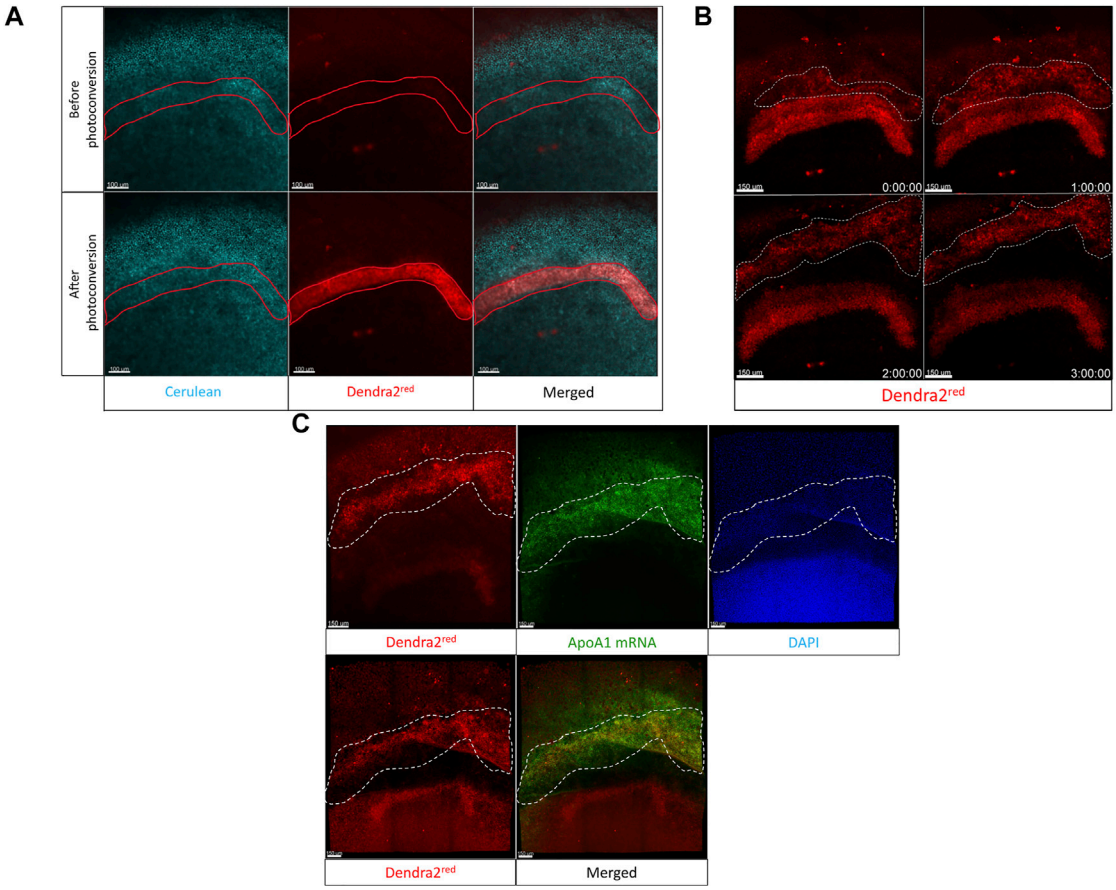


FIGURE 6 | The Egg-in-cube system enables imaging of hypoblast migration. **(A)** Panels show the putative hypoblast region of interest in the anterior germinal crescent of an HH2 [Tg(hUbC:H2B-Cerulean-2A-Dendra2)] quail embryo before and after photoconversion. The 405 nm UV laser is used to photoconvert a region of interest using the "Regions" and "bleaching" function in Zen Black. Nuclei are labeled by Cerulean fluorescent protein (Cyan). Photoconverted cells are labeled by Dendra2^{red} fluorescent protein (red). Scale: 100 μm. **(B)** The migration of cells (red cells, region bounded by a white dotted line) in the photoconverted region of interest is shown over different time points (see **Supplementary Video S6**). Captured frames from live confocal microscope imaging from the dorsal side of an HH2 [Tg(hUbC:H2B-Cerulean-2A-Dendra2)] quail embryo. Images are maximum intensity projections of ×20 tiled images (24 slices of 6 μm) with ×0.6 optical zoom acquired every 10 min. Scale: 100 μm. **(C)** The last frame of the time-lapse (Dendra2^{red}, upper left panel, 3 h) is compared to the Dendra2^{red} channel (Lower left panel) to demonstrate the position of the hypoblast layer (region bounded by a white dotted line in all panels) before and after ApoA1 HCR staining. Images are maximum intensity projections of ×20 confocal Z stacks (20 slices of 8 mm) from whole-mount *in situ* hybridization for hypoblast specific ApoA1 (green) staining within an HH2 [Tg(hUbC:H2B-Cerulean-2A-Dendra2)]. DAPI stain in the nuclei is shown as blue. Scale: 150 μm.

Cerulean-2A-Dendra2)] quail line and transferred multiple embryos to cubes. Embryos were screened for the brightest Cerulean-Dendra2 fluorescence, and the selected embryo was then transferred to the stage of the inverted confocal microscope. We photoconverted cells (Huss et al., 2019) in an arc-shaped region of interest (ROI) in the putative migrating hypoblast front (**Figure 6A**). This photoconverted cell layer was then tracked over time. The time-lapse analysis showed that a group of cells migrating like a collective sheet separated from this photoconverted ROI (white dotted selection, **Figure 6B**, 1 h–3 h, **Supplementary Video S6**) and migrated towards the anterior germinal crescent of the embryo. An *in situ* hybridization staining with probes against ApoA1 mRNA immediately following the time-lapse, allowed us to validate the identity of the tracked cells as hypoblast cells from the photoconverted region (white arrows, **Figure 6C**, top panel, left-right). Using the egg-in-cube system along with the photoconvertible [Tg(hUbC:H2B-Cerulean-2A-Dendra2)] quail embryos, we were able to track the anterior migration of hypoblast cells.

Electroporation of the Quail Neural Tube "in Cubo"

To demonstrate the increased accessibility of the developing embryo in the cube, we electroporate mRNA encoding membrane-GFP into an HH10-11 [Tg(PGK1:H2B-mCherry)] embryo unilaterally into the dorsal neural tube/midbrain-hindbrain region and image the resulting electroporation after 2 h of incubation at 37°C. Images from two embryos are presented in **Figure 7**, the top panel shows the left side of the midbrain region of one of the H2B-mCherry embryos (red nuclei) electroporated with membrane eGFP mRNA (green) and the bottom panel shows a region caudal to the midbrain

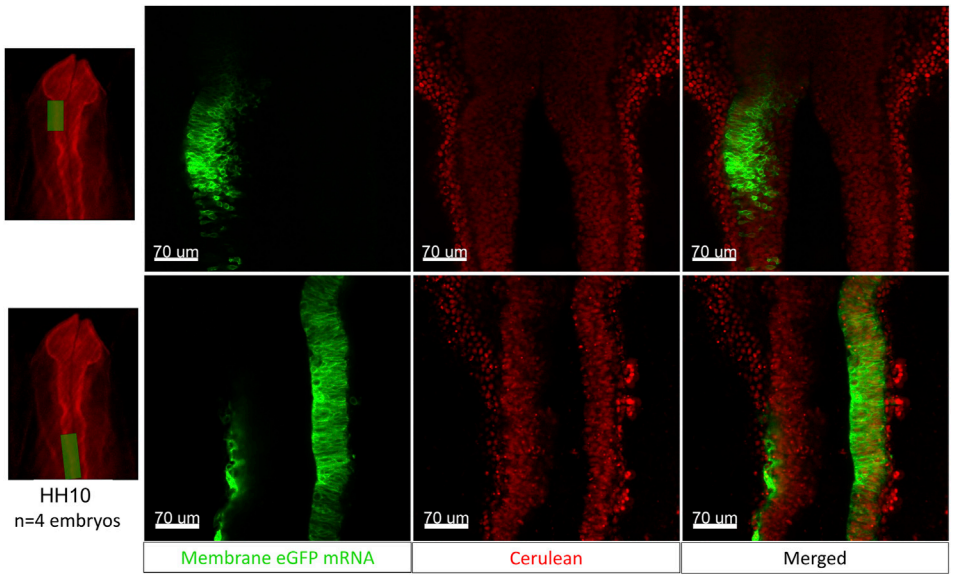


FIGURE 7 | The egg-in-cube system enables electroporation of the neural tube with fluorescent mRNA. The images in the top panel show a representative midbrain region of the [Tg(PGK1-H2B-mCherry)] embryo (red nuclei) electroporated with membrane-eGFP mRNA (green) and the bottom panel shows a region caudal to the midbrain (closer to the hindbrain and otic placode). These images show a small proportion of H2B-mCherry expressing cells colocalizing with membrane eGFP post electroporation. Scale: 70 µm.

from the second electroporated embryo (closer to the hindbrain and otic placode).

Optimizing the Use of the Cube on Different Microscopes

We observed that quick lateral movements cause the embryo to slosh about both in cubo and in ovo. This is not unexpected since the yolk is surrounded by thick and thin liquid albumen layers. Embryo displacement can cause serious image registration issues if the movements occur during time-lapse imaging that might prevent accurate post hoc cell tracking and analysis. For instance, motorized stages are often used during time-lapse imaging experiments to cover and collect larger regions of interest (ROI) with high spatial resolution. Indeed we noticed during post hoc analysis that stage movements on some microscopes induced embryo displacements that we did not notice by eye during the experiments.

The stage speed on our Zeiss 780LSM inverted confocal microscope in the lab was seen to be slower than the Zeiss 780 LSM upright version at a different location. This slower stage speed helped to prevent the sloshing of the yolk in the X-Y direction and leads to seamless stitching between tiles after the time-lapse images have been acquired. To circumvent this problem on other microscopes, we came up with the heat fixation method for stabilizing the yolk. Using a hot soldering iron, we can denature the yolk in contact with the PDMS membranes to bind it to the four sides of the cube and make it more resistant to sloshing even at faster stage speeds.

Another essential consideration for the choice of imaging modality for any egg-in-cube experiment is the developmental stage of the embryo. The embryo in the cube is more stable and resistant to yolk rotation in the first 24 h of development, and hence either an inverted or upright microscope can be used for imaging in these stages. As the embryo grows in 3D and becomes heavier, yolk rotation becomes a significant factor, and an upright imaging microscope is better suited for imaging post 24 h of incubation.

It is well-known that egg composition can vary in weight significantly with different factors like breed, flock age, strain, and even within the flock breed (Friese, 1923; Scott & Warren, 1941; Tolman & Yao, 1960; Fletcher et al., 1981; Hussein et al., 1993). This variability in egg component weight, specifically the size of egg yolk causes some of the embryos with a smaller yolk volume to lose contact with one or more surfaces of the cube when mounting the embryo into the cube for imaging and makes it prone to sloshing as described above. To deal with this problem, we added sterilized glass beads into the cube after adding the egg contents to raise the egg yolk and maintain the embryo close to the imaging surface of the cube. Another method that can be applied to raise the height of the embryo, is to add ~1 ml of molten bacto agar before adding the yolk. This bacto agar bed solidifies and essentially reduces the height of the cube temporarily and can be used for eggs with smaller yolk sizes. Another advantage of the bacto-agar method is the flexibility of decreasing the available cube height as desired by the user. Both the sterilized beads and the bacto agar bed, marginally flatten the yolk from its equator, increasing contact with the cube surface and allowing heat fixation for smaller yolks to provide better stability in case of inverted confocal imaging.

CRITICAL OBSERVATIONS FROM THE EGG-IN-CUBE SYSTEM

We have engineered an egg-in-cube system that permits embryogenesis to be dynamically imaged in its native state

atop the yolk. In the experiments shown here, we have used the quail egg-in-cube system to explore different developmental events occurring in avian embryos using different imaging platforms. The egg-in-cube system provides easy access to the developing embryo to carry out a variety of experiments, including tissue transplantation, microinjection, a viral infection of early embryos, electroporation of DNA/RNA or morpholinos into the embryos, and setup dynamic imaging in a short interval of time.

We first imaged the development of the quail embryo from EGK-X until 11.25 days of incubation using the egg-in-cube system, which corresponded to a developmental stage of HH35 (~E8) for the embryo. This is the first report of an ex ovo culture system being able to image the first 8 days of quail embryo development. Several studies in the literature report the effects of hyperoxia on avian embryos at different levels of O_2 supply above the normoxic condition (ranging from 25 to 100% O_2) (Stock and metcalfe, 1984; Metcalfe et al., 1981; Höper and Jahn, 1995; Lourens et al., 2007). Experiments by Stock and Metcalfe. (1984) have described an accelerated embryo growth with chick embryo incubation at 60% O_2 compared to controls incubated in the air (20.9% O_2). We reasoned that we might be able to decrease the lag in embryo development and improve the embryo survival rates observed in the egg-in-cube system using the 60% O_2 condition. Kaplan Meier's Survival curve analysis showed that the embryos incubated in 60% O_2 had a significantly higher chance of surviving longer with a median survival time of 14 days. This system, in its current form, can achieve a 50% embryo survival rate up to 14 days of incubation in the cube initiated from EGK-X (8/15 embryos, **Figure 1B**) at 60% O_2 with minimal interference to the system during the culture period. The lag in embryo growth when compared to an in ovo control also reduces from ~3 days (Air incubation, 20.9% O_2, **Figure 1A**) to ~24 h (60% O_2, **Supplementary Figure S6A**). Additional characterization of the embryo culture conditions with a hyperoxic incubation environment will be needed to further minimize the embryo development lag and enable the hatching of embryos using the egg-in-cube system. This system proves to be a powerful tool to understand the development of avian embryos through long-term culture and longitudinal imaging.

We demonstrate that the egg-in-cube system with a custom incubator can be used for embryo culture and dynamic imaging of the fluorescent quail embryo starting from the egg-laying stage (EGK-X). Though a modified ex ovo New culture has been successfully used to image the development of the chick embryo from EGK-XII (2 h of incubation) (Voiculescu et al., 2007), the cube system makes it possible to study the cell movements in the freshly laid avian blastoderm. Also, when the cube is used in an upright imaging modality, we can recapitulate the proper tissue tension and mechanical forces that occur in the embryo during its development in the egg.

During the first few hours of incubation, the primary hypoblast arises from the upper epiblast layer by the process of polyingression (Vakaet, 1962, 1970). This primary hypoblast is carried by the anterior migrating endoblast and spreads anterio-laterally, giving rise to the developing extra-embryonic (yolk sac) endoderm (Eyal-Giladi and Kochav, 1976; Sanders et al., 1978). One of the disadvantages of the ex ovo culture system for imaging development of the hypoblast is the difficulty in maintaining the lower layer (hypoblast) of cells intact while isolating the embryo at stages earlier than HH2. Using the fluorescent transgenic [Tg(hUbC:H2B-Cerulean-2A-Dendra2)] quail line in the egg-in-cube system, we photoconvert and track the anterior migration of the putative hypoblast layer. The time-lapse is immediately followed by *in situ* hybridization staining against a specific hypoblast marker ApoA1 to confirm the identity of the tracked cells as the hypoblast. The egg-in-cube system along with photoconvertible fluorescent transgenic quail embryos opens up the avenues to better understand the process of hypoblast polyingression and migration dynamically in living embryos.

Along with the formation of the hypoblast, primordial germ cells are specified in the epiblast layer and also undergo delamination to meet the anterior migrating hypoblast and reach the extra-embryonic space by HH4 (Swift, 1914; Eyal-Giladi et al., 1981). The specification of primordial germ cells has only been studied using static snapshots over development. With the combined power of the egg-in-cube system and fluorescent transgenic quail, it will become easier to image these different developmental events using multiphoton microscopy, which would be technically challenging to accomplish by ex ovo culture.

The in ovo electroporation technique for chick embryos has been developed as an essential method to introduce plasmids/viral vectors for gain or loss of function studies in development (Muramatsu et al., 1997; Funahashi et al., 1999). In this work, we demonstrate the electroporation of the quail neural tube with *in vitro* transcribed membrane-GFP mRNA followed by a short 2-hour embryo incubation and imaging of the transfected embryo. The egg-in-cube system may also be useful for dynamic imaging of fluorescent protein maturation, and translocation into targeted organelles post electroporation *in vivo*. In our previous study (Tran et al., 2019), we have shown that when we electroporate ex ovo cultured HH5 quail embryos, mRNA encoded fluorescent proteins (FPs) are expressed within 22 min of electroporation with 75% efficiency as compared to DNA encoded FPs (~3–6 h post electroporation with 25% efficiency). This technique of "in cubo" electroporation makes it easier to transfect cells in the avian embryo with mRNA encoded fluorescent proteins, dominant-negative expressing constructs & introduce knockdown reagents, followed by "in cubo" culture. The egg-in-cube system will enable dynamic/static microscopy of the transfected embryos to observe the phenotype caused by the perturbation.

In the first 3 days of incubation, the avian embryos can easily be manipulated by a "window" in the eggshell and re-incubated until the desired stage for observation. This property of avian embryos has led to the development of several classical techniques for surgical manipulations, tracing cell migration by DiI injections (Kulesa & Fraser, 2000), tissue transplantation/ablation studies (Le Douarin, 1973; Goldstein, 2006; Lwigale & Schneider, 2008) for studying morphogenesis and cell fate. The egg-in-cube system makes the embryo accessible to all these manipulation techniques and enables the direct observation of its effects on the embryo through static/

dynamic imaging. We have shown that the cube is easily amenable to the insertion of surgical tools and manipulation of blood vessels and tissues in the chick system. We have also used the cube as a platform to study angiogenesis and induction of blood vessels in the chick CAM (Huang et al., 2017). This system will also make it convenient to study the function of lipid uptake/metabolism by the introduction of fluorescent lipid tracers or chemical inhibitors through yolk injection in the cube. One of the few disadvantages of this culture system for dynamic imaging is the inability to directly access the ventral side of the avian embryo as it remains in yolk for the first few days of development.

In this study, we have demonstrated the use of the egg-in-cube platform using the simple phone camera, an upright fluorescent stereoscope, and the upright/inverted confocal microscopes for imaging quail embryo development. The cube can potentially also be used on any other imaging platform in the upright/inverted modalities like the light sheet microscope, with minor stage modifications. We have adapted the egg-in-cube system developed for the chick embryo in Huang et al. (2015) to the quail embryo here. The concept of the cube can be extended easily to other avian species to empower research in these model systems, potentially making it valuable to other oviparous model systems too.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Southern California Institutional Animal Care and Use Committee (Protocol # 11944-CR001) and the Children's Hospital Los Angeles Institutional Animal Care and Use Committee (Protocol # 351-16).

AUTHOR CONTRIBUTIONS

RL, TK, and MD conceived and designed the study. MD collected the data, carried out statistical analysis, and drafted the manuscript. JL participated in the data collection. SR provided critical inputs and expertise on dynamic confocal imaging of embryos. YS and SF provided critical inputs in the experimental design and execution of the studies. TK and RL participated in the data collection and revised the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Incubation Temperature and Lighting: Effect on Embryonic Development, Post-Hatch Growth, and Adaptive Response

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During incubation, the content of the egg is converted into a chick. This process is controlled by incubation conditions, which must meet the requirements of the chick embryo to obtain the best chick quality and maximum hatchability. Incubation temperature and light are the two main factors influencing embryo development and post-hatch performance. Because chicken embryos are poikilothermic, embryo metabolic development relies on the incubation temperature, which influences the use of egg nutrients and embryo development. Incubation temperature ranging between 37 and 38°C (typically 37.5–37.8°C) optimizes hatchability. However, the temperature inside the egg called "embryo temperature" is not equal to the incubator air temperature. Moreover, embryo temperature is not constant, depending on the balance between embryonic heat production and heat transfer between the eggshell and its environment. Recently, many studies have been conducted on eggshell and/or incubation temperature to meet the needs of the embryo and to understand the embryonic requirements. Numerous studies have also demonstrated that cyclic increases in incubation temperature during the critical period of incubation could induce adaptive responses and increase the thermotolerance of chickens without affecting hatchability. Although the commercial incubation procedure does not have a constant lighting component, light during incubation can modify embryo development, physiology, and post-hatch behavior indicated by lowering stress responses and fearful behavior and improving spatial abilities and cognitive functions of chicken. Light-induced changes may be attributed to hemispheric lateralization and the entrainment of circadian rhythms in the embryo before the hatching. There is also evidence that light affects embryonic melatonin rhythms associated with body temperature regulation. The authors' preliminary findings suggest that combining light and cyclic higher eggshell temperatures during incubation increases pineal aralkylamine N-acetyltransferase, which is a rate-limiting enzyme for melatonin hormone production. Therefore, combining light and thermal manipulation during the incubation could be a new approach to improve the resistance of broilers to heat stress. This review aims to provide an overview of studies investigating temperature and light manipulations to improve embryonic development, post-hatch growth, and adaptive stress response in chickens.

Keywords: chick embryo, incubation, temperature, light, adaptive response, growth

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INTRODUCTION

Over the past 10 years, global chicken meat production has increased from 83 million tons in 2012 to 100.5 million in 2020. The projected global chicken meat production is 102 million tons in 2021 (Shahbandeh, 2021). Optimizing production is important to meet this demand for chicken meat. Incubation conditions seem the first step to maximizing meat production because commercial fast-growing broiler chickens spend 33%–38% of their total life period in the incubator environment. This period is 20.5%–26.5% for slow-growing broilers, which is aimed to reach the slaughter weight between 58–81 days. It is well known that incubation conditions, such as temperature, humidity, gas exchange, turning, and light have been shown to affect embryo growth and development. Among these factors, incubation temperature is the most critical. Overall, the related literature review shows that lighting during incubation is not crucial as other incubation factors do. Therefore, under commercial conditions, embryos are incubated in the dark. However, studies have shown that variations in incubation temperature and light affect hatchability, chick quality, and post-hatching growth. Indeed, variations in temperature and light occur during the natural incubation conditions; the mother hen leaves the nest an average of 8.2 times (ranging from 2 to 13) for food and water (Archer and Mench, 2014), or the hen rises to turn the eggs as a response to embryos' call (Rogers, 1995). This environmental stimulus during the embryonic period may be useful to prepare the embryo for post-hatching life. In other words, these changes in temperature and light would contribute to the capacity of the chicken to combat the post-hatching environment through adaptive response. An adaptive response mechanism has three stages: 1) detecting threats, 2) responding physiologically or genetically to present threats, and 3) preparing the body for future threats. Epigenetic changes during embryogenesis in chicken embryos are the main mechanism for adaptation to the post-hatching environment. Therefore, incubation temperature and light may be a tool to improve the performance and adaptive response of birds. The present review will address the main effects of 1) incubation temperature and lighting on embryonic development and broiler growth, and 2) manipulations in temperature and lighting as a tool to improve the adaptive response of chicks to postnatal rearing conditions.

Temperature During Incubation

During the first 18 days of incubation, the chicken embryos show poikilothermic reaction; i.e., they are susceptible to changes in incubation temperature. A rise in incubation temperature increases embryonic heat production and eggshell temperature while lowered incubation temperature decreases heat production and eggshell temperature (Romjin et al., 1955; Whittow and Tazawa, 1991). Many researchers have tried to determine the optimum temperature for embryonic development. The minimum temperature for blastoderm development is reported as 27°C which does not result in embryonic differentiation to the point of vascular system establishment (Funk and Biellier, 1944). Early research suggests a 38.8–39.4°C and 39.4–40°C for the first and second half of the incubation, respectively, based on imitating the incubation temperature under natural conditions (Eycleshymer, 1907). Later, the requirement of an embryo with regards to optimum temperature is reported as 37.5–37.7°C, indicating temperatures higher than 38°C and lower than 37°C reduce hatchability (Ramanoff, 1936; Barott, 1937). Further studies state that incubator temperature should be fixed between 37.5 and 37.8°C from 1 to 18 days and between 36.1 and 37.2°C during the hatching period (Decuypere et al., 2001). In all these studies, suggested incubation temperatures are based on incubator temperature, however, the eggshell temperature (EST) which is a reflection of embryo temperature, is slightly different than the incubator temperature, being approximately 1–1.5°C higher than the surrounding air temperature at the egg level, due to the metabolic rate of the embryo (Tazawa and Rahn, 1987; Leksrisompong et al., 2007).

It is questionable whether these values are still valid for existing breeds and commercial strains, as the above-mentioned temperatures are based on studies from many years ago. Because selection for rapid growth and high body weight for broiler lines over the last 60 years has resulted in an increase in metabolic rate including the embryonic stage and affected embryonic development pattern (Druyan 2010), which might require reconsideration of incubation temperatures.

Effects of Incubation Temperature on Embryo Development and Post-hatching Growth

In recent years, relatively slight variations (1–1.5°C below or above) from optimum temperature have been extensively used to examine its effect on embryonic development (Lourens et al., 2005; Yalcin and Siegel, 2003; Yalcin et al., 2007; Oviedo-Rondon et al., 2008; van der Pol et al., 2014) (**Tables 1**, **2**). Since the yolk is a primary nutrient source for a developing embryo, the utilization of yolk nutrients is one of the main factors affecting embryo development (Vieira and Moran, 1998; Wagt et al., 2020). High or low incubation temperatures from embryonic day (ED) 1 throughout hatch lower yolk sac utilization and absorption, affect the utilization of egg yolk nutrients by changing the expression of the yolk sac tissue genes, which are responsible for the absorption, and digestion of yolk lipids and peptides, glycogenesis, and gluconeogenesis, and in turn affect chick quality (Dayan et al., 2020). It was shown that both the high and low incubation temperatures (1.5°C below or above 37.8°C) decreased the expression of PEPT1 (a gene involved in oligopeptides uptake), ApoA1 (a gene involved in lipid metabolism), and altered glycogen stores of yolk sac tissue toward the hatch (Dayan et al., 2020).

It is known that embryos are more sensitive to moderate changes in EST during the early development period. Low (36–36.6°C) ESTs during the first week of incubation reduce hatchability, saleable chick number, and increase chick weight compared to control (37.5°C) (Joseph et al., 2006). Hamidu et al. (2018) demonstrated that low (36–36.5°C) EST when applied from ED15 to hatch, increased external pipping time and delayed

TABLE 1 | Higher than optimum incubation temperature: Effects on the embryo's physiology and post-hatch growth.

References	Strain	Embryonic age (days) and temperature	Differences in compared to control (37.5–37.8°C) incubation				
			Incubation duration	Hatchability	Yolk sac/yolk-free chick W[a]/chick W/ chick length	Morphological and physiological effects	Post-hatch growth performance
Lourens et al. (2005)	ND[b]	D15-21/39.9°C (EST)[c]	ND	↓	= /↓/ND/↑	ND	ND
Joseph et al. (2006)	Ross 308	D 18–21/ 39.5°C (EST)	ND	↑	=/↓/↓/ =	ND	= BW[e] at 21 and 42 days = FCR[f], CW[g], BrstW[h]
Oznurlu et al. (2010)	Ross 308	D 11–21/38.8°C (IT)[d]	ND	ND	ND/ND/ND/ND	↓ Thymus and bursa fabricius W	ND
Werner et al. (2010)	Cobb 500	D 7–10/38.5°C (IT)	ND	=	ND/ND/ = /ND	ND	= BW, FCR at 36 d
Willemsen et al. (2010)	Cobb 500	D 16–18.5/ 40.6°C (IT)	=	↑	= /ND/↓/ND	↓ Blood T3 &T4, triglycerides	ND
Janisch et al. (2015)	Cobb 500	D 7–10 or 10–13/ 38.8°C (IT)	ND	ND	ND/ND/ND/ND	ND	= BrstW, LegW
Maatjens, et al. (2016)	Ross 308	ED15-21/ 38.9°C (EST	↓	=	↑/↓/ND/ND	= Liver and spleen W ↓ Stomach and intestine W	ND
Almeida et al. (2016)	Cobb 500	D13-21/39°C (IT)	=	↑	↑/=/ND/ND	= Blood cholesterol = Adipocytes size	ND
Lin et al. (2017)	Ross 708	D0-5/38.1°C (IT)			↓/ = /↓/	ND	= BW at 49 days
Molenaar et al. (2010)	Hybro	D7-19/38.9°C (EST)	↓	↓	↓/ = /ND/↓	ND	ND
Morita et al. (2016)	Cobb 500	D 13–21/39°C (IT)	=	ND	= / = / = /ND	↑ Blood vessel number	ND
Wijnen et al. (2020a)	Ross 308	D7-14/38.9°C (EST)	↓	ND	=/=/=/↑	↓Blood glucose	= BW, FCR, CW at 40 days
Avşar et al. (2022)	Ross 308	D 0–3/38.6°C (EST)	↓	=	=/=/= /ND	ND	= BW, FCR at d7
		D 3–6/38.6°C (EST)	↓	↓	=/=/= /ND	ND	= BW, FCR at d7
		D 0–6/38.6°C (EST)	↓	↓	=/=/=/ND	ND	↓BW = FCR at 7 d

[a]*W: weight.*
[b]*ND: not determined.*
[c]*EST: eggshell temperature.*
[d]*IT: incubation temperature.*
[e]*BW: body weight.*
[f]*FCR: feed conversion ratio.*
[g]*CW: carcass weight.*
[h]*BrstW: breast weight.*
[i]*DL: drip loss.*

hatching compared to control (37.5°C). Higher incubation temperatures than optimum affect embryo development in the opposite direction. Lin et al. (2017) observed that a high EST of 38.1°C during the first 5 days of incubation decreased day-old chick weight and residual yolk sac weight, increased chick length, which is one of the indicators of chick quality (Lin et al., 2017). However, another study showed a 38.6°C EST during the first 6 days of incubation did not affect chick and yolk sac weight but reduced hatchability (Avsar et al., 2022). High EST (38.9°C) applied at the second week of incubation, might accelerate embryo development, shorten the hatch window, and decrease incubation duration without affecting day-old chick weight (Wijnen et al., 2020a). Molenaar et al. (2010) and Maatjens et al. (2016) observed that when high EST is applied in the last week of incubation, chick quality decreases by shortening the time for the embryo to use yolk nutrients, reducing protein productivity and reducing egg yolk-free body mass. These differences in the literature suggest that 1) small changes in eggshell temperature may affect the absorption of egg nutrients, 2) sensitivity to temperatures lower or higher than the optimum incubation temperature also depends on the embryonic developmental stage, and 3) incubation temperature influences the metabolism and physiology of the embryo.

Indeed, lengthening or shortening of the incubation duration by lowered or increased incubation temperature, respectively, is a reflection of changes in the metabolic rate, physiological processes, and their regulation (Black and Burggren, 2004; Molenaar et al., 2010; Maatjens et al., 2016; Hamidu et al., 2018). During the late stages of embryogenesis where most of the physiological systems are under rapid maturation, continuous low temperatures decrease plasma triiodothyronine (T_3) at the external pipping stage, plasma triglycerides, and non-esterified fatty acids (NEFA) at hatch, and increase plasma corticosterone level at hatch (Willemsen et al., 2010). All these changes link to a slower metabolic rate and prolonged internal pipping, which is

TABLE 2 | Lower than optimum incubation temperature: Effects on the embryo's physiology and post-hatch growth.

References	Strain	Temperature treatment during incubation (Day/ temperature)	Differences in compared to control (37.5–37.8°C) incubation				
			Incubation duration	Hatchability	Yolk sac/yolk free chick W[a]/Chick W/chick length	Morphological and physiological effects	Post-hatch growth performance
Lourens et al. (2005)	ND[b]	D1-7/36.7°C (EST)[c]	ND	=	=/↓/ND/↓	ND	ND
Joseph et al. (2006)	Ross 308	D 0–10/36.6°C (EST)	ND	↓	↑/=/↑/↓	ND	↓ BW[e] at 21 and = FCR, CW ↑Abdominal fat
Willemsen et al. (2010)	Cobb 500	D 16–18.5/34.6°C (IT)[d]	↑	↑	↓/ND/ = /ND	↓ Blood T3 and triglycerides	ND
Janisch et al. (2015)	Cobb 500	D 7–10 or 10–13/ 36.8°C (IT)	ND	ND	ND/ND/ND/ND	ND	= BW, CW, DL
Maatjens, et al. (2016)	Ross 308	ED15-21/35.6°C (EST)	↑	=	=/↑/ND/ND	↑ Liver W = Spleen and intestine W ↓Stomach W	ND
		ED15-21/36.7°C (EST)	↑	=	=/↑/ND/ND	↑ Liver W =Spleen, stomach and intestine W	ND
Almeida et al. (2016)	Cobb 500	D13-21/36°C (IT)	↑	=	=/=/ND/ND	↑Blood cholesterol ↓Adipocytes size	ND
Hamidu et al. (2018)	Ross 708	D15-21/36°C (IT)	↑	ND	=/=/=/=	↓ O2 consumption at E16 and 17	ND
		D15-21/36.5°C (IT)	=	ND	= / = / = / =	↓ O2 consumption at E16 and 17	ND
	Ross 308	D15-21/36°C (IT)	=	ND	=/=/=/=	=O2 consumption E15–21	ND
		D15-21/36.5°C (IT)	=	ND	=/=/=/=	=O2 consumption E15–21	ND
Morita et al. (2016)	Cobb 500	D 13–21/36°C (IT)	↑	ND	=/=/=/ND	↓ Blood vessel number, T_3 and growth hormone	ND
Wijnen et al., (2020)	Ross 308	D15-21/36.7°C (EST)	↑	ND	=/=/=/↓	↑ Blood glucose, heart and stomach W	= FCR at 40 d

[a]*W: weight.*
[b]*ND: not determined.*
[c]*EST: eggshell temperature.*
[d]*IT: incubation temperature.*
[e]*BW: body weight.*
[f]*FCR: feed conversion ratio.*
[g]*CW: carcass weight.*
[h]*Brst: breast.*
[i]*DL: drip loss.*

mainly due to the energy needed to grow being directed into existing body tissue and a longer hatching process under low temperatures (Yalcin et al., 2012a). The slower metabolic rate of the embryo leads to higher O_2 availability relative to metabolic rate and an increase in liver glycogen level (Willemsen et al., 2010; Yalcin et al., 2012a; Morita et al., 2016). The prolonged incubation duration together with the higher liver glycogen content and increased yolk sac use promotes embryonic development, resulting in heavier yolk-free body weight at hatch. Therefore, a lower EST than 37.5°C after ED14 may be considered to be beneficial for embryonic development (Maatjens et al., 2016). On the contrary, continuous high incubation temperatures accelerate the growth rate and increase the metabolic rate, oxygen, and energy demand of embryos. The accelerated growth increases glucose oxidation and depletes glycogen stores thus amino acids are used as metabolic fuel leading to lower protein retention (Maatjen et al., 2016). This results in lowered yolk-free body weight, organ weights, chick quality, and retarded lymphoid organs development. Furthermore, limited O_2 availability in the last stages of incubation triggers the chicks to hatch (Mortola and Labbe, 2005; Piestun et al., 2009; Molenaar et al., 2013; Maatjen et al., 2016; Nangsuay et al., 2016). Contradictory to these results, higher hatchability, similar chick weight, and no differences in the morphology of the small intestine and nutrient transporters gene expression in chicks from optimum and high temperature were reported (Barri et al., 2011; de Barros Moreira Filho et al., 2015).

Aside from affecting embryonic development, incubation temperature also affects broiler growth. It is reported that early incubation temperatures changing from 36.5 to 39°C for a short period (2–3 days) have no effect on slaughter weight and feed conversion ratio however may affect muscle and bone development (Werner et al., 2010; Oksbjerg et al., 2019). Contradictory to these results, Janisch et al. (2015) reported that a 1–1.5°C higher EST than the optimum for 3 days during the first week of embryogenesis positively influenced body weight, but reduced meat quality while low temperature during the first

10 days of incubation reduced body and breast weights (Joseph et al., 2006). Several other reports showed that low or high (36.7 or 38.4–39°C, respectively) EST during the last week of embryogenesis lowered broiler growth rate, body weight, and feed intake at slaughter age, and increased mortality rate (Hulet et al., 2007; Sözcü and İpek, 2015; Wijnen et al., 2020b). It has been also shown that high incubation temperature (38–39°C) reduces tibia weight and increases relative asymmetry of leg weights in broiler chicks and turkey poults affecting growth plate maturation, which may have implications on tibial dyschondroplasia incidence (Yalcin et al., 2007; Oviedo-Rondon et al., 2008). Recently Muir and Groves (2018) concluded that slow start incubation from 37.2°C at ED1 reaching 37.8°C EST at ED13 resulted in higher hatchability with more late-hatched chicks and higher bone ash.

The effect of incubation temperature on the immune system has received limited attention. Nevertheless, studies on incubation temperature's effect on the post-hatch immune system are inconsistent. No interference was found in the humoral immune response against NDV and IBDV vaccine in broilers incubated at 36.8 or 38.8°C from ED14 of incubation to hatch (Santin et al., 2003). High temperature (38.7°C) from ED10 to hatch was shown to delay thymus and *bursa of Fabricius* development (Oznurlu et al., 2010). Contradictory to this finding, de Barros Moreira Filho (2015) showed that high temperature from 10 days of incubation to hatch induced resistance to *Salmonella* infection and improved intestinal integrity and mucus production whereas low temperatures at the same period resulted in a smaller villus: crypt ratio. More recently, it has been reported that low incubation temperature (36.7°C) during the last week of incubation would negatively affect immune organ development and later-life resilience to necrotic enteritis (Wijnen et al., 2020b, 2021). However, the biological mechanism that underlies the association between incubation temperature and immunity is not entirely clear. A deeper understanding of the mechanism will be needed to understand incubation temperature's impact on immunity, meriting further studies to clarify this issue.

These discrepancies in the literature on the effects of hatching temperature could explain that temperature interacts with other factors such as humidity, egg position, eggshell quality, egg weight, and breeder age (Yalcin et al., 2005; Hulet et al., 2007). Breeder age influence eggshell temperature, which can be explained by the higher heat production of embryos from heavier eggs. Comparing 30 and 60 weeks old breeders, Gualhanone et al. (2012) showed that day-old chick weight interacted with incubation temperature when eggs were exposed to 36.8, 37.8 and 38.8°C incubation temperatures. It should be noted that the developmental differences between the strains are also important in response to incubation temperature (**Tables 1**, **2**). Differences between Ross and Cobb embryos have been demonstrated under the same incubation conditions (Druyan 2010; Tona et al., 2010). Therefore, the response of strains to early or late incubation temperature manipulations should be investigated under the same experimental conditions in further studies.

Incubation Temperature and Post-Hatching Adaptive Response

There is evidence that changes in temperature during embryonic development play an important role in the adaptive response of physiological systems such as thermoregulation (Nichelmann et al., 2001; Tzschentke and Batsa, 2002) and stress response (Loyau et al., 2015). It is a hypothesis that exposing embryos to short-term cyclic or constant lower or higher than optimum results in an epigenetic memory making the chicks more resistant to lower or higher ambient temperatures, respectively, during the postnatal period. This memory is linked to changes in hormonal profiles and alterations in gene activity and expression that control the thermoregulatory system (Nichelmann et al., 2001; Tzschentke and Batsa, 2002). In agreement with this hypothesis, higher or lower incubation temperatures during the critical periods of embryonic development may have a training effect and result in changes in the preoptic area of the anterior hypothalamus neurons (PO/AH) thereby controlling their temperature sensitivity (Tzschentke and Batsa, 2002). Neurons in the PO/AH lead to the secretion of corticotropin-releasing factor (CRF) and thyrotropin-releasing hormone (TRH) from the hypothalamus. CRF stimulates the synthesis and the secretion of ACTH, which in turn leads to the secretion of corticosterone from the adrenal. CRF also plays a role in the activation of TRH, which stimulates the release of thyroid-stimulating hormone (TSH) secretion. TSH, in turn, results in increased thyroid hormones, mainly T_4 (thyroxine), synthesis then circulating T_4 is converted into the biologically active form of T_3 (Decuypere and Kühn, 1988). Because the hypothalamus-pituitary-thyroid (HPT) and hypothalamus-pituitary-adrenal (HPA) axes play an important role in the adaptation of an individual's thermoregulation (Bohler et al., 2021; Ruuskanen et al., 2021), changes in incubation temperature during the development of these axes may improve the thermotolerance of birds and cause long-term effects on the responsiveness of these axes (Nichelmann and Tzschentke, 2003; Piestun et al., 2008). The available evidence clearly shows that the changes in incubation temperature have to be linked to the development of the HPT and HPA axes, which are formed between ED10.5 and 11.5 and ED14 and 15 days, respectively (de Groef et al., 2008).

Therefore, studies have addressed the timing of alterations in temperature, temperature level to which the embryo is exposed, and duration of exposure (Yahav et al., 2004a; Collin et al., 2005; Yalcin et al., 2005; Yalcin et al., 2008a; Piestun et al., 2008). The period from ED10 to ED16 of embryogenesis has been used to test the effect of daily 3–24 h, 1–2°C increases or decreases from an incubation temperature on thermotolerance and postnatal heat or cold stress response, respectively. The first studies were conducted to test the potential of adaptive body functions of day-old chicks after embryonic heat treatments. The studies revealed the potential of temperatures of 38.6 and 39.6°C for 3–12 h/d between ED10 to 18 had no effect on hatchability, decreased plasma T_3 and corticosterone concentrations, oxygen consumption, heat production, and body temperature of day-old chicks (Yahav et al., 2004a; Yalcin et al., 2008a; Piestun et al., 2008; Tona et al., 2008; Piestun et al., 2009). These changes

obtained in day-old chicks could be accepted as an indication of learning and long-lasting cell memory of broiler chickens (Yahav and Tzschentke, 2006; Yalcin et al., 2008b; Halle and Tzschentke, 2011). Indeed, embryonic heat-treated broilers show a lower body temperature, T_3, and corticosterone levels when expose to post-natal chronic or acute heat stress indicating an improvement in heat tolerance and adaptive stress response linking to prenatal plasticity in the HPT and HPA axes (Yahav et al., 2004b; Yalcin et al., 2008b). The reduced body temperature and T_3 under heat challenge lead to a reduction in metabolic rate, which, in turn, lowers susceptibility during heat exposure. On the other hand, Collin et al. (2007) reported that a 39.5°C for 3 h/d during early (ED8 to 10) and late (ED16 to 18) embryogenesis failed to improve long-term thermotolerance in chickens at 6 weeks indicating that the timing and duration of incubation temperature manipulation are critical parameters to set adaptive response.

The effect of temperature manipulation during the embryogenesis on post-hatch adaptive stress response may be explained by mRNA expression of genes involving stress response, thermoregulatory and metabolic programming (Loyau et al., 2016). Comparing thermally manipulated and control chicks under heat stress conditions showed that 759 genes were differently expressed (Loyau et al., 2016). Heat shock proteins (Hsp) involve in the biochemical response of cells to cope with heat stress and maintain the integrity of structural proteins. Al-Zghoul. (2018) found an increase in Hsp70 expression in heat-stressed chickens exposed to incubation temperatures of 38.5–39.5°C for 18 h from ED12 to 18 of embryogenesis. Because heat stress causes inhibition of protein synthesis, an increase in Hsp70 mRNA expression in heat-stressed chickens would be associated with an improvement in protecting cell integrity in chickens (Al-Zghoul et al., 2013). It was also shown that genes coding components of the CRF signaling pathway change their expression in the hypothalamus in thermally manipulated chicks providing evidence that thermal manipulation involves epigenetic changes in the hypothalamus (David et al., 2019).

The studies also attempt to evaluate lower incubation temperature and its effect on adaptive response. Shinder et al. (2011) reported that at ED18 and 19, a short (30 min) cold exposure (15°C) did not affect hatchability, but improved growth rate and reduced ascites incidence. Similarly, 6 h/d low temperature (36.6°C) from ED10 to 18 induced an increase in body weight and a better cold tolerance in broilers when subsequently subjected to cold and resulted in long-term changes in antioxidant defenses and energy metabolism in broilers (Aksit et al., 2013; Loyau et al., 2014). Alterations in antioxidant and fatty acid profiles in brain and liver tissues of embryos and day-old chicks were found at an incubation temperature of 36.6°C, 6 h/d from ED10 to 18. These changes may be accepted as coordinated adaptive reactions of chicks (Yalcin et al., 2012b).

Several studies have also shown that high temperatures promote muscle development and myoblast proliferation in day-old chicks. Piestun et al. (2009) showed that during late-term embryogenesis (ED16 to 18), high incubation temperature (39.5°C for 3 or 6 h daily) increased muscle insulin-like growth factor I (IGF-I), which enhanced muscle cell proliferation and differentiation, and myofibers diameter. However, as the study was ended at post-hatch d 13, if muscle development was affected at slaughter age is unknown. Our recent finding (Yalcin et al., 2021) suggested that exposing Ross308 and Cobb embryos to 38.8°C between ED10 and 14 resulted in heavier body weight and higher insulin-like factor-1 (IGF-I) expression, and larger fiber area in breast muscle of broiler chickens at slaughter age. However, breast muscle properties of strains, i.e., expression of vascular endothelial growth factor-A and myogenin, carcass part yields, pH_{24}, and water holding capacity of strains responded differently to temperature manipulation (Yalcin et al., 2021). This result supports further evidence that the effect of thermal manipulation is strongly related to the strain.

In conclusion, the studies showed that the effect of incubation temperature during embryonic development is undoubtedly crucial for adaptive stress response. Incubation temperature could program the chick to construct traits in adaptation to a post-hatching temperature environment. This response may be explained by the imprinted epigenetic changes in the hypothalamus that trigger a response when the chickens are again exposed to high or low temperatures (David et al., 2019). The studies tell us that interaction among timing, duration, and temperature shape embryo development and adaptive stress response. Indeed, Wilsterman et al. (2015) showed that exposure of embryos to slightly higher temperatures either early, late, or whole incubation period had an impact on the pattern of glucocorticoid release, however, the specific response of chicks and broilers varied with the timing. Currently, it is unclear how the sensitive period and temperature interact with the other environmental factors in the incubator and maternal factors (strain, breeder age, egg composition, and egg quality). Nevertheless, during the second half of incubation, the embryo may be more sensitive to temperature manipulation signals to have a long-lasting post-hatch effect. Further studies are needed to understand the effect of epigenetic modifications during embryonic development, their molecular mechanisms underlying these changes, and their long-term effects.

Light During Incubation

Light controls many of the physiological and behavioral processes including growth, reproduction, and migration in birds. Recent studies have had evidence showing that exposure of developing embryos to light could play an important role in hatching performance and embryonic growth rate, reduce stress responses to the post-hatch environment, and ultimately affect the performance, behavior, and welfare of birds. Therefore, providing light during incubation has been introduced as a practice to improve hatching and post-hatching performance, and adaptive response to the post-hatch environment (Shafey and Al-Mohsen, 2002; Özkan et al., 2012a,b; Rozenboim et al., 2004; Archer and Mench, 2013; Archer, 2017; Tainika and Bayraktar, 2021).

The effect of light on these processes is mediated through the detection of light by photoreceptors located in the retina of the eye and extraretinal photoreceptors in the pineal, and

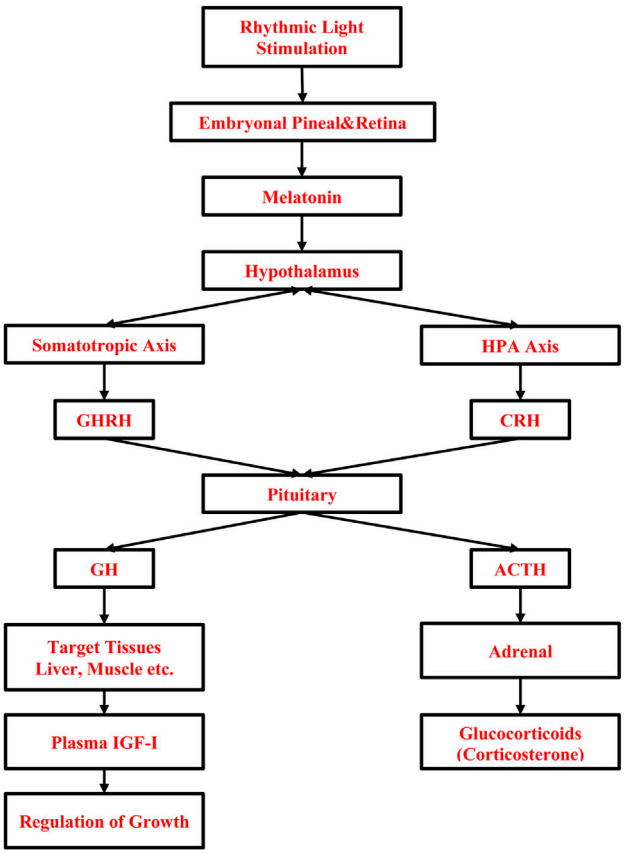

FIGURE 1 | Role of light stimulation during incubation on embryonic muscle growth and stress. HPA, Hypothalamus-Pituitary-Adrenal; GHRH, Growth Hormone-Releasing Hormone; CRH, Corticotrophin Releasing-Hormone; ACTH, Adrenocorticotropic Hormone; TRH, Thyrotropin-Releasing Hormone; GH, Growth Hormone; IGF-1, Insulin-like Growth Factor-1.

hypothalamus (Kumar, 2015; Kuenzel et al., 2015). Embryonic eye development starts with differentiation in the neurons of optic vesicles on ED2, the connection between retinal ganglion cells and optic chiasma is completed by ED4 (Rogers, 1995). By ED14, embryonic eye growth completes, light-sensing proteins (opsins) in photoreceptor cells, which respond to different wavelengths of the light spectrum (Perez et al., 2019) are expressed (Bruhn and Cepko, 1996). The visual system of chicken embryos becomes functional at ED18 (Rogers, 1995). Besides the embryonic visual system, the formation of primary structures of pineal on ED3 is important because it is the main secretory organ for the melatonin hormone, which is one of the candidates to explain the effect of lighted incubation on embryonic development and to maintain entrainment of rhythmic biological functions of embryos by photoperiod (Hill et al., 2004; Zeman et al., 2004). It has been shown that embryonic pineal melatonin rhythm is established between ED16-18 (Zeman et al., 1992; 2004; Csernus et al., 2007). It is accepted that the rhythmic production of melatonin, which is produced in vertebrates at high concentrations during the night and at low concentrations during the day, is transferred to the endocrine system (Cassone et al., 2009). The effect of light on embryonic growth might be also related to the activation of the HPT and HPA coinciding with the rhythmic melatonin hormone production (Tong et al., 2018) and the somatotropic axis, i.e., growth hormone (GH), IGF-1 (Bai et al., 2019; Wang et al., 2017; Zhang et al., 2014). The role of light stimulation during incubation on the somatotropic and stress systems is given in **Figure 1**. Light-induced muscle proliferation is linked to blood IGF-1 (Halevy et al., 2006), which is mainly secreted by the liver in association with melatonin (Wang et al., 2014) and upregulation of genes involving myogenic regulatory factors (MYF5, MYOD), paired box 7, which maintain adult skeletal satellite cell integrity, and muscle-specific regulatory factor 4 through the melatonin hormone (Bai et al., 2019).

While many of the early research reported that photostimulation accelerates embryo development and usually shortens the incubation time in chickens (Siegel et al., 1969; Walter and Voitle, 1972), it has been speculated that the heating effect of light could have been confounded by the effect of light thus observed effects may partly be related to increased embryo temperature (Gold and Kalb, 1976). Therefore, studies considered the confounding effect of heat from light sources and tried to minimize it either by changing the light source from incandescent to light emitted diode (LED) known to have lower heat production, using intermittent lighting (Rozenboim et al., 2004; 2013; Dishon et al., 2017) or photoperiodic lighting schedules with fluorescent lamps instead of continuous lighting (Archer et al., 2009; Özkan et al., 2012a) or combined LED and photoperiod (Archer, 2016, 2017; Van der Pol et al., 2017, 2019; Güz et al., 2021). Many of them have confirmed optimum incubation temperature by measuring eggshell temperature and adjusting incubator temperature accordingly (Rozenboim et al., 2004; Özkan et al., 2012a; Van der Pol et al., 2017, 2019; Güz et al., 2021).

Therefore, photostimulation at early or late periods of embryonic development has been investigated in the studies to see if lighted incubation affected embryo development and hatching performance. However, not only the critical periods but also the duration of photostimulation per day and characteristics of light including intensity, color (wavelength), and color temperature of light are important. In this part of the paper, we review the effect of light provision during incubation on embryo development and post-hatching growth, and the post-hatching adaptive response of chicken, taking into account timing, duration, color, and intensity.

Effects of Light on Embryo Development and Post-Hatching Growth

Chicken embryos can detect color differences. Several studies have been conducted to investigate the effect of light color on embryonic development and post-hatching growth. As compared with blue light and dark incubation conditions, continuous green light during the incubation enhances the post-hatch body weight of male broilers, improves the feed conversion ratio, increases the satellite cell mitotic activity of the pectoral muscle with

upregulation of MyoD, myogenin, and myostatin mRNA expression in late embryos and newly hatched chicks, and muscle growth with no noticeable changes in chemical composition and meat quality characteristics (Zhang et al., 2012, 2014). Providing green light intermittently (light/dark cycles of 15 min) during incubation also increases hypothalamic expression of growth hormone-releasing hormone (GHRH), liver growth hormone receptor (GHR), levels (Dishon et al., 2017). Dishon et al. (2021) compared intermittent green light stimulation throughout the incubation (ED0-21) with different stimulation periods starting from ED15, 16, and 18 of incubation and observed a higher expression of the somatotropic axis genes in all lighting treatments than in dark incubation. They suggested that photostimulation of embryos only last 3 days of incubation would be enough to stimulate the somatotropic axis since photostimulation of embryos from ED18 to hatch resulted in similar expression levels of hypothalamic GHRH, liver GHR, and IGF-1 genes and GH plasma levels to the positive control group (lighted from E0-21). These findings deserve to be investigated further to establish a clear conclusion regarding the critical period for the growth-stimulating effect of green light on broiler embryos.

The pineal gland of the chick embryo shows a selective sensitivity to different wavelengths (color) of the light spectrum. Drozdova et al. (2019) found a higher biosynthesis of pineal melatonin during scotophase under red (632 nm) and white (a peak wavelength of 448 nm) lighting compared to green (517 nm) and blue (463 nm). Further research from the same group showed that red-lighted incubation resulted in higher body weights in broiler chicks during the post-hatch rapid growth phase (from 18 to 21 days) compared to blue light (Drozdova et al., 2021). Although there is not much information regarding the effect of red light on the somatotropic axis, increased growth of chicks incubated under red light may be related to the early entrainment of melatonin rhythms. Not only the light color but also the color temperature of polychromatic light would be important. The cool white LED (5,000 K) containing more blue wavelength could improve weight gain and reduce stress and fear responses of broilers as compared to warm white LED (2,700 K) (Archer, 2018). However, it was shown that incubation in warm and cold white light did not significantly influence embryonic melatonin biosynthesis in the pineal, T_3, T_4, corticosterone hormone levels in the blood and immune system-related genes, presenilin-1, and avian betadefensin1, in the duodenum and *bursa Fabricius* (Drozdova et al., 2020). The authors concluded that selective effects of distinct wavelengths on embryonic and post-embryonic development might be more profound than the effects of change in the color temperature of polychromatic light.

Limited research is available regarding the effect of lighting during the incubation on bone growth and leg health, and the results are not consistent. An improvement in leg health of broilers was found using a 16L:8D (van der Pol et al., 2017) or 12L:12D (van der Pol et al., 2019) at 500 lux white LED lighting compared to continuous light or dark incubation conditions. However, in a recent study, Güz et al. (2021) did not find any significant effect of green LED light on tibia bone parameters when they used a 16L:8D photoschedule. It is necessary to clearly reveal whether the light color will affect bone development.

The intensity of light has also been the subject of interest. In a recent study, Yu et al. (2018) reported that 50 lux intensity using green LED light (16L:8D) increased chick length, weight, hatchability, testosterone, and T_4 hormone levels and reduced hatching time, i.e., an earlier peak of 12 h, in newly hatched chicks compared to 150 and 300 lux. However, the transmission of light into the eggs significantly varies with the level of pigmentation and the conductance of eggshells (Shafey et al., 2002). Shafey et al. (2002) compared the spectral absorption rate of pigmented and non-pigmented eggshells over the wavelength range between 200 to 1,100 nm. Brown pigmented eggs had a max absorption rate of 99.96% for the near-ultraviolet region (wavelength ≤380 nm) of the light spectrum, which was higher than the absorption rate of 99.88% for long wavelengths, about 1,075 nm at the near-infrared region. Shafey et al. (2005) also compared two high intensities changing between 1,430–2,080 and 900–1,380 lux using a green fluorescent light source and different pigmentation levels of brown eggshells. They reported that higher intensity resulted in higher embryo mortality and decreased hatchability in light pigmented brown eggshells while there was no negative effect for dark drown eggshells (Shafey et al., 2005). Yu et al. (2016) confirmed that eggshell pigmentation and the region of the eggshell determine the transmission of visible wavelength (380–780 nm) into the egg. These findings support the hypothesis that the evolution of eggshell pigmentation for selective transmission of different wavelengths into eggs is based on preventing the negative effects of ultraviolet and infrared light (Maurer et al., 2011). Maurer et al. (2015) supplied further evidence from wild birds and concluded "avian eggshell properties, including eggshell structure and pigmentation, which are consistent with an evolutionary pressure to both enhance and protect embryo development". Huth and Archer (2015) investigated the effect of eggshell pigmentation on the spectrum of light filtered by eggshell. They reported that the spectrum of light filtered by white eggshells was quite similar to unfiltered light; however brown eggshells produced a redder spectrum as evidence of higher transmission of long wavelengths into eggs. Recently Güz et al. (2021) observed that a green LED light source with a peak light spectrum of 522 nm yielded a 536 nm peak in the light spectrum after passing through the eggshell of broiler breeder eggs showing that pigmented eggshell may change wavelength reach into the egg. It is clear that the wavelength and intensity of light that reach the embryo are limited by eggshell properties. It should also be considered that the lux unit is based on human spectral sensitivity. Bird's spectral sensitivity to short (400–480) and long (580–700) wavelengths is higher than humans due to their additional cone type of photoreceptor cells (Lewis and Morris, 2006). Therefore, eggshell properties, wavelength, and intensity of light both outside and inside of the egg should be taken into account in future studies to have a finely tuned lighting program for broiler embryos.

Since melatonin modulates immune responses in poultry (Markowska et al., 2017), the effect of lighted incubation on broiler immunity has also been studied. Both 12L:12D or 24L:0D white LED (5,000 K) lighting with 250 lux intensity significantly increases NDV titers and spleen weights of 35 days old Hubbard broilers compared to dark incubation (Yameen et al., 2020). A stronger humoral immune response to keyhole limpet hemocyanin (KLH), which is a non-pathogenic protein antigen and often used to assess humoral immunity, was reported in broilers compared to dark incubation when eggs were exposed to a 12L:12D white fluorescent light (Archer and Mench, 2013). Drozdova et al. (2020) further investigated if the color temperature of white light affects the immune system and did not find any significant effect on the expression of genes involved in innate immune responses in the duodenum and bursa of Fabricius. However, distinct effects of different wavelengths have been reported. A study comparing red, blue, and white light and the dark incubation conditions reveals that red-lighted incubation (12L:12D) increased total IgG concentration in broiler chicks on d 14 post-hatch and bursa weights of 35 days old male broilers as compared to blue light (Li et al., 2021a). A 12L:12D red-lighting also upregulates the expression of avian β-defensin-1 (AvBD-1) in the duodenum of day-old chicks and IL-6 in two-week-old broiler chickens compare with blue LED light (Kankova et al., 2022). AvBD-1 is an important peptide for innate immunity in birds (Cuperus et al., 2013; Zhang et al., 2016), and IL-6 acts as both pro-inflammatory and anti-inflammatory, stimulating intestinal epithelial proliferation and repair (Fasina et al., 2008). Thus this finding might be interesting to further research. In a recent study effect of green light (250 lux, 24L:0D) was investigated by Ibrahim et al. (2021). They supplied promising information regarding the activation of the Nuclear factor kappa-light-chain-enhancer of activated B-cell (NF-kB) and sirtuin signaling pathways in 18 days of embryos and acute phase response signaling (APR) pathway in 7 days old chicks in comparison to dark incubation (Ibrahim et al., 2021). NF-kB pathways control the regulation of various biological responses including immune responses and inflammation (Dabek et al., 2010), sirtuins influence many metabolic, inflammation, and stress responses (Zhao et al., 2020), and APR is responsible for early defense responses to the stressors (Cray et al., 2009). Thus, activation of all these pathways provides evidence for an improvement in the immune response of birds when the light is provided during incubation. These results suggest that the light source and light wavelength may be responsible for the different effects on the immune response.

The available data presented above show that there is no accepted standard lighting procedure in the incubator until now. It can be concluded that green light is the most effective for stimulating growth and improving muscle development. However, homogenous intensity should be kept with lower intensities inside the incubator. Red light may have a more profound effect on innate immunity while green light may affect the immune and inflammation, and stress response of broiler chicks. However, further research is needed underlying mechanism for early immune programming and interactions between the light source and embryo development.

Incubation Light and Post-hatching Adaptive Response

The effect of light on post-hatch adaptive response has also been evaluated. One of the approaches to how light affects the post-hatch stress response is that light induces changes in lateralized brain functions through asymmetrical development of visual pathways in chickens (Rogers, 1995). The right eye is known to be important in examining and assessment of potential danger (Rogers et al., 2004). The embryo has a position within the egg so light can only affect the embryo's right eye and the development of the left hemisphere of the brain. The left hemisphere of the brain is associated with the control of behavior with focused attention and positive emotions, e.g., specialized for visual discrimination tasks, food-searching, and vocal production and recognition. It is a hypothesis that the role of the left hemisphere in positive cognitive bias may be important in the post-hatch adaptation of broilers to stressful environments (Rogers, 2010). Lighted incubation may lead to behavioral changes *via* lateralized brain functions, i.g., discrimination of non-food material, a more specialized visual perception of fearful stimuli resulting in long-term reductions in fearfulness in broilers (Sui and Rose, 1997; Rogers et al., 2004, Rogers et al., 2007; Dayıoğlu and Özkan, 2012; Rogers, 2012; Archer and Mench, 2017). Thus lateralized birds may habituate more quickly and react less strongly to stressors than non-lateralized birds. This result is confirmed by Chiandetti et al. (2013). They showed that the chicks incubated under the lighted incubation either first or last 3 days of incubation would ignore the barrier, in a test environment, on the way to access the food source compare to chicks incubated in the dark. The authors further suggested that lighting during the first 3 days of incubation where pineal starts to form may result in cerebral lateralization through the molecular changes in the neural system (Chiandetti et al., 2013). This finding supplies evidence that lighted incubation let to a better spatial ability to deal with the different stimuli at the same time such as "finding food and being vigilant to predators" (Rogers et al., 2004). Available research evidence is quite clear and it could be expected that incubation lighting might be a promising tool to decrease the fear and stress responses of birds by affecting the development of visual lateralization in brain functions.

The second approach to how light affects the post-hatch stress response is *via* melatonin, which also acts as a modulator of the stress response by inhibiting the HPA axis (**Figure 1**) thus preventing peripheral elevation of corticosterone hormone (Saito et al., 2005). There are indications that a cyclic light/dark schedule during incubation can modify the chick's post-hatch stress response and may improve the growth and welfare of broilers through better adaptation of birds to a novel environment as compared to dark incubation (Archer et al., 2009; Özkan et al., 2012a, Özkan et al., 2012b; Archer and Mench, 2013). Day-old chicks incubated under 16L:8D using white light during the entire incubation period show lower corticosterone response to 8 h holding at the hatchery compared with dark incubated ones (Özkan et al., 2012a). The effect of lighting on stress response seems long-lasting as reported by Archer and Mench (2013); a 12:L12D lighting schedule results

in a lower corticosterone response of broilers to post-hatch 1 h of crating stress at 3 weeks compared to dark or 1L:23D, 6L:18D lighting (Archer and Mench, 2013). Moreover, 12L:12D incubation is found to reduce asymmetry and heterophil-to-lymphocyte ratio in broilers at slaughter age (Archer et al., 2009; Riaz et al., 2021) as compared to dark incubation. It can be concluded that proving light during the incubation has stress reliving effect on birds through early entrainment of melatonin rhythm that alters the HPA axis and could allow the birds to better adapt to post-hatch stressors (Rasmussen et al., 2003; Özkan et al., 2012a, Özkan et al., 2012b; Archer and Mench, 2013).

In mammals and chickens, melatonin is known to regulate daily and seasonal cycles in physiological systems including the thermoregulatory system (Pevet and Challet, 2011). Although this review aims the literature on broilers, Saarela and Heldmaier (1987) reported that short photoperiod (8L:16D) gives a cue for the thermoregulatory system and increases cold tolerance of quails in natural conditions maintaining body temperatures *via* increased heat production. They used long and short photoperiods to investigate cold tolerance of quails with or without melatonin administration and concluded that cold tolerance of quails is related to melatonin hormone; because daily melatonin administration results in improved cold resistance even under long photoperiod conditions (16L:8D) as evidence of pineal control of cold acclimation in quails. Melatonin administration by feed reduces body temperature (Zeman et al., 2001) and thus has been used as a management tool to combat heat stress in broilers (Gharib et al., 2008). There is not much information regarding the effect of lighted incubation on the body temperature of broiler chicks. However, there may be a regulatory effect of photoperiodic lighting during incubation on the thermoregulatory responses of broiler chicks. Hill et al. (2004) investigated if light cues during embryonic development may entrain the circadian rhythm of body temperatures in chicks. When embryos are exposed to a 12L:12D photostimulation either through ED0-21 or between ED13-15 and ED16-18, a circadian rhythm of the chick's body temperature, which is higher in the morning than in the afternoon, has been recorded during the first 5 days post-hatch. Authors suggested that photoperiodic light cues after ED13 can establish the circadian rhythm of body temperature in chicks. However, they did not note a difference in body temperatures of lighted and dark incubated chicks (Hill et al., 2004). Recently higher and less fluctuating cloacal temperatures at 36 h post-hatch have been observed in chicks incubated under white, red, or blue light as compared to dark incubation suggesting a better thermoregulatory ability (Li et al., 2021a). This finding may associate with a higher feed consumption of birds resulting from a better orientation to feeders due to lateralized brain functions (Rogers et al., 2007; Chiandetti et al., 2013). In any way, lighted incubation may have positive effects on the development of thermoregulation in broiler chicks. It is not known if wavelength would affect embryonic heat production and post-hatch body temperature of broilers. The only information is from Li et al. (2021b) who reported that compared to white, blue light, or darkness, red-light reduced air cell temperatures, measured in the incubator between ED8-18, suggesting red light had a more prominent effect on the energy metabolism of embryos (Li et al., 2021b). The authors speculated that red light may increase embryonic melatonin production, which in turn reduces air cell temperature as compared to white or blue light. Considering that the suppressive effect of white and green light on pineal melatonin production is stronger than red light (Zawilska, et al., 1995) this finding deserves to be further investigated.

Simultaneous Light and Temperature Manipulations

Since melatonin administration is used as a tool to improve thermoregulation of birds at both low and high ambient temperatures due to its anti-stress properties, it would be interesting to examine together the effect of incubation temperature and light on improving the adaptive response of broilers to ambient temperature. Because the effect of light and temperature are mostly together in nature it seems logical to consider both effects with possible interactions. It is known that temperature is an effective zeitgeber in poikilotherms to entrain pineal melatonin rhythm (Underwood and Calaban, 1987). Barret and Takahaski (1995) first showed that a rapid increase in the temperature of the pineal cell culture of chicken significantly reduces pineal melatonin release, as does light. Therefore, it was concluded that light and temperature may eventually have similar effects on the chick pineal circadian clock. Further studies revealed that rhythmic temperature changes in the environment could entrain pineal melatonin production rhythm in broiler embryos, i.e., incubation of broiler breeder eggs on ED19 at a low temperature of 4°C for 1 h during the scotophase resulted in an increased melatonin content in the pineal gland of embryos but not during the photophase (Zeman et al., 2004). In addition, under dark conditions using a daily temperature rhythm of 33°C for 8 h from ED13 to hatch, they suggested that pineal and plasma melatonin was higher during the low-temperature period indicating that embryo pineal melatonin rhythm was shaped by temperature rhythms in the incubation environment (Zeman et al., 2004). Therefore, it would be interesting to examine the effect of lighting in combination with temperature manipulation to elucidate the epigenetic thermal adaptation phenomenon. Earlier studies indicate that a 39°C for 3 h/d between ED16-18 with green LED lighting from ED6 to hatch stimulates the proliferation of myoblasts (Stojanovic et al., 2014; Kanacki et al., 2017). However, there has been no study investigating the effect of both light and temperature applied at the same period of incubation on hatch-related traits, growth performance, and adaptive responses of broilers at post-hatch. Our recent findings suggest that both a 38.5°C EST for 6 h/d between ED11-16 together with a 16L:8D photostimulation increased chick length and liver weight which may be a positive approach towards better chick quality (Shah and Özkan, 2022), increased the resilience of broilers to acute heat stress at slaughter age (Shah, 2021). Furthermore, our study supplied the first evidence that cyclic thermal manipulation could modify melatonin hormone synthesis *via* pineal

aralkylamine N-acetyltransferase (AANAT) expression, which is a rate-limiting enzyme of melatonin (Shah, 2021). Indeed, day-old chicks exposed to cyclic high incubation temperature showed lower pineal AANAT expression but pineal AANAT expression increased at both hatch and slaughter age when lighting combined with temperature manipulation. These results may indicate that melatonin may also have a long-lasting positive role in the development of thermal adaptation to a post-hatching environment. Therefore, we may speculate that thermal manipulation together with a photoperiodic lighting schedule is worthy to study further regarding combining both improving growth and adaptive response to acute high temperature.

CONCLUSION

Our understanding of the effects of incubation conditions has become more important in recent years. This review aims to summarize and discuss studies on the effects of manipulations in incubation temperature and light on embryonic development, post-hatching growth, and adaptive response. Studies can provide a general understanding that temperature and light manipulations in the incubator can have a positive effect on growth and adaptation to the post-hatching environment by reducing the stress response. It seems that broiler embryos are sensitive to constant high temperatures during the second half of the incubation, which can be partly explained by the increased heat production. The increment in embryonic heat production may depend on breeder age/egg weight and/or incubation conditions. Therefore, differences among studies reported here might be due to the maternal effects, i.e., differences in energy demands between embryos from young and old breeders which should be taken into account to optimize hatching conditions. In addition, different commercial broiler strains are used in different studies, which may also affect the results. On the other hand, short-time cyclic high- or low-temperature manipulations during the second half of incubation alter the temperature tolerance capacity of broiler chickens by epigenetic changes affecting the metabolic process, threshold response to temperature, and stress-responsive pathways. It can be concluded that short-term temperature manipulations would modify the response of broiler chickens to post-hatch temperature adaptation. The details of this mechanism on how these changes interact with other incubation conditions remain to be elucidated.

Available information suggests that intermittent lighting with short periods of light and dark stimulates muscle growth both in embryo and broiler chicks post-hatching. Monochromatic green light seems superior to other wavelengths in the stimulation of muscle growth however, the effects on leg health of broilers are contradictory and need further investigation. Recent findings regarding the effects of lighted incubation on the immune system bring us new research questions on the interactions between different wavelengths and critical times for the development of the physiological systems of embryos. However, when considering adaptive responses of broilers to the post-hatching environment including stress, immune, behavioral, physiological, and fear-related responses associated with either entrainment of melatonin rhythm in embryos or development of cerebral lateralization through the visual pathways at late stages and/or early molecular changes during the pineal formation, photoperiodic lighting may be more promising to stimulate adaptive responses of broilers. However, there is still a need to investigate connections between retinal and extraretinal photoreceptors and activation of somatotropic, HPT, and HPA axes before implementation of embryonic lighting programs for broilers on a commercial scale. Further, we may speculate that thermal manipulation together with a photoperiodic lighting schedule is worthy to study regarding combining both improving growth and adaptive responses of broiler chickens to post-hatching temperature challenges.

AUTHOR CONTRIBUTIONS

SY and SÖ contributed to the design of the whole manuscript, the interpretation of the literature, and reviewing before submission for its content. TS helped to draft the manuscript. All authors read and approved the final manuscript.

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Avian Embryonic Culture: A Perspective of *In Ovo* to *Ex Ovo* and *In Vitro* Studies

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The avian embryos growing outside the natural eggshell (*ex ovo*) were observed since the early 19th century, and since then chick embryonic structures have revealed reaching an in-depth view of external and internal anatomy, enabling us to understand conserved vertebrate development. However, the internal environment within an eggshell (*in ovo*) would still be the ideal place to perform various experiments to understand the nature of avian development and to apply other biotechnology techniques. With the advent of genetic manipulation and cell culture techniques, avian embryonic parts were dissected for explant culture to eventually generate expandable cell lines (*in vitro* cell culture). The expansion of embryonic cells allowed us to unravel the transcriptional network for understanding pluripotency and differentiation mechanism in the embryos and in combination with stem cell technology facilitated the applications of avian culture to the next levels in transgenesis and wildlife conservation. In this review, we provide a panoramic view of the relationship among different cultivation platforms from *in ovo* studies to *ex ovo* as well as *in vitro* culture of cell lines with recent advances in the stem cell fields.

Keywords: avian embryo, embryonic development, *ex ovo* cultivation, *in ovo* cultivation, *in vitro* culture, pluripotency

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INTRODUCTION

In mammals, it is infeasible to observe the development of embryos outside the womb from fertilization to birth. Possibly, aves as a mammalian counterpart provide us an excellent model to be able to observe such a thing. Their advantages are low-cost without the need of feeding the embryos, ease of handling the eggs, and easy visualization of the embryonic development (Wolpert, 2004; Stern, 2005). Several studies have observed morphological changes in the development of the embryo within the eggshell by windowing or injecting compounds into the egg to observe changes in hatchability or physiological response in *in ovo* studies, or by removing the embryos from natural eggshells to new surrogate eggshells in semi-shell-less studies; however, there is a limitation in each method (Silver, 1960; Fisher and Schoenwolf, 1983; Andacht et al., 2004). Alternatively, completely removing embryos from their natural eggshells to various types of recently developed artificial vessels for further observation of developmental changes and genetic manipulation is called *ex ovo* studies. To provide in-depth studies into avian embryogenesis, parts of embryos were taken to derive cell lines in *in vitro* culture including embryonic stem cells (ESCs) and primordial germ cells (PGCs), and become an essential tool to understand pluripotency network regulating early development (van de Lavoir et al., 2006; Choi et al., 2010; Aubel and Pain, 2013; Whyte et al., 2015; Altgilbers et al., 2021).

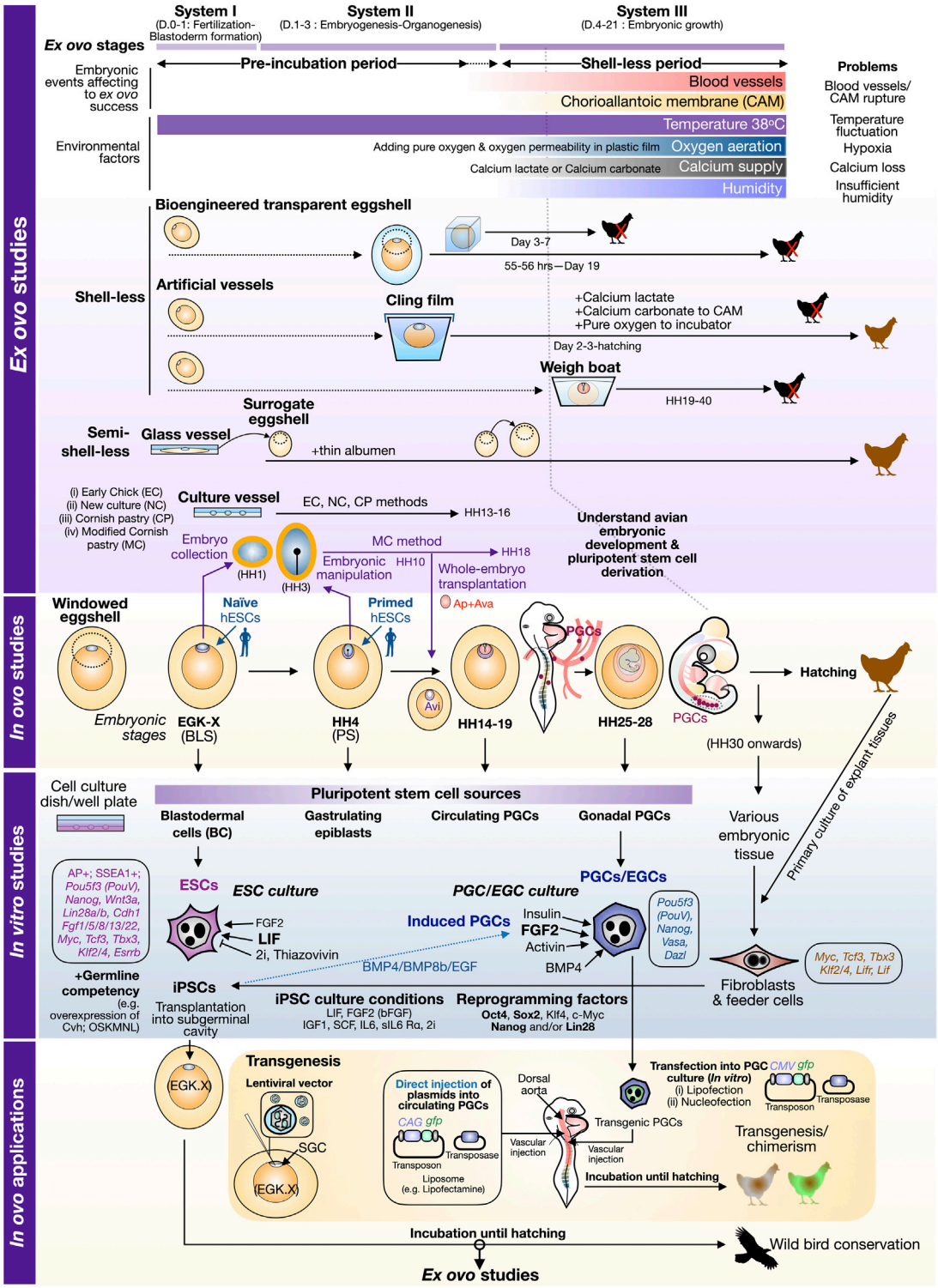

FIGURE 1 | Perspective view of avian embryonic culture with *ex ovo–in ovo* cultivation and *in vitro* cell culture. In the schematic illustration of *ex ovo* studies, avian embryos at early stages can be collected into culture vessels (e.g., Petri dish) and cultured under chemically defined solutions (as listed in **Table 1**). In "shell-less" culture, experiments aimed to nourish embryos without natural eggshells until hatching. Specific types of shell-less cultures are described in **Table 1**. In "semi-shell-less" culture, early embryos are temporarily cultured in the culture vessels and later transferred to surrogate eggshells with the addition of thin albumen. A bigger eggshell is also required at the transition from embryonic system II to III. Environmental factors and potential problems affecting the success of *ex ovo* culture are noted in relation to embryonic systems I–III. *In ovo* culture depicts the early stages (EGK X to HH28, day 0–5) of avian embryos using a chick as a model. The early embryonic stage of a chick

(*Continued*)

FIGURE 1 | embryo at the freshly laid egg and stage HH4 can be incorporated well with naive and primed ESCs, respectively (Akhlaghpour et al., 2021). Dark magenta dots in the embryo stages HH14–19 and HH25–28 represent primordial germ cells (PGCs) circulating in blood vessels and migrating to the genital ridges which later become gonads (Nakamura et al., 2013). The embryos described in *in ovo* are also used to establish pluripotent stem cells in *in vitro* culture. Specific culture conditions to support avian ESCs/EGCs are also noted with a brief list of gene expression profiles from Katayama et al. (2018). Fibroblasts are a common source for cellular reprogramming. The list of reprogramming factors, Oct4 (O), Sox2 (S), Klf4 (K), c-Myc (M), Nanog (N), and Lin28 (L), are listed as well as cytokines and inhibitors used for avian iPSC induction. The bottom panel shows the connection from *in vitro* studies to *in ovo* works, in particular how to produce transgenic birds *via* injection of lentiviral vectors in the subgerminal cavity (SGC), transgenic PGCs, and direct transfection of plasmids into the dorsal aorta of early embryos. Brown chicks indicate embryonic progression into hatching. Black chicks with red cross indicate embryos incapable of hatching or terminated before hatching. Brown-white/brown-green chicks indicate the current success of chimera and transgenic birds. Abbreviation: AP+, positive alkaline phosphatase staining; Ap, area pellucida; Ava, area vasculosa; Avi, area opaca vitellina; D., embryonic day; EGC, embryonic germ cells; EGK, Eyal-Giladi and Kochav (1976) Chick Embryonic Stages; HH, Hamburger and Hamilton (1951) Chick Embryonic Stages; hESCs, human embryonic stem cells; GC, germline competency.

In vitro culture with genetic manipulation also enables us to study the gain/loss of functions and perform cellular reprogramming to produce induced pluripotent stem cells (iPSCs) capable of differentiation into all cell types, similar to ESCs (Dai et al., 2014; Katayama et al., 2018). Thus, in this review, we highlight the recent development of techniques used to study avian embryos in both *in ovo* and *ex ovo* cultivation as well as environmental parameters affecting hatchability and also the roles of *in vitro* culture to understand avian early development; in particular, pluripotency and germline-competent stem cells are also discussed.

IN OVO CULTIVATION: ESSENTIAL TOOL FOR AVIAN DEVELOPMENT INSIGHT

The avian embryo developed within a calcium carbonate-containing eggshell to provide protection and to be part of normal development. This enclosing environment of the eggshell provides sufficient nutrients from the yolk and osmotic pressure from albumen to generate a new live young chick, unlike receiving continuing support within the mother's body in eutherian mammals. The isolated and complete system of avian eggs to progress into full development without further needs of materials from the mother, and high availability with low cost in poultry markets enables us to easily study the development of avian species, in particular the domestic chick (*Gallus gallus domesticus*). In recent years, studies have shown the great advantages of using developing chicken eggs in biotechnology including *in ovo* delivery of biological supplements and vaccination *via* amniotic inoculation or embryo body inoculation (reviewed in Saeed et al., 2019). *In ovo* feeding also plays an important role to improve the health of gastrointestinal tracts and immunity, increasing hatchability and resistance to pathogens (reviewed in Das et al., 2021). From day 4 to 5 post laid egg, the chorioallantoic membrane (CAM), a vascularized membrane responsible for gas exchanges that attaches directly to the eggshell, develops and this *in ovo* CAM structure provides a great platform to study diverse fields (reviewed in Nowak-Sliwinska et al., 2014) including tissue engineering [e.g., biomaterial and biosensors (Borges et al., 2003; Valdes et al., 2003; Azzarello et al., 2007)], testing of various compounds in angiogenesis (Nowak-Sliwinska et al., 2014), drug screening, and tumor growth treatment (Dupertuis et al., 2015).

To address the mechanism of development in avian embryos, chick embryos in *in ovo* cultivation have been used to understand the changes in embryonic morphology over time through the open windowed eggshell (windowing) method (Speksnijder and Ivarie, 2000; Andacht et al., 2004; Korn and Cramer, 2007), **Figure 1**. The key question in development is to understand pluripotency networks orchestrating early development and how these networks are conserved across species, in particular mammalian versus avian models. The timeframe to study early events in pluripotency acquisition [indicated by the presence of *Pou5f3* (*PouV*), *Sox2*, and *Nanog*] in avian species is limited due to the onset of development that has already occurred since intrauterine development (Han et al., 2018) requires the sacrifice of hens. At the freshly laid egg stage, pluripotency has been shown to differ between finch and chick embryos, as the finch blastoderm in the laid egg (EGK V–VIII) expresses more naive-like state genes similar to naïve mouse embryonic stem cells (mESCs) while the chick blastoderm at the laid egg (EGK IX–XI) has primed bias similar to the mouse epiblast stem cells (EpiSC). This suggests that the pluripotency mechanism in different avian species is diverse at the early stage of freshly laid eggs and cannot be relied only on a chick model. A recent study of *in ovo* interspecies chimerism also shows the striking result to highlight the pluripotent state at equivalent points between human versus chick at an early embryonic development in that the EGK X blastodermal cells matched well with naïve human pluripotent stem cells (hPSCs), while the primed hPSCs can incorporate into the gastrulating epiblasts of chick embryos (Akhlaghpour et al., 2021) (**Figure 1**). In addition, CAM is used to understand pluripotency properties in an interspecies manner. Traditionally, the mouse model has been used as a platform for tumor formation from ESCs or induced pluripotent stem cells (iPSCs) established from various species of mammals to ensure the true nature of cells possessing "pluripotency"; the method is called teratoma assay. To replace mouse as an animal model for teratoma assay, CAM in the *in ovo* cultivation was used as a platform for seeding human iPSCs, which can grow on CAM and form a three-germ layer containing tumors within 9 days at 37°C (Weber et al., 2021). Taken together, *in ovo* study is still an essential tool for an ideal environment to unravel the developmental state and can be used to understand

TABLE 1 | Timeline of *ex ovo* techniques developed to understand the embryonic development in avian species.

Ex ovo Technique	Species	Objectives and key benefits	References
Embryonic manipulation at early stages			
Watch-glass	Chick and duck	To cultivate an avian blastoderm on a clot of fowl plasma and embryo extract which remained alive for 2–3 days	Waddington (1932)
Albumen-agar	Chick	To explant the blastoderm on a semi-solid substratum containing clot with more stiffness by agar and diluted albumen	Spratt (1947)
New culture	Chick	To support the explant and expansion of the blastoderm up to 48 h of incubation (appearance of primitive streak and blood circulation)	New, (1955)
Early chick (EC) culture	Chick	To culture the whole embryo using a filter paper carrier	Chapman et al. (2001)
Cornish pasty	Chick and quail	To grow chick and quail embryos from stage 3HH to stage 18HH with normal morphology	Connolly et al. (1995); Nagai et al. (2011)
Semi-shell-less			
Surrogate eggshell	Domestic fowl and turkey	To be able to transfer the cultured embryos into different species	Rowlett and Simkiss (1987)
Shell-less			
Baggie	Chick	To grow the embryos in artificial membranes using polyethylene bags	Elliott and Bennett (1971)
Petri dishes	Chick	To grow the embryos in Petri dishes from the 3rd to the 20th day of incubation	Auerbach et al. (1974)
The plastic wrap/culture tripod	Chick	To be able to access the embryo and its membranes for experiments	Dunn and Boone, (1976)
Beaker	Chick	To observe the embryos from the unincubated stage to the 19th day of incubation	Barnett (1982)
Modified Callebaut's method	Chick and quail	To investigate development events of the single-cell stage fertilized egg taken from the maternal oviduct	Perry (1988)
Polytetrafluoroethylene (PTFE) membrane	Quail	To use a gas-permeable membrane to increase the viability of culture embryos	Kamihira et al. (1998)
Polyurethane (PU) membrane	Chick	To culture whole embryos on hexagonal weigh boats for up to 10 days	Yalcin et al. (2010)
Polymethylpentene (PMP) film	Chick	To establish a simple method for culturing the embryos with high hatchability using a plastic film	Tahara and Obara (2014)
Egg-in-cube (polydimethylsiloxane: PDMS and polycarbonate: PC)	Chick	To generate artificial transparent eggshell with functionalized surface allowing better observation of chick embryo development	Huang et al. (2015)
Bioengineered transparent eggshell (polydimethylsiloxane: PDMS polymer)	Chick	To generate artificial transparent eggshell retaining a natural shape that can support normal chick development; enhance the visibility of imaging in 3D	Ishak et al. (2020)

mammalian development mechanisms to reduce the use of animals, in particular mice, in the 3R model.

EX OVO CULTIVATION OF AVIAN EMBRYOS: ENVIRONMENTAL PARAMETERS FOR SUCCESSFUL HATCHING

Ex ovo culture is a system in which the original eggshell is removed, and the embryos are transferred to the new culture milieus, including a surrogate eggshell, Petri dishes, and artificial eggshell-like vessels (Ono, 2000; Borwompinyo et al., 2005; Liu et al., 2012; Tahara and Obara, 2014). By removing the eggshells, the *ex ovo* culture allows us to manipulate developing embryos at certain stages for surgical methods (Cloney and Franz-Odendaal, 2015). Of course, it also paves the way to seek how the embryo develops without a protective layer (Buskohl et al., 2012). Many studies have used this technique in several aspects such as angiogenesis (Hockel et al., 1987), intravasation assays (Uchibayashi et al., 1992), grafting, and tumor formation (Dohle et al., 2009; Villanueva and Sikora, 2022). Moreover, this technique can be applied to the lessons in high school and advanced developmental biology research fields (Buskohl et al., 2012; Dorrell et al., 2012; Cloney and Franz-Odendaal, 2015).

Since a previous study has shown that metabolism and internal environment within eggshells change dramatically at different stages (Givisiez et al., 2020), *ex ovo* cultivation aimed to follow embryonic development until hatching needs to find the optimal balance of several factors, in particular oxygen demand, humidity, and calcium requirement. Generally, the development of chick embryos can be divided into three phases: fertilization to blastoderm formation (day 0–day 1), embryogenesis (day 1–day 3), and embryonic growth (day 4–day 21) (Perry, 1988). Hitherto, three different aforementioned systems can be categorized into three systems with roman numerals: system I (for day 0–day 1), system II (day 1–day 3), and system III (day 4–day 21). The details of different techniques or modified methods described in previous studies are summarized in **Table 1**. Here, we also provide insights into each parameter (i–v) required for successful *ex ovo* studies and hatchability in relation to three phases of avian development (**Figure 1**).

Incubation Period and Embryonic Stage

Significantly, the embryonic age is the key factor for manipulating *ex ovo* cultured embryos. To increase viability during culture, transferring the embryos to the new culture vessels after stage HH15-16 (Hamburger and Hamilton, 1951) is recommended (Tahara and Obara, 2014). Those embryos developing in a polymethylpentene film and supplemented with calcium lactate and distilled water resulted in a 90% survival rate (Tahara and Obara, 2014). Tahara and Obara (2014) also showed that the embryos transferred to the culture vessel after stage HH16 showed viability on day 8 of incubation. Hence, the timing of the incubation period before transferring to the new environment plays a major role in enhancing the viability. Ideally, the embryos should be transferred to the new culture vessel at stage HH19 (Rowlett and Simkiss, 1989; Borwompinyo et al., 2005). Even though different egg preincubation periods between stages HH13 and 16 affected embryonic survival, higher viability was still observed before stage HH15 (Tahara and Obara, 2014). In addition to the embryonic age, it was also reported that care had to be taken with the vitelline membrane as it could be easily damaged while transferring to the new culture vessel (Tahara and Obara, 2014). This can be seen particularly in manipulating after-stage HH17 embryos. Dohle et al. (2009) reported that the stage HH35 (at around 9 days of incubation) embryos were less sensitive to agitation when compared to those at earlier stages. In addition, the survival rate of *ex ovo* cultured embryos can be higher up to 90–100% when they reached stage HH35, but then declined at the later stages, that is at stages HH40–41 (Cloney and Franz-Odendaal, 2015). Altogether, handling the right time of incubation period to acquire the right embryonic stage to proceed with hatching is essential for the *ex ovo* culture technique.

Temperature and Humidity

Practically, the temperature inside the incubator should not be lower than 38°C, which could cause a developmental delay (Dohle et al., 2009), and the ideal humidity was reported at 40% (Cloney and Franz-Odendaal, 2015). However, it was reported that covering the top of culture vessels with a polystyrene plastic lid could maintain 100% humidity and could be varied to 38°C with 80% humidity (Tahara and Obara, 2014). When the CAM is formed, which can be recognized on day 3 of incubation, the humidity is vital to preventing the desiccation of embryonic structures (Dohle et al., 2009). Importantly, the humidity of the system at the stage of the yolk sac formation is crucial to decreasing the humidity, to lower than 60%, which caused the CAM rupture by sticking to the eggshells (Dohle et al., 2009). Regarding the concern with water circulation systems, Yalcin et al. (2010) recommended that the culture should be kept at an optimal temperature. The design of the vessel structure is not only to prevent water loss but to facilitate oxygen supply (Tahara and Obara, 2014). The installation of a circulating water bath within the culture system helps to maintain the incubation temperature for developing embryos (Yalcin et al., 2010). In addition, a resistance heater was also used to regulate the water temperature (Yalcin et al., 2010).

Calcium Supplementation

Calcium, primarily calcium carbonate, is one of the major constituents of the eggshells (Scanes and Christensen, 2020) that serves as a tertiary envelope to protect developing embryos (Bellairs and Osmond, 2014). The majority of calcium provided to the embryos until hatching derives from the eggshell (Rowlett and Simkiss, 1987; Kamihira et al., 1998). Furthermore, calcium from the eggshells is also needed for ossification, and its deficit results in limb deformity (Cloney and Franz-Odendaal, 2015); therefore, supplying calcium to developing embryos is required (Elliott and Bennett, 1971; Rowlett and Simkiss, 1987). During the *ex ovo* culture, the embryos develop with no eggshells as the protective structure leading to a high mortality rate (Tahara and Obara, 2021). Therefore, adding calcium carbonate to the CAM in shell-less cultured embryos helped to increase more than 40% hatchability (Tahara et al., 2021). Kamihira et al. (1998) reported that various forms of calcium including eggshell powder and calcium lactate could also be added to the albumen as a calcium source following the eggshell removal. Although calcium carbonate is the main substance found in the eggshells, it failed to increase the hatchability rate for the shell-less system (Tahara et al., 2021). Alternatively, calcium lactate was shown to improve the hatchability rate with less toxicity to the cultured embryos (Kamihira et al., 1998; Tahara and Obara, 2014). However, supplementing calcium carbonate could increase hatchability up to 40% if it was added to the CAM (Tahara et al., 2021). These indicate that calcium supplementation with the minimum requirement is necessary for developing embryos to hatch in the shell-less system. Furthermore, information to understand the details of calcium availability and dynamics during embryonic development in this system still needs functional and molecular studies.

Oxygen Supply

Aeration in the culture vessels is mandatory for the survival of the embryos due to the loss of moisture by embryonic transpiration (Dohle et al., 2009). Importantly, the efficiency of the hatching rate can be improved by adding pure oxygen to culture vessels (Kamihira et al., 1998; Tahara and Obara, 2014). Previous studies reported that the number of embryos survived at the later stages in the pure oxygen culture system is higher than 50%, indicating the achievement of a high hatchability by culturing in such an artificial vessel (Kamihira et al., 1998; Tahara and Obara, 2014). Practically, the aeration in this culture system can be made to the ventilation holes on the top of the sealing film (Tahara and Obara, 2014). Ideally, the rate of aeration with pure oxygen installed plastic tube in the vessel should be 500 ml/h, and it should be well prepared, as the embryos do not survive to hatch without an oxygen supply (Tahara and Obara, 2014). Rowlett and Simkiss (1989) reported that chick embryos cultured in an artificial vessel made of a "cling-film" were susceptible to hypoxia and hypocapnia. Moreover, oxygen aeration at the early stages of culture should be a concern due to its toxic effect that can reduce embryonic viability (Tahara and Obara, 2014). Considerably, for the later stages (stage HH43–44), at around 17 days of incubation, oxygen insufficiency can be noticed by changing the color of the

CAM vessels (Dorrell et al., 2012; Tahara and Obara, 2014; Tahara et al., 2021), indicating that oxygen aeration to the later stages is indispensable for the *ex ovo* culture.

Sealing Film

Favorably, in the *ex ovo* culture, the use of a transparent plastic film allows the embryos to reside as a cradle, namely, a hammock (Kamihira et al., 1998; Yalcin et al., 2010; Tahara and Obara, 2014) as mentioned in **Table 1**. The adhesive property of this material with the culture vessels is relatively low. A rubber band was carefully used to tighten the film with culture vessels firmly averting embryonic mortality during manipulation (Cloney and Franz-Odendaal, 2015). A previous study reported that the positioning of the eggs and the sealing material in culture vessels affect the hatchability rate (Borwompinyo et al., 2005); the sealing materials including Handi and Saran plastic films were able to increase the percentage of hatchability (Borwompinyo et al., 2005). There are different types of plastic packaging such as polyethylene, polyvinylidene chloride, and polymethylpentene. Tahara and Obara (2014) demonstrated that these materials should possess oxygen permeability. One important point that also increases viability and hatchability is smoothening of film surface since film wrinkles caused a low survival rate of culture embryos (Tahara and Obara, 2014, 2021). Although using transparent films would help to facilitate whole-embryo observation, some disadvantages of their properties should be ameliorated to increase the hatchability rate.

IN VITRO CULTURE: ESSENTIAL TOOLKIT FOR AVIAN PLURIPOTENCY STUDIES, TRANSGENESIS, AND CONSERVATION

As pluripotent cells in embryos are restricted in some periods and in some embryonic tissues, the understanding of the pluripotency mechanism in embryos is limited. The advents of *in vitro* culture from avian embryos enable us to examine the pluripotency network and possible use of germline-competent stem cells (GCSCs) in avian biotechnology, in particular gene editing, transgenesis, and wild bird conservation (Han et al., 2015). There are four main types of GCSCs that can be derived from the *in vitro* culture of avian embryonic tissues: blastodermal cells/embryonic stem cells (ESCs), embryonic germ cells (EGCs), primordial germ cells (PGCs), and spermatogonial stem cells (SSCs) (Han et al., 2015). The discovery of chick ESCs and blastodermal cell culture emphasizes the conserved network regulating pluripotency required at least leukemia inhibitory factor (LIF) (Etches et al., 1997; Pain et al., 1996), a cytokine used to culture naïve mouse ESCs and iPSCs (Niwa et al., 1998; Takahashi and Yamanaka, 2006). In addition, basic fibroblast growth factor (bFGF), a cytokine used to cultivate primed human ESCs/iPSCs (Thomson et al., 1998; Takahashi et al., 2007), is required in several avian ESCs/iPSCs studies (Pain et al., 1996; van de Lavoir et al., 2006; Whyte et al., 2015; Choi et al., 2016; Katayama et al., 2018). Thus, this suggests that pluripotent stem cells from avian species exhibit some bias in a naïve-primed direction which could depend on other culture supplements. Several studies have used (Aubel and Pain, 2013) protocol to culture chick iPSCs in DMEM/F12 containing other several cytokines in addition to LIF and/or bFGF (FGF2) including IGF1, SCF, IL6, and sIL6 Rα (Dai et al., 2014; Fuet and Pain, 2017; Fuet et al., 2018). To force pluripotent cells to bias toward a naïve state, two inhibitors' (2i) cocktail including CHIR99021 (GSK3 inhibitor) and PD0325901 (MEK inhibitor), generally used in naïve mouse ESCs (Ying et al., 2008) can also support the establishment of chick iPSCs (Dai et al., 2014; Katayama et al., 2018; Yuan et al., 2021), as shown in **Figure 1**. In avian cellular reprogramming, choices of delivery and expression system and reprogramming factors are crucial for induction success of iPSCs. Exogenous gene delivery can be done *via* using a virus (retrovirus, lentivirus, and Sendai virus) (Dai et al., 2014; Fuet et al., 2018) and liposome-based transfection of a piggyBac transposon carrying a single polycistronic reprogramming cassette (Modified Oct4, Sox2, Klf4, c-Myc, Lin28, and Nanog) (Katayama et al., 2018) (**Figure 1**). Reprogramming factors used for induction of avian iPSCs varied in several studies but at least Oct4 and Sox2 were used in all studies (Dai et al., 2014; Fuet and Pain, 2017; Fuet et al., 2018; Katayama et al., 2018), while Klf4 and c-Myc were replaced with Nanog and Lin28 in Yuan et al.(2021) and Zhao et al. (2021). Fuet et al. (2018) also showed that Nanog is essential for long-term iPSC culture. In addition to pluripotent stem cells, *in vitro* culture allows the exploration of the primordial germ cell (PGCs) specification mechanism. It has been shown that avian PGCs do not require LIF for self-renewal (Whyte et al., 2015) while mammalian PGCs need it (Leitch et al., 2013). But, instead, bird PGCs, which resemble more the mammalian gastrulating epiblast (or *in vitro* mouse EpiSCs), can be supported under FGF2, insulin, and activin-BMP4 to induce SMAD signaling (Whyte et al., 2015). Also, chick iPSCs can differentiate into induced PGCs (iPGCs) which can be transplanted into another strain of a chick embryo to produce viable offspring (Zhao et al., 2021).

In vitro culture also supports the study of transcriptomic analysis of established ESCs/iPSC cell lines and sheds light on the conservation and uniqueness of pluripotency-related transcriptional networks in the avian species (Jean et al., 2015; Katayama et al., 2018). The list of ESCs/PGCs versus fibroblast markers is also shown in **Figure 1**. Finally, the key advantage of *in vitro* culture is the application of established cell lines including GCSCs back into *in ovo* cultivation for producing interspecific avian chimeras (within avian species or even mammalian to avian species), as shown in **Figure 1**. At the early stage of transgenic avian technology, transgenesis in chick embryo was done using a virus-dependent approach, e.g., injection of lentiviral vectors into the subgerminal cavity of the early embryo (Chapman et al., 2005). The advent of PGC culture allows easy manipulation of gene delivery *via* transfection (lipofection or nucleofection) as shown in the success of stable integration of *gfp* transgene into the host genome and later transgenic PGCs can be transplanted back to the embryos to eventually generate chimera and transgenic chicks (Park and Han, 2012). Recently, the direct injection of plasmids carrying the gene of interest, together with a liposome-based reagent, into the dorsal aorta of chick embryo containing

circulating PGCs was performed with the successful production of transgenic quail offspring (Serralbo et al., 2020). Overall, the achievement of *in vitro* culture with *in ovo* application and avian embryo plasticity to accept xenograft cells provide a hope of using chick embryos as a platform to propagate endangered wild birds under stem cell-based conservation.

FUTURE RECOMMENDATIONS AND CONCLUSION

For *ex ovo* milieu, exploration of possible avenues on how to culture avian embryo without a natural shell continues with the further development of imaging, chemistry, and nano-biomaterial technology, which can provide better-engineered eggshells resembling natural ones and fit to in-demand experiments such as in-depth imaging of embryos or transgenic embryos with artificial eggshells intact. In addition to *in vitro* culture, research on cellular reprogramming and ESCs derivation of various avian species is still required to understand the nature of naïve-primed pluripotency in relation to germline competency-the benefit of this understanding is that one day it can provide long-term security and prevent the extinction of avian species.

AUTHOR CONTRIBUTIONS

WS and SI conceived the idea. WS, AT, and SI contributed to the writing of the manuscript. All authors contributed to the articles and approved the submitted manuscript.

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REVIEW
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Chicken Incubation Conditions: Role in Embryo Development, Physiology and Adaptation to the Post-Hatch Environment

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The chicken hatching egg is a self-contained life-supporting system for the developing embryo. However, the post-hatch performance of birds depends on several factors, including the breeder management and age, egg storage conditions and duration before incubation, and the incubation conditions. Studies have determined the effect of incubation factors on chick post-hatch growth potential. Therefore, chick physical quality at hatch is receiving increasing attention. Indeed, although incubation temperature, humidity, turning and ventilation are widely investigated, the effects of several variables such as exposure of the embryo to high or low levels, time of exposure, the amplitude of variations and stage exposures on embryo development and post-hatch performance remain poorly understood. This review paper focuses on chick quality and post-hatch performance as affected by incubation conditions. Also, chick physical quality parameters are discussed in the context of the parameters for determining chick quality and the factors that may affect it. These include incubation factors such as relative humidity, temperature, turning requirements, ventilation, *in ovo* feeding and delay in feed access. All these factors affect chick embryo physiology and development trajectory and consequently the quality of the hatched chicks and post-hatch performance. The potential application of adapted incubation conditions for improvement of post-hatch performance up to slaughter age is also discussed. It is concluded that incubation conditions affect embryo parameters and consequently post-hatch growth differentially according to exposure time and stage of exposure. Therefore, classical physical conditions are required to improve hatchability, chick quality and post-hatch growth.

Keywords: chicken, chick quality, physiology, metabolism, post-hatch growth, photo-incubation, circadian rhythm

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1 INTRODUCTION

The growth and metabolism of a 1-day-old chick are mostly determined by processes that occur during embryonic development. The major goal of farmers is to develop a chick with good hatchability, viability, and post-hatch performance. To reach this goal, it is vital to determine the sources of variable factors as well as the repercussions of these factors for optimal embryonic development and hatching outcomes. Physiological changes occur during embryonic development

and the hatched day-old chick results in 21 days of development (Decuypere and Bruggeman, 2007). As a result, the endocrine system is absolutely necessary for appropriate embryonic development and hatching success.

The relationship between several physiological parameters such as corticosterone and thyroid hormones balances, heat production and metabolism, and gas exchange (O_2, CO_2) is crucial for the development of embryos and their survival under the incubation process (Decuypere et al., 1990; Tona et al., 2004). Furthermore, incubation conditions such as temperature, hypoxia (low oxygen), hyperoxia (high oxygen), and hypercapnia (high CO_2) can alter these physiological parameters and influence embryonic development in various ways. This could have an impact on embryo general growth trajectory and, as a result, flock uniformity. In literature, the relationships between physiological parameters and incubation conditions with embryonic development in time are scarce, and a better understanding of these parameters that affect chick quality and post-hatch growth is highly desired. The link between the initial feeding and post-hatch chick performance is crucial. It is well known that denying 1-day-old chicks access to nutrition decreases post-hatch growth. It is widely known that the first feeding stimulates a variety of molecular and cellular targets, including enzymes and hormones, which affect general growth and a variety of physiological processes, including the yolk utilization, metabolic level, and gastrointestinal development (Decuypere and Bruggeman, 2007). As a result, the relationship between the initial feeding and post-hatch chick performance is particularly intriguing. *In-ovo* feeding was examined in-depth to understand how exogenous nutrients could affect embryonic growth and hatching. Additionally, photo-incubation, a process of stimulating developing embryos with light is also reviewed. During embryogenesis, the growth-promoting effect of photo-incubation has been reported and there are shreds of evidence that photo-incubation influences hatch events (Tong et al., 2018), post-hatch growth performance parameters (Zhang et al., 2016), fear responses (Archer et al., 2009), stress level and adaptability to novel post-hatch environment (Ozkan et al., 2012). The role of light in the physiological process of poultry ontogenesis is essential to synchronize knowledge and scientific findings. This review focuses on the effects of incubation conditions such as ventilation, light, temperature, relative humidity and *in ovo* feeding on embryo and post-hatch parameters.

2 VENTILATION

Hypoxia (low O_2), hyperoxia (high O_2), and hypercapnia (high CO_2) during incubation are known to have a positive impact on embryonic development, depending on the extent to which the embryo is exposed to these conditions and the stage of the embryo development. As a result, hatchery managers must understand the impacts of low O_2, high O_2, and high CO_2 on embryo growth trajectory during incubation.

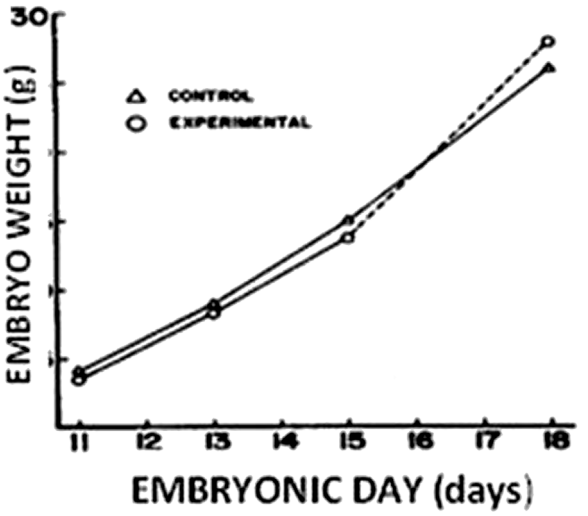

FIGURE 1 | Chicken embryo weight (g) according to hyperoxia treatment (60% O_2 on d 16–d 18) [adapted from Stock and Metcalfe (1987)].

2.1 Effect of Hypoxia/Hyperoxia or Hypercapnia on Embryonic Development

2.1.1 Effect of Hypoxia

It is widely known that the level of O_2 in the atmosphere varies with altitude, implying that the risk of hypoxia exists. With higher altitudes, the oxygen rate declines, affecting incubation time and hatchability (Hassanzadeh et al., 2002). According to (Smith, 1933), incubation of eggs at high altitudes caused a delay in embryo growth. Zhang and Burggren (2012) reported that variations in normal chick embryo growth are dependent on the timing of hypoxia and on its severity, with lower O_2 levels having a greater impact on growth and size.

Mild hypoxia (15 percent O_2) is the most studied level of hypoxia because it poses a major hypoxic threat to the embryo without causing severe mortality. This interpretation is backed by Chan and Burggren (2005) findings show that embryo growth is reduced but smaller when exposed to 15% O_2 hypoxia for 6 days (E1 to E6, E6 to E12, and E12 to E18) compared to controls. Furthermore, mild hypoxia (15 percent O_2) during internal pipping reduced O_2 intake and altered chick weight at hatching, but it had a minimal morphological influence on chicken embryos, whereas severe hypoxia (10 percent O_2) compromised embryo viability (Szdzuy et al., 2008).

During the external pipping, responses to both levels of hypoxia increased (Menna and Mortola, 2003). Depending on the timing, short periods of hypoxia exposure throughout different time frames have varying impacts on embryo viability. During hypoxic incubation, Zhang and Burggren (2012) found that mortality was higher from E0 to E10 than from E11 to E18. This finding suggests that the first eleven days of incubation is the critical phase for the deleterious impact of hypoxia on embryonic development, whereas the last ten days

is the crucial phase for the organs' compensatory response to hypoxia.

2.1.2 Effect of Hyperoxia

The demand for oxygen surpasses the oxygen diffusion capacity of the egg-shell pore system and chorio-allantoic membrane in the last half of the incubation phase (Rahn et al., 1974), resulting in a decrease in O_2 consumption (Prinzinger et al., 1995), and the development rate (Vleck et al., 1980). Internal pipping and the commencement of pulmonary respiration restore these modifications on day 19 (Prinzinger et al., 1995). As a result, the embryo outgrows the egg shell's oxygen diffusion capability, and its growth may be restricted by the availability of oxygen during regular air incubation. As a result, raising O_2 levels during the final stage of incubation can help the embryo grow faster. According to Stock and Metcalfe (1987), exposure to hyperoxia (60% O_2) late in the incubation period (days 16–18) produces accelerated foetal development (**Figure 1**). Furthermore, Van Golden et al. (1998) found that exposing the embryo to acute hyperoxia (60% O_2 for 48 h) on days 10–11, 14–15, and 18–19 increases the embryo's and all organs' mass. However, it should be highlighted that previous studies on hyperoxia are outdated, and there are insufficient investigations on the impact of hyperoxia on embryo physiology and later performance. As a result, further investigations are needed in this area.

2.1.3 Effect of Hypercapnia

Decuypere et al. (2006) found that chicken embryos become less susceptible to high incubator CO_2 levels as they become older, similar to hypercapnia. Although hypercapnia during incubation was traditionally thought to be harmful to embryo development, recent research suggests that, depending on the timing of its occurrence, elevated incubator CO_2 levels may be advantageous to the growing embryo (Onagbesan et al., 2007). Özlü et al. (2018) found that a higher CO_2 concentration of 0.70% during the first three days of incubation lowered viable hatchability by 2 percent due to increased early embryonic mortality. This finding backs with Taylor and Kreutziger's (1965, 1966) findings that indicated CO_2 concentrations surpassing 1, 3, 6, 9, 8, and 7% between ED 0–4, 3–5, 9–12, 13–16, and 17–20 decreased hatchability. In a more recent study, El-Hanoun et al. (2019) found that duck breeder eggs incubated in a closed incubator with a carbon dioxide concentration of 1% at the end of the first 10 days of incubation had higher hatchability and embryonic growth. Everaert et al. (2007) discovered that exposing embryos to high CO_2 (4%) during the second half of the incubation (d10–d18), had no influence on hatchability or hatch time but did increase embryonic weights. These reports show that the susceptibility of the chick embryo to CO_2 changes with age, the same as it does with O_2.

2.1.4 Synergistic Effect of Hypoxia/Hyperoxia and Hypercapnia

It is proposed that CO_2 levels of more than 6–7% have been demonstrated to drastically reduce O_2 levels in the incubator, exacerbating the negative consequences of these high CO_2 levels

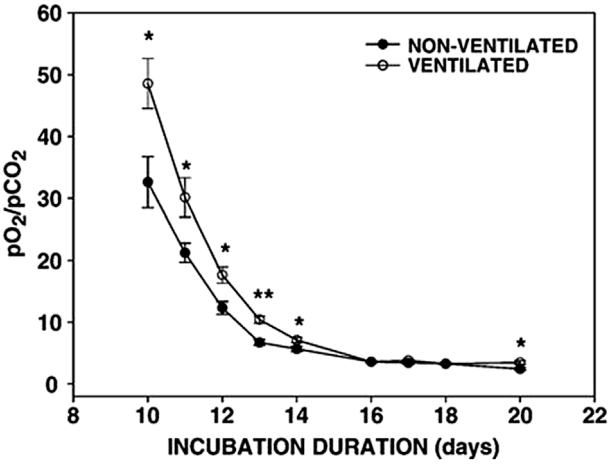

FIGURE 2 | Changes in the ratio of the partial pressure of O_2 and CO_2 in the air cell of the developing egg. *$p < 0.05$; **$p < 0.0001$, De Smit et al. (2006).

(Taylor et al., 1971). Restoring O_2 levels to normoxic levels with high CO_2 levels was observed to restore optimum hatchability in the later part of the incubation period, but restoration to hyperoxic levels induced an increase in hatchability relative to control incubations (Taylor and Kreutziger, 1969). This shows that high amounts of CO_2 and O_2 have a synergistic impact that may be beneficial to the growing embryo. These studies found that manipulating O_2 or CO_2 levels during incubation can influence the development of certain physiological regulating systems, producing alterations in the embryo's development trajectory. As a result, one can wonder about the impact of hypoxia or hypercapnia on embryonic physiology during embryogenesis.

2.2 Effect of Hypoxia or Hypercapnia on Embryonic Physiology

Hypercapnia or hypoxia can cause changes in the physiology of embryos with respect to the control and timing of the hatching events The physiological changes can be induced in the pulmonary and circulatory system by chronic hypercapnia. This observation has resulted in the view that higher levels of CO_2 can shorten the effects of hypoxic conditions on developing embryos. Increasing CO_2 early or at the end of incubation acts as a hatching stimulus but also the hypoxic condition of high-altitude incubation also affects hatching events as well as hormonal levels. In fact, Hassanzadeh et al. (2004) showed that embryos incubated at high altitude had higher plasma triiodothyronine (T_3), thyroxin (T_4), and corticosterone levels and hatched earlier than those incubated at low altitude. El-Hanoun et al. (2019) reported that duck breeders' eggs incubated under hypercapnic conditions hatched earlier than those incubated under normal conditions, and the hatch window was narrower. The authors demonstrated that this phenomenon is strongly related to increased levels of

TABLE 1 | Hormone levels (ng/ml) at day 18 of incubation according to incubation treatments (IT) and dexamethasone administration at day 16 (Dex 16) (n = 24) of incubation or control eggs (Cont) (n = 40).

IT	Groups	T_3	T_4	Ratio,T_3/T_4	Corticosterone
NV	Count	0.11 ± 0.01^c	3.94 ± 0.26^b	0.03 ± 0.01^c	8.19 ± 1.04^a
	Dex 16	0.32 ± 0.04^a	5.47 ± 0.52^a	0.08 ± 0.02^a	2.91 ± 0.99^b
V	Count	0.11 ± 0.01^c	3.52 ± 0.57^b	0.05 ± 0.02^b	7.72 ± 1.15^a
	Dex 16	0.23 ± 0.04^b	5.51 ± 0.90^a	0.05 ± 0.01^b	4.61 ± 1.44^b

Within columns, data sharing no common letters (a–c) are different (p < 0.05). Adapted from Tona et al. (2004).

corticosterone, T_3 and T_4 as a result of increased pCO_2 (De Smit et al., 2006) in the air cell at internal pipping in hypercapnic condition (**Figure 2**). Thus the positive effects of hypercapnic incubation suggest an increase of T_3 and air cell pCO_2 resulting in the early hatch and enhanced hatchability.

The study of Tona et al. (2004) on non-ventilation during early incubation in combination with dexamethasone administration at two stages of development (d16 or d18) in embryos has elucidated the importance of timing in manipulating the hypothalamic-pituitary-adrenal (HPA) axis. Indeed, the authors reported that dexamethasone injected on day 18 raised the plasma T_3 levels (**Table 1**) at internal pipping (IP) and advanced hatching and reduced the hatching process. However, injection on day 16 had no effect on the plasma T_3 levels at IP. Also, dexamethasone injection on day 16 resulted in a rebound effect on the functioning of the HPA axis in early postnatal life, which was not observed in chickens injected on day 18. This disturbance in HPA axis establishment may cause an increased functioning and has been reviewed earlier (Decuypere and Michels, 1992).

Moreover, exposure of embryos to low O_2 or higher CO_2 resulted in significantly higher haematological parameters (Hb, PCV %, and RBC counts). Increased Hb under hypercapnia or hypoxia conditions is known to raise the oxygen-carrying capacity of the blood and represent an adaptive physiological response. The findings of Mortola (2004) indicated a stimulatory role of CO_2 on the chemoreceptors that enhance breathing efficiency and that hyperoxia at this period decreased the effect of hypercapnia. Hence, hypercapnia can achieve a similar effect as hypoxia on lung function during internal and external pipping and hatching.

These findings indicate that embryos adapted to hypoxic or hypercapnic conditions by enhancing angiogenesis processes, which subsequently increases their blood oxygen-carrying capacity, which positively affects their growth development and maturation. Such alterations may induce permanent phenotypic changes in the embryo, which may have a long term epigenetic effect on post-hatch performance.

2.3 Effect of Hypoxia or Hypercapnia on Embryonic Post-Hatch Growth

2.3.1 Effect of Hypoxia

The use of moderate to high hypoxia is beneficial to chicken embryos during incubation as it supports the cardiovascular development chorioallantoic membrane, leading to an enhanced oxygen-carrying capacity and resulting in developmental plasticity which can influence the tolerance and performance of chicks to stressful conditions during their post-hatch growth.

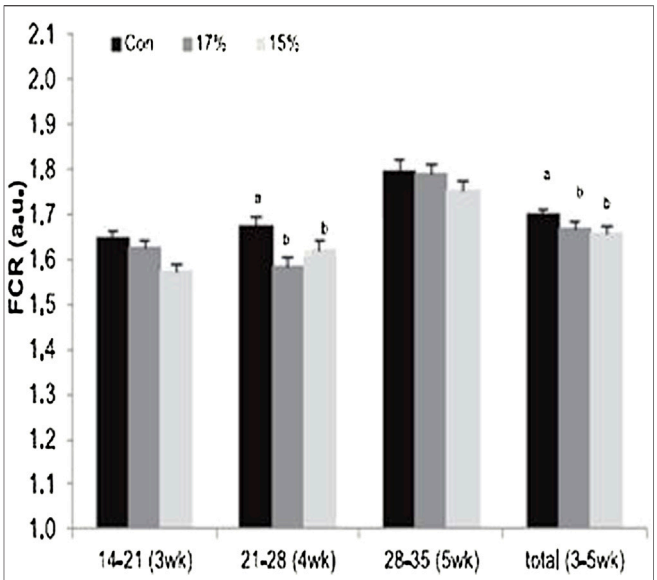

FIGURE 3 | Effect of hypoxia (17 or 15% O_2) on feed conversion ratio [adapted from Druyan et al. (2018)]. FCR, feed conversion ratio.

Chronic hypoxic conditions retarded growth rate in the initial phase in the first 14 days post-hatch but there was no difference in the body weights in the later phase of growth (Hassanzadeh et al., 2004). Also, the findings of Huang et al. (2017) showed that chronic hypoxia condition adversely affected survivability, feed conversion ratio and growth in broiler chickens. On the contrary, the findings of Druyan et al. (2018) showed that hypoxic conditions did not alter the juvenile growth performance of broiler chickens using a hypoxic condition of 15 or 17% O_2 during a short period of embryonic development. The authors reported that hypoxic conditions improved the body weights of the birds at the market age. At weeks 3 and 4, the treated birds had higher growth and a better feed conversion ratio (FCR) (**Figure 3**).

2.3.2 Effect of Hypercapnia

Relative to hypercapnia, El-Hanoun et al. (2019) reported higher body weights, better feed intake and FCR in the first 6 weeks of age of ducks incubated under hypercapnic conditions induced by non-ventilation during the first 10 days of incubation. These findings were in agreement with the results reported by Fares et al. (2012) and De Smit et al. (2006). The findings of De Smit et al. (2006) showed that the differences in body weight were due to the higher growth speed of chicks from nonventilated incubated eggs in the first week post-hatch compared to those from the ventilated incubation. They maintained higher bodyweight during the entire post-hatch growing period which suggests a long term epigenetic effect of non-ventilation. Based on

this epigenetic effect, in literature, it has been hypothesized that the negative impact of long-term storage can be compensated by increasing CO_2 levels in the incubator during incubation.

Developmental changes induced by increasing dioxide carbon or oxygen level during embryonic development may play a role in post-hatch performance, affecting growth and metabolism (Decuypere, 2002). Although later prenatal hypoxia, as well as hypercapnia, may be beneficial for a lower incidence of ascites during the growing period of broilers, early hypercapnia as induced by non-ventilation during the first 10 days of incubation may result in increased sensitivity for ascites-inducing factors (De Smit et al., 2008). This shows that the timing of the treatment influences the lasting epigenetics of this condition.

3 LIGHT

Photo-incubation is a complex phenomenon whose outcome can be determined by a certain number of factors which can be categorized as the bulb type, wavelength and correlated colour temperature (CCT), time of initiation of photo-incubation, light duration and light intensity. During embryogenesis, the growth-promoting effect of photo-incubation has been reported and there are pieces of evidence that light-dependent factors influence hatch events (Tong et al., 2018), post-hatch growth performance parameters (Zhang et al., 2016), fear responses (Archer et al., 2009), stress level and adaptability to novel post-hatch environment (Ozkan et al., 2012). It is known that the ability of birds to adapt to the prevailing post-hatch environment has been linked to physiological roles played by biological rhythm established during embryogenesis (Ozkan et al., 2012). Therefore, reviewing the importance of light dependent factors on developing embryo and their effect on post-hatch growth is essential to synchronize knowledge and scientific findings.

3.1 Effect of Light Characteristics on Embryonic Development and Physiology

3.1.1 Effect of Bulb Type on Embryonic Development and Physiology

Bulb type, which serves as a light source, is a crucial factor that can potentially enhance or disrupt the process of photo-incubation. For instance, the problem of secondary heating associated with incandescent (ICD) due to its high heat emitting capability could engage the mechanism of thermal physiology in the process. The usage of ICD is highly discouraged if the bulb is not intended to be used as a primary source of heat in the incubator. Studies have demonstrated that other bulb types such as fluorescent and LED posed lower (Rozenboim et al., 2004) or no secondary heating effect during incubation (Zhang et al., 2016). A comparative study on light sources revealed that fluorescent light enhances the embryonic weight of quail over ICD. Incandescent light decreased hatch weight and hatchability but increased early and late embryonic mortality in contrast to fluorescent (Hanafy and Hegab, 2019). It was also reported that small-sized eggs develop faster under ICD while the rate of development under fluorescent was not influenced by egg size (Hanafy and Hegab, 2019).

3.1.2 Effect of Light Duration on Embryonic Development and Physiology

Duration of light exposure or photoperiod is an essential photo-incubation parameter. Non-continuous or intermittent lighting (12 h of light) seems beneficial over other photoperiods. Studies have shown a reduction in embryonic mortality (Riaz et al., 2021) and an increase in melatonin hormone under intermittent lighting on day 19 of incubation (Archer and Mench, 2014a) compared to continuous or dark incubation. Continuous photo-regimen (23 or 24 of light) has been reported to elevate eggshell temperature (Rozenboim et al., 2004) and a destructive effect of the regimen has also been reported on avian eyes (Archer et al., 2009). Interestingly, Raiz et al. (2021) reported a shorter hatch window and improved hatchability under both continuous and intermittent in contrast to dark incubation. In contrast, Archer and Mench (2014b) reported no impact of lighting duration on hatchability relative to dark incubation. A factor confounding these studies might be the differences in intensities used by the authors.

3.1.3 Effect of Light Intensity on Embryonic Development and Physiology

An existing study on light intensity showed that the use of the fluorescent green light at 900–1,380 lux and 1,430–2,080 lux, had no significant influence on the embryonic weight and hatch weight of broiler eggs (Shafey et al., 2005). Embryonic response to wavelength differs. Rozenboim et al. (2004) found an increase in embryonic weight of chicken eggs stimulated under green LED (at 0.1 W/m2 intensity, phot-incubated from day 5–21) relative to dark incubated eggs. Zhang et al. (2016) noted that light colours (White LED, green LED-560 nm supplied at 30 lux) had no influence on hatchability or hatching weight as compared to dark incubation. Tong et al. (2015) and Wang et al. (2020) reported that green LED (522 nm, 520–525 nm) shortens hatch time over dark incubation. In contrast, no effect of incubation condition (green LED or darkness) was recorded on hatchability (Zhang et al., 2016) hatching weight (Tong et al., 2015; Zhang et al., 2016) and chick quality (Tong et al., 2015). Sabuncuoglu et al. (2018) reported no differences in hatch weight, hatch time, and hatchability of quail hatching eggs incubated in the dark, blue (480 nm) or green (560 nm) LED. Green LED (565 nm at 15 lux) has been reported to increase growth hormone (GH) and insulin-like growth factor (IGF-I) during embryonic development (Zhang et al., 2014) while the prehatch level of T_3 and T_4 remains unchanged. Differences in intensities due to light distribution might influence embryonic response on an individual level. Thus, it is essential for researchers to report light intensities based on average measurements recorded at the egg level. It is also imperative for researchers to report light intensities in gallilux or chicken

lux rather than lux as poultry birds and humans perceive light differently (Oso et al., 2022a,b). This is important, especially when photo-incubation extends till hatch or birds are light stimulated post-hatch.

3.1.4 Effect of Onset of Photo-Incubation/Total Duration of Photo-Incubation on Embryonic Development and Physiology

The onset of photo-incubation/total duration of photo-incubation is another factor due for consideration during photo-stimulation. Archer and Mench (2014b) demonstrated that initiating photo-incubation either from day 1 or day 7 or day 14 till hatch had no impact on hatchability. Similarly, Hannah et al. (2020) proved that initiating photo incubation on days 0, 9, and 17 till hatch had no influence on hatchability and embryonic mortality. Exposing eggs to light from day 1–18 or day 1–21 has been demonstrated to have no effect on embryonic weight and hatch weight of chicks in comparison to dark incubation (Archer, 2015). Scientific knowledge is limited on the effect of the varying onset of photo-incubation on physiological indices, especially hormones during embryonic development and at hatch.

3.1.5 Other Factors That can Influence the Photo-Incubation Process

Photo-incubation is a complex phenomenon whose outcome cannot only be determined by light-dependent factors but also by other factors known as egg dependent factors. Egg dependent factors include egg internal qualities, shell characteristics, specie, breed and strain, storage duration of the egg before incubation, age of parent stock, size of an egg, and stage of embryonic growth/development at the time of photo-initiation (Shafey et al., 2005; Hannah et al., 2020). A crucial egg-dependent factor is the shell qualities. Eggshell thickness and shell pigmentation are capable of changing an embryo's perception of light, thus influencing its response to photo-stimulation (Shafey et al., 2005). Darker eggshells change the wavelength perceived by the embryo in contrast to lightly-coloured eggs (Hannah et al., 2020). The differences in the outcome of photo-incubation based on the factors above suggest that the mechanism of photo-incubation may slightly differ under varying conditions; however, the site(s) of photo-stimulation during embryogenesis remains the same.

3.2 Effect of Photo-Incubation Light Factors on Post-Hatch Growth Development and Physiology

3.2.1 Effect of Bulb Type on Post-Hatch Growth Development and Physiology

The effect of bulb type used during photo-incubation on post-hatch growth and physiology of many poultry species remains un-elucidated. More often than not, photo-incubated chicks are reared under lights that differ from the incubation light source. In Japanese quails, Hanafy and Hegab (2019) reported no significant influence of ICD and fluorescent bulb type used during incubation on post-hatch body weight, weight gain and FCR at 6 weeks; however, birds belonging to the fluorescent group had significantly higher feed intake group compared to those in ICD group. The bulb type used during post-hatch was not stated by the author, although the author noted that the post-hatch lighting condition was the same. It is not known if maintaining the incubation light source during post-hatch could influence the growth and physiology of photo-incubated birds differently, thus, a comparative study is necessary in this regard.

3.2.2 Effect of Light Duration on Post-Hatch Growth Development and Physiology

The effects of incubation photo-period on post-hatch growth performance reported in the literature are contradicting. Both intermittent and continuous lighting were reported to have no influence on feed intake, weight gain (Archer and Mench, 2013; Riaz et al., 2021) and feed conversion ratio (FCR) (Archer and Mench, 2013) in contrast to dark incubation. On the contrary, both continuous and intermittent duration had similarly been demonstrated to reduce post-hatch feed intake over dark incubation. Interestingly, continuous lighting during incubation had been shown to significantly reduce post-hatch weight gain in contrast to dark incubation and non-continuous (Yameen et al., 2020). Riaz et al. (2021) highlighted that FCR was significantly better under intermittent photo-incubation over continuous or dark incubation. A likely interactive effect between pre-hatch and post-hatch photo-period and other lighting conditions might have confounded the results of these studies and this appears to be another promising area of research. The effect of incubation light duration on melatonin appears to wane with time or fades out due to prevailing post-hatch lighting duration. Archer and Mench (2014a) demonstrated that changes in pre-natal melatonin level were not sustained till 5 weeks post-hatch as no significant difference was observed in the melatonin level between the continuous, non-continuous or dark incubation groups. It is indistinct if post-hatch photoperiod over-rides the effects of pre-hatch photoperiod, but certainly, the circadian rhythm established during the last phase of photo-incubation under an established non-continuous regimen is beneficial to post growth and development either directly or indirectly. Before exposure of birds to a stressful situation (crating exercise), Archer and Mench (2013) recorded a similar level of corticosterone in chickens exposed to continuous lighting, intermittent and near intermittent (6 h of light). After exposure, a lower level of corticosterone was reported in birds belonging to the intermittent group when compared to other groups. This suggests that the incubation photo-period has a vital role to play in post-hatch stress management. The Interactive effect of pre-hatch photo-period and prevailing post-hatch photoperiod on growth and physiological indicators in poultry birds has not been explored and this might be a promising area of research requiring considerable attention.

3.2.3 Effect of Light Intensity on Post-Hatch Growth Development and Physiology

The effect of pre-hatch light intensity on post-hatch growth, physiology and adaptation of poultry birds is not established in the literature and a probable interaction between pre-hatch light intensity and post-hatch intensity has not been researched. Different light intensities are required by different poultry

birds at different stages of post-natal growth and this might extend to photo-incubation. Establishing intensity specifications required by each poultry species for a maximum photo-incubation outcome would further broaden photo-related scientific horizons.

It appears that the impact of photo-incubation wavelength on post-hatch growth performance varies between species, breeds or strains. Wang et al. (2020), who experimented with layer breeder eggs reported an increase in 8–12 weeks body weight of Rhode Island Red photo-incubated with green LED in contrast to dark incubated, but these differences disappeared from 14 weeks, whereas body weights of other strains (Columbia Rock, White Leghorn, Barred rock) remained unchanged throughout regardless of the incubation treatment. Sabuncuoglu et al. (2018) demonstrated that pre-hatch light colour (blue, green) does not influence the post-hatch bodyweight of quails throughout the rearing period. Rozenboim et al. (2003) showed that female turkeys earlier photo-incubated under green LED had higher body weight from day 28 till day 59 compared to those incubated in the dark. In another experiment published in the same article, the author recorded no difference in post-hatch body weight between male turkeys photo-incubated under green LED, white mini-ICD, or dark incubated. These results suggest that sex might play an important role in post-hatch growth response to incubation light colour. Zhang et al. (2016) highlighted that at 30 lux, green LED photo-incubation enhanced the weight gain of broiler chicks at 6 days old over dark incubation though the result was similar to those obtained in the white LED group. Furthermore, the author noted that feed intake and FCR were not influenced by the photo-incubation condition (Zhang et al., 2016). Prevailing post-hatch light colour might overwrite the effect of pre-hatch light colour. Rozenboim et al. (2004) found an increase in body weight of broilers photo-stimulated under green LED and reared under the green light (green-green) compared to those incubated in the dark and reared under white LED (dark-white). Although, the author demonstrated that green-green birds and green-white birds (photo-incubating with green and rearing under white light) had similar body weights. The significant differences emanating when comparing green-green and dark-white suggest that rearing birds under their incubation light colour might be more beneficial. Studies on hormones showed that green light enhances GH and IGF-I during post-hatch life (Zhang et al., 2014) compared to dark incubation and at slaughter age, but no differences within treatment groups were found in the T3 and T4 levels post-hatch. Archer (2017) reported a lower level of corticosterone and serotonin hormones in birds incubated under green, red and white LED compared to dark incubated birds.

3.2.4 Effect Onset of Photo-Incubation on Post-Hatch Growth Development and Physiology

The onset of photo-incubation seems to have a more pronounced effect on hormones rather than growth performance. At post-hatch, photo-stimulation initiated from day 1 or day 7 or day 14 till hatch does not influence feed intake, weight gain and FCR (Archer and Mench, 2014b). Similarly, exposing eggs to light from day 1–18 or day 1–21 has no impact on weight gain and FCR at slaughter age. Before and after exposure to a stressful situation, Archer and Mench (2014a) recorded a significant reduction in corticosterone levels of birds exposed to light from day 1–21 and day 7–21 when compared to those incubated in the dark. Corticosterone level was found to be similar within the photo-incubated treatments but significantly lower than in the dark incubated groups (Archer, 2015). Dishon et al. (2021) demonstrated that exposing eggs to green LED from day 18 till hatch and photo-incubating them from day 1 till hatch significantly improve body weight at slaughter age. Also, at 5 days post-hatch, secretion of GH was higher in birds photo-incubated from day 18–20 in contrast to those incubated and hatched in the dark.

4 INCUBATION TEMPERATURE AND RELATIVE HUMIDITY

One of the crucial determinants of the development of chickens' embryos and hatchability is incubation temperatures (Decuypere and Michels, 1992). According to Molenaar et al. (2011), a constant temperature of 37.8°C during incubation allows for optimal embryo development, the best hatchability and the highest chick quality. It has been shown that a 1°C change from the optimum temperature can have a significant effect on hatchability (French, 1994). Additionally, the period of temperature change and its intensity and the age of the embryos during incubation will determine its impact on the developing embryos (French, 2002).

4.1 Effect of Low and High Incubation Temperature on Embryo Development and Hatching Performances

Joseph et al. (2006) highlighted that a continuous low eggshell temperature of 36.6°C during the first 10 days of incubation reduced embryonic weight, hatchability and chick quality. Nideou et al. (2019) observed an increase in albumen utilization and growth of Isa brown embryos subjected to elevated temperature (38.5°C) during the first 10 days of incubation while the incubation time was shortened. According to Maatjens et al. (2017), a negative effect of an eggshell temperature of 38.9°C was observed from E15 onward and emphasize that an eggshell temperature of 35.6 and 36.7°C from E15 onward might be beneficial for chick embryo physiology. However, Yildirim and Yetisir (2004) studied the effect of different hatcher temperatures (36.1; 37.2; 38.3, and 39.4°C from 17 days of incubation until hatch with relative humidity at around 75% in all groups) on hatching traits. They found that the control group (37.2°C) had a better hatchability compared to the low-temperature group (36.1°C) which had low metabolic activity and then a high late embryo mortality rate. Wilson (1991) reported that in the last third of the

incubation phase, a decrease in the temperature of incubation did not have a significant effect on hatchability but elongated incubation duration and decreased water loss. According to Tzschentke and Hall (2009), a temperature of 36.2°C from day 18–21 of incubation improved hatchability. Concerning the effect of a high temperature in the hatcher; Yildirim and Yetisir (2004) found that a temperature of 38.3°C in the hatcher allows for a hatchability similar to the control group (37.2), but a very high temperature (39.4°C) negatively affect hatchability by increasing late embryo mortality. Their results are not similar to those of Joseph et al. (2006) who found a better hatchability with eggs subjected to a higher hatcher temperature (39.5°C) compared to the control group (37.8°C).

4.2 Effect of Low and High Incubation Temperature on Post-Hatch Performances

Joseph et al. (2006) found that a continuous low eggshell temperature of 36.6°C during the first 10 days of incubation reduced live weight at 6 weeks of age and carcass yields. But increasing the incubation temperature (38.5°C) during the first 10 days of incubation did not impact the T_3 concentration of the hatched chicks and their post-hatch performance. Maatjens et al. (2016) showed that a high eggshell temperature (38.9°C) applied from day 15 of incubation on Ross eggs negatively impacted chick's growth and FCR during the first week of rearing, while this post-hatch performance was improved compared to control batches when eggs were subjected to a temperature of 36.7°C from day 15 of incubation. According to İpek and Sözcü (2015), the carcass weight and yield are negatively affected by higher hatcher temperature but slaughter weight at higher hatcher temperature was similar to the control group. Joseph et al. (2006) stated in their study that high eggshell temperature in the hatcher reduced bodyweight and one-week weight gain. However, by three weeks of age, there was no difference in body weight between chicks in high eggshell temperature and control eggshell temperature treatments.

4.3 Effect of Duration of Thermal Treatment During Incubation on Embryo Development and Chickens' Post-Hatch Performance

Increasing incubation temperature at 39.5°C during 24 h/day from E7 to E16 reduced hatchability by 25% and negatively affected the quality and the bodyweight of the hatched chicks while a thermal treatment at 65% during 12 h/day from E7 to E16 did not affect hatchability and bodyweight of cobbs hatched chicks (Piestun et al., 2008a). Tzschentke and Hall (2009) revealed that neither short-term (38.2°C–38.4°C, 2 h daily) nor chronic (38.2°C–38.4°C, 24 h daily) increase in incubation temperature in the hatcher (during the last four days of incubation) adversely affected hatchability and chick quality in broiler chickens. The body temperature was significantly reduced in broiler chicks subjected to thermal treatment (12 and 24 h/day) compared to the control group, but those subjected to a thermal treatment during 24 h/day had a body temperature lower than those of 12 h/day (Piestun et al., 2008a). This aligns with the reduced plasma triiodothyronine and thyroxine of the birds of 24 and 12 H groups and the plasma corticosterone increased with time.

4.4 Effect of Duration of Thermal Treatment During Incubation on Post-Hatch Performance and Physiology

Increasing incubation temperature at 39.5°C during 12 h/day from E7 to E16 improved the feed conversion ratio of broilers compared to the control (Piestun et al., 2013; Meteyake et al., 2020) without affecting broilers' growth compared to 24 h/day group which had lower body weights (Piestun et al., 2008b). According to Tzschentke and Hall (2009), the FCR of short-term warm stimulated broilers (38.2–38.4°C, 2 h per day, from d17 onward) was significantly lower than in broilers of the control (37.2–37.4°C) and chronic warm (38.2–38.4°C, 24 h per day, from d17 onward) incubated groups. The daily feed intake and weight gain were significantly lower in the short-term warm stimulated ducks than those of the control group in the first three weeks while short-term cold stimulation improved feed conversion ratio during the whole growing period exclusively in male ducks (Halle et al., 2012). According to Sgavioli et al. (2016), incubating eggs at 39°C compromises the body and heart development of layer chicks and reduces the availability of blood ionized calcium for bone mineralization during embryo development. Morita et al. (2016) concluded in their study that changes in chicken stickiness and vascularity as well as changes in thyroid and growth hormone levels are the results of embryonic strategies to cope with higher or lower than normal incubation temperatures. Overall, Madkour et al. (2021) claimed that thermal acclimation at the postnatal stage or throughout the embryonic stages has been considered as a novel promising strategy to mitigate the detrimental effects of heat stress in poultry. These authors suggested that, for large-scale application, this strategy needs further investigation to determine the suitable temperature and poultry age.

4.5 Relative Humidity

There is a loss in egg weight during incubation due to the evaporation of water (Rahn et al., 1977). This is crucial to make available ample air needed for the lung ventilation of the embryos sequel to internal pipping and ultimately hatching (Ar and Rahn, 1980). The best hatchability has been attained with 12–14% water loss at embryonic day 18 (Ar and Rahn, 1980). The relative humidity in the incubator can be manipulated to control the water evaporation of the eggs during incubation (Buhr, 1995). Lower or higher relative humidity could have variable effects on hatching and post-hatch performances.

Van der Pol et al. (2013), investigated the effect of low or high relative humidity and showed showed that incubating eggs at a higher or lower relative humidity negatively influenced hatching performances. They found that at the same incubation temperature (37.8°C), the reduction of relative humidity increased egg weight loss at E18 and reduced hatchability by increasing late embryo mortality. But it did not affect the chick's weight, quality and hatching time. They found that incubating

eggs at a low RH compared with a high RH and maintaining the EST at 37.8°C decreased the hatch of fertile eggs. Bruzual et al. (2000) reported that relative humidity of less than 63% during incubation decreased chick weight. Reducing relative humidity during incubation had little effect on post-hatch performance according to Van der Pol et al. (2013). El-Hanoun et al. (2012), investigating the effect of incubation humidity and flock age on hatchability traits and post-hatch growth in Pekin ducks found that the optimal relative humidity depends on breeders' flock age.

4.6 Interaction Between Temperature Incubation and Relative Humidity

It is well known that a temperature of 37.5–37.8°C and relative humidity of 55–60% are optimal environmental conditions for efficient embryo development, and the best hatching and post-hatch performances. Any change of one of these two factors without changing the second accordingly could have variable effects on incubation results. (Boleli and Queiroz, 2012) found an interaction of these two incubation parameters on hatchability. They also found a moderate negative correlation between hatchability and temperature (r = −0.41), hatchability and egg weight loss (r = −0.31) and a positive correlation between hatchability and relative humidity (r = 0.47). The effect of the interaction of temperature and relative humidity was also significant on incubation duration and chicks' body weight.

5 EGG TURNING DURING INCUBATION

5.1 Effect on Physiology and Embryo Development

Egg turning involves four major factors that can be taken into account; the position of eggs, angle of turning, frequency (times/day) and stage of incubation in whom this turning occurs. These different factors of turning diversely influence the physiology of the embryo, incubation parameters and post-hatch performances.

The lack of turning during incubation has been reviewed (Lundy, 1969; Baggott et al., 2002). A complete absence of turning during the first but not the second week of chicken eggs incubation leads to an increase in mal-positioned embryos and mortality (Elibol and Brake, 2004). The results of New (1957) showed that days 3 to days 7 of incubation were critical and failure to turn eggs during this critical period leads to a decrease in hatchability and rates of embryonic growth (Deeming, 1989). Moreover, the lack of turning of chicken eggs between days 12 and days 19 of incubation (last stages of embryonic development) leads to less embryonic growth as a result of impaired O_2 consumption through the chorioallantoic gas exchanger (Pearson et al., 1996). Tona et al. (2003a) noted that turning of eggs until 12, 15, and 18 d of incubation did not affect the levels of plasma corticosterone in the developing embryo or newly hatched chicks. The author inferred that corticosterone might not be involved in the mechanism by which turning affect hatching and production parameters. However, they observed that turning beyond 15 days increased pCO_2 in air cells (hypercapnia) and plasma levels of T_3 and T_4 at the internal pipping. In contrast to Tona et al. (2003b) who showed a correlation between this increase in metabolism, hatching time and hatchability, these authors observed that these parameters remain similar between eggs turned until 12 days and 18 days, suggesting the involvement of other intrinsic factors.

Egg turning at 90° and 45° on either side of the vertical using as a standard practice in the industry was linked to considerable research during the 1930s–1950s (Baggott et al., 2002). Lesser turning angles increase mal-positioned and embryo mortality in domestic fowl (Funk and Forward, 1953; Elibol and Brake, 2006; Cutchin et al., 2009). Schalkwyk et al. (2000) noticed that hatchability amounted to 1·83% for an increase of 1° (R^2 = 0·96) when ostrich eggs were rotated hourly through angles ranging from 60° to 90°. In a recent study, Guo et al. (2021) recorded a shortened incubation duration and improvement in hatchability and goslings' quality when eggs were turned at a wider angle (60° compared with 50°). They also observed an increase in late embryos and goslings' weight correlate with a significant upregulation of genes in the somatotropic axis (GHRH, GH, and IGF-1 mRNA expression) and muscular development (pax7, MyoD, MYF5, and MRF4 mRNA expression). The authors concluded that wider angle turning made full use of the albumen content in goose eggs and recommend adjusting the angle of turning to the ratio of albumen in eggs according to avian species as previously stated by other researchers (Baggott et al., 2002; Elibol and Brake, 2006; Deeming, 2009).

Static incubation impairs the expansion of the *area vasculosa* during the critical period of sub-embryonic fluid production in the domestic fowl (Baggott et al., 2002). This absence of egg turning delayed the formation of extra-embryonic fluids and reduced rates of embryonic growth later in embryonic development (Deeming, 1989). Turning once an hour is commonly used in the industry concerning fowl eggs. The increase of this frequency up to 96 times daily does not significantly improve incubation results (Freeman and Vince, 1974). By contrast, lower frequencies decrease these results. Indeed, Oliveira et al. (2020) showed a decrease in hatchability due to a gradual increase percentage of early and late mortality with less turning frequency (**Table 2**). Elsewhere, a study conducted by Elibol and Brake (2006) showed that increasing turning frequency is a good way to reduce mal-positioned embryos associated with less turning angle during incubation. These data suggest possible interactions between the different factors of turning that are not well explored.

Moraes et al. (2018) incubated Japanese quail eggs in different positions (vertical position with the small end up, vertical position with the small end down, horizontal position) without turning and found that hatchability is best when eggs were set with the small end down although the outcome was similar to those set horizontally (65.3 ± 6.4% vs. 59.3 ± 9.2). In a 2 × 2 factorial design trial, Schalkwyk et al. (2000) recorded an increase in hatchability when ostrich eggs were set horizontally for 2 weeks and vertically for the remainder of the incubation period compared to those set vertically for the entire incubation period irrespective of angles of rotation (60° or 90°). Nevertheless,

TABLE 2 | Fertility, hatchability of fertile eggs, and embryonic mortality according to the turning frequency (Oliveira et al., 2020).

Turning frequency (time/D)	Fertility[2] (%)	Hatchability of set eggs[3] (%)	Hatchability of fertile eggs[4] (%)	Early dead (%)	Mid dead (%)	Late dead (%)
24	93.00 ± 3.393[a]	85.34 ± 2.30[a]	91.84 ± 2.73[a]	2.84 ± 1.89[b,c]	1.41 ± 0.87[a]	3.57 ± 1.39[b]
12	91.33 ± 1.96	78.34 ± 2.30[b]	85.77 ± 3.05[b]	6.22 ± 1.99[b]	2.19 ± 0.73	5.46 ± 0.69[a,b]
6	90.67 ± 2.53	70.33 ± 5.3.31[c]	77.75 ± 3.89[c]	12.45 ± 2.05[a,b]	2.59 ± 0.83	7.37 ± 3.37[a,b]
3	91.56 ± 4.27	67.55 ± 5.5.82[c]	73.75 ± 3.89[c]	14.31 ± 1.82[a]	2.92 ± 0.64	8.05 ± 1.24[a]
p value	0.13	<0.0001[5]	<0.0001[5]	<0.0001[5]	0.11	0.02[5]
CV (%)	3.89	4.04	3.74	22.12	38.69	31.02
R^2 adjust	0.23	0.81	0.84	0.81	0.25	0.38

Within columns, data sharing no common letters (a–c) are different (p < 0.05).

FIGURE 4 | Broiler growth according to day-old chick quality. From Tona et al. (2003a).

the results of these trials encourage the vertical setting despite having no obvious advantage over the eggs set horizontally then vertically with the appropriate angle of rotation. This is consistent with practices in the industry supported by the work of Funk and Forward (1960) who recorded better hatchability when fowl eggs are set vertically with their air sac up. This position prevents hatching failure due to mal-positioning or pipped eggshells on the narrow end.

5.2 Effect on Post-Hatch Performances

Studies on the effect of these different factors on egg turning on post-hatch growth are not well documented. However, many hypotheses can be made based on the results of turning on incubation parameters. Then, Takeshita and McDaniel (1982) observed that although chicks from eggs in horizontal or vertical with small end up required less time to exhibit initial pips, they required longer to emerge from the shell than those in vertical with the small end down. Usually, the spread of hatching can affect the time of first feeding. Shortness of hatching windows when eggs were set with the small end down would lead to improved growth performances through early access to feed, which is crucial for post-hatch performances (Gaglo-Disse et al., 2010; Wang et al., 2014). In addition to short hatching windows, Guo et al. (2021) recorded an increase of goslings of high quality with a proper turning angle. According to Tona et al. (2003a), day-old chick quality and relative growth up to 7 days as well as slaughter performance are positively correlated (**Figure 4**). The authors concluded that the quality of chicks was better with an adequate turning angle (45°) because these chicks were able to use more nutrients to produce body mass tissue. An adequate turning angle could improve the feed efficiency of birds during the growth period.

6 *IN OVO* FEEDING PRACTICE DURING THE INCUBATION PROCESS: EFFECTS ON EMBRYO DEVELOPMENT AND HATCHING PERFORMANCES

In literature, embryos have been fed with a variety of nutrients with a diversity of results depending on the stage of incubation when *in ovo* feeding occurs, the nature and quantity of nutrients inoculated and the route of injection. This paper does not aim to review all the nutrients inoculated in eggs. It focuses on the way *in ovo* feeding of some critical substances that could affect embryo development, physiology and post-hatch growth depending on the different factors mentioned above.

6.1 Effect on Physiology, Embryo Development and Hatching Performances

Chicken eggs contain a small amount of carbohydrate (less than 1%) that supplies glucose, the most important source of energy needed for embryo growth (Starck and Rickelefs, 1998). This amount of carbohydrates initially available in the egg could not cover all the needs of the embryo until hatch. Therefore, glucose and glycogen are preferentially utilized as energy sources over lipids and protein because the limited oxygen available is mainly generated *via* gluconeogenesis and glycogenesis (Pearce, 1971). These carbohydrates were important for the final stage of embryonic development especially for pipping and chick emergence from the shell (Christensen et al., 1993; Moran, 2007).

Glucose inoculated in albumen at days 7 during organogenesis failed to improve hatchability while chicks' weight was significantly improved at hatch (Salmanzadeh et al., 2012). In contrast, *in ovo* injection of carbohydrates (glucose, maltose)

during the late stage of incubation (at days 17.5 or days 18) did not affect hatchability or newly chick weight (Ipek et al., 2004; Dos Santos et al., 2010, Eslami et al., 2014). However, Zhai et al. (2011) recorded a decrease in hatchability with glucose, fructose, sucrose or maltose *in ovo* injected at d 18.5 of incubation although body weight or body weight relative to set egg weights were significantly increased. A combination of carbohydrates (maltose, sucrose and dextrin) inoculated *in ovo* on day 19 also increase the bodyweight of newly hatched chicks (Tako et al., 2004). Thus, *in ovo* injection of carbohydrates at the appropriate time during incubation seem to be a good way to improve embryo growth and then chick weight at hatch. In addition, an insufficient amount of glycogen in late-term embryo forces the embryo to mobilize more muscle protein for gluconeogenesis until replenishing of glycogen reserves with the access of newly hatched chicks to feed (Vieira and Moran, 1999a,b; Moran, 2007). Uni et al. (2005) showed that injection of a solution containing β-hydroxy-β-methylbutyrate (a leucine metabolite) into the amniotic fluid of broiler embryos on day 17.5 spare the use of pectoral muscle leads to an increased body-weight at hatch. The *in ovo* inoculation of amino acids mixture seems to be also a good way to improve significantly chick weight at hatch if the amino acids used were identical to the amino acids pattern of egg protein (Al-Murrani, 1982). In chicken embryos, days 19 of embryonic development is an important point when the risk of lipid peroxidation is very high because tissues are characterized by comparatively high levels of polyunsaturated fatty acids. But at this time, the natural antioxidants level is not sufficient for innate protection. This risk is more when internal piping occurs with increasing oxygen availability as pulmonary respiration begins. Thus, low antioxidant status increases the embryo's susceptibility to lipid peroxidation. However, *in ovo* injection of vitamins (A, B1, B2, B6, and C or E) at days 14 of incubation failed to improve hatchability and chicks' weight at hatch (Nowaczewski et al., 2012; Goel et al., 2013). In contrast, extract from plants like *Moringa oleifera, Nigella sativa, etc*. are rich in carotenoids, an antioxidant naturally present in eggs, increase significantly hatchability (N'nanle et al., 2017; Oke et al., 2021). The advantage of some plant extract use is to concentrate different nutrients like vitamins and trace minerals (selenium, copper, zin, and iron) that act as co-factor of many enzymes involves in hatching success (Malheiros et al., 2012).

6.2 Effect on Post-Hatch Performances

Administration of exogenous nutrients and other agents *in ovo* can advance the development of the embryo and post-hatch growth (Uni and Ferket, 2003). Some nutrients *in ovo* injected lead to an increase of body weights at hatch (Al-Murrani, 1982; Tako et al., 2004; Uni et al., 2005; Zhai et al., 2011; N'nanle et al., 2017). Like chick quality, hatching weight is a major predictor of marketing weight in chickens. Thus, advantages observed at hatch in the *in ovo* feeding treatment were maintained during rearing (Al-Murrani, 1982; Uni et al., 2005). This gain could be attributed to changes that occur earlier in the gut. For example, Smirnov et al. (2004) and Tako et al. (2004) showed an increased surface area, length and width of villus at hatch and 3 days after with carbohydrates inoculated at days 17.5 in chicken eggs. In the same way, manna oligosaccharides injected *in ovo* at days 17 resulted in the newly hatched chick with more mature enterocytes in the small intestine that can enhance digestive capacity and epithelial barrier (Cheled-Shoval et al., 2011).

Although chicks' weight at hatch was unaffected, Joshua et al. (2016) demonstrated that *in ovo* inoculation of nano form of selenium (0.075 or 0.15 μg/egg) and zinc (40 μg/egg) significantly improve weight gain, body weight at market age and feed efficiency (only with nano form of Zn). This could be explained by the involvement of these minerals in immunity (McKenzie et al., 1998; Cardoso et al., 2007). Immunity was also improved by *in ovo* inoculation of probiotics and synbiotics which led to better post-hatch resistance against pathogens (De Oliveira et al., 2014; Sławinska et al., 2014) while prebiotics have been reported to increase the number of beneficial bacteria and promote their early colonization in the intestine of neonatal chicks (Tako et al., 2014).

7 CONCLUSION

There have been remarkable advances in the incubation of chickens. Incubation conditions affect embryo parameters and consequently post-hatch growth differentially according to exposure time and stage of exposure. In addition, the amplitudes of changes in these conditions need to retain attention during incubation. Therefore, classical physical conditions are required to improve hatchability, chick quality and post-hatch growth.

AUTHOR CONTRIBUTIONS

KT organized the different sections of the article, wrote the abstract, read and approved the submitted version. KV wrote the introduction and conclusion and performed the article on journal design and submitted the manuscript. ON wrote **Section 5**, OEO and EK read the first draft of the manuscript and made significant contributios, AB wrote **Section 1**, HM wrote **Sections 3**, **4**, OO wrote **Section 2**.

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Physiological Regulation of Growth, Hematology and Blood Gases in Chicken Embryos in Response to Low and High Incubation Humidity

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Variations from a relative humidity (RH) of ~50–60% can unfavorably alter chicken embryo development, but little is known of whether the embryo can mitigate these effects through physiological regulation. We examined effects of Low RH (25–35%), and High RH (85–93%) compared to Control RH (50–60%) on hatchability, embryonic growth, hematology and blood gases and pH. Mean hatchability was not affected by RH. Yet, Low RH decreased wet body mass of advanced embryos (days 17–19; d17-19), with lowered body water content compared with embryos of Control and High RH. However, dry body mass of developing (d11-19) embryos was not different between the three RH groups. Mean blood osmolality across development was higher in Low RH embryos and lower in High RH embryos compared with Control embryos. Mean blood lactate was higher in both Low and High RH embryos compared to Control embryos. Unexpectedly, hematological respiratory variables (Hct, [RBC], MCV, [Hb]) and blood gas variables (Po_2, Pco_2, pH, $[HCO_3^-]$) across development were not affected by RH. Mean wet body mass at hatch (d20-22) was larger in High RH embryos compared with Low RH embryos, but mean wet and dry body mass upon euthanasia on d22 was unaffected. The ability of the three populations to physiologically regulate blood respiratory variables and blood acid-base balance was then examined by observing their responses to intrinsic hypoxemia and hypercapnia created by controlled partial egg submersion in water. Hct and [RBC] responses were less disturbed by submersion in High RH embryos compared with both Control and Low RH embryos, which showed major disturbance. Acid-base regulatory responses did not differ between RH groups. We conclude that, while different incubation RHs cause large differences in tissue water content and body mass, most hematological and acid-base regulatory capabilities are regulated near Control values.

Keywords: chicken embryo, incubation, relative humidity, embryonic development, acid-base balance, hematology, hydration, dehydration

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1 INTRODUCTION

Chicken eggs are typically incubated at ~37–38°C of temperature and a ~50–60% relative humidity (RH). Deviation of incubation temperature and/or humidity from these standard ranges produces abnormal embryonic development and hatchability (Noiva et al., 2014). While major deviations in incubation temperature are invariably lethal, it is less likely that even large deviations of RH created during artificial incubation causes total mortality, because embryos in the egg are protected by the finite water vapor conductance created by the eggshell. In typical desktop (non-commercial) egg incubators, RH falls no lower than ~20–30% (Low RH) or higher than ~85–93% (High RH). Even these relatively dry or wet conditions are not always lethal, but mortality is much increased in comparison with incubation at RH of ~50–60% (Control) due ultimately to the degradation of embryonic physiology. A classic study showed that estimated mortality in chicken embryos increasing to 1/3—½ of all eggs incubated at a RH of ~30–20% and to ½-3/4 incubated at a highly elevated RH of ~85–93% (Ar and Rahn, 1980). These values compare to estimated minimal mortality of ~1/5 in Control eggs incubated at 50–60% RH. Even when chicken embryos survived these extreme incubation RHs, some physiological functions are likely to be degraded even as others are strengthened to cope with altered RH. In an example of enhanced physiological function to meet this environmental challenge, presumably advantageous renal morphological and physiological remodeling occurs in chicken embryos surviving incubation in low humidity (Bolin et al., 2017).

In the current study, we examined potential effects of both elevated and reduced RH on embryonic growth, hematology, respiratory blood gases and acid-base variables. We created an additional physiological challenge by transient, partial water submergence in d17 eggs to judge the efficiency of physiological regulation in altered humidity conditions. We first hypothesized that, predictably, d17-21 embryos incubated in High RH will be hydrated and Low RH will be dehydrated, with lower hatchability in both groups compared with Control embryos. Additionally, we hypothesized that embryos incubated in High and Low RH will show altered physiological regulatory capabilities that could enhance survival.

2 MATERIALS AND METHODS

2.1 Egg Incubation

All experiments with chicken embryos were performed at the University of North Texas in accordance with the protocol approved by the UNT Institutional Animal Care and Use Committee. Fertile eggs of the chicken (*Gallus gallus domesticus*, layer strain) were obtained weekly from a local hatchery. Upon arrival at the laboratory, eggs were weighed to 0.01 g with an electronic balance. Very small (<50 g) or very large (>70 g) eggs were excluded from the study. Eggs were numbered in order of egg mass and divided equally into the three groups according to egg mass. The eggs in individual groups were placed at 12:00 in three Hova-Bator desk-top incubators (GQF Manufacturing, Savannah, Georgia) set to a temperature of 38 ± 0.5°C. Control eggs were incubated at ~50–60% RH (Control RH), while other two groups were incubated at ~20–35% RH (Low RH) and ~85–93% RH (High RH), respectively. The Low RH conditions was created by providing no water to the incubator, while the High RH was created by filling all available pools in the incubator with water. Relative humidity was monitored by Baro-Thermo-Hydrometers (Oregon Scientific, Tualatin, OR, United States).

This study was completed in a series of six incubation experiments between February 15th and November 20th in the same calendar year. **Table 1** outlines these experiments, providing n numbers and additional information on hatchability.

Water loss from eggs was determined as the difference of egg mass from d0 to a given day.

2.2 Protocols

Examination of incubation RH effects focused on three categories of variables: hatching success, changes of physiological variables during the developmental process, and physiological regulatory capabilities in the face of environmental challenges (intrinsic progressive hypercapnia and hypoxia). On the day of experiment, individual egg mass was measured in the morning prior to blood sampling. Individual embryos were then euthanized after blood sampling and measured for wet and dry body mass.

2.2.1 Hatchability

Fertile eggs with growing embryos were confirmed by candling on d4 of incubation and continuously incubated in their assigned RH level. Hatchability was determined as the percentage of successful hatchlings from the initial fertile eggs.

2.2.2 Hatchling Wet and Dry Body Mass

On d19 of incubation, the viable eggs in the three RH groups were measured for mass to nearest 0.01 g. Water loss from the beginning of the incubation period was determined. The eggs in the three RH groups were transferred sequentially to hatchers consisting of desk-top incubators provided with 25 partitions equally divided by cardboard partitions and maintained at ~37°C and ~60–65% RH. These standardized hatching conditions were created to ensure that all embryos hatched under the same conditions, thus removing effects of different environmental humidity conditions during the hatching process. This assumed that any permanent changes related to development in high or low RH that had appeared during the entire process of development would not be quickly reversed during a final day leading up to hatching.

Hatchers were monitored every h from 0:700 to 21:00 every d until d22 to obtain newly hatched chicks and determine their wet body mass to the nearest 0.01 g. On d22, hatchlings were transferred to a closed container and euthanized with isoflurane, immediately followed by determination of wet body mass to the nearest 0.01 g. The carcasses were then placed on tared plastic trays and placed in a desiccating oven at 65°C. The dry body mass was determined when the mass of body and tray changed by < 0.01 g during three consecutive days of dry mass

TABLE 1 | Hatchability of eggs incubated in Low, Control and High relative humidity determined from six separate experiments.

		Hatching variable	Egg set date						
			Feb. 15th	Apr. 30th	Sep. 19th	Oct. 9th	Oct. 29th	Nov. 20th	Mean ± se
Relative Humidity (%)	CONTROL (50–60%)	# of Eggs Incubated	28	28	28	28	28	28	–
		# of Fertilized Eggs	24	23	23	23	21	18	22 ± 1
		# of Hatchlings	18	17	20	16	13	9	16 ± 2
		Hatchability of Set Eggs (%)	64%	61%	71%	57%	46%	32%	55 ± 6%
		Hatchability of Fertilized Eggs (%)	75%	74%	87%	70%	62%	50%	70 ± 5%
	LOW (25–35%)	# of Eggs Incubated	28	28	28	28	28	28	–
		# of Fertilized Eggs	19	20	20	15	21	23	20 ± 1
		# of Hatchlings	10	14	13	7	15	12	12 ± 1
		Hatchability of Set Eggs (%)	36%	50%	46%	25%	54%	43%	42 ± 4%
		Hatchability of Fertilized Eggs (%)	53%	70%	65%	4%7	71%	52%	60 ± 4%
	HIGH (85–93%)	# of Eggs Incubated	28	28	28	28	28	28	–
		# of Fertilized Eggs	24	22	22	24	21	19	22 ± 1
		# of Hatchlings	16	18	13	15	15	15	15 ± 1
		Hatchability of Set Eggs (%)	57	64	46	54	54	54	55 ± 2%
		Hatchability of Fertilized Eggs (%)	67	82	59	63	71	79	70 ± 4%

measurement. Typically, this occurred after ~17–19 days of drying in the oven.

2.2.3 Daily Water Loss

During the period from incubation d11 to d19, water loss from individual embryonated eggs on the target day was determined from the egg mass difference between d0 and the morning of the day prior to blood sampling.

2.2.4 Blood Sampling

The allantoic vein was located by candling and the location marked on the eggshell 1 day before the target developmental day. On the target developmental day, a ~0.8 cm square of eggshell was removed at the marked region. The now-exposed underlying allantoic vein was gently lifted by forceps through the hole in the eggshell. Arterialized blood from the allantoic vein was drawn into a 25-gauge needle attached to a 1 ml sampling syringe which had been flushed in advance with heparinized saline. 200 μL of blood was withdrawn on d11, and 250 μL of blood withdrawn on each of all subsequent days. Since the allantoic vein of d19 embryos could not be candled accurately, arterialized blood was not always collected on d19.

Once blood had been collected, the sampled eggs were placed for at least 30 min into an anoxic bag vented with N_2 to euthanize the embryos. Embryos minus allantoic membranes were then removed from the eggs, blotted with paper to remove surface fluids and wet body mass (WBM) measured to the nearest 0.01 g. Dry body mass was then determined as described above for embryos. Body water content (% of embryo body) was calculated as: Water content = 100 x (WBM—DBM)/WBM.

2.2.5 Hematology and Blood Gases

Immediately after collection, blood was gently transferred to a 0.15 ml plastic vial. From this vial ~120 μL of blood was withdrawn and injected into a blood gas analyzer (ABL5, Radiometer Medical A/S, Copenhagen, Denmark) to determine blood Po_2 (mmHg), Pco_2 (mmHg), pH and [HCO_3^-] (mmol L^{-1}). The blood remaining in the sampling syringe was transferred to the vial, thoroughly mixed and then injected in a blood cell counter (Coulter analyzer, A^c·10T, Beckman, United States) to measure red blood cell concentration ([RBC], 10^6 μL^{-1}) and hemoglobin concentration ([Hb], g%). A further ~10 μL of blood was used to measure blood osmolality (Osm, mmol kg^{-1}) using a vapor pressure osmometer (Vapro 5520, Wescor, United States). With one drop of blood lactate ion concentration ([La^-], mmol L^{-1}) was determined with a lactate meter (Nova Lactate Plus Meter, Nova Biomedical, MA, United States). Finally, ~60 μL of blood was transferred into each of two hematocrit tubes. The tubes were sealed and centrifuged for 5 min at 10,000 rpm to determine hematocrit (Hct, ±0.1%) (Readacrit Centrifuge, Becton Dickinson, United States). The mean value of duplicate determinations was Hct for this embryo. From the measured values of Hct, [RBC] and [Hb], the following mean corpuscular variables were calculated; mean corpuscular volume (MCV = 10 x Hct/[RBC] in μm^3), mean corpuscular hemoglobin (MCH = 10 x [Hb]/[RBC] in pg) and mean corpuscular hemoglobin concentration ([MCHb] = 100 x [Hb]/Hct in g%).

Blood acid-base status was represented on a Davenport diagram, constructed by plotting Pco_2 isopleths calculated from the Henderson-Hasselbalch equation using a CO_2 solubility factor of 0.0308 mmol L^{-1} $mmHg^{-1}$ and a serum carbonic acid pK' according to pH (Severinghaus et al., 1956a, b). A mean slope of -16 mmol L^{-1} pH^{-1} was used for the buffer line of chicken embryo blood (Burggren et al., 2012).

2.2.6 Experimental Challenge to Assess Physiological Regulation of Hematological and Acid-Base Disturbances

Embryos under the various RH incubation regimes were assessed for altered regulation of hematological variables, blood respiratory gases and acid-base balance when experimentally exposed to progressive intrinsic hypoxia and hypercapnia. These conditions were created by partial egg submersion in water, previously established as a method for testing embryonic physiological regulation (Andrewartha et al., 2014;

Branum et al., 2016). On d16 of incubation, the viable eggs in the three RH groups had a red line drawn on the egg's mid-line. Details of the submersion process are described in Andrewartha et al. (2014) and Branum et al. (2016). Each RH group was divided into four subgroups; 0 h (Control without water submersion), 2, 6 and 24 h of submersion. Submersion of the 24 h group began on d16 so that it would end on d17, as for the other groups. All eggs were submersed with air cell down.

After the designated time of water submersion (2, 6 and 24 h), each egg was removed from the water and the submerged half of the egg was immediately covered with Parafilm to preserve blood gas values during blood collection by continuing to eliminate gas exchange across the egg shell between environment and air cell. An area ~1 cm across was created in Parafilm over the site of the allantoic vein and a small hole ~0.8 cm across opened in the eggshell. Arterialized blood was immediately collected and analyzed for Pco_2, pH and [HCO_3^-] with the blood gas analyzer and subsequently analyzed for all of the hematological variables described above.

2.3 Statistical Analysis

Data were tested for normality and equal variance. Differences between means of multiple groups were tested by one-way ANOVA with post-hoc multiple comparison analysis to determine differences between individual group means. Multiple group means consisting of several subsets individually were examined by two-way ANOVA for the significance of difference between group means and between individual subsets. Significance was assumed at $p < 0.05$. All data were presented as means ±1 S.E.M.

3 RESULTS

3.1 Hatchability and Hatchling Gross Morphology

Overall hatchability was 70 ± 5% for fertilized Controls ($n = 132$), 60 ± 4% for Low RH ($n = 118$) and 70 ± 4% High RH ($n = 132$) (**Table 1**). There were no significant differences in % hatchability between the three RH groups ($p = 0.203$). There was also no significant relationship between incubation time and hatching time between the three experimental groups.

While hatchlings were not subjected to necropsy, we observed no gross morphological developmental disturbances, such as wing, beak or limb abnormalities, edema or abnormal yolk sac resorption.

3.2 Daily Egg Water Loss

Egg water loss during d11 to 19 of incubation significantly increased with development ($p < 0.001$) and was significantly different between the three RH groups ($p < 0.001$) (**Figure 1**). Average water loss during the designated period of incubation was 7.61 ± 0.08 g (13.5 ± 0.1% of egg mass) for Low RH, 4.66 ± 0.08 g (8.2 ± 0.1%) for Control RH, and 2.26 ± 0.08 g (4.0 ± 0.1%) for High RH. On d19 of incubation, the average water loss was 10.17 ± 0.24 g (17.6 ± 0.4%), 6.50 ± 0.24 g (11.2 ± 0.4%) and 2.92 ± 0.21 g (5.0 ± 0.4%) for Low, Control and High RH, respectively.

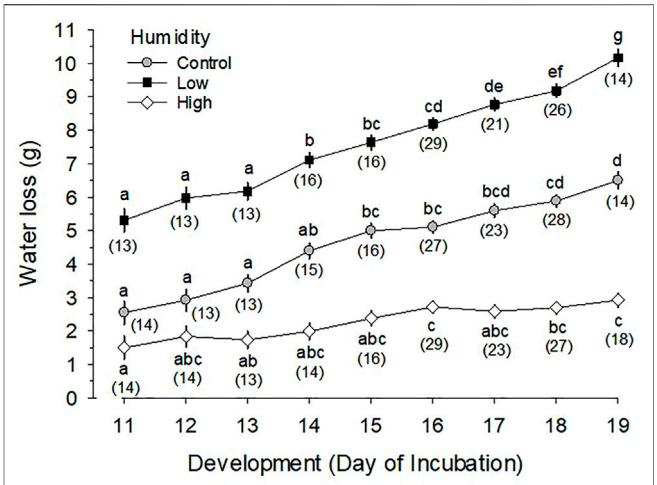

FIGURE 1 | Daily water loss (g) from eggs incubated at Control (~50–60%), Low (~25–30%) and High (~80–93%) relative humidity during the last half of egg incubation. Different letters for means in each of the three relative humidity groups indicate significant differences, while number of measurements (n) is shown in parentheses. Mean values at any given day of incubation (i.e., vertical comparison) for each of the three relative humidities are significantly different from each other ($p < 0.001$). Daily water loss (WL in g) is expressed by the following linear regression equations; Low RH, WL = 0.59·Day-1.19 ($p < 0.001$, $t = 14.17$ for coefficient); Control RH, WL = 0.46·Day-2.22 ($p < 0.001$, $t = 13.69$); and High RH, WL = 0.17·Day-0.32 ($p < 0.001$, $t = 9.03$).

3.3 Wet and Dry Body Mass of Hatchlings

3.3.1 Wet Body Mass

Developmental changes in wet body mass were significantly different between the three RH groups ($p < 0.001$ for effect of humidity) and were significant ($p < 0.001$ for development) with significantly different rate between groups ($p < 0.001$ for interaction of humidity and development) (**Figure 2A**). The increase in wet body mass during development was not different between Control and High RH groups, but mean wet body mass of Low RH group was significantly lower than that of Control and High RH groups during the last 3 days of incubation (d17-19). Mean wet body mass at hatch was 43.20 ± 0.71 g (Control), 40.65 ± 1.07 g (Low RH) and 46.09 ± 1.18 g (High RH), which differed significantly among the three groups ($p = 0.006$). Pairwise multiple comparisons indicated a significant difference between Low and High RH groups ($p < 0.005$). At hatchling euthanasia on d22, mean wet body mass had decreased to 37.02 ± 1.01, 35.73 ± 1.08 and 38.55 ± 1.42 g for of Control, Low and High RH groups, respectively, with no significant difference between them ($p = 0.271$).

3.3.2 Dry Body Mass

Changes in dry body mass were significant across development ($p < 0.001$), but were not significantly different between the three RH groups ($p = 0.695$ for effect of humidity), with no significant interaction of development and humidity ($p = 0.877$) (**Figure 2A**). The mean dry body mass at hatching was 9.44 ± 0.24, 9.41 ± 0.33 and 9.62 ± 0.36 g for Control, Low and High RH groups, respectively, and the difference between RH groups was not significant ($p = 0.876$).

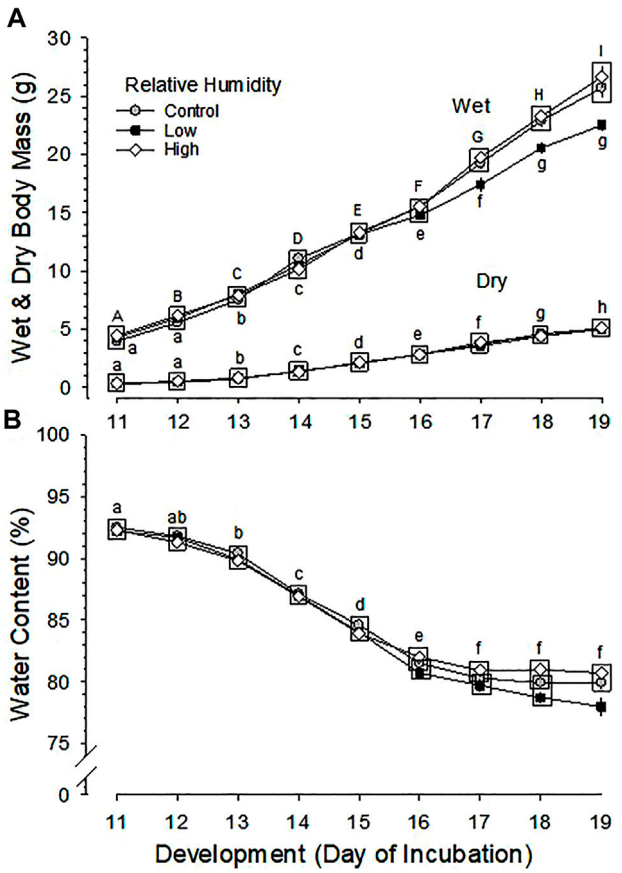

FIGURE 2 | Developmental changes in **(A)** wet and dry body mass and **(B)** body water content of embryos incubated at Control, Low and High relative humidity. Means ± standard error are presented. In most cases error bars are smaller than the symbol size. Means on any given day of incubation that are not significantly different from each other are enclosed by a box. **(A)** Wet and Dry Body Mass: Effect of RH and development on wet body mass and their interaction are all significant ($p < 0.001$ for all). Different uppercase letters indicate significant difference between development of embryos incubated at Control and High RH and lowercase letters indicate significant difference between development of embryos incubated at Low RH. Effect of development is significant ($p < 0.001$), but there is no significant effect of RH on dry body mass of the three groups ($p = 0.695$), and with no significant interaction between RH and development ($p = 0.877$). **(B)** Body Water Content: Effect of RH, development and their interaction on water content are all significant ($p < 0.001$ for all). Lowercase letters indicate significant differences between developments of embryos incubated at Control RH. *n* values are as in **Figure 1**.

3.4 Body Water Content

Daily changes in water content of embryos were significant across development ($p < 0.001$) and between the three RH groups ($p < 0.001$), with significant interaction of development and RH ($p < 0.001$) (**Figure 2B**). The daily changes of water content were not different between Control and High RH groups, but mean water content of Low RH group was significantly lower than that of High RH group during d16-19 and that of Control group on d19 of incubation.

3.5 Hematology

Developmental changes in Hct and [RBC] were not different between the three RH groups ($p = 0.616$ and $p = 0.539$ for effect of humidity on Hct and [RBC], respectively), but were significant across development ($p < 0.001$) for both Hct and [RBC]), with no significant interaction between humidity and development ($p = 0.365$ and $p = 0.395$ for Hct and [RBC]) (**Figures 3A,B**).

Changes across development in MCV were not different between the three RH groups ($p = 0.106$ for effect of humidity), nor were significant across development ($p = 0.054$) with no significant interaction ($p = 0.508$) (**Figure 3C**).

Changes across development in [Hb] were significant ($p < 0.001$), but were not different between the three RH groups ($p = 0.799$), with no significant interaction ($p = 0.226$) (**Figure 3D**).

Changes across development in MCH and [MCHb] were significant ($p < 0.001$) and these variables different significantly between the three RH groups ($p = 0.011$ and $p = 0.027$ for MCH and [MCHb], respectively), with significant interaction for MCH ($p = 0.036$) or with no significant interaction for [MCHb] ($p = 0.661$) (**Figures 3E,F**).

3.6 Blood Chemistry

3.6.1 Osmolality

Blood osmolality (Osm) did not change significantly across development in the three RH groups ($p = 0.787$), but mean values of Osm were significantly different between the three groups ($p < 0.001$ for effect of humidity), with no significant interaction of development and humidity ($p = 0.671$) (**Figure 4A**). The mean Osm during the period d11-18 was 273 ± 1, 267 ± 1 and 263 ± 1 mmol kg^{-1} for Low, Control and High RH, respectively.

3.6.2 Lactate Concentration

Blood lactate ion concentration ($[La^-]$) changed significantly across development in all three RH groups ($p < 0.001$). Mean values of $[La^-]$ were significantly different between three RH groups ($p = 0.001$) with no significant interaction of development and humidity ($p = 0.060$), but there was no clear pattern of change associated with incubation RH (**Figure 4B**). The mean $[La^-]$ of Low and High RH groups were 1.52 ± 0.07 mmol L^{-1} and 1.47 ± 0.07 mmol L-1, respectively, which were not different from each other, but were both significantly higher than that of the Control group (1.20 ± 0.06 mmol L^{-1}).

3.6.3 Blood gas Pressures and Acid-Base Balance

Changes across development in arterialized blood Po_2 and Pco_2 were both significant ($p < 0.001$), but were no significant differences between the three RH groups ($p > 0.35$) and no significant interaction between development and humidity ($p = 0.691$ and $p = 0.134$ for Po_2 and Pco_2, respectively) (**Figure 5A**).

Changes across development in the pH and $[HCO_3^-]$ of arterialized blood were both significant ($p < 0.001$), but were not significant between the three RH groups ($p > 0.136$), with no significant interaction between development and humidity ($p > 0.49$) **(Figure 5B)**.

3.7 Hematological and Acid-Base Disturbances Associated With Hypoxemia and Hypercapnia

3.7.1 Hematological Variables

In response to half submersion of eggs in water with egg air cell down, Hct significantly increased ($p < 0.001$) in all three RH

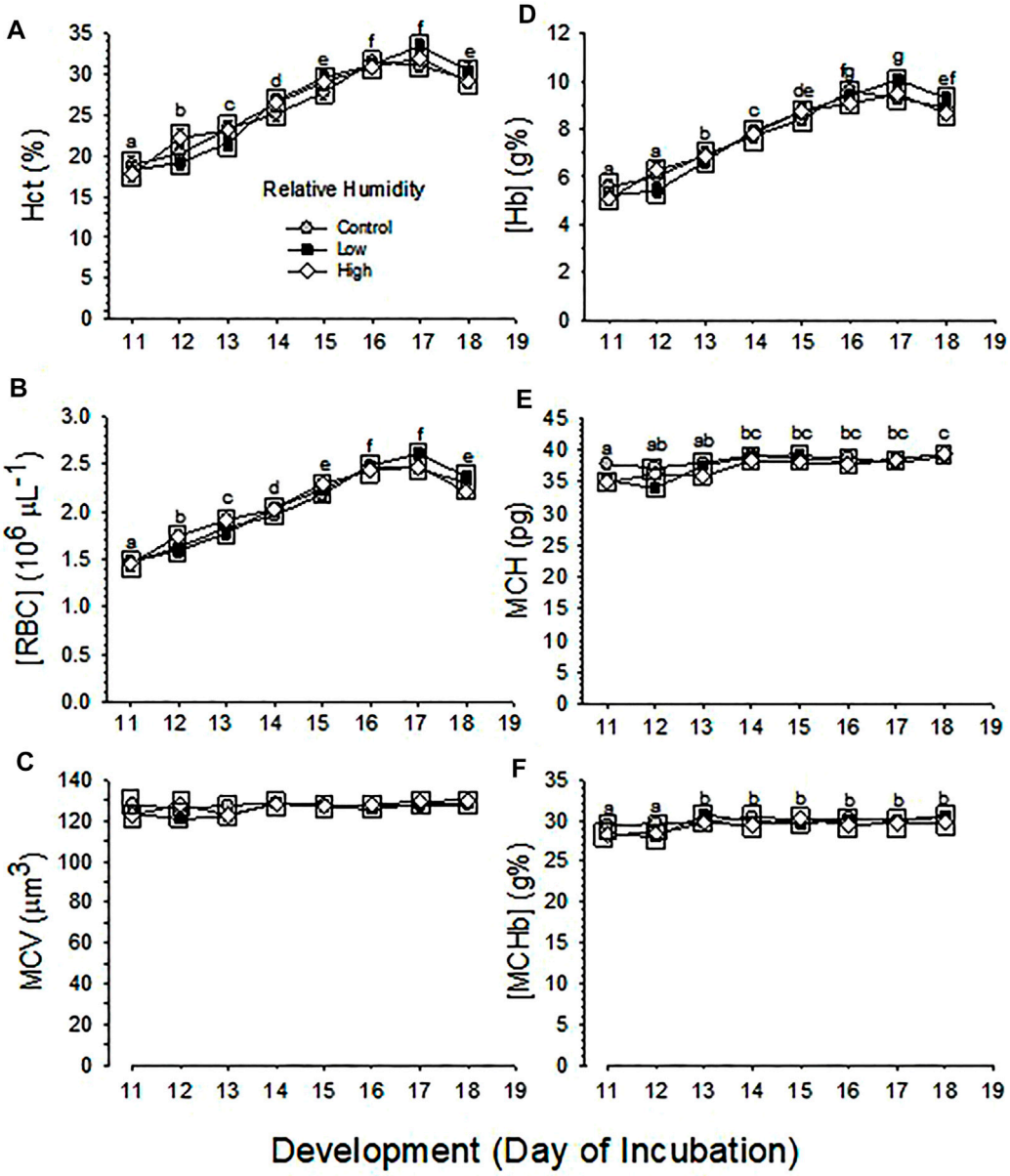

FIGURE 3 | Developmental changes in hematological variables of embryos incubated at Control, Low and High relative humidity. Means ± standard error are presented. Error bars are smaller than the symbol size. Different lowercase letters indicate significant differences across development. Boxes enclosing statistically identical means are omitted for clarity. **(A)** Hct **(%)** is not significantly ($p = 0.616$) affected by RH (vertical comparison), but is significantly affected by day of development ($p < 0.001$), with no significant interaction between the two variables ($p = 0.365$). Different lowercase letters indicate significant differences across development. **(B)** [RBC] is not significantly ($p = 0.539$) affected by RH (vertical comparison), but is significantly affected by development ($p < 0.001$), with no significant interaction between the two variables ($p = 0.395$). Different lowercase letters indicate significant difference. **(C)** MCV is not significantly affected by RH ($p = 0.106$) or development ($p = 0.054$) and their interaction is not significant ($p = 0.508$). **(D)** [Hb] is not significantly affected by RH ($p = 0.799$), but is affected by development ($p < 0.001$), with no significant interaction ($p = 0.226$) between the two variables. **(E)** MCH is significantly affected by both RH ($p = 0.011$) and development ($p < 0.001$), and the interaction between these two variables is significant ($p = 0.036$). **(F)** [MCHb] is significantly affected by both humidity ($p = 0.027$) and development ($p < 0.001$), with no significant interaction ($p = 0.661$) between the two variables. n values are as in **Figure 1**.

groups ($p = 0.005$) (**Figure 6A**). Mean Hct during submersion was 35.6 ± 0.5, 36.1 ± 0.5 and 34.0 ± 0.5% for Control, Low and High RH groups, respectively. The increase in High RH group was significantly less compared with the increases in Control and Low RH groups, which did not differ from each other. However, the extent of increase in Hct with submersion length was not different between the three RH groups ($p = 0.232$) (**Figure 6A**).

[RBC] in the three RH groups was not significantly changed by any length of submersion ($p = 0.185$) (**Figure 6B**). However, mean [RBC] during submersion (2.51 ± 0.03, 2.51 ± 0.03 and

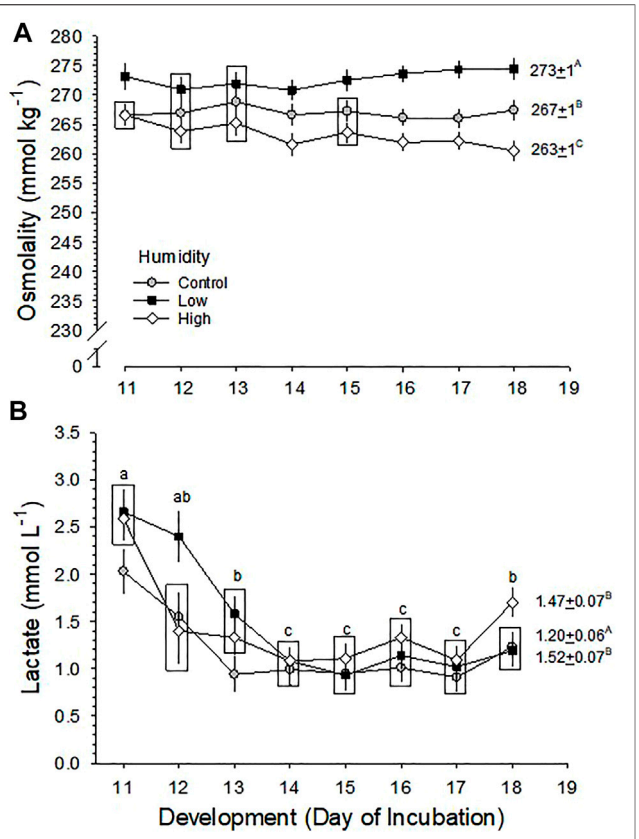

FIGURE 4 | Developmental changes in **(A)** blood osmolality (Osm) and **(B)** [Lactate] in embryos incubated at Control, Low and High relative humidity. Means ± standard error are presented. Means on any given day of incubation that are not significantly different from each other are enclosed by a box. **(A)** Blood Osmolality: The effect of RH on Osm (vertical comparison) is significant ($p < 0.001$), but effect of development ($p = 0.787$) and their interaction ($p = 0.671$) are not significant. Mean Osm across development is shown to the right of each RH plot, and these values are significantly different from each other ($p < 0.001$ for any two comparisons), as indicated by uppercase letters. **(B)** [La^-]: [La^-] was significantly affected by both humidity ($p = 0.001$) and development ($p < 0.001$), with no significant interaction ($p = 0.060$). Mean [La^-] during the last half of incubation (D11-D19) is shown to the right of each RH plot, with uppercase letters indicating differences between means. Mean [La^-] values of Low and High RH groups are significantly higher than the Control value ($p = 0.002$ for Low RH *vs.* Control RH, $p = 0.010$ for High RH *vs*. Control RH, $p = 0.610$ for Low RH *vs*. High RH). Lowercase letters indicate significant differences across development. *n* values are as in **Figure 1**.

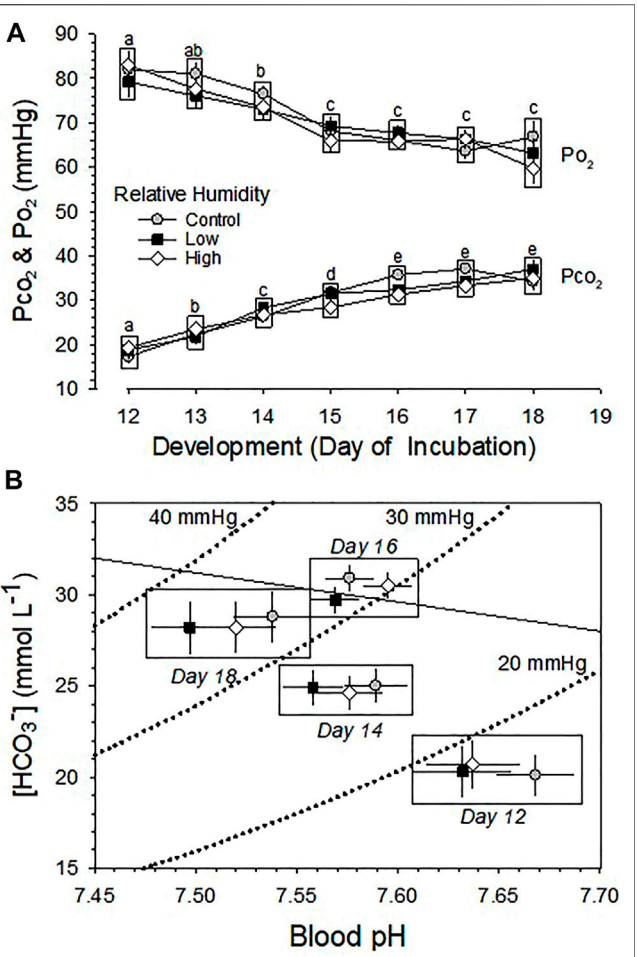

FIGURE 5 | Developmental changes in blood gas pressures and acid-base balance in embryos incubated at Control, Low and High relative humidity. Means ± standard error are presented. In some cases error bars are smaller than the symbol size. Means on any given day of incubation that are not significantly different from each other are enclosed by a box. **(A)** Arterialized blood Po_2 and Pco_2. Arterialized blood Po_2 and Pco_2 are not significantly affected by RH ($p \geq 0.359$). Means on any given day of incubation that are not significantly different from each other (vertical comparison) are enclosed by a box. However, there is a significant effect of development on both Po_2 ($p < 0.001$) and Pco_2 ($p < 0.001$), with no significant interactions ($p = 0.691$ for Po_2 and $p = 0.134$ for Pco_2). Different lowercase letters indicate significant difference between development. **(B)** Acid-base balance. For clarity, acid-base variables for incubation days 13, 15 and 17 are omitted from this Davenport diagram. RH did not significantly affect blood pH ($p = 0.133$) or blood [HCO_3^-] ($p \geq 0.241$), but there was a significant effect of development on pH ($p < 0.001$) and [HCO_3^-] ($p < 0.001$), with no significant interaction between these variables ($p = 0.491$ for pH and $p = 0.781$for [HCO_3^-]). n values are as in **Figure 1**.

$2.39 \pm 0.03 \times 10^6$ μL^{-1} for Control, Low and High RH groups, respectively), was significantly different ($p = 0.001$). [RBC] of High RH group was significantly lower compared with [RBC] of Control and Low RH groups, which were not different. The significant difference in [RBC] between the three RH groups was not changed by submersion ($p = 0.179$).

MCV significantly increased with submersion time ($p < 0.001$) in all three RH groups, with no significant difference of magnitude of increase between groups ($p = 0.780$) (**Figure 6C**). The interaction between effects of RH and submersion was not significant ($p = 0.096$).

[Hb] was not significantly changed by any length of submersion ($p = 0.438$) in the three RH groups, but mean [Hb] during submersion; 9.6 ± 0.1, 9.7 ± 0.1 and 9.1 ± 0.1 g% for Control, Low and High RH groups, respectively, was significantly different ($p < 0.001$) (**Figure 6D**). [Hb] of High RH group was significantly low compared with [Hb] of Control and Low RH groups that were not different. There

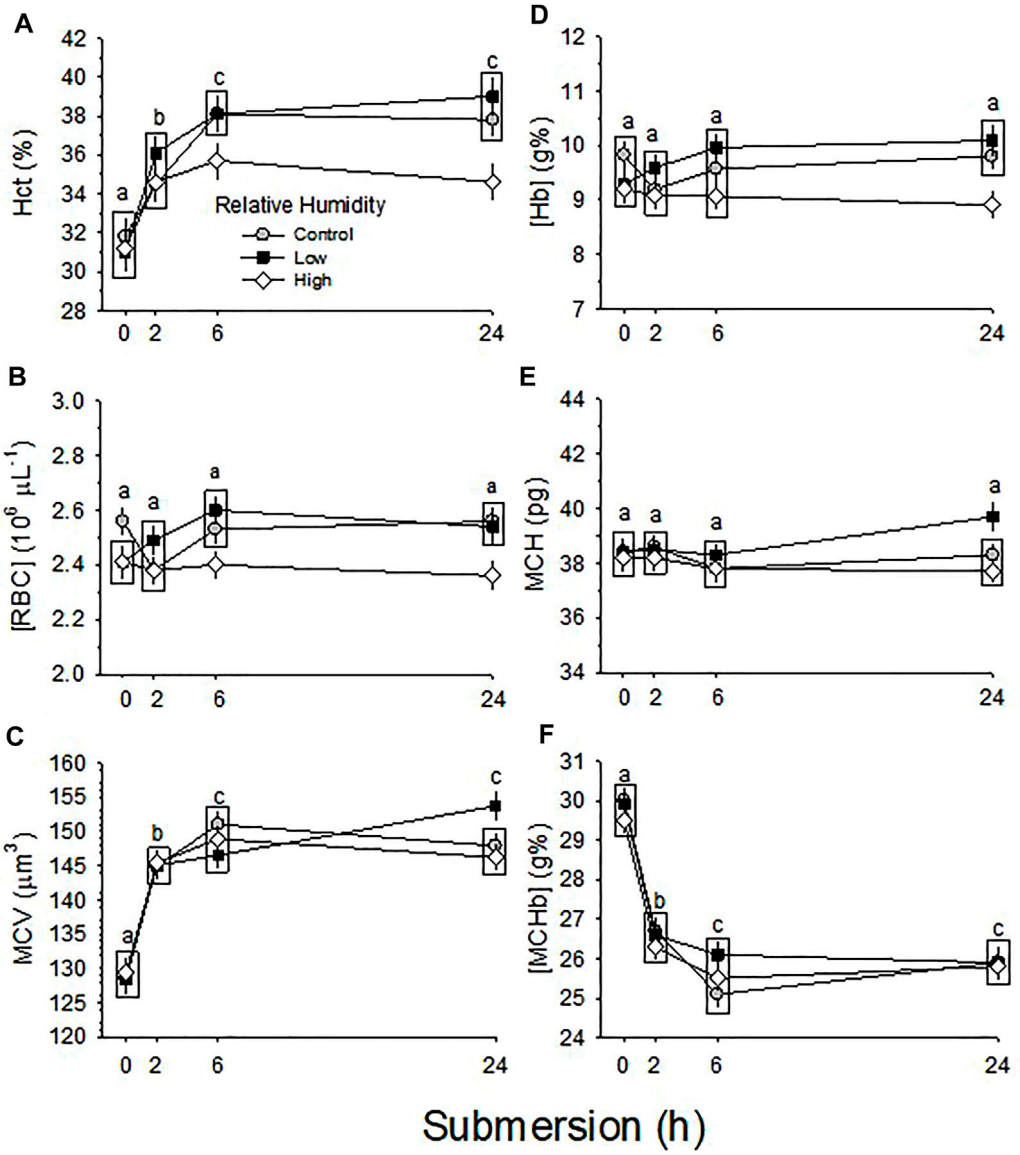

FIGURE 6 | Hematological changes in response to 24 h partial water submersion at d17 of incubation. Means ± standard error are presented. In some cases error bars are smaller than the symbol size. Means on any given day of incubation that are not significantly different from each other are enclosed by a box. Different lowercase letters indicate significant difference between submersion times. **(A)** Hct **(%).** Hct was significantly affected by both RH ($p = 0.005$) and partial water submersion ($p < 0.001$), with no significant interaction ($p = 0.232$). **(B)** [RBC]. [RBC] was significantly affected by RH ($p = 0.001$) but not partial water submersion ($p = 0.185$), with insignificant interaction ($p = 0.179$) occurring between the two variables. **(C)** MCV. MCV was not significantly affected by RH ($p = 0.780$), but was significantly affected by submersion ($p < 0.001$), with no significant interaction between the two variables ($p = 0.096$). **(D)** [Hb]. [Hb] was significantly affected by humidity ($p < 0.001$) but not partial water submersion ($p = 0.438$), with no significant interaction ($p = 0.226$) between the two variables. **(E)** MCH. MCH was not significantly affected by either RH ($p = 0.072$) or submersion ($p = 0.419$), with no significant interaction ($p = 0.463$) between the two variables. **(F)** [MCHb]. [MCHb] was significantly affected by submersion ($p < 0.001$) but not by RH ($p = 0.333$), with no significant interaction ($p = 0.550$) between the two variables. *n* values for each condition and each hour of submergence are indicated in **Figure 8**.

was no significant interaction between the three RH groups for submersion ($p = 0.226$ for interaction) (**Figure 6D**).

MCH was not changed by any length of submersion ($p = 0.419$) and mean MCH was not different between the three RH groups ($p = 0.072$) with no significant interaction between effects of RH and submersion ($p = 0.463$) (**Figure 6E**).

[MCHb] was significantly decreased by at all submersion times ($p < 0.001$) in the three RH groups, but there was no significant difference of magnitude between RH groups ($p = 0.333$) (**Figure 6F**). The interaction between RH and submersion was not significant ($p = 0.550$).

[La^-] was significantly affected by submersion in three RH groups ($p < 0.001$). [La^-] increased sharply after just 2 h of submersion in all

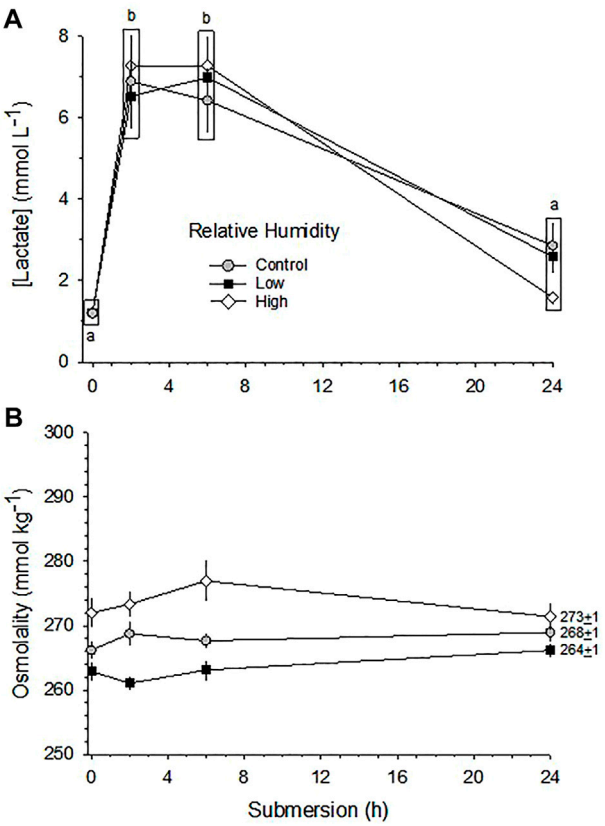

FIGURE 7 | Changes in [La^-] **(A)** and [Osm] **(B)** in response to 24 h partial water submersion at d17 of incubation. Means ± standard error are presented. Means on any given day of incubation that are not significantly different from each other are enclosed by a box. Different lowercase letters indicate significant difference between submersion times. n values for each condition and each hour of submergence are indicated in **Figure 8**.

three humidity groups and stayed elevated through hour 6. By hour 24, however, [La^-] had declined in the Control and Low RH groups, but were still significantly elevated in the High RH group (**Figure 7A**).

While the differences in [Osm] due to relative humidity differences was maintained throughout the submersion period, in no group did submersion *per se* significantly affect [Osm] ($p > 0.54$) (**Figure 7B**).

3.7.2 Acid-Base Balance

Arterialized blood pH and [HCO_3^-] of all three RH groups were significantly different across time submerged in water ($p < 0.001$) (**Figure 8**). However, there were no significant differences ($p > 0.36$) between the responses of the RH groups at any point during submersion with no significant interaction between effects of RH and submersion time ($p > 0.86$). All three RH groups initially showed an immediate mixed metabolic and respiratory acidosis at 2h, with blood PCO_2 before submersion (35 ± 1) rising significantly by ~30 mmHg–68 ± 3 mmHg. Concurrently, the pH before submersion, (7.58 ± 0.2), fell ~0.3 units to 7.28 ± 0.1 at 2 h. However, from 2 to 24 h there was a partial compensation of the metabolic and respiratory acidosis, with PCO_2 declining significantly from its peak by ~10 mmHg–56 ± 2 mmHg over this period and pH rising by ~0.15 mmHg–7.42 ± 0.1.

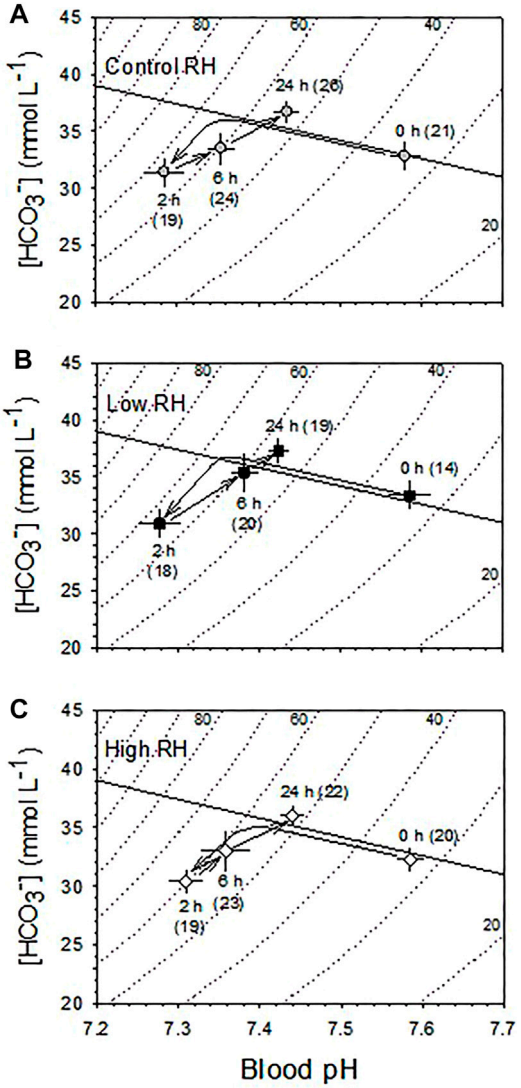

FIGURE 8 | Blood acid-base balance changes during the course of 24 h partial water submersion at d17 of incubation. **(A)** Control, **(B)** Low and **(C)** High humidity. Means ± standard error are presented in this Davenport Diagram. N values are indicated in parentheses. Measurements were made at 0, 2, 6, and 24 h following partial water submersion. Arrows indicate direction of change in mean values during the course of submersion. Both pH ($p < 0.001$) and [HCO_3^-] ($p < 0.001$) were significantly affected by partial water submergence in all three RH groups. However, there was no significant effect of incubation RH on either blood pH ($p = 0.762$) or [HCO_3^-] ($p = 0.359$), and there was no significant interaction between RH and submersion hour ($p > 0.87$). n values indicated in parentheses.

4 DISCUSSION

4.1 Hatchability

The hatching success of eggs incubated in commercial incubators is determined, in part, by incubator relative humidity. The relationship between embryo mortality and RH has been empirically determined and used to generate a bimodal curve (Ar and Rahn, 1980). In the literature on chicken egg incubation,

effects of incubation RH on hatchability vary somewhat. For example, in several studies the RH for optimum hatchability ranged from ~40 to 65%, maximum hatchability occurred at 50% RH, and hatchability was decreased at ~35–20% RH (Robertson I. S., 1961; Snyder and Birchard, 1982; Tullet and Burton, 1982; van der Pol et al., 2013). In contrast, there were no differences in hatchability among eggs incubated at ~40–70% RH (Buhr, 1995) and, similarly, in our study mean hatchability was unaffected by Low or High RH. There are clear physiological differences between different strains of chickens (Flores-Santin and Burggren, 2021) and these differences may extend back to the embryo, perhaps accounting for hatching variability in different relative humidities. Further experiments are required to determine the mechanisms underlying humidity-affected hatchling success.

4.2 Body Mass

Although RH did not affect hatchability in the current experiment, hatchling body mass was altered, with body mass increasing in proportion to incubation RH. Again, results of RH differences in the literature are variable. There was no effect of RH on wet body mass of hatchlings hatched from eggs incubated at 40 and 70% RH and wet body mass at hatch was not different between Control and 85–90% RH groups (Robertson I. S., 1961; Davis et al., 1988). Wet body mass at hatch decreased in eggs incubated at 20 and 33% RH and increased with increasing RH (Tullet and Burton, 1982; Peebles et al., 1987; Hamdy et al., 1991; Bruzual et al., 2000). Bruzual et al. (2000) reported that wet body mass at hatch increased significantly with increasing RH treatment, but BM at pull (removal from machine) was not different. That result resembles as our observations. Dry body mass was unaffected by RH in the current study (**Figure 2A**) as well as that of Tullet and Burton (1982). Collectively, these data suggest that variation in wet body mass under different conditions results from hydration/dehydration of the embryos, rather than actual changes in growth.

Since dry body mass of embryos was not different between the three groups with larger body water content in Control and High RH groups compared with Low RH group, embryos in Control and High RH groups are assumed to be becoming hydrated late in incubation toward hatching.

4.3 Blood Variables

Blood Osm reflected tissue water content as influenced by incubation RH, as expected, with blood Osm being ~4% higher in Low RH compared to High RH groups (**Figure 4A**). Similarly, lactate concentration was different between RH groups at different points in development (**Figure 4B**). Increased blood [La^-] has traditionally been viewed to be the result of stress, particularly if aerobic metabolism is affected, although increasingly lactate is viewed as an intermediary in aerobic metabolism, as well (Brooks, 2018, 2020). In the present study, the highest lactate levels were found in the High RH group later in development. Since blood Po_2 was unaffected by RH (**Figure 5**), it is unlikely that changes in glycolysis under different incubation RHs was contributing to any changes in [La^-]. Noteworthy is that no consistent patterns emerged during incubation and even the largest [La^-] differences were only in the order of 50–70%, compared to as much as 16-fold increases that can occur in chicken embryos when experiencing hypoxia (Tazawa et al., 2012).

Developmental changes in hematological respiratory variables in the developing chicken embryo have been reported in many studies (Yosphe-Purer et al., 1953; Barnes and Jensen, 1959; Temple and Metcalfe, 1970; de W Erasmus et al., 1970; Tazawa, 1971; Tazawa et al., 1971a, 1971b; Tazawa, 1984; Dzialowski et al., 2002; Black and Burggren, 2004; Khorrami et al., 2008; Bolin et al., 2017). Some studies and reviews summarizing several reports indicated that hematological variables plateaued towards the end of incubation (Johnston, 1955; Macpherson and Deamer, 1964; Jalavisto et al., 1965; Romanoff, 1967; Tazawa, 1980; Davis et al., 1988; Tazawa et al., 2011). Although the current study did not collect arterialized blood after d19, Hct, [RBC] and [Hb] reached maximal values on d16-17 with subsequent decreases on d18 of development (**Figure 3**), rather than plateauing. This decrease is consistent with other studies showing a temporary decrease in red blood cells and related hematological variables prior to hatching (Clark, 1951; Bartels et al., 1966; Ackerman, 1970; Freeman and Misson, 1970; Lemez, 1972). The reason for decreases of Hct, [RBC] and [Hb] remain to be studied.

There was no influence of incubation RH on hematological variables (**Figure 3),** on blood PO_2, PCO_2 and acid-base balance variables (**Figure 5).** Gas diffusion conductance of the eggshell and shell membranes remains unchanged during the last half of incubation when the chorioallantoic membrane has fully developed, so arterialized blood Po_2 decreases and Pco_2 increases due to increasing metabolism of developing embryos (**Figure 5A**). Simultaneously, as dissolved CO_2 increases in blood with development of embryos, acid-base status of older embryos develops a respiratory acidosis relative to younger embryos (**Figure 5B**). Yet, these significant developmental changes in blood gases and acid-base balance were completely unaffected by incubation RH, even as water content and body mass were greatly affected.

4.4 Incubation RH and Regulation of Hematological and Acid-Base Balance

Partial water submersion of embryonated eggs is a useful tool for decreasing gas exchange through the eggshell, resulting in intrinsic O_2 deficiency (hypoxemia) and CO_2 accumulation (hypercapnia) in the embryo (Andrewartha et al., 2014; Kohl et al., 2015; Branum et al., 2016). In the current study, hypoxemia and hypercapnia induced swelling of the red blood cells as a result of hypoxemia and hypercapnia—i.e. Hct and MCV both increased. The maximum change was typically reached at 6 h of submergence, with no further change through to 24 h of submergence (**Figure 6**). Interestingly, no hematological variables at the 6 h point of submersion differed between the Control and Low RH groups. Notably, however, the High RH group at 6 h and especially at 24 h had a significantly lower Hct, lower [Hb], lower [RBC] compared with the Control and Low RH groups, which still showed a greater disruption caused by

hypoxemia and hypercapnia. That is, the High RH group at 6 and especially 24 h showed the smallest deviation from pre-submergence values of all hematological variables (**Figure 6**). Thus, we posit that the additional tissue water content associated with High RH incubation provides some degree of protection against hematological disturbance induced by submergence-related intrinsic hypoxemia and hypercapnia. Identification of the mechanism affording this red blood cell protection awaits further experimentation.

The initial development of large mixed metabolic and respiratory acidosis of the blood upon partial water submergence was unaffected by incubation RH, and was reflected by the very large and immediate increase in blood [La^-] (**Figure 7**). Similarly, the partial recovery through primarily metabolic mechanisms after 2 h of submergence developed to the same extent in all three RH groups (**Figure 8**). These data suggest that the regulatory capability to at least partially restore blood acid-base balance exists independently of the severe disturbances to tissue water content caused by incubation in High or Low RH. To what extent other physiological systems—e.g., renal, cardiovascular—retain the ability to carry out their physiological functions awaits additional experimentation.

4.5 Implications of Non-Ideal Incubation Humidity

This study has revealed that chronic relative humidities above or below optimum can to some extent influence both morphological and physiological factors at the time of hatching. Several questions arise: Do hatchlings showing alterations at birth as a result of incubation in non-ideal humidity subsequently 'grow out of' these modifications into adulthood, or are they retained as life-long effects? Do hatchlings with normal morphological and physiological variables despite Low and High RH-incubation actually develop abnormalities during subsequent development to adulthood? While entirely speculative could there be effects of RH incubation that, through epigenetic inheritance, carry over into the offspring of chickens incubated in non-ideal conditions? Certainly, in the King quail (*Coturnix chinensis*) there can be transgenerational effects of pollutant exposure (Bautista et al., 2021). Could this also be the case for RH-induced changes on the embryo? Finally, could different breeds of chicken have different response to incubation RH, and might they thus be better adapted to low or high humidity climates? Answering these and other questions warrants additional study in chickens and other birds.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, University of North Texas.

AUTHOR CONTRIBUTIONS

SB; Conceptualization; Experiments, Analysis, Manuscript Preparation. HT; Experiments, Analysis, Manuscript Preparation, Figure Preparation; WB, Conceptualization, Manuscript Preparation.

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How Egg Storage Duration Prior to Incubation Impairs Egg Quality and Chicken Embryonic Development: Contribution of Imaging Technologies

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Storing fertilised eggs prior to incubation is a frequent practice in commercial hatcheries to coordinate activities and synchronise hatchings. However, the conditions used to store eggs can have major impacts on egg quality and the subsequent viability of chicken embryos. While storage temperatures of 16–18°C are classically used in hatcheries, the duration of storage varies from three to more than 10 days. We explored the effect of storage duration (zero, three or 10 days; D0, D3 and D10, respectively) at 16°C, 80% relative humidity (RH) on egg quality (Broiler, Ross 308), using computed tomography (CT) and classical measurements (egg weight, eggshell strength, egg white pH, Haugh units, yolk index and colour). The results revealed that a storage duration of up to 10 days negatively affected some egg quality traits (yolk index and volume, air chamber volume and egg white pH). Eggs stored for three or 10 days were further incubated for 11, 13 or 15 days (37.8°C, 55% RH). Eggs were analysed by magnetic resonance imaging (MRI) and CT to assess the development of the embryo and internal egg changes occurring during incubation. First, data showed that the fertility and sex ratio of eggs were not affected by storage duration. However, the mortality of viable eggs was increased in the D10 group compared to the D3 group. Results of non-invasive imaging technologies revealed that the storage of eggs for 10 days impaired embryo growth as early as 11 days of incubation (decrease in brain and embryo volumes). Collectively, these data provide new evidence that the duration of egg storage negatively affects embryonic growth. They further corroborate that this parameter is likely to be crucial to synchronising embryonic stages and maybe reducing the hatching window, hence limiting the time spent by newborn chicks in hatchers. In addition, our results highlight that CT and MRI imaging technologies are useful non-invasive tools to evaluate egg quality prior to incubation and the impact of storage (or incubation) practices on developmental growth of the embryo.

Keywords: chicken, egg, storage, duration, egg quality, embryonic development, magnetic resonance imaging, computed tomography

Abbreviations: CT, computed tomography; D, day; EID, embryonic incubation day; MRI, magnetic resonance imaging; RH, relative humidity.

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INTRODUCTION

Egg storage prior to incubation is an common practice in the broiler industry (Fasenko, 2007). It allows coordinating hatchery activities, considering the time between laying and the arrival of eggs in hatcheries. It supplies a certain flexibility towards demands and facilitates synchronisation of hatchings.

Egg storage prior to incubation does not negatively affect hatchability when the duration of storage does not exceed 7 days (Fasenko, 2007). It is noteworthy that incubating freshly laid eggs is, unexpectedly, not associated with higher embryo viability compared to that of stored eggs, and eggs incubated the day of laying tend to hatch later compared with eggs stored for one or 2 days (Reis et al., 1997). The freshly laid egg contains a high concentration of carbon dioxide that may be detrimental to initiating the first stages of embryo development, while the thickness of the egg white is assumed to slow vital gas diffusion and limit access to egg nutrients (Benton and Brake, 1996). On the other hand, extended storage can have a dramatic impact on blastoderm reactivation, even if it is conducted at 17–18°C under controlled relative humidity. Storage beyond 7 days is usually associated with decreased hatchability rates compared to short periods of storage (Lapão et al., 1999; Elibol et al., 2002; Hamidu et al., 2011; Goliomytis et al., 2015; Bakst et al., 2016b; Abioja et al., 2021) and egg storage time was evidenced to be the most important factor (among genotype, hen age, setter and hatcher type) associated with early embryonic mortality (Grochowska et al., 2019). Long storage induces an alteration of many egg quality features including a decrease in yolk and albumen quality parameters and water loss, but also impairs the quality of the blastoderm (increased diameter, small shift of its position on the yolk likely due to the progressive disintegration of the chalazae, decreased number of viable cells, increased necrosis and apoptosis, etc.) (Burkhardt et al., 2011; Bakst et al., 2012; Abioja et al., 2021) (**Figure 1**). These combined alterations result in an increase of early and late embryo mortality. Similar observations have been reported in the literature for other domestic avian species (Fasenko et al., 2001; Hassan et al., 2005; Nowaczewski et al., 2010; Hyánková and Novotná, 2013; Kouame et al., 2019; Taha et al., 2019).

Upon egg storage at cooled temperature, the embryo metabolism changes (Christensen et al., 2001) and the embryonic development pauses. The embryo (blastoderm) enters a temperature-induced diapause (Pokhrel et al., 2021), also termed dormancy, which is characterised by reduced cellular activity and suppressed apoptosis (Tona et al., 2003b; Bakst et al., 2016b; Ko et al., 2017). However, lengthening the storage period of eggs irreversibly impairs embryo survival. Prolonged storage (over 10 days) has been shown to activate mechanisms of apoptotic cell death at the blastodermal level (upregulation of pro-apoptotic genes), resulting in decreasing blastodermal cell viability (Hamidu et al., 2011). Long storage also affects the ability of the embryo to resume development once incubated, while embryos that survive long storage treatment undergo delayed hatching by several hours (Christensen et al., 2002; Tona et al., 2003a; Tona et al., 2003b). Long storage of eggs can affect the intestinal morphology of the chicks, the expression of nutrient transporters (Yalcin et al., 2016; Yalcin et al., 2017), chick immunocompetence (Goliomytis et al., 2015) and hormonal metabolism (Tona et al., 2003b). Long storage may also have long-term negative effects on the quality and physiology of hatched chicks (Tona et al., 2003a; Tona et al., 2003b; Reijrink et al., 2010; Yalcin et al., 2017; Mróz et al., 2019).

Many studies have been published on the effects of prolonged storage on egg quality and blastoderm characteristics, but also on the chick after hatching. However, only few articles address the impact of egg storage on embryonic development. Long storage conditions have been shown to affect the development of the embryo, which exhibits lower overall weight (Christensen et al.,

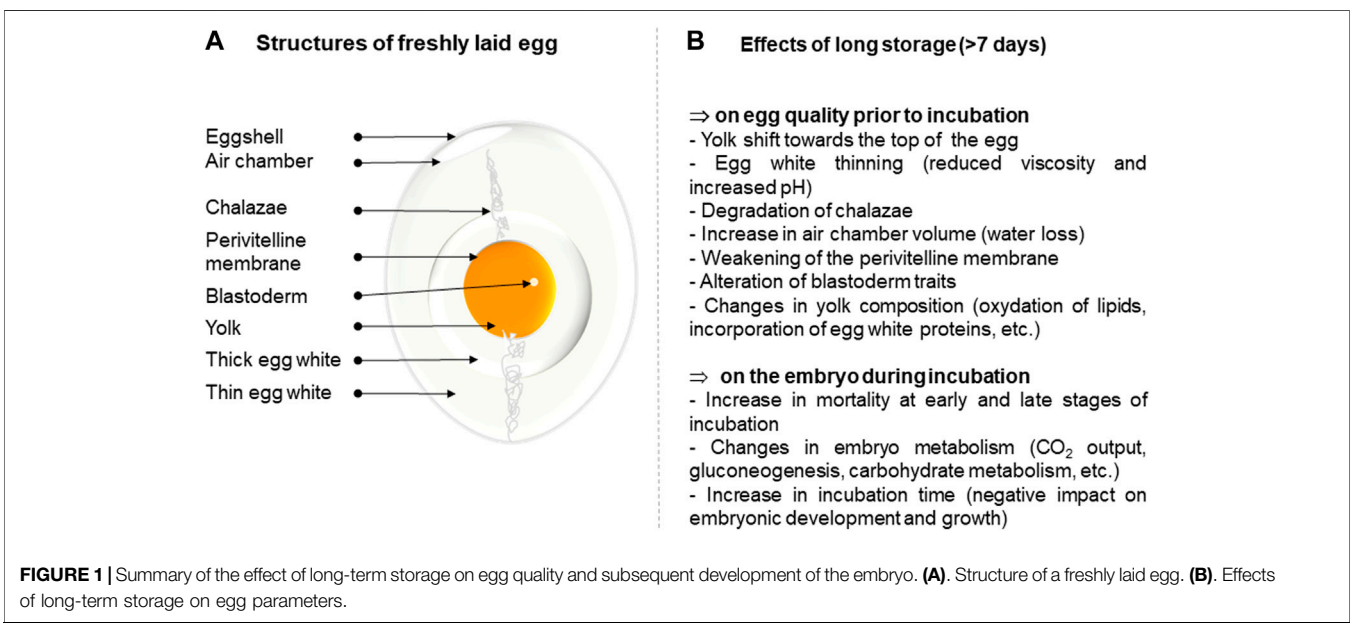

FIGURE 1 | Summary of the effect of long-term storage on egg quality and subsequent development of the embryo. **(A)**. Structure of a freshly laid egg. **(B)**. Effects of long-term storage on egg parameters.

2002; Hamidu et al., 2011; Bakst et al., 2016a), lighter heart, liver and thigh muscle (Christensen et al., 2002) and smaller leg bones (Yalcin and Siegel, 2003), compared to those stored for one to 4 days. All these observations strongly support that the development of embryos after long storage is slowed down, which likely explains the delayed hatching observed in several studies (Christensen et al., 2002; Tona et al., 2003a; Tona et al., 2003b) when compared to eggs stored for only few days. It seems that embryos from eggs stored for a long period require more time in the incubator to reach the developmental maturity that is necessary for hatching, compared with eggs stored for a short period.

Based on these data, which were mostly obtained after egg opening, we evaluated whether computed tomography (CT) and magnetic resonance imaging (MRI), as non-invasive technologies, could be used to monitor internal changes that occur in eggs upon short and long storage. CT has been previously applied to localise the germinal disc *in ovo* (Bartels et al., 2008) and MRI techniques have been used to study the egg yolk structure (Hutchison et al., 1992), the localisation of the germinal disc (Klein et al., 2002) and to monitor the development of the embryo under conventional conditions (Bain et al., 2007; Boss et al., 2008; Zhou et al., 2015; Lindner et al., 2017; Kantarcioglu et al., 2018). However, the use of such techniques to assess the effect of storage time on egg quality and embryo development has not been reported to date.

MATERIALS AND METHODS

The experimental workflow is illustrated in **Figure 2**. Using CT, we analysed the quality of eggs collected on the day of laying (D0) or after three and 10 days of storage (D3 and D10, respectively) at 16°C, 80% RH. Eggs were then opened and classical egg quality parameters were measured. Remaining D3 and D10 eggs were incubated (37.8°C, 55% RH) for 11, 13 and 15 days and analysed by MRI and CT, followed by egg opening to weigh the embryo.

Incubation Procedures and Sampling

Freshly laid fertilised eggs were obtained from 30-week-old broiler breeder hens (ROSS 308, Boyé Accouvage, La Boissière en Gâtine, France). Eggs were all weighted and ten-egg batches of similar egg weight (56.5 ± 0.52 g) were formed. The egg weight in each batch ranged from 53 to 60.4 g to illustrate natural egg weight heterogeneity. Ten eggs were kept (D0) for analyses, while the remaining eggs were stored in the Poultry Experimental Facility (PEAT) UE1295 (INRAE, F-37380 Nouzilly, France, DOI: 10.15454/1.5572326250887292E12) in a dedicated room at 16°C, 80% RH for three (D3) or 10 days (D10). Ten eggs were collected at D0, D3 and D10 and were analysed by computed tomography, followed by the measurement of some egg quality parameters. The day before incubation 60 D3 and 60 D10 eggs were placed at room temperature (45% hygrometry), and then transferred into a 3900-egg incubator (Bekoto B64-S, Pont-Saint-Martin, France) set at 37.8°C, 55% RH (automatic turning every hour, large end of eggs on top). After 11, 13 and 15 days of incubation (Embryonic incubation day 11, 13 and 15 or EID13, EID13, and EID15, respectively), D3 and D10 eggs containing viable embryos were analysed by computed tomography while others were selected for MRI analyses. After CT and MRI acquisitions, all eggs were weighed and opened to collect embryos that were killed by decapitation. This experimental procedure meets the guidelines approved by the Institutional Animal Care and Use Committee (IACUC). Dead embryos were weighed and a small piece of the liver was collected, kept at −20°C for molecular sexing.

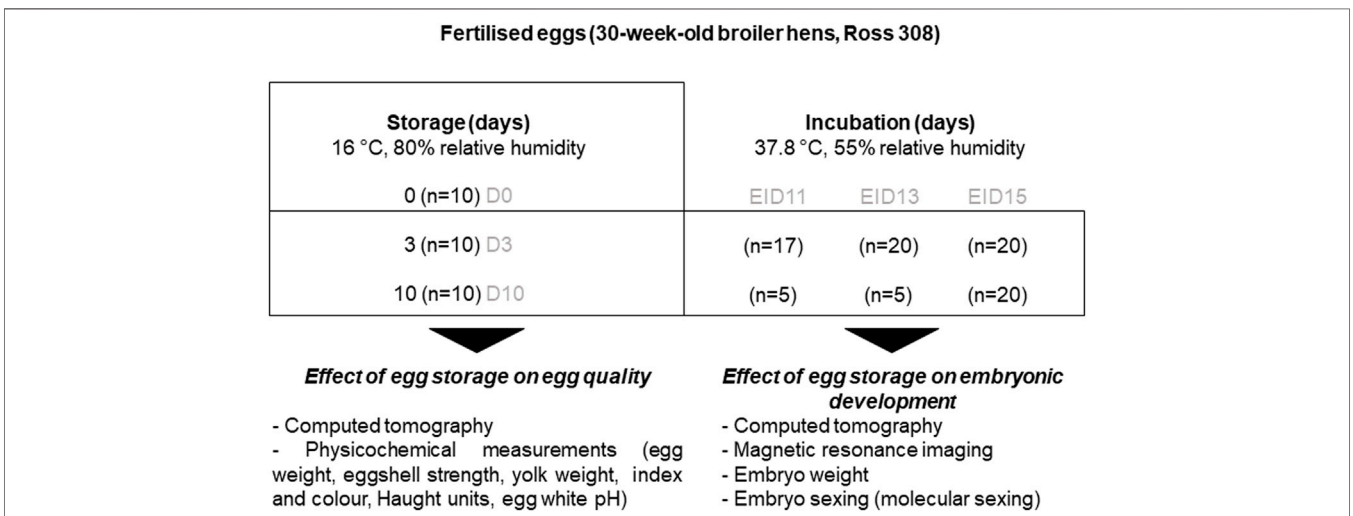

FIGURE 2 | Experimental design. The internal quality of freshly laid eggs (day of laying, D0) and eggs stored for three (D3) or 10 days (D10) was measured to evaluate changes occurring during storage (left panel). A series of eggs stored for three or 10 days were further incubated for 11 (EID11), 13 (EID13) or 15 (EID15) days to analyse the impact of storage on embryonic development (right panel).

Measurements of Egg Quality Parameters During Egg Storage and Incubation (CT and Physicochemical Measurements)

A series of ten eggs were collected the day of laying, three and 10 days after storage. Eggs were analysed by clinical computed tomography (Siemens Somatom® Definition AS 128, Siemens, Germany). The X-ray source was set at 140 kV and 400 mA/s. The image acquisition mode was 16 cm * 16 cm, 512 pixels matrix size with a slice thickness of 0.4 mm and a resolution of 312.5 μm. Safire U 40 and V80 reconstruction filters were used to characterise the internal components of the egg and the shell, respectively (**Table 1**). The analysis of egg structures was performed with the Syngo. Via software (Siemens, Germany). The volume of the air chamber and the albumen was estimated automatically using ITK Snap software (Yushkevich et al., 2006).

Following CT acquisition, eggs were characterised for their quality. Egg weight, eggshell strength, Haugh units, yolk colour and index were measured using a Digital Egg Tester 6,000 (Nabel, Kyoto, Japan). The egg yolk was weighed and egg white pH was measured (Eutech pH metre, Thermo Fisher Scientific, Illkirch, France).

MRI Analyses During Incubation

The eggs stored for 3 days (D3) or 10 days (D10) were incubated. After 11, 13 or 15 days (EID11, EID13, EID15), eggs were collected and refrigerated at 4°C for 1 h and 10 min at −20°C prior to analyses with 3 T (T) MRI scanner (Siemens Magnetom®, Verio, Erlangen, Germany). Such an egg cooling was necessary to anesthetise the chick and thus avoid movements of the embryos during MRI acquisition.

We used one radio frequency (RF) 'loop' coil, with an inner diameter of 7 cm, to analyse eggs independently. Each egg was inserted in the middle of the loop coil.

Two separate MRI image sequences (T_1: spin-lattice or longitudinal relaxation time and T_2: spin-spin or transverse relaxation time) were performed on the whole brain, in order to get two distinct image contrasts. The T_1 3D and T_2 3D were the Magnetisation Prepared Rapid Acquisition Gradient Echo (MPRAGE) and the Sampling Perfection with Application-optimised Contrasts (SPACE), respectively.

The acquisition parameters for these T_1 and T_2 anatomical analyses were as follows:

-T_1 3D: repetition time (TR) = 1970 ms; echo time (TE) = 3.34 ms; inversion time (TI) = 900 ms; flip angle = 9°; field-of-view (FOV): 81 * 81 mm^2; matrix: 192 * 192^2; and a slice thickness of 0.4 mm resulting in a voxel size of 0.42 * 0.42 * 0.40 mm^3. A bandwidth of 150 Hz/Px and two number of excitations (NEX) producing an acquisition time of 9 min 29 s were used.

-T_2 3D: TR = 1860 ms; TE = 140 ms; flip angle = 140°; bandwidth of 296 Hz/Px; and a turbo factor of 99. The inter echo space was 7.38 ms. The FOV was 70 * 70 mm^2. The matrix was 192 * 192^2 and slice thickness was 0.35 mm, which ended with a voxel size of 0.36 * 0.36 * 0.35 mm^3. The acquisition time was 8 min 57 s.

Volumes on the MRI images were estimated based on T_1 images of the yolk and the albumen, and on the T_2 images for the brain, eyes, yolk sac, allantoic fluid and embryo, as T_2 contrast clearly allows distinction of water content between egg compartments (**Table 1**). At EID13 and EID15, the yolk sac and embryo signals were merged, as the tissue aspect of the yolk sac as a very dense and vascularised tissue (Wong and Uni, 2021) resembled the embryo and both structures could not be distinguished.

For volume estimation, the Digital Imaging and Communication in Medicine (DICOM) images were converted into the Neuroimaging Informatics Technology Initiative (NIfTI) format.

The NIfTI images were read with ITK Snap, which is a free, post-processing software generally used to segment 3D medical image structures (Yushkevich et al., 2006). The segmentation of "area growing" type was done automatically and then corrected manually.

After MRI analyses, eggs were weighed, embryos were removed from the eggs and decapitated and embryo weight was determined. Small pieces of the liver were collected and stored at −20°C for further analysis (molecular sexing).

Molecular Sexing

Molecular sexing was performed as previously published with minor adjustments (He et al., 2019). Small pieces of EID11, EID13

TABLE 1 | Egg analysis *via* imaging methods.

Traits	Volume [min; max] mm^3		Imaging method
	During storage (D0, D3, D10)	During incubation (EID11, EID13, EID15)	
Egg white	[31,950; 39,970]	[1,512; 4,664]	CT/T_1 MRI
Egg yolk	[8,371; 11,740]	N/A	CT/T_1 MRI
Air chamber	[144.0; 1,110.0]	[3,249; 5,342]	CT
"Grey zone" including the blastoderm[a]	[153.3; 502.9]	N/A	CT
Allantoic fluid	N/A	[4,026; 12,680]	T_2 MRI
Amniotic fluid	N/A	[3,125; 3,762]	T_2 MRI
Eyes	N/A	[336.6; 632.6]	T_2 MRI
Brain	N/A	[260.5; 781.5]	T_2 MRI
Embryo	N/A	[4,429; 6,524]	T_2 MRI
Embryo + Yolk	N/A	[14,010; 27,690]	T_2 MRI

[a]*This trait was estimated based on pixels that could not be assigned to the egg yolk or egg white. N/A. not applicable.*

and EID15 embryo livers were lysed in 150 µl of lysis buffer containing 10% of chelating beads (Chelex 100), 0.2% SDS, 10 mM Tris pH 8 and 0.2 mg/ml Proteinase K). Samples were incubated for 3 hours at 55°C followed by a 15-min incubation at 95°C. Samples were then centrifuged for 3 min at room temperature at maximum speed with a Mini centrifuge 6K (ExtraGene, Taichung City, Taiwan). Supernatants were recovered and stored at −20°C until use. DNA lysate quantification was assessed by reading the 260 nm absorbance with a micro volume spectrophotometer (Nanodrop One Thermo Scientific, Wilmington United States). Embryo lysates were diluted ten times in nuclease free water and 1 µl of dilution was mixed on ice with primer SWIM (forward: 5′- GAGATCACGAACTCAACCAG -3′/ reverse: 5′- CCAGACCTAATACGGTTTTACAG -3′), which is female specific and primer 12S (forward-5′ CTATAATCGATA ATCCACGATTCA- 3′, reverse: 5′- CTTGACCTGTCTTATTAG CGAGG -3′) and Dream Taq PCR Master Mix (2X), according to the manufacturer's recommendations (Thermo Fisher Scientific, Illkirch, France). Amplification by polymerase chain reaction (PCR) was performed using a thermocycler (Eppendorf, Montesson, France), as described previously (He et al., 2019). PCR products were loaded on a 2% agarose gel containing 0.01% gel Red in 1X TAE buffer, and separated by electrophoresis at 100 V. Gels were imaged using a Bio-Print imager (Vilber Lourmat, Marne-la-Vallée, France). Female samples exhibited two amplification products (131 bp for 12S and 212 bp for SWIM gene), while male samples exhibited only one amplification product (131 bp for 12S gene).

Statistical Analyses

All statistical analyses were performed using XLSTAT software (Data Analysis and Statistical Solution for Microsoft Excel, Addinsoft, Paris, France 2017). For most parameters, normality of the samples was not achieved (Shapiro–Wilk test). Thus, all statistical analyses (except for embryo weight) were performed using a Kruskal–Wallis test ($p < 0.05$), followed by a pair comparison using a Mann–Whitney test ($p < 0.05$), when required. For embryo weight, we used an ANOVA test.

RESULTS

Impact of Egg Storage for Zero, Three or Ten Days on Egg Quality

CT images that are representative of each egg storage group (D0, D3 and D10) are shown in **Figure 3**. Analysis of principal components clearly demonstrated that all three groups of eggs, freshly laid (D0) or stored for three or 10 days (D3 and D10, respectively) were distributed distinctly (**Figure 4**). The D3 group was intermediate between the D0 and D10 groups, as expected. Combined results from CT and egg quality parameter measurements are presented in **Table 2**. No statistical difference was observed for egg weight, eggshell strength, yolk colour, yolk weight and egg white volume. Yolk index, yolk volume and Haugh units tended to decrease over storage, while egg white pH and the volume of the air chamber increased. Although not statistically significant between days of storage ($p = 0.129$, ANOVA test), the volume of the "grey zone" (that includes the blastoderm) tended to increase over time. The trend was confirmed when comparisons were performed between two groups (Mann-Whitney test). The difference between D3 and D10 groups was shown to be significant (p-value = 0.031), not significant between D0 and D3 groups ($p = 0.297$), and close to statistical significance between D0 and D10 groups ($p = 0.060$). It is noteworthy that volumes of the yolk and the "grey zone" were expected to be negatively correlated: the more pixels attributed to the "grey zone", the less pixels assigned to the yolk.

Impact of Egg Storage for Three or Ten Days on Embryo Viability and Development

The time of storage prior to incubation did not significantly affect fertility (98.1% for the D3 group and 98.5% for the D10 group). However, when considering the whole period of experimentation (from EID0 to EID15), the duration of storage was shown to impair embryo viability: 4.7% mortality on the 65 viable D10-eggs vs. 1% mortality on the 105 viable D3-eggs.

In addition, the sex ratio determined on viable eggs was not equilibrated, especially at EID11 and EID15 after 10 days of incubation (**Figure 5A**). However, due to the small number of eggs analysed, we could not conclude on the effect of storage on sex ratio at each incubation day. After 15 days of incubation, the

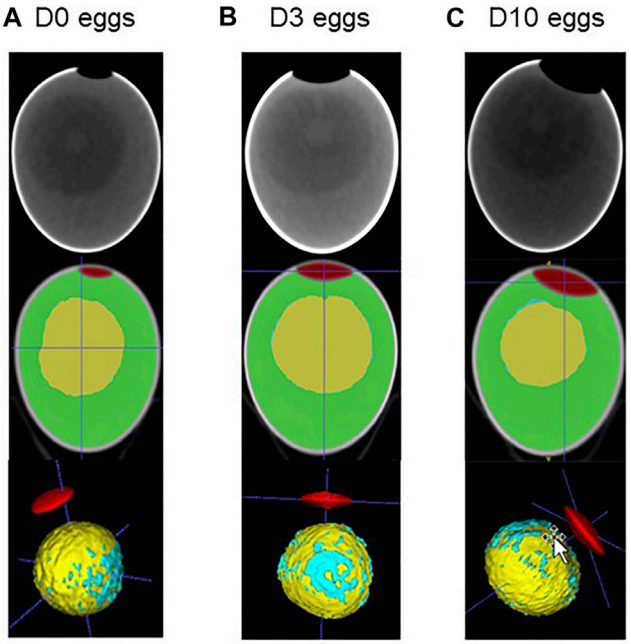

FIGURE 3 | CT imaging of fertilised eggs stored for zero, three or 10 days (D0 **(A)**, D3 **(B)** and D10 **(C)**, respectively). Using ITK. snap tool (Yushkevich et al., 2006), images obtained after CT imaging (upper panel) were analysed for 3D reconstruction, segmentation and coloured for illustration. The air chamber is illustrated in red, the yolk is shown in yellow, the white in green and the blastoderm in blue. It is noteworthy that for the latter parameter, we considered all blue spots distributed on the yolk surface to avoid any bias between groups. A concentration of blue spots that likely corresponds to the blastoderm is clearly visible, while blue spots are also sporadically distributed on the yolk surface.

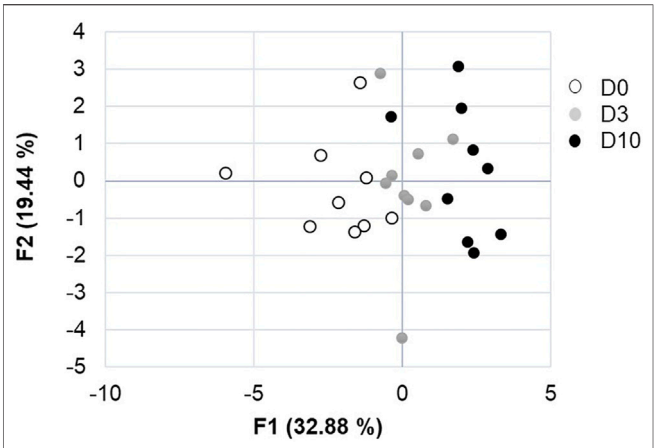

FIGURE 4 | Analysis of principal components of the effect of storage duration on egg parameters. F1 and F2 axes explain 52.2% of the variability between D0, D3 and D10 groups.

sex ratio of the 20 viable eggs was in favour of males (65 and 70% for D3-EID15 and D10-EID15 groups, respectively). Analysis of D3 and D10 groups (after combination of EID11, EID13 and EID15 eggs) indicated that sex ratios were comparable between the two groups, with a slight predominance of female embryos, regardless of the storage time (56/57% females vs. 43/44% males, **Figure 5B**). Therefore, from this experiment and considering the entire incubation period studied (EID11 to EID15), we concluded that there was no effect of storage duration on the sex ratio of the embryos.

MRI images were analysed to measure volumes of internal egg components and the embryo (**Figure 6**).

Independent of storage time, the volumes of egg white, air chamber, allantoic fluid were shown to decrease over time ($p < 0.05$) during embryonic development, while those of eyes, brain and embryo increased ($p < 0.05$) (**Table 3**).

Pair comparisons between D3 and D10 groups at each developmental stage revealed a decrease in eye, brain and embryo (or embryo + yolk for EID13 and EID15 stages) volumes at EID11 and EID15 for D10 eggs, compared with D3 eggs (**Table 3**). Similarly, these embryo volumes at EID13, tended to be lower in the D10 group compared with the D3 group, although no statistical difference was observed (**Table 3**). Collectively, these data support that the embryonic development is delayed after a 10-days storage but not altered (absence of visible malformations).

Embryo weight increased similarly between D3 and D10 groups (**Figure 7**). However, embryos from D10 eggs were significantly lighter than embryos from D3 eggs, especially after 13 (EID13, $p < 0.0001$) and 15 (EID15, $p < 0.0001$) days of incubation.

DISCUSSION

In birds, the embryo can pause its development until incubation, when the temperature is too cold or until the clutch size is optimal for brooding. This dormancy is characterised by cell arrest in the G (2) phase and suppression of apoptosis (Ko et al., 2017). However, extended storage impairs hatchability due to its deleterious effects on blastoderm viability. The detrimental effect of extended storage (over 7 days) on egg quality, embryo mortality and hatchability has been extensively reviewed in the literature (Asmundson, 1947; Brake et al., 1997; Fasenko, 2007; Bakst et al., 2012; Bakst et al., 2016a; Branum et al., 2016; Abioja et al., 2021; Melo et al., 2021; Özlü et al., 2021). Such negative effects are very well known to turkey and chicken breeders but they can have difficulties avoiding long storage practices for logistical reasons. Prolonged egg storage alters many egg quality parameters and

TABLE 2 | Effect of egg storage on egg parameters.

	Storage duration (days)			*p* Value	Trend during storage
	D0	D3	D10		
Egg weight (g)	56.73 ± 2.29	56.35 ± 2.05	56.05 ± 2.21	0.787	—
Eggshell strength (N)	37.35 ± 5.82	39.51 ± 6.19	34.85 ± 4.06	0.196	—
Yolk index	0.43 ± 0.03^{a}	0.41 ± 0.03^{ab}	0.30 ± 0.03^{b}	**0.023**	↘
Yolk colour	6.27 ± 0.48	6.25 ± 0.37	6.35 ± 0.44	0.935	—
Egg yolk weight (g)	15.53 ± 0.99	15.82 ± 0.78	16.26 ± 0.74	0.130	—
Haugh units	87.30 ± 4.34^{a}	80.01 ± 3.06^{b}	74.57 ± 0.44^{c}	<**0.0001**	↘
Egg white pH	8.53 ± 0.32^{a}	9.20 ± 0.08^{b}	9.32 ± 0.09^{c}	<**0.0001**	↗
Yolk (cm^3)	10.68 ± 0.94^{a}	10.31 ± 0.78^{ab}	9.61 ± 0.99^{b}	**0.048**	↘
Egg white (cm^3)	34.24 ± 1.53	34.27 ± 1.63	35.11 ± 2.08	0.636	—
Air chamber (cm^3)	0.27 ± 0.07^{a}	0.52 ± 0.07^{b}	0.79 ± 0.17^{c}	<**0.0001**	↗
Blastoderm (cm^3)	0.27 ± 0.11	0.28 ± 0.05	0.33 ± 0.06	0.129	—

Values with different letters indicate statistical differences between eggs stored for zero, three or 10 days (D0, D3 and D10, respectively; p < 0.05). As data normality was not observed for yolk index, egg white volume and eggshell strength (Shapiro–Wilk test), statistical analyses were performed using the Kruskal–Wallis test. Significant p Values are indicated in bold.

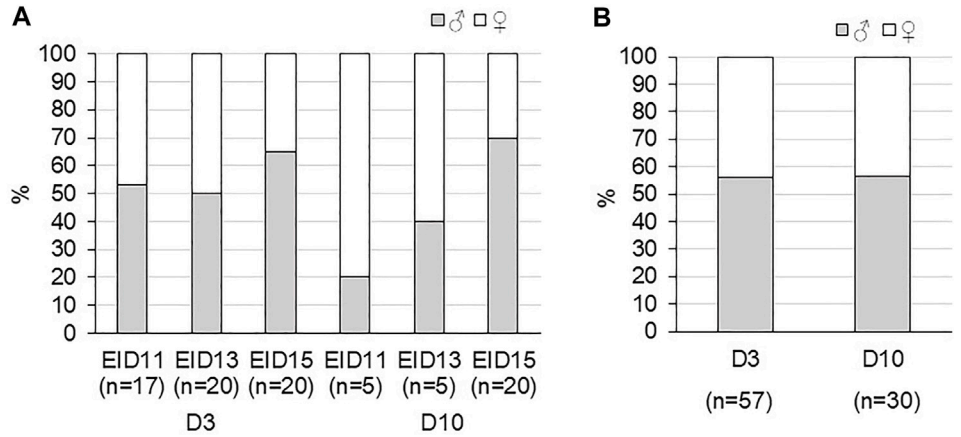

FIGURE 5 | Sex ratio of fertilised eggs during incubation after three or 10 days of storage (D3 and D10, respectively) after 11 (EID11), 13 (EID13) and 15 (EID15) days of incubation. **(A)**. Sex ratio at each stage of incubation. **(B)**. Sex ratio in D3 and D10 groups (considering all EID11, EID13 and EID15 eggs).

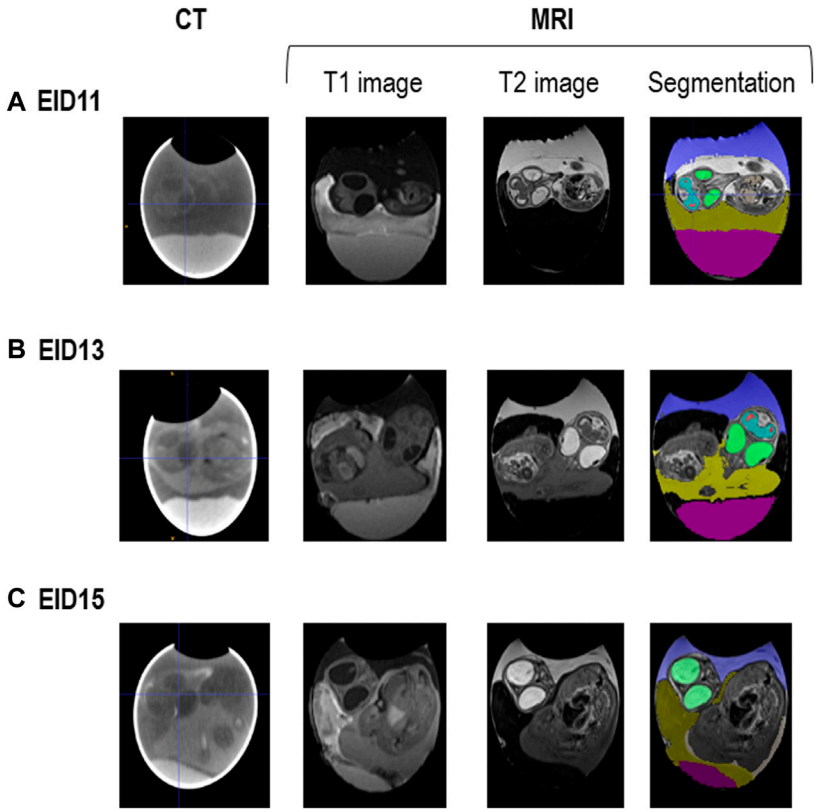

FIGURE 6 | CT and MRI representative images of fertilised eggs during incubation. **(A)**. After 11 days of incubation (EID11). **(B)**. After 13 days of incubation (EID13). **(C)**. After 15 days of incubation (EID15). For segmentation (right panel), colours are as follows: in green, eyes; in blue and red spots, brain; in dark blue/purple, allantoic fluid; in pink, egg white; in yellowish colour, yolk. The amniotic fluid corresponds to the white zone between the embryo and the allantoic fluid on A (EID11, right panel) and to the dark zone between the embryo and the allantoic fluid on B (EID13, right panel). The change in the MRI contrast of the amniotic fluid between EID11 and EID13/EID15 (white to black), is due to the transfer of egg white into the amniotic cavity from EID12 onwards.

may deteriorate the blastoderm to the point that it cannot resume upon incubation. Several studies have reported a decrease in viable blastodermal cells after storage over 8 days (Bakst et al., 2012). To mitigate such a negative effect of prolonged storage, strategies have been developed. These include short periods of incubation during egg storage (SPIDES) that reactivate embryo

TABLE 3 | Effect of egg storage on embryonic development.

Method	Volume (cm^3)	Storage Duration (days)						Trend between D3 and D10 groups
		D3			D10			
		Incubation (days) EID11	EID13	EID15	Incubation (days) EID11	EID13	EID15	
CT	Egg white	9.22 ± 0.93	6.12 ± 1.07	2.93 ± 0.96	ND	ND	3.19 ± 0.86	—
	Air chamber	3.02 ± 0.29	3.70 ± 0.53	4.24 ± 0.53	ND	ND	4.23 ± 0.44	—
MRI	Allantoic fluid	10.97 ± 0.84	7.85 ± 0.69	5.08 ± 0.95	11.04 ± 0.51	8.35 ± 0.47	5.86 ± 1.67	—
	Amniotic fluid	3.42 ± 0.25	ND	ND	3.41 ± 0.26	ND	ND	—
	Eyes	0.38 ± 0.03	0.53 ± 0.04	0.59 ± 0.05[a]	0.35 ± 0.01	0.48 ± 0.03	0.54 ± 0.03[b]	D3>D10
	Brain	0.34 ± 0.01[a]	0.50 ± 0.03	0.73 ± 0.03[a]	0.28 ± 0.02[b]	0.47 ± 0.03	0.70 ± 0.02[b]	D3>D10
	Embryo	6.00 ± 0.40[a]	ND	ND	4.83 ± 0.30[b]	ND	ND	D3>D10
	Embryo + Yolk	ND	16.84 ± 1.13	25.08 ± 1.61[a]	ND	14.98 ± 0.99	22.12 ± 1.59[b]	D3>D10

Values with different letters indicate statistical differences between eggs stored for three (D3) vs. 10 days (D10) (p < 0.05). ND: not determined due to reduced number of eggs (CT, EID13 and EID15, D10) or technical constraints (amniotic fluid at EID13 and EID15; embryo at EID13 and EID15). n = 10, except for MRI, analyses of D10-EID11 and D10-EID13 eggs (n = 5 for each group). A Kruskal–Wallis test was first performed followed by a Mann–Whitney test for comparison between D3 and D10 groups. Values with different letters indicate statistical differences between eggs stored for three (D3) or 10 days (D10) (p < 0.05). Where statistics revealed significant differences between D3 and D10 groups, the trend is indicated in the last column of the table.

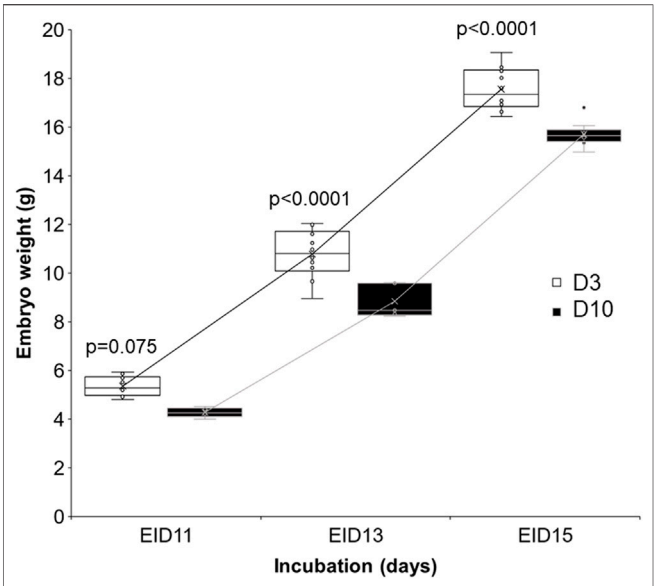

FIGURE 7 | Effect of storage time on embryo weight after 11, 13 and 15 days of incubation. D3 and D10 eggs were opened at EID11, EID13 and EID15 after CT or MRI analyses and weighed. Statistical analysis was performed using one-way ANOVA with $p < 0.05$.

metabolism prior to incubation, thereby increasing the viability of embryos until hatching (Dymond et al., 2013; Damaziak et al., 2018). Some authors also reported a positive hatchability effect of storage at cooled temperatures [15°C, (Bakst and Gupta, 1997); 11.6°C (Guinebretière et al., 2022)], egg turning (Melo et al., 2021) or of *in ovo* injection of biological buffers to reinforce the buffering capacity of the albumen (Akhlaghi et al., 2013). The mechanisms underlying the reduced viability of blastodermal cells during storage have been partly elucidated: increases in expression of genes associated with apoptosis, oxidative stress and fatty acid metabolism (Hamidu et al., 2011; Bakst et al., 2016b). However, there are only few studies that investigated the impact of prolonged storage on the development of viable embryos. Some authors have described a reduced femur and tibia length being positively correlated with the duration of preincubation storage (Yalcin and Siegel, 2003), while others reported an altered growth of heart and liver (Christensen et al., 2002) and a slower metabolic rate (Christensen et al., 2001; Fasenko, 2007). In this study, we used non-invasive imaging approaches (MRI and CT) to investigate the impact of prolonged storage on egg quality, and on the growth and development of the embryo. Such imaging methods have previously been used to assess egg quality (Hutchison et al., 1992), to identify the localisation of the blastoderm (Bartels et al., 2008) and to monitor brain, liver or eye development (Bain et al., 2007; Boss et al., 2008; Zhou et al., 2015; Lindner et al., 2017).

We first explored how storage duration at 16°C, 80% relative humidity (classical hatchery conditions) for zero, three and 10 days affects egg quality. We used a combination of non-invasive CT on intact eggs and classical measurements on internal egg quality traits after egg breakage. In accordance with numerous articles and reviews (Benton and Brake, 1996; Brake et al., 1997; Abioja et al., 2021), we showed that the length of storage affects several egg parameter traits including egg white pH, Haugh units, yolk index and air chamber volume (**Table 2**). The volume assigned to the blastoderm tends to increase upon storage, especially when comparing three and 10 days of storage (Mann–Whitney test, $p = 0.060$), which corroborates previous studies (Bakst et al., 2012; Abioja et al., 2021). The analyses of the 30 eggs (10 eggs at D0, D3 and D10) revealed that the position of the blastoderm is always localised at the surface of the yolk, roughly in the middle (**Figure 3**), when the large end of the egg is maintained on the top (air chamber located at the top). Only one egg exhibiting a blastoderm oriented towards the bottom was noticed. It would have been interesting to put this egg back into the incubator to assess how this specific orientation impacts embryo development and whether it was still associated with a viable chick or not. Anyway, this observation suggests that CT imaging of eggs before storage to verify the localisation of the blastoderm may help the development of new experiments to determine whether this parameter (blastoderm location) is a predictor of hatchability.

Knowing that the increase in blastoderm volume is unlikely correlated with cell proliferation or the number of viable cells (Bakst et al., 2012), we hypothesised that it may correspond to the dispersion of the blastoderm cells as the vitelline membrane is losing its mechanical strength. Such a hypothesis is in accordance with the decrease of yolk index (**Table 2**) and the changes in yolk/white physicochemical properties. Further studies on the impact of extended storage (egg white physicochemistry, ultrastructure of the vitelline membrane that supports the embryo) may be useful to identify the determinant parameters that alter embryo survival and restarting. We believe that the quality of the vitelline membrane is crucial in the early stages of incubation as its inner layer contains many proteins that are assumed to support the development of the embryo and the expansion of the yolk sac during embryogenesis (Brégeon et al., 2022). Alteration of the perivitelline membrane is supposed to be a key determinant that can explain very early mortality. Although the duration of storage is detrimental to embryo survival, it is noteworthy that incubation of freshly laid eggs (that are characterised by high viscosity and neutral pH) is not correlated with higher embryo survival (Benton and Brake, 1996). Altogether these data highlight the major role of egg components that surround the blastoderm, on cell survival and hatching success. This observation is partly corroborated by our data, where storage up to 10 days at 17°C, 80% RH did not affect fertility (the number of eggs that restarted was comparable between D3 and D10 eggs) but impaired embryo viability (4.7% mortality for D10 eggs vs. 1% mortality for D3 eggs during incubation). The reason why some eggs resist extended storage more is not known but it probably results from many variables including genetics, egg quality (including composition) and embryo specificities. Christensen et al. reported that embryos from a genetic line that resisted storage mortality maintained greater glycogen concentrations in muscle and heart tissues than those from a line and old hens associated with reduced survival rates (Christensen et al., 2001).

Hence, similar to MRI (Hutchison et al., 1992; Klein et al., 2002; Burkhardt et al., 2011), CT imaging can help to localise the position of the blastoderm, as confirmed in this study and others (Bartels et al., 2008), which might be particularly interesting to develop tools that require information from the embryo. For example, identifying the exact position of the blastoderm on the yolk may help in developing sexing methods, as suggested previously (Burkhardt et al., 2011) and to differentiate an early-dead embryo from an unfertilised germinal disc (Bakst et al., 2016a). In this study, CT has been used to estimate the localisation of the blastoderm during storage but it might be interesting to further explore how the CT signal associated with the blastoderm is changing during the first 3 days of incubation, and whether this method can be used to monitor early stages of embryonic development.

Interestingly, the sex ratio of viable embryos was shown to be comparable between the two experimental (D3 and D10) groups (**Figure 5**). The verification of this parameter was essential to avoid any bias associated with the sex of the embryo, knowing that the maturation/growth of the embryo may differ between males and females, even during early stages (Tagirov and Golovan, 2015; Hirst et al., 2018). Such information remains important in the context of the development of methods to avoid the culling of male chicks. Indeed, storage or incubation methods that could imbalance the sex ratio in favour of females would diminish the number of male embryos and chicks to eliminate (Gautron et al., 2021).

Data related to embryo weight and brain, embryo and eye volume (**Table 3**; **Figure 7**) all support that storing eggs for 10 days negatively affects embryo growth. Similar to our results, body mass of EID15 embryos was previously shown to be significantly affected by a storage duration of up to 3 weeks ($p < 0.001$) (Branum et al., 2016). It was also reported that the acid-base balance of embryos was modified according to storage duration (Branum et al., 2016). However, storing eggs for 10 days does not seem to affect the kinetics of embryo development (similar growth curves, **Figure 7**). Further studies on a higher number of eggs are needed to increase the statistical significance of some parameters including egg white and allantoic fluid volumes (**Table 3**). Notably, at EID15, the egg white volumes tended to be higher in D10 eggs than D3 eggs (2.93 ± 0.96 and 3.19 ± 0.86 in D3 and D10 eggs, respectively). A similar trend was observed for the volume of allantoic fluid, which was higher in D10 than D3 eggs (5.08 ± 0.95 and 5.86 ± 1.67 in D3 and D10 eggs, respectively). These observations corroborate the aforementioned conclusion that the growth/developmental stage of D10 eggs was delayed compared with D3 eggs. Indeed, the decrease in egg white volume, which is located at the bottom of the egg, is concomitant with its transfer into the amniotic cavity between EID11 and EID12 (Da Silva et al., 2019). From EID13 onwards, the amniotic fluid/egg white mix will start to be absorbed orally by the embryo as a source of amino-acids, to accompany its growth. The higher volume of egg white noticed in D10 eggs compared to D3 eggs suggests that the egg white transfer into the amniotic cavity was also delayed in D10 eggs.

Further research should include analyses of other physiological/phenotypical traits including organ growth, embryo positioning within the egg and initiation of skeletal mineralisation, to complete the story and facilitate the identification of indicators of normal or abnormal development/growth. To our knowledge, only a few articles report such experimental studies (Christensen et al., 2001; Christensen et al., 2002; Yalcin and Siegel, 2003; Fasenko, 2007). Our data suggest that the physiological stages of EID11, EID13 and EID15 embryos are more advanced for D3 eggs than D10 eggs and that the development of D10 embryos is delayed compared with D3 embryos. Although our experimental design did not include incubation up to hatching, other studies reported that chicks originating from eggs stored for a long period hatched later than embryos from eggs stored for a short period (Christensen et al., 2002; Tona et al., 2003a; Tona et al., 2003b). However, we have no evidence to date that the duration of storage induces abnormalities.

Additional data on the volume of embryo organs and supporting structures, the orientation of the embryo inside the egg and the movements of egg structures during incubation should also help to revise the atlas of chicken development

(Hamburger and Hamilton, 1951). Indeed, most modern chicken lines have been selected for decades on performance, egg or meat quality, and there is an increasing number of articles that alerts on differences in metabolism and health between commercial genotypes (Koenen et al., 2002; Tona et al., 2004; Buzała et al., 2015). Comparisons of the embryonic development (organogenesis, growth, kinetics) between several contrasted phenotypic lines should help to investigate the impact of genetic selection on embryos, whose proper development predetermines health and welfare of chicks and adult chickens. Integrative studies considering the normal development of the embryo using several levels of structural organisation (from the embryo within the egg to the molecular profiling of egg/embryo contents) are lacking. New data from experiments addressing the impact of genetics, age and nutrition of reproductive hens, egg storage and incubation conditions on egg and embryo specificities may be useful for the development of predictive tools (Yimenu et al., 2017) to model egg quality (for both table and fertilised eggs) and developmental kinetics of embryos, upon exposure of eggs to suboptimal conditions.

CONCLUSION

In this work, we demonstrated: 1) that the storage of fertilised eggs up to 10 days is associated with a decrease in several egg quality parameters; and 2) that the development of embryos exposed to extended egg storage is delayed compared to those stored for a shorter period. These data combined with published works suggest that eggs exposed to different storage durations are likely associated with differences in embryo maturity and hatching time. This observation underlines the necessity to improve the homogeneity of egg batches in terms of storage conditions to narrow the hatching window, thereby limiting the time spent by new hatched chicks in hatchers (without access to water and food). This work also evidences the relevance of imaging techniques to monitor the development of bird embryos during incubation but also to visualise and quantify how egg components (egg white, extraembryonic fluids) are modified throughout incubation. Classical measurements of egg quality parameters and embryo development usually require the egg opening and embryo killing. In this respect, CT and MRI techniques are non-invasive approaches. Although some egg quality parameters cannot be determined using these techniques (egg white pH and viscosity, yolk colour and index), they facilitate the analysis of some egg and embryonic components that are usually difficult to measure/evaluate (volume of the air chamber and extraembryonic fluids, embryo positioning, and movement of egg structures during storage and incubation).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the embryo study. The experimental procedure meets the guidelines approved by the Institutional Animal Care and Use Committee (IACUC). All experiments were conducted in compliance with the European legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" (2010/63/UE) and under the supervision of an authorised scientist (S. Réhault-Godbert, Authorisation no. 37-144).

AUTHOR CONTRIBUTIONS

HA performed MRI image acquisition and supervised data analyses. VP analysed MRI and CT data, performed segmentation for volume determination and participated in data interpretation. IC contributed to segmentation, statistical analyses and participated in discussions. NB and MH performed experiments on eggs and embryos after egg opening and contributed to data analyses. NB conducted molecular sexing experiments. FL performed CT experiments and contributed to discussions. SR-G conceived, designed, participated in experiments and supervised the entire study. SR-G and HA wrote the first draft of the manuscript. All authors reviewed the manuscript and approved the submitted version.

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Long-Lasting Effects of Incubation Temperature During Fetal Development on Subcutaneous Adipose Tissue of Broilers

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Increasing evidence indicates that fetal programming may cause permanent effects on offspring adipose tissue and body composition. Previous study showed reduction in newly-hatched broiler chick adiposity by manipulating incubation temperature during fetal development. The present study examined whether incubation temperature during fetal development has long-term effects on post-hatching fat deposition in broilers. Broiler breeder eggs (Cobb-500®) were incubated under constant low (36°C, LT), control (37.5°C, CT) or high (39°C, HT) temperature from day 13 onward, giving to eggshell temperature of 37.3 ± 0.08°C, 37.8 ± 0.2°C, and 38.8 ± 0.3°C, respectively. Male chicks were reared under recommended temperatures until 42 days old. LT 21 days old broilers exhibited higher blood cholesterol than CT broilers, and higher triglycerids, VLDL, and LDL, and lower HDL than CT and HT broilers. LT broilers presented higher liver cholesterol than CT broilers and lower ether extract percentage than CT broilers. Adipocyte count was lower in the abdomen than in the thigh. Until day 21 of age, feed intake was higher in LT than in HT broilers. At day 42 of age, blood cholesterol and LDL were higher in HT broilers than in CT and LT broilers. Liver cholesterol was higher in LT than in HT broilers. LT treatment reduced neck and increased thigh adipocyte size compared to CT treatment, while the HT treatment reduced abdomen and neck adipocyte size compared to other two treatments and in the thigh compared to LT treatment. In CT broilers, thigh adipocytes were smaller than abdomen and neck adipocytes. HT treatment increased adipocyte number per area in the neck compared to LT and CT treatment, and LT and HT treatments reduced adipocyte count in the thigh compared to CT treatment. CT broilers presented higher adipocyte count in the thigh than the abdomen and neck, while HT broilers presented higher adipocyte count in the neck than the abdomen and thigh. Cell proliferation was lower in the abdomen than in the thigh. The results show incubation temperature manipulation during fetal development has long-term and distinct effects on regional adiposity, and can be used to modulate broiler fat deposition.

Keywords: adipose tissue cellularity, body composition, fat deposition, fetal programming, incubation temperature, lipid prolife

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INTRODUCTION

Broiler lines genetically selected for higher and faster growth and higher feed efficiency and meat yield also exhibit greater liver growth and excessive adiposity (Tavárez and Solis de los Santos, 2016; Zaefarian et al., 2019) and higher sensitive to chronic heat stress (Abo Ghanima et al., 2020; Ibrahim et al., 2021). Due to its low thermal conductivity and increased insulator capacity, adipose tissue provides an insulating barrier to heat conduction and reduces the animal's ability to respond to ambient temperature changes (Cooper and Trezek, 1971; Jequier et al., 1974; Savastano et al., 2009). Thus, although fat is important to structural and functional processes in the animal, excessive fat can be harmful depending on the environmental conditions to which the animal is exposed. It is well known that heat stress occurs when the ambient temperature exceeds the comfort zone for poultry species and/or metabolic heat production exceeds the bird's ability to dissipate heat (Diarra and Tabuaciri, 2014; Akbarian et al., 2016). These studies indicate that very high body fat deposition can makes it difficult for broilers to lose body heat and maintain homeothermy when exposed to thermal stress, and this can lead to death. In addition, excessive fat is the main source of waste in the slaughterhouse and an important factor increasing costs (Tůmová and Teimouri, 2010). However, fat deposition and fatty acid composition in muscles contribute to attributes of meat quality, including juiciness, flavor, tenderness and other organoleptic properties (Wood, 1990; Wood et al., 1999; Jeremiah et al., 2003; Williams, 2007; Pereira and Vicente, 2013). Although the presence of fat in the muscle and skin confers high eating quality, and meat provides essential fatty acids and other nutrients for humans, there is an increased demand for high-quality meat consumption that is conducive to human and animal health and well-being, with the implication of a reduction in fat deposition in poultry (Wood et al., 2008; Del Gobbo et al., 2016; Forouhi et al., 2018). Thus, a reduction in fat deposition is desirable to the broiler meat production industry.

Increasing evidence suggests programming or imprinting of several tissues, including adipose tissue, takes place during fetal development, with long-term effects on post-born phenotype of mammals (Hanson and Gluckman, 2014). Tissue programming occurs during specific developmental windows, determined by developmental time throughout fetal development. In layer chickens and broilers, adipose tissue hyperplasia takes place during the second week of egg incubation (between E12 and E14), followed by a reduction of hyperplasia and increasing rates of hypertrophy through the subsequent days of incubation (Jo et al., 2009; Chen et al., 2014). In previous studies, we verified that manipulation of incubation temperature during fetal development affects skin thickness and vascularity, blood hormonal concentration and thermal preference of broiler chicks (Morita et al., 2016a; Morita et al., 2016b). We demonstrated that broiler chicks present differences in adipocyte size and proliferation across different regions of the body, and that continuously high incubation temperature during fetal development reduces abdominal and cervical adipocyte size (Almeida et al., 2016). These studies indicated fetal responsivity to manipulation of incubation temperature in broilers occurs from day 13. However, the application of this thermal manipulation during egg incubation to reduce or modulate post-hatch fat deposition in broilers has yet to be investigated.

The aim of the present study was to evaluate the long-term effect of manipulation of incubation temperature, from day 13, on adiposity of broilers after hatching. The effects of egg incubation at higher (39°C) and lower (36°C) incubation temperatures than the usual (37.5°C) on hyperplasia and hypertrophy of subcutaneous adipose tissue for three parts of the body, body weight and chemical composition, blood lipid profile and liver weight and lipid and cholesterol content of 21 and 42 day-old broilers was analyzed. External development of the avian embryo together with rapid prenatal and postnatal periods make broilers an excellent experimental model to investigate fetal tissue programming without maternal influences. Excessive adiposity compromises the health of many other animals, including domestic animals and humans, making long-lasting effects of incubation temperature during fetal development on subcutaneous adipose tissue of broilers studies on adipose tissue of interdisciplinary interest.

MATERIALS AND METHODS

Experimental Procedures

A total of 900 fresh fertile eggs of 59-week-old broiler breeder flocks (Cobb500®) were obtained from a commercial hatchery (Globoaves, Itirapina, São Paulo, Brazil). All eggs were individually weighed before incubation. Eggs were then homogeneously distributed by weight (67–72 g) over six incubators (Premium Ecológica- IP200, Belo Horizonte, MG, Brazil) with automatic control of temperature and egg turning (1 turning per hour), kept at 37.5°C and 60% RH until day 12 of incubation. From day 13 to hatching day, incubation temperature protocols were similar to Almeida et al. (2016), i.e., the incubation temperature was maintained at 37.5°C (control), lowered to 36°C, or increased to 39°C, resulting in two incubators per incubation temperature profile. The average eggshell temperature under continuous incubation at 36°C (low temperature: LT), 37.5°C (control temperature: CT), and 39°C) high temperature: HT) from day 13 was 37.4 ± 0.1°C, 37.8 ± 0.1°C, and 38.8 ± 0.3°C. Eggshell temperatures were measured due to its relationship with embryo temperature (van der Pol et al., 2014) and was measured every 30 min by thermistors (Alutal Type T; São Paulo, Brazil) attached to the equatorial region of the eggshells of 5 eggs per incubator. Data were recorded in data loggers connected to a computer. Manipulation of incubation temperature from the 13rd day was based on the knowledge that functional maturation of the neuroendocrine hypothalamic-pituitary-thyroid axis is established around the 13th-14th day of incubation (McNabb and Olsen, 1996; Jenkins and Porter, 2004), as well as the fetal timing of adipose tissue hyperplasia and hypertrophy (Jo et al., 2009; Chen et al., 2014). In all incubators, relative humidity was maintained at 60% throughout the incubation period, including hatching phase, to avoid effects on fetal development and survival. Incubation was considered finished after 504 h.

All chicks were removed from the hatchers and sexed by examining the feathers. Male chicks were then weighed and 115 chicks per incubation treatment were homogeneously distributed by average body weight (51 ± 2 g) into 5 replicate groups (23 chicks each). Only males were used because in previous studies we verified incubation temperature effects on thermal preference, skin thickness and vascularity, body surface temperature, and plasma concentration of T3 and GH of male broiler hatchlings (Morita et al., 2016b). The chicks were reared until 42 days post-hatching in thermal chambers containing 16 boxes each (2.5 × 1.5 m) and equipped with a monoblock refrigerant system, heaters, and exhaust fans, with automatic control of ambient temperature and a daily light regime (22L:2D) with the dark period occurring from 4 to 6 a.m. (Morita et al., 2020) and maintained at temperatures recommended for the strain (Cobb Broiler Management Guide, 2013). Average weekly values of temperature and RH in the climatic chambers were 33.3°C, 29.8°C, 27. 3°C, 25.4°C, 23.0°C, and 21.0°C and 65, 76, 68, 57, 65, and 51%, from the first to the 6 week of the experiment, respectively. Throughout the experimental period, broilers received water and feed *ad libitum*. The broilers received two diets based on corn and soybean meal formulated in accordance with the requirements established for broiler chickens by Rostagno et al. (2011): starter diet (1–21 days: ME = 2,883 Kcal/kg, PB = 21.27%) and grower diet (22–42 days: ME = 3,121 Kcal/kg, PB = 18.86%). The chicks were vaccinated against Marek and Poxvirus disease while in the hatchery, against infectious bursal disease (intermediate strain Lukert) and Newcastle (La Sota strain) on the 8th day post hatching and against infectious bursal disease (strong strain Australia v-877) on the 18th day.

Blood Lipid Profile

Blood contents of cholesterol, triglycerides, high-density lipoprotein (HDL, low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were analyzed at day 21 and 42. Blood samples were collected from 12 birds/incubation temperature/age by jugular puncture (two *ca* 3 ml samples per bird) and stored in plastic tubes without anticoagulant for 1 h at room temperature. They were then centrifuged at 3,500 rpm and 4°C for 15 min for serum separation. After collection, serum (supernatant) was maintained in a freezer at −70°C until analysis. Measurements of total serum cholesterol, HDL, and triglycerides were performed using commercial kits (Labtest Diagnóstica S.A., Belo Horizonte, MG, Brazil). All serum samples were prepared and analyzed according to manufacturer's specifications and three absorbance readings per birds/treatment with a spectrophotometer (Beckman Coulter, DU-800; Minnesota, United States) at a wavelength of 500 nm. VLDL and LDL concentrations were calculated by the equations: VLDL = Triglycerides/5; LDL = total cholesterol—(HDL + VLDL).

Adipocyte Size, Number and Proliferation

On day 21 and 42 post-hatching, and after blood collection, male broilers were killed by cervical dislocation, and samples of adipose tissue collected from the abdomen, neck and thigh. The samples were fixed for 48 h in 10% formaldehyde at room temperature and then processed by standard histological procedures for optical microscopy. Briefly, samples were washed with distilled water, dehydrated in a series of increasing ethanol concentrations [70, 80, 90, 95, and 100% (2x)], diaphanized in a mixture of ethanol : xylene (1:1) and subsequently xylene (100%, 2x), infiltrated with a mixture of xylene:paraffin (1:1) followed by paraffin (100%), and embedding in paraffin (100%) (Almeida et al., 2016). For analysis of adipocyte size and number, deparaffinized semi-serial sections (7 μm thick) were rehydrated and stained with hematoxylin-eosin (HE) using standard procedures. Adipocytes area (μm^2) and number (cell number/area) were determined using an image capture and analysis system (LAS Leica® type V4.3, Wetzlar, Hessen, Germany). Adipocyte area (μm^2) was obtained by measuring 180 adipocytes per sample. Adipocyte number per area was counted on 10 microscopic fields with 202,800 μm^2 each per sample. For adipocyte proliferation analysis, two 8-μm thick sections per sample were placed on electrically charged histological slides (Tissue-Tek R©AutoWrite R©Starfrost R©Green) and kept at 30°C until processing to allow them to settle on the slide. Histological sections were then submitted to an indirect immunohistochemical technique to mark and identify adipocytes expressing proliferating cell nuclear antigen (PCNA) protein (Starr Trek Universal HRP Detection System, Biocare Medical, Concord, California), following the manufacturer's instructions (see Almeida et al., 2016). Briefly, after deparaffinization followed by hydration, histological sections were incubated with 15% hydrogen peroxide in methanol at room temperature for 30 min to inactivate endogenous peroxidase, washed with 1M Tris-HCl buffer pH 7.4 (3x for 1 min/each), and incubated for 90 min with non-specific horse serum (15 μL serum + 85 μL Tris-HCl buffer). After draining the non-specific serum, sections were incubated with primary monoclonal mouse anti-PCNA antibody (PCNA Clone PC10, 1:100 in Tris-HCl buffer, Concord, California) for 1 hour. After removing the primary antibody, sections were washed with Tris-HCl buffer, incubated with secondary antibody universal (Starr Trek Universal HRP Detection) at room temperature for 20 min and then washed with Tris-HCl buffer (2x, 2 min/each). Sections were then incubated with Avidin-HRP Trek at room temperature for 10 min and washed with Tris-HCl (2x for 2min/each). Following, diaminobenzidine-betazoid chromogen solution (DAB) [one drop (~ 15 μl) + substrate buffer (4 μl), at room temperature for 3 min] was used to reveal antigen-antibody binding. The reaction was stopped by immersing the sections in distilled water and the sections were then dehydrated (ethanol 70, 80, 90, and 100%) and diaphanized (100% xylene, 2x), and the slides were mounted with Entellan. Negative controls were generated by omitting the section incubation with primary antibody. All procedures were performed in a humid chamber at 4°C. Mitotic rates were determined as the average number of adipocytes expressing PCNA present in 16 fields per sample (each field: 202,800 μm^2) using the previously mentioned image capture and analysis system.

Liver Lipids

Liver samples were obtained from the same birds used for blood and adipose tissue analyses (12 birds per treatment per age; 21

and 42 days old). Soon after collection, livers were weighed and divided in two, labelled and frozen and stored in a freezer at −20°C until analyses. Total lipid content was determined following the protocol established by Bligh and Dyer (1959). Cholesterol content was determined by enzymatic measurement (Saldanha et al., 2004). Briefly, 4 ml of KOH 50% and 6 ml of ethanol were added to 2 g of liver in falcon tube. Tubes containing the samples were kept in water bath at 40°C for 2 h, followed by an additional 10 min at 60°C. 5 ml distilled water was then added to each tube. For cholesterol extraction, 10 ml of hexane was added and vortexed for 1 min. After allowing separation of the mixtures into two phases, the hexane phase was removed and transferred to another tube. This extraction procedure was repeated three times. 3 ml of the hexane extract was then dried in N_2 and 0.5 ml of isopropanol was added to dissolve the residue. Following the procedures recommended by the enzymatic method cholesterol kit (Cholesterol cod.74305 Laborclin, Pinhais, PR, Brazil), absorbance readings were made at 499 nm in a spectrophotometer (Beckman Coulter DU-800- GMI, Minnesota, United States).

Body Weight and Chemical Composition

At day 21 and 42 after hatching, an additional 12 broilers/incubation temperature were weighed and killed by stunning with CO_2 followed by bleeding, and stored at −20°C before analyses of moisture, dry matter, ether extract, crude protein, and ash. Broilers were ground and a homogeneous sample dried at 55°C for 4 days, and remaining pre-dried matter weighed, ground and homogenized. After pre-drying, samples (1 g each) were dried at 105°C for 16 h and the remaining dry matter weighed. The moisture was calculated as the total difference between wet and dry weights. Moisture and dry matter were given in total grams and percentage in relation to body weight. For ash content determination, the dried samples were heated in a muffle furnace at 600°C for 4 h. Organic matter was calculated as the difference between dry matter and ash weights. Ether extract content (crude fat) was determined by extraction with petroleum ether for 8 h using 1 g of dry matter per sample and corresponded to difference between the weights before and after extraction. Following the crude fat extraction, the fat-free dry matter (100 mg per samples) was used for crude protein determination by the Kjeldahl method for detection of total N content, and crude protein was calculated as N content x 6.25. Two replicates per bird of broiler body composition were used following the methods of Silva and Queiroz (2002). Crude protein, ether extract and ash contents are expressed in g and % in relation to dry matter contents.

Broiler Performance

The broiler body weights were determined at 21 and 42 days of age (g), and feed intake (g), weight gain (g) and feed conversion (g/g) for two periods: 1–21 and 22–42 days were calculated. Feed intake was calculated by difference between the amounts of feed offered and uneaten feed in each replicate. Weight gain corresponded to the difference between body weight at the beginning and end of the rearing period. Feed conversion was calculated by dividing the feed consumed by the body weight gain (gram per gram).

Statistical Analyses

Serum lipid profile, body weight and chemical composition, total lipid and cholesterol concentrations in the liver, and performance, were analyzed, comparing the effects of the three incubation temperatures (IT: 36°C, 37.5°C and 39°C) on the grow out phase. The model $Y = \mu + IT + e$, in which Y is the dependent variable, μ is the overall mean and e is the residual error, was applied to the data. Adipocyte size and proliferation rate were analyzed with respect to the effects of incubation temperatures (IT) and body regions (BR) (neck, abdomen and thigh) and of the respective interactions (IT x BR), using the following model: $Y_{ijk} = \mu + IT_i + BR_j + (IT \times BR)_{ij} + e_{ijk}$, where μ is the overall mean and e_{ijk} is the residual error term. All data were analyzed using the GLM procedure of SAS (SAS-Institute Inc, 2009). When necessary, differences among means were tested by Tukey`s test. In all cases, p-values ≤ 0.05 were considered statistically significant.

RESULTS

Serum Lipid Profile and Liver Lipid and Cholesterol Levels

At day 21 of age, broilers from eggs incubated at 36°C exhibited higher serum triglycerides, VLDL and LDL concentrations and lower HDL concentration than broilers reared from eggs incubated at 37.5°C or 39°C, that did not differ, as well as higher cholesterol concentration than broilers from egg incubation at 37.5°C (**Table 1**). At 42 days of age, incubation temperature influenced cholesterol and LDL concentrations only, values were higher in broilers from egg incubated at 39°C than at 37.5 and 36°C, which did not differ. Incubation temperature had no significant effect on the weight and lipid concentration of the liver at 21 or 42 days of age. However, incubation temperature changed the liver cholesterol concentration, which was higher in 21 day-old broilers reared from eggs incubated at 36°C than at 37.5°C and in 42 day-old broilers reared from eggs incubated at 36°C than 39°C (**Table 2**).

Adipocyte Size, Number and Proliferation Rate

At day 21 of age, no significant effects of incubation temperature, body region and their interaction were observed on adipocyte size, number per area. Incubation temperature did not affect mitotic rate, and, independently of treatment, the thigh presented higher cell proliferation than the abdomen ($p < 0.05$; **Table 3**). At 42 days of age, significant effects of body region for cell proliferation remained apparent, with significantly higher mitotic rate in the thigh than in the abdomen. Significant interactions between incubation temperature and body region for adipocyte area and number per area (**Table 3**) also occurred. As showed in **Table 4**, adipocyte area in the abdomen was smaller in broilers from eggs incubated at 39°C than at 36°C or 37.5°C,

TABLE 1 | Blood lipid profile of male broilers at day 21 and 42 of age, according to incubation temperatures.

Variables	Incubation temperatures			*p*-values	CV(%)
	36°C	37.5°C	39°C		
		21 d. old broilers			
Cholesterol	184.79 ± 29.3^{A}	141.07 ± 28.6^{B}	159.62 ± 18.0^{AB}	0.0372	16.04
Triglycerids	39.54 ± 3.2^{A}	30.63 ± 3.8^{B}	31.03 ± 5.9^{B}	0.0015	21.42
VLDL	7.91 ± 1.7^{A}	6.12 ± 0.8^{B}	6.21 ± 3.2^{B}	0.0001	21.43
HDL	62.61 ± 24.4^{B}	99.47 ± 18.4^{A}	92.31 ± 16.5^{A}	0.0088	23.45
LDL	114.27 ± 39.6^{A}	35.48 ± 23.6^{B}	61.11 ± 24.8^{B}	0.0008	45.02
		42 d. old broilers			
Cholesterol	126.51 ± 30.93^{B}	115.34 ± 22.51^{B}	153.97 ± 20.6^{A}	0.0013	15.44
Triglycerids	35.54 ± 5.54	35.95 ± 6.68	34.09 ± 9.3	0.8184	21.77
VLDL	7.11 ± 3.2	7.18 ± 1.15	6.82 ± 2.0	0.8147	21.78
HDL	71.43 ± 18.7	60.84 ± 17.98	71.56 ± 19.2	0.2090	20,78
LDL	47.98 ± 5.3^{B}	47.32 ± 6.2^{B}	75.59 ± 1.,2^{A}	0.0073	32.56

$^{A–B}$: Means with distinct superscript letters differ significantly (rows) (p < 0.05).

TABLE 2 | Liver weight and lipid and cholesterol contents of male broilers at 21 and 42 days of age, according to incubation temperatures.

Variables	Incubation temperatures			*p*-values	CV(%)
	36°C	37.5°C	39°C		
		21 d. old broilers			
Liver Weight (g)	20.9 ± 3.4	20.7 ± 2.9	20.6 ± 3.6	0.4055	16.87
Liver Lipids (%)	4.8 ± 1.2	4.5 ± 1.3	4.3 ± 1.7	0.7851	29.92
Liver Cholesterol (µg/ml)	108.8 ± 18.9^{A}	75.6 ± 2.7^{B}	89.5 ± 16.8^{AB}	0.0028	16.61
		42 d. old broilers			
Liver (g)	40.8 ± 5.2	36.03 ± 4.8	34.7 ± 5.1	0.2343	16.87
Liver Lipids (%)	4.1 ± 1.3	3.35 ± 1.5	3.70 ± 0.7	0.5519	30.52
Liver Cholesterol (µg/ml)	136.9 ± 13.6^{A}	123.2 ± 10.9^{AB}	106.8 ± 12.2^{B}	0.0025	16.05

$^{A–B}$: Means with distinct superscript uppercase letters differ significantly (rows) (X ± SD, p < 0.05).

TABLE 3 | Adipocyte size and counts (number per area) and proliferation rate of 21 and 42 d old male broilers, according to incubation temperature and body region.

	Area (µm^2)	Count (cells/area)[1]	Proliferation (cells/area)[2]	Area (µm^2)	Count (cells/area)[1]	Proliferation (cells/area)[2]
	21 d-old broilers			42 d-old broilers		
Incubation Temperature (IT)						
36°C	1,419 ± 463	82 ± 35	18 ± 6	6,209 ± 1,602	75 ± 12	9 ± 3
37.5°C	1,332 ± 169	92 ± 21	18 ± 6	5,112 ± 3,320	96 ± 35	10 ± 3
39°C	1,472 ± 426	102 ± 30	18 ± 6	1,950 ± 1,010	83 ± 27	9 ± 3
Body Region (BR)						
Abdomen	1,496 ± 466	78 ± 25^{b}	18 ± 4	5,584 ± 2,424	78 ± 11	8 ± 2^{b}
Neck	1,464 ± 283	87 ± 22^{ab}	17 ± 7	4,875 ± 3,013	81 ± 24	9 ± 2^{ab}
Thigh	1,273 ± 336	106 ± 36^{a}	19 ± 6	2,763 ± 2,536	95 ± 37	11 ± 3^{a}
p-values						
IT	0.4082	0.3938	0.9420	<0.0001	0.0140	0.5724
BR	0.1363	0.0594	0.7695	<0.0001	0.0152	0.0064
IT x BR	0.1612	0.2082	0.8253	<0.0001	<0.0001	0.9775
CV (%)	26	31	27	23	20	27

[1]Average adipocyte number counted in 10 areas of 202,800 µm^2.

[2]Average number of adipocytes presenting immunostained nuclei for PCNA (proliferating cell nuclear antigen) counted in 16 areas of 202,800 µm^2.

$^{a-b}$: Means with distinct superscript lowercase letters differ significantly (columns) (X ± SD, p < 0.05).

TABLE 4 | Unfolding of the interaction between incubation temperatures and body regions on the adipocyte area and number of 42 d. old male broilers.

Body regions	Incubation temperatures			*p*-values
	36°C	37.5°C	39°C	
Adipocyte Area (μm^2)				
Abdomen	6574.44^{Aa}	7310.80^{Aa}	2436.12^{Ba}	<0.0001
Neck	5460.24^{Ba}	7841.20^{Aa}	1322.70^{Ca}	<0.0001
Thigh	6970.92^{Aa}	1001.99^{Bb}	2247.72^{Ba}	<0.0001
p-values	0.0557	<0.0001	0.1950	
Adipocyte Number (no cells/area)[1]				
Abdomen	85.50^{Aa}	74.67^{Ab}	74.61^{Ab}	0.3356
Neck	65.99^{Ba}	74.43^{Bb}	106.80^{Aa}	0.0004
Thigh	78.34^{Ba}	139.27^{Aa}	71.67^{Bb}	<0.0001
p-values	0.2230	<0.0001	0.0018	

[1]*Average adipocyte number counted in 10 areas of 202,800 μm^2.*
$^{A–B, a-b}$*: Means with distinct superscript uppercase (rows) and lowercase (columns) letters differ significantly (p < 0.05).*

which did not differ. Adipocyte area in the neck was also smaller in broilers from eggs incubated at 39°C than at 36°C or 37.5°C, and also smaller in broilers from eggs incubated at 36°C than at 37.5°C. In the thigh, adipocyte area was larger in broilers from egg incubated at 36°C than at 37.5°C or 39°C, which did not differ. Significant regional differences in adipocyte size only occurred in broilers from eggs incubated at 37.5°C. In these broilers, the area of thigh adipocytes was smaller than the area of abdomen and neck adipocytes, which did not differ. Incubation temperature influenced adipocyte number per area in the neck and thigh, with higher adipocyte number in the neck in broilers from egg incubated at 39°C than at 37.5°C or 36°C, which did not differ. In the thigh, adipocyte number was similar in broilers from eggs incubated at 36°C and 39°C, and both presented lower adipocyte number compared to broilers of eggs incubated at 37.5°C. In addition, no significant difference in adipocyte number was observed among the body regions in broilers from egg incubated at 36°C. However, in broilers from eggs incubated at 37.5°C, adipocyte was more numerous in the thigh than in the other two body regions, that did not differ. In broilers from egg incubated at 39°C, adipocyte number was larger in the neck compared to in the abdomen and thigh, which did not differ.

Body Chemical Composition

Ether extract content was the only variable influenced by incubation temperature in 21 days old broilers (**Table 5**). Ether extract content was lower in broilers from eggs incubated at 36°C than at 37.5°C and 39°C, and these did not differ. 42 days after hatching no significant effect of incubation temperature on the analyzed variables was found.

Broiler Performance

Table 6 presents feed intake, weight gain, body weight and feed conversion obtained for two rearing periods: 1–21 days and 21–42 days of age. Feed intake until 21 days of age was the only variable significantly affected by incubation temperature,

TABLE 5 | Body weight and composition of male broilers at day 21 and 42 of age, according to incubation temperature.

Variables		Incubation temperatures			*p*-values	CV(%)
		36°C	37.5°C	39°C		
			21 d. old			
Body Weight	(g)	822 ± 33	799 ± 24	802 ± 22	0.3968	24.47
Dry matter	(g)	256.44 ± 13.5	259.97 ± 19.6	255.29 ± 16.5	0.3037	12.58
	(%)[1]	31.20 ± 0.6	32.52 ± 0.4	31.83 ± 0.7	0.2110	1.17
Moisture	(g)	565.24 ± 21.4	539.01 ± 12.6	546.93 ± 19.2	0.2037	21.42
	(%)[1]	68.80 ± 0.6	67.47 ± 0.4	68.17 ± 0.7	0.2110	1.17
Ash	(g)	19.98 ± 1.1	17.12 ± 2.3	16.46 ± 3.1	0.1027	2.77
	(%)[2]	7.79 ± 0.2	6.58 ± 0.7	6.47 ± 1.3	0.5098	1.07
Ether Extract	(g)	69.28 ± 5.8	81.00 ± 12.1	72.63 ± 6.4	0.2577	9.75
	(%)[2]	27.02 ± 1.7^{B}	31.04 ± 2.3^{A}	28.42 ± 2.1^{AB}	0.0448	2.71
Crude Protein	(g)	150.83 ± 6.6	149.97 ± 7.5	148.09 ± 7.7	0.8343	6.86
	(%)[2]	58.84 ± 1.1	57.80 ± 2.5	58.09 ± 4.3	0.3210	3.02
			42 d. old			
Body Weigth	(g)	2.385 ± 124.0	2.355 ± 97.6	2.305 ± 109.1	0.4168	5.43
Dry Matter	(g)	975.65 ± 54.4	937.43 ± 100.7	914.83 ± 55.2	0.4089	9.83
	(%)[1]	40.92 ± 1.2	39.29 ± 2.7	39.68 ± 4	0.4942	2.16
Moisture	(g)	1409.12 ± 81.9	1447.18 ± 17.8	1390.26 ± 103.2	0.5225	76.53
	(%)[1]	59.08 ± 1.2	60.71 ± 2.7	60.32 ± 2.4	0.4942	2.16
Ash	(g)	60.91 ± 6.3	55.56 ± 5.2	55.20 ± 4.7	0.2245	5.76
	(%)[2]	6.24 ± 0.3	5.99 ± 1.0	6.04 ± 0.4	0.8279	0.61
Ether Extract	(g)	329.69 ± 36.9	294.82 ± 89.2	264.14 ± 54.1	0.3206	6.70
	(%)[2]	33.86 ± 4.1	30.98 ± 6.4	28.66 ± 4.3	0.3399	5.41
Crude Protein	(g)	1305.20 ± 144.8	1358.01 ± 96.6	1310.63 ± 244.8	0.3485	16.55
	(%)[2]	54.84 ± 2.57	58.83 ± 5.96	54.87 ± 4.85	0.6410	7.25

[1,2]*: Calculated with respect to body weight and dry matter, respectively.*
$^{A–B}$*: Means with distinct superscript uppercase letters differ significantly (rows) (p < 0.05).*

TABLE 6 | Broiler body weight at 21 and 42 days of age and performance from day 1 to 21 and from day 22 to 42 of age, according to incubation temperature.

Variables	Incubation temperatures			*p*-values	CV(%)
	36°C	37.5°C	39°C		
		1–21 days of age			
Feed Intake (g)	983 ± 54^{A}	953 ± 100^{AB}	902 ± 55^{B}	0.0317	52.51
Weight Gain (g)	757 ± 48	738 ± 41	758 ± 52	0.5099	26.41
Body Weight (g)	822 ± 48	799 ± 104	802 ± 52	0.3967	27.47
Feed Conversion (g/g)	1.30 ± 0.12	1.29 ± 0.11	1.22 ± 0.21	0.7493	0.75
		22–42 days of age			
Feed Intake (g)	2,729 ± 261	2,798 ± 300	2,818 ± 434	0.9123	10.02
Weight Gain (g)	1,515 ± 115	1,538 ± 91	1,455 ± 82	0.4081	7.07
Body Weight (g)	2,385 ± 123	2,325 ± 107	2,245 ± 82	0.4168	5.43
Feed Conversion (g/g)	1.81 ± 0.18	1.82 ± 0.11	1.94 ± 0.31	0.5734	11.57
		1–42 days of age			
Feed Intake (g)	3,713 ± 286	3,751 ± 333	3,720 ± 422	0,6217	6,97
Weight Gain (g)	2,272 ± 124	2,275 ± 107	2,196 ± 81	0,5365	5,21
Feed Conversion (g/g)	1.63 ± 0.12	1,65 ± 0.08	1,69 ± 0.19	0,5945	8,98

$^{A–B}$: Means with distinct superscript uppercase letters differ significantly (rows) ($p < 0.05$).

and this was lower for broilers from eggs incubated at 39°C than 36°C.

DISCUSSION

Low-fat deposition in broilers is currently desirable to preserve and/or to promote their well-being and survival in the face of heat stress, to increase feed-to-muscle conversion, to maximize production efficiency of the industry, and to address the market's requirement for lean meat. Previous study showed continuously high incubation temperature during fetal development reduces abdominal and cervical subcutaneous adipocyte hypertrophy in broiler hatchlings (Almeida et al., 2016), causes variations in subcutaneous adipose tissue deposition across different body areas and in skin temperature and thickness of broiler hatchlings (Bai et al., 2015; Almeida et al., 2016; Morita et al., 2016b). The current study assessed whether low (36°C) and high incubation temperatures (39°C) from day 13, exerted a long-term and regional-related effect on fat deposition in broilers by analyzing subcutaneous adipose tissue hyperplasia and hypertrophy in three anatomically distinct adipose deposits (neck, abdomen, and thigh), liver weight and content of lipid and cholesterol, blood lipid profile, body weight and chemical composition, and performance of 21-day-old and 42-day-old male broilers.

We evaluated the effects of incubation temperature on regional cellularity pattern of adipose tissue depots by measuring adipocyte size and number per area. Effects of incubation temperatures and differences among the three body regions on these adipocyte characteristics were apparent in 42-day-old broilers. At this age, egg incubation at 39°C reduced the abdominal adipocyte size by approximately 67% compared to egg incubation at 36°C or 37.5°C, but without change in the number of adipocytes per area. It also reduced neck adipocyte size by approximately 83 and 76% compared to 37.5°C and 36°C, respectively, resulting in an increase in the adipocyte number per area by approximately 43 and 38% under incubation temperature of 37.5°C and 36°C, respectively. These findings showed that high incubation temperature greatly reduced the hypertrophic growth of adipocytes in the abdomen and neck, with much more intense effects on fat deposition and cell density (cells per area) in the adipose tissue of the neck. In contrast to that observed for the abdomen and neck, no significant effect of high incubation temperature on adipocyte size was recorded in the thigh. However, there was an increase in the cell density of the thigh adipose tissue, as showed by the increase in the number of adipocytes/area. These region-specific effects of high incubation temperature may be related to distinct metabolic rate existing among the body adipose tissue depots (Xiao et al., 2019). Contrary to the high incubation temperature, low incubation temperature (36°C) did not influence the abdominal adipocyte size nor the counts, and markedly increased thigh adipocyte size, which was about 86 and 68% greater compared to 37.5°C or 39°C, respectively, resulting in a decrease of approximately 56% in the cell density compared to 37.5°C. However, like 39oC, egg incubation at 36°C also reduced the size of neck adipocytes, but its effect was less pronounced because adipocytes were only approximately 30% smaller compared to 37.5°C. Since adipocyte hypertrophy is directly related to fat deposition (Tchernof et al., 2006; Kou et al., 2012; Choe et al., 2016), this supports the idea that high incubation temperature induced a decrease in fat deposition in neck and abdominal adipocytes, while a low incubation temperature induced increased deposition in thigh and abdominal adipocytes and a decreased deposition in neck adipocytes. From a developmental view, our results are of great interest and importance because they reveal a close link between egg incubation temperature during fetal development and postnatal adiposity in broilers. This leads us to recognize adipose tissue as a key target of fetal developmental programming for birds, as has been observed for mammals (Ravelli et al., 1999; Lecoutre et al., 2019; Lecoutre et al., 2021). We previously

reported abdominal and/or cervical adipocyte size reduction in response to egg incubation at 36°C or 39°C in broiler hatchlings (Almeida et al., 2016). Thus, as expected, the present study confirmed that incubation temperature during fetal development has a long-term effect on adiposity of broilers. The occurrence of effects of incubation temperature on adipose tissue only at 42 days of age may be related to a significantly higher cumulative fat deposition between 21–42 days than between 1–21 days of age (Hen et al., 2014). Comparison among the three body regions showed broilers reared from eggs incubated at 37.5°C had similar size and number of adipocytes per area in the abdomen and neck, but these two regions presented adipocyte size approximately 86–87% larger and adipocyte number per area 50 % smaller than the thigh, indicating higher adipocyte hypertrophy in the abdomen and neck adipose tissue than the thigh. When eggs were incubated at 36°C or 39°C, no differences in the adipocyte size and number per area were found among the three body adipose depositional sites, showing the same hypertrophy rate for the three regions.

In this study, we evaluated the effects of incubation temperature on regional subcutaneous adipocyte hyperplasia by analyzing adipocyte proliferation. At 42 days of age, independently of incubation temperature, differences among the three regions were apparent. The rate of adipocyte proliferation was lower in the abdomen than the thorax and thigh (abdomen > thigh). An effect of incubation temperature on adipocyte proliferation was also not previously found in broiler hatchlings, but in contrast to what we observed for 42 day-old broilers, the cervical region exhibited higher cell proliferation than the abdomen and thigh (Almeida et al., 2016). Regional differences in adipocyte size and proliferation was reported for chicken and/or broiler lines in other studies (Guo et al., 2011; Chen et al., 2014; Xiao et al., 2019). Heterogeneity in adipocyte size among adipose tissue deposits also occurs in mammals, including humans (Sbarbati et al., 2010). However, to the best of our knowledge, for the first time in the literature, comparison between body regions revealed that manipulation of incubation temperature during fetal development also reprograms differences in size and number of adipocytes per area among different adipose tissue deposits in later life of broilers.

Temporal analysis showed, from day 21 to 42 of age, broilers reared from eggs incubated at 36°C, 37.5°C, and 39°C increased their adipocyte area by approximately 437, 384, and 132% and reduced rate of adipocyte proliferation by approximately 50, 45, and 50%, respectively. During the same period, differences in the rate of growth and proliferation of adipocytes were also recorded for regional subcutaneous adipose tissue deposits: the size of adipocytes increased by 273% in the abdomen, 233% in the neck and 117% in the thigh, while the proliferation of adipocytes decreased around 55% in the abdomen, 47% in the neck and 42% in the thigh. This showed that, independently of incubation temperature and body region, both hyperplasia and hypertrophy contributed to adipose tissue growth throughout the broiler age, in agreement with Cartwright (1991), but the magnitude of contribution of hyperplasia was larger than hypertrophy from day 21. Our data differ from those obtained for chicken by Cartwright (1991), who demonstrated adipocyte hyperplasia and hypertrophy increase with age. They differ from those reported for goose by Huo et al. (2021), who verified the average number of adipocytes had no increase after birth. We previously reported the adipocyte size and proliferation rate in abdominal, cervical and thigh adipose deposits of broiler hatchlings from eggs incubated at 36°C, 37.5°C, and 39°C in Almeida et al. (2016). Comparing the data obtained in the present study with those in Almeida et al. (2016), we verified that broilers have a low rate of adipocyte hypertrophy and a marked increase of adipocyte hyperplasia from hatching to 21 days of age, in contrast to what occurs between 21 and 42 days of age. White adipose tissue is characterized by its capacity to adapt and expand, or not, in response to energy availability or demand by changing adipocyte hypertrophy and hyperplasia (Öst and Pospisilik, 2015; Pellegrinelli et al., 2016). Positive links between adipose tissue growth by hyperplasia and hypertrophy and body weight was recorded in chickens (*Gallus domesticus*) (Cartwright, 1991). Despite that, in the present study, no incubation temperature-related changes in broiler body composition (ether extract, crude protein, ash, water contents) at 21 and 42 days of age and in performance (body weight, feed intake, feed conversion, body gain) that showed a relationship with marked hyperplasia and reduced hypertrophy after 21 days of age, were found. However, although cumulative fat deposition in broilers is greater at 21–42 days than at 1–21 days of age, the weekly rate of fat deposition decreases with age after 21 days (Henn et al., 2014). Furthermore, broilers decrease their activity levels with age (Yang et al., 2020), which has been associated with increased resting and high locomotion-associated metabolic costs, and results in heavier and less active broilers (Tickle et al., 2018). Thus, independently of incubation temperature during fetal development, the occurrence of increased adipocyte hyperplasia and reduced adipocyte hypertrophy in the three adipose deposits from day 21–42 of age that were observed in the present study appear to result from simultaneous increased energy availability and decreased metabolic expenses associated with locomotion, as a function of age.

Lipid droplets of an adipocyte are composed of a hydrophobic core of triacylglycerides and cholesterol esters, which, in birds, result from *de novo* lipogenesis occurring predominantly in the liver, where glucose is catabolized to acetyl-CoA. This is subsequently converted into fatty acids and cholesterol, incorporated into very low density lipoproteins (VLDLs) and transported to other tissues, including adipose tissue, via blood circulation (Walther and Farese, 2012; Wang et al., 2017). We further analyzed blood lipid profile and liver weight and cholesterol and lipid contents to assess the effects of incubation temperature on lipid metabolism. Egg incubation at 36°C and 39°C influenced blood and liver lipid profile of 21 and 42 day-old broilers. At 21 days of age, a low egg incubation temperature (36°C) increased liver cholesterol content compared to 37.5°C, as well as increased the blood contents of cholesterol, triglycerids, VLDL, and LDL and diminished blood HDL content compared to 37.5°C and 39°C. These effects may be a result of the increase in energy intake due to higher feed

consumption from day 1 to 21 exhibited by broilers reared from eggs incubated at 36°C. Although these data indicate an increase in broiler lipid metabolism induced by low incubation temperature (36°C), this increase was not converted into larger adipocyte size and proliferation at day 21 in any of the three subcutaneous adipose tissue deposits, as appears to be demonstrated by the absence of incubation temperature-dependent differences in the area and proliferation of adipocytes at this age. However, Almeida et al. (2016) demonstrated that broiler hatchlings from eggs incubated at 36°C exhibited smaller adipocytes in the neck than the abdomen and leg, due to the higher rate of cell proliferation reported in the neck. This led us to raise the possibility that the absence of differences in adipocyte size and proliferation between regional adipose tissue deposits and incubation temperatures observed in 21-day-old broilers results from an increase in lipid metabolism, from hatchling to 21 days age, as indicated by the highest values recorded for lipid profile at this age. 42 days after hatching, liver cholesterol content decreased as incubation temperature increased, resulting in lower cholesterol values in broilers from eggs incubated at 39°C than at 36°C. In contrast, broilers hatched from eggs incubated at 39°C exhibited increased blood cholesterol and LDL contents compared to broilers hatched from eggs incubated at 36°C or 37.5°C. At this age, adipocyte size in the three body regions analyzed were significantly smaller in broilers reared from eggs incubated at 39°C than at 36°C and 37.5°C, and there was no difference in the rate of cell proliferation between broilers hatched from eggs incubated at different temperatures (our data). Thus, our results suggest a lack of links between increased blood cholesterol and LDL and liver cholesterol with adipocyte size at 42 days of age when egg incubation occurred at 39°C. Lipids are important structural and functional components of animal cells, particularly playing an important role in membrane stabilization, permeability and signaling (Santos and Preta, 2018). As a component of cell membranes, cholesterol is a neutral lipid that acts as a buffering molecule of membrane fluidity despite deviations in body temperature. Membrane stability is required for the continued action of membrane proteins in the transport of molecules across the lipid bilayer. Cholesterol decreases membrane fluidity at high temperatures and increases fluidity at low temperatures (Kroes and Ostwald, 1971; Bloom and Mouritsen, 1988; Yeagle et al., 1988). This makes cholesterol an essential molecule for cell proliferation during growth and cell renewal. Consequently, temperature an important determinant of animal cholesterol content and high ambient and acclimation temperatures induce an increase in cholesterol content, while low temperatures induces a reduction (e.g., Crockett, 1998; Hassett and Crockett, 2009). A link between ambient temperature and blood lipid profile has been also reported. Halonen et al. (2011), for example, found decreased HDL levels and increased LDL level in association with each 5°C increase in ambient temperature. To our knowledge, the long-term associations between egg incubation temperature during fetal development and blood lipid profile and total liver cholesterol have not been previously reported for broilers or other birds.

In conclusion, our data demonstrate that incubation temperature during the fetal phase 1) reprograms postnatal ontogenetic development of adipose tissue and lipid metabolism and 2) influences the hypertrophy and hyperplasia rate of regional adipocyte deposits in different ways. These findings on the occurrence of fetal reprogramming of postnatal adipose tissue development point to the potential use of manipulation of incubation temperature as a management for modulation of adiposity in broiler lines.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Use (CEUA, protocol n° 021086/11) of the College of Agricultural and Veterinary Sciences, of the São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil.

AUTHOR CONTRIBUTIONS

Conceptualization: AA and IB. Data curation: AA, JM, TV, VM, and IB. Formal analysis: AA, VM, SS, and IB. Funding acquisition: IB. Investigation: AA, VM, JM, SS, TV, and IC. Methodology: AA, VM, and IB. Project administration: AA, VM, and IB. Resources: IB. Supervision: IB. Writing—original draft: AA, VM, and IB. Writing—review and editing: IB. Writing—review and editing: IB. All authors have read and agreed to the submitted version of the manuscript.

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REVIEW
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The Role of Incubation Conditions on the Regulation of Muscle Development and Meat Quality in Poultry

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Muscle is the most abundant edible tissue in table poultry, which serves as an important source of high protein for humans. Poultry myofiber originates in the early embryogenic stage, and the overall muscle fiber number is almost determined before hatching. Muscle development in the embryonic stage is critical to the posthatch muscle growth and final meat yield and quality. Incubation conditions including temperature, humidity, oxygen density, ventilation and lighting may substantially affect the number, shape and structure of the muscle fiber, which may produce long-lasting effect on the postnatal muscle growth and meat quality. Suboptimal incubation conditions can induce the onset of myopathies. Early exposure to suitable hatching conditions may modify the muscle histomorphology posthatch and the final muscle mass of the birds by regulating embryonic hormone levels and benefit the muscle cell activity. The elucidation of the muscle development at the embryonic stage would facilitate the modulation of poultry muscle quantity and meat quality. This review starts from the physical and biochemical characteristics of poultry myofiber formation, and brings together recent advances of incubation conditions on satellite cell migration, fiber development and transformation, and subsequent muscle myopathies and other meat quality defects. The underlying molecular and cellular mechanisms for the induced muscle growth and meat quality traits are also discussed. The future studies on the effects of external incubation conditions on the regulation of muscle cell proliferation and meat quality are suggested. This review may broaden our knowledge on the regulation of incubation conditions on poultry muscle development, and provide more informative decisions for hatchery in the selection of hatching parameter for pursuit of more large muscle size and superior meat quality.

Keywords: poultry, incubation condition, myofiber formation, muscle development, meat quality

Abbreviations: E, embryonic day; D, day of age; T3, thiothyrotropine; T4, thyroxine; GHRH, growth hormone-releasing hormone; GH, growth hormone; IGF-1, insulin-like growth factor 1; GHR, growth hormone receptor; MyoD, myogenic differentiation antigen; Myf5, myogenic factor-5; MyoG, myogenin; Pax7, paired box 7; MRF4, myogenic regulatory factor-4.

1 INTRODUCTION

Poultry is major source of protein and the demand of poultry meat and other by-products are increasing day-by-day throughout the world (Scanes, 2007). With the rapid development of modern society, consumers are demanding more and more quality poultry meat. In the past decades, great progress in genetic selection and management has been made in meat type poultry, which contribute to the fast growth rates and large muscle mass (Petracci et al., 2015). However, the increasing production of poultry meat has been accompanied by increasingly serious meat quality problems including white striping, woody breast and spaghetti meat (Baldi et al., 2018; Oviedo-Rondón et al., 2020; Che et al., 2022), which will negatively affect the desire of people to consume. As a result, there is growing interest in searching for new approaches to improve poultry meat quality while increasing the meat yield.

Incubation phase is a critical stage in the life cycle of poultry, which covers the phase from the start of embryogenesis to the beginning of the young bird stage or birth (Gilbert and Knisely, 2000) and play an important role in the skeletal muscle growth and final meat quality in poultry (Nyuiadzi et al., 2020; Guo et al., 2021). Skeletal muscle is the dominant component of poultry meat. It is well established that poultry muscle fiber number is determined during embryonic development (Stockdale and Miller, 1987). Evidences have shown that regulation of myofiber development during myogenesis can increase the number and fiber diameter of muscle fiber (Gonzalez and Jackson, 2020; Ma et al., 2020). Therefore, environmental regulation during the incubation phase may be a novel way out to ameliorate the muscle development and meat quality in poultry.

Incubation conditions including temperature, humidity, lighting, ventilation and other regimen affects the development of muscle. The effect of changing incubation conditions on embryonic development and muscle growth in birds has gained much attention in the fields of poultry science in recent years. It was demonstrated that lighting and thermal adjustment during the incubation phases improves the number and activity of satellite cells, promotes muscle fiber development, increases the embryonic muscle fiber area (Zhang et al., 2014) and improved meat quality (Piestun et al., 2013). Incubation of chicken embryos in a hypoxic environment with 17% oxygen content improves posthatch broiler body weight and increases pectoral muscle production (Druyan et al., 2018). Green light exposure during broiler incubation increased the number and proliferative activity of myogenic and satellite cells, promoted embryonic muscle fiber development (Bai et al., 2019), and increased pectoral muscle production (Zhang et al., 2012). Heating at 39.5°C for 12 h per day from embryonic day E7 to E16 increased the weight and ratio of embryonic pectoral muscle and improved the number and proliferative activity of adult myoblasts (Piestun et al., 2013). Stimulation of embryonic eggs at 39.5°C for 3 or 6 h per day during the E16–E18 stages increased broiler pectoral muscle production myofiber diameter (Piestun et al., 2009). When the incubation temperature was adjusted upward to 38.8°C at mid-embryonic stage, the pH values and water holding capacity of the breast muscle after slaughter were improved (Janisch et al., 2015). Increasing the temperature to 39.5°C can reduce the severity of many microscopic features of myopathies common in the broiler industry and have a positive effect on improving muscle quality (Clark et al., 2017). If the incubation environment is suboptimal, it is not conducive to the formation of excellent chicken meat quality. Irradiation of the embryos at 39.2°C for 3 h per day at E8–E10 and E16–E18, respectively, decreased the water holding capacity and pH values of the broiler breast meat (Collin et al., 2007). It is clear that regulation of the incubation environment can be a potential solution for improving poultry muscle quality during poultry breeding.

Therefore, the purpose of this paper is to present scientific evidence that incubation conditions may convey substantial and lasting effects on embryonic muscle fiber and posthatch muscle growth as well as meat quality at market age. The first part will describe the muscle fiber growth in poultry, with an emphasis on the hormone and growth factor. The second section will provide scientific results of incubation parameter-induced changes of muscle growth and meat quality, and discuss the modes of action for diverse incubation conditions. The third portion will summarize the overall evidences and recommend future directions for incubation condition modulation on embryonic muscle fiber development and meat quality in poultry.

2 PHYSIOLOGICAL PROCESSES OF MUSCLE FIBER DEVELOPMENT

2.1 Developmental Characteristics of Embryonic Muscle Fibers

The embryonic stage is an important period for the formation of muscle fibers. Embryonic muscle fibers are differentiated from myogenic precursor cells. Myogenic precursor cells proliferate and differentiate into myogenic fibers, move toward the site of muscle formation, align and fuse to form myotubes, and finally fuse into multinucleated myofibers, which in turn fuse with existing muscle fibers to form new myofibers, and further differentiate into skeletal muscle (Swartz et al., 1994). Myoblasts can be designated as embryonic, fetal and adult (Stockdale, 1992), embryonic myoblasts differentiate into primary fibers, fetal myoblasts form secondary fibers and adult myoblasts plays an important role in the formation and growth of muscle fibers during late incubation period and after hatching of poultry (Stockdale and Miller, 1987). On E14, a third type of myogenic cells (satellite cells) emerge, which attach to myofibrils beneath the basement membrane of muscle fibers (Mauro, 1961; Hartley et al., 1992). Satellite cells have the potential to promote postnatal skeletal muscle growth in vertebrates, and are the main source of myoblasts in neonatal skeletal muscle growth (Moss and Leblond, 1970). The number of satellite cells rapidly decreases 1 week after hatching. When muscle is stimulated, the activated satellite cells proliferate and differentiate to fuse with the original myofibers to form new muscle fibers (Hawke and Garry, 2001; Halevy et al., 2004; Halevy et al., 2006a; Allouh et al., 2008).

2.2 Effects of Hormones and Myogenic Regulatory Factors on Muscle Fiber Development

Hormones also have the potential to promote the development and growth of skeletal muscle during embryonic development. In addition, hormones can also influence embryonic development through multiple mechanisms, such as muscle cell growth, proliferation, and differentiation (Dishon et al., 2017, 2018). It has been found that hypertrophy of muscle fibers during late hatching may be associated with increased levels of thiothyrotropine (T3) and thyroxine (T4), thyroid hormones. Among them, T3 and T4 are involved in many physiological processes, including embryonic muscle growth (Christensen et al., 1996) and stimulation of embryonic hatching (Christensen et al., 2003, 2004). Regulating specific environmental factors can ameliorate physiological response at the critical stages of embryonic development. Fertilized duck eggs placed in environment with 1% CO_2 before incubation had a positive effect on posthatch body weight by increasing T3, T4 and corticosterone levels in plasma of embryos (El-Hanoun et al., 2019). In mammals and birds, the proliferation and differentiation of muscle cells are controlled by growth factors and other factors that play an important role in promoting skeletal muscle growth (Adams et al., 1999). The mechanism controlling muscle growth and development is the pro-growth axis, which includes hypothalamic growth hormone-releasing hormone (GHRH), growth hormone (GH) produced by the anterior pituitary, insulin-like growth factor 1 (IGF-1) produced by the liver and skeletal muscle, and the corresponding receptors. GH and IGF-1 induce proliferation and differentiation of muscle cells (Halevy et al., 2006b). In later incubation period, GH levels are high in blood and decline with age (Buyse and Decuypere, 1999; Kim, 2010). GH affects growth by activating its receptor (Edens and Talamantes., 1998). The growth hormone receptor (GHR) is a transmembrane protein that begins to develop on E12 (Kocamis et al., 1999), and can be found in a variety of tissues, including liver, muscle, and adipose tissue (Mao et al., 1998). Receptors in muscle can be found on satellite cells, and irradiating embryonic eggs with green light during incubation increases GHR expression on satellite cells (Halevy et al., 2006b). IGF-1 is one of the most important growth factors regulating satellite cell proliferation, and it plays a role in muscle growth and hypertrophy through its effects on satellite cells (Adams and Mccue., 1998; Adams et al., 1999). IGF-1 has also been found in amniotic fluid and may play a role in embryonic regulation of amino acid utilization (Karcher et al., 2005). IGF-1 belongs to a family of insulin-related peptide hormones with multiple metabolic and anabolic properties. IGF-1 play an important role in the metabolism of carbohydrates, lipids and proteins in a variety of tissues, including liver, muscle and adipose tissue (Kanacki et al., 2012), and is able to stimulate hepatic glycogen, RNA and protein synthesis (Mcmurtry, 1998). In addition, it also plays a key role in muscle cell proliferation and differentiation. Similar to GHR in satellite cells, muscle IGF-1 was also significantly elevated in birds exposed to light stimulation in the early post-hatching period (Halevy et al., 2006b). The expression level of *IGF-1* gene in broiler pectoral muscle was higher when the incubation temperature is increased to 39.5°C at E12 to E18 embryonic ages (Piestun et al., 2009).

The proliferation and differentiation process of myogenic cells is controlled by a family of muscle-specific basic helix-loop helix transcription factors containing four basic helix-loop-helix *myogenic differentiation antigen* (*MyoD*), *myogenic factor-5* (*Myf5*), *myogenin* (*MyoG*) and *myogenic regulatory factor-4* (*MRF4*) that positively regulate myogenesis. These factors are sequentially expressed when satellite cells are activated (Weintraub, 1993) and are essential for satellite cell activation, proliferation and differentiation (Schultz and Mccormick., 1994). *MyoD* and *MyoG* are only continuously expressed in activated satellite cells (Hernández-Hernández et al., 2017). Numerous studies have shown that *MyoD* is a marker of satellite cell activation and proliferation, while *MyoG* is a marker of cells entering the terminal differentiation program (Smith et al., 1994; Seale and Rudnicki, 2000). Higher myogenic inhibitor mRNA levels are accompanied by lower *MyoD* and *MyoG* mRNA levels, and myogenic inhibitor inhibits muscle growth by downregulating the gene expression of *MyoD*, *Myf5*, and *MyoG* (Thomas et al., 2000; Langley et al., 2002). Initially, *Myf5* and *MyoD* are expressed in proliferating zygotes, and then *MyoG* is also expressed as cells begin to differentiate (Beauchamp et al., 2000). Increasing the incubation temperature (39.5°C, 3 and 6 h) between E12 and E18 embryonic has been reported to promote cell differentiation and increase muscle myostatin expression levels (Piestun et al., 2009). Hypoxia during embryonic development inhibits myogenic cell differentiation, decreases the expression of *MyoD*, *Myf5* and myosin heavy chain, and hinders the formation of multinucleated myotubes (Beaudry et al., 2016; Yang et al., 2017). *Paired box 7* (*Pax7*) is considered an early marker of myogenesis during post-hatching muscle growth, and is selectively expressed in quiescent and proliferating satellite cells and is essential for their self-renewal (Halevy et al., 2004; Allouh et al., 2008). Furthermore, the expression of *Pax7* was increased during myogenic cell proliferation and decreased during differentiation (Halevy et al., 2004). Previous studies have reported that increasing the incubation temperature to 39.5°C during E12–E18 increased the proliferative activity of adult myoblasts, thymidine DNA content, the number of muscle cells with PCNA in the muscle, and *Pax7* protein expression level (Piestun et al., 2009).

2.3 The Effect of Muscle Fiber Development on Meat Quality

The growth and development rate of muscle tissue before and after hatching has an important influence on muscle yield and meat quality after hatching and even at market age (Gratta et al., 2019; Ma et al., 2020). The number and morphology of different fibers are closely related to meat quality, when the number of small diameter fibers in the muscle is high and the intramuscular fat is abundant, the meat more tender. Conversely, when the muscle has a higher proportion of fibers with larger cross-

sectional area and increased glycogen content, the pH values of muscle decreases rapidly after slaughter, the final pH values and tethering power are lower, and the color and quality of the meat may be slightly worse (Joo et al., 2013; Cong et al., 2017). During mid-incubation, increasing the incubation temperature to 38.8°C increased post-slaughter pectoral muscle pH values and tethering power and improved final meat quality in broilers (Janisch et al., 2015). Poultry meat quality can be affected by pectoral muscle diseases, including pectoral woody meat, white striping and spaghetti meat, which are manifested in poor muscle microvascularization, leading to muscle fiber degeneration during muscle regeneration, with a rounded shape and internalized nuclei (Soglia et al., 2016a; Sihvo et al., 2018; Petracci et al., 2019). These lesions can be observed in broilers during the first week after hatching (Chen et al., 2019), with diffuse thickening of the endomysium and perimysium connective tissue of granulation tissue, increased deposition of connective tissue (fibrosis), increased fat deposition or infiltration, leading to varying degrees of muscle damage in the pectoral muscle. (Soglia et al., 2016b; Clark and Velleman, 2016). Among them, woody meat showed the appearance of sclerotic and uniform pale areas in the muscle, white striping meat showed the presence of white streaks parallel to the direction of the muscle fibers, and spaghetti meat showed the tendency to separate the fiber bundles that make up the muscle tissue of the breast muscle (Soglia et al., 2019). Increasing the incubation temperature from 37.8 to 39.5°C for 12 h a day during the late incubation period can reduce the severity of many microscopic features of myopathies common in the broiler industry (Clark et al., 2017), which may have a positive effect on improving meat quality and processing functions.

2.4 Environmental Regulation Effect on Muscle Fiber and Meat Quality

2.4.1 Temperature on Muscle Development

Temperature is the most important environmental factor to regulate the embryonic development (Fasenko, 2007). Variations in incubation temperature in stages of embryonic muscle fiber development have diverse effects on muscle fiber development and posthatch muscle growth. Generally, mild increase of temperature can stimulate muscle fiber growth, while severe high temperature tends to cause embryo death (Reyna and Burggren, 2012). Piestun et al. (2015) demonstrated that temperature elevation during mid-term embryogenesis results in enhanced muscle growth in the embryo and posthatch by induction of myoblasts proliferation. Temperature stimulation aims to improve embryonic muscle fiber and posthatch muscle development especially in mid-incubation period, when the myogenic cells proliferate rapidly, which can determine the final muscle fiber number, morphology, and size (Stockdale, 1992). Broiler muscle yield was reported to be improved when E7-E16 embryos were indirectly incubated at high temperature (39.5°C) for 12 h per day, due to the elevation of number and proliferative activity of pectoral myoblast. (Piestun et al., 2013). From E7 to E16, the 24 h continuous high temperature exposure at 39.5°C reduced broiler body weight, but it increased breast muscle percentage and yield (Piestun et al., 2015). This may be related to the difficulty of dissipating excess heat in the embryo, which leads to teratogenic consequences and growth retardation regardless of the increased proliferation of myoblasts, compared to the sustained effect of intermittent high-temperature stimulation on myofiber development at mid-hatch without affecting the final body weight of the broiler, and increased pectoral muscle production. Krischek et al. (2018) found that increased the incubation temperature from 37.8 to 38.8°C from E7 to E16 increased the muscle fiber area at hatching and body weight of broiler at 35 D, indicating that the positive effect of increasing the temperature at mid-incubation on embryonic muscle fiber development could persist until market age.

Similarly, exposed embryonic eggs to 38.5 and 39.5°C conditions for 18 h per day between E12 and E18 was observed to increase the posthatch broiler body weight and pectoral muscle production by upregulating muscle growth factor (IGF-1 and GH) and muscle marker gene (*MyoD*, *MyoG*, *Pax7*, and *PCNA*) expression during incubation and posthatch (Al-Zghoul and El-Bahr, 2019). Late hatching is a critical period for the development of satellite cells, the only myogenic cells capable of repairing damaged muscle fibers and promoting post-muscle growth, and they are able to re-enter the cell cycle under different muscle stresses, proliferate, and then fuse into existing or newly formed muscle fibers (Hawke and Garry, 2001). At E16–E18, stimulating embryonic eggs at 39.5°C for 3 or 6 h per day increased the number and activity of embryonic and number of myoblasts in broilers before and after hatching, and pectoral muscle fiber diameter on 13 day of age (D) and 35D, as well as the final body weight and pectoral muscle production in broilers (Piestun et al., 2009). Thus, it is clear that increasing the incubation temperature appropriately during the critical stage of muscle fiber development has a direct and lasting positive effect on embryonic muscle fiber development and post-hatch muscle growth. In another study, heating (38.1°C) the embryos between embryonic E0 and E5 of incubation, increased the body weight of broiler chicks on the day of hatching and at 35D, and significantly increased pectoral muscle production and plasma testosterone levels (Lin et al., 2017). As an important sex hormone, testosterone levels are positively correlated with broiler growth, and high plasma testosterone levels are associated with faster growth and greater body weight (Buyse et al., 1996), whether raising high incubation temperatures in the pre-hatching period is associated with the ability to promote muscle development by increasing plasma testosterone levels needs further study.

Studies regarding the effect of incubation temperature on meat quality are fewer and results vary. Pre-hatching thermal stimulation at 39.5°C for 3 h per day reduced breast muscle pH values, while continuous stimulation between E 8–10 and E16–E18 reduced pH values and water holding capacity of chicken meat after slaughter (Collin et al., 2007). Temperature adjustment upward to 38.8°C in the middle stage of incubation, improved the broiler breast meat quality as evidenced by the increased post-slaughter breast muscle pH values and reduced grill loss (Janisch et al., 2015). Increasing the incubation

temperature from 37.8 to 39.5°C for 12 h per day during late incubation reduced the severity of many microscopic features of myopathy common in the broiler industry (Clark et al., 2017), and myopathy severity and muscle degradation had a significant negative effect on meat quality and processing characteristics (Soglia et al., 2016a). Therefore, increasing the incubation temperature to below 39.5°C for intermittent thermal manipulation during late embryonic development can be a viable management strategy to improve meat quality and processing functions.

2.4.2 Lighting on Muscle Development

Light is an important exogenous environmental factor that affects embryonic development during incubation. As early as the late 1960s, it was found that embryonic development was accelerated when fertilized eggs were stimulated by continuous light (Siegel et al., 1969). Studies have shown that providing light in a dark incubation environment has a positive effect on embryo development, while different colors of light during incubation has diverse effects on embryo development and muscle growth after emergence (Rozenboim et al., 2003, 2004).

Green light stimulation during incubation is known to be a method to increase poultry meat production. Green light illumination to embryos during incubation increases turkey body weight at 28 D, and this positive effect can be sustained up to 79 D, increasing breast muscle production (Rozenboim et al., 2003). Green light stimulation given during the incubation period had strongest effect on muscle growth (Rozenboim et al., 2004). Green light during incubation of chicken embryos increased daily weight gain and pectoral muscle weight in the week before fledging, which was beneficial for early development and muscle growth of broiler chicks. (Özkan et al., 2012a). Green light stimulation of chicken embryos during incubation increased pectoral muscle and embryo weight during incubation, and also increased the proportion of pectoral muscle during early chick emergence (Rozenboim et al., 2004).

Monochromatic green light irradiation at different incubation periods increased GH levels in plasma and up-regulated hypothalamic growth-promoting axis gene expression of chicken embryos (Dishon et al., 2021), Green light stimulation at E0 increased GH levels in the plasma of chicken embryos from E14 to E20, and upregulated GHR and insulin growth factor I gene expression in the liver, GH can act through GHR in muscle and adipose tissues, and through pro-growth axis mechanisms to influence embryonic development, such as muscle cell growth, proliferation and differentiation (Dishon et al., 2017). Intermittent light irradiation from E5 to E14, followed by continuous green light stimulation, up-regulated Pax7 and myostatin expression, increased the number of pectoral myogenic cells and the proliferation activity of satellite cells, promoted the proliferation and differentiation of myogenic cells, and increased the posthatch breast muscle weight of broiler chicks (Halevy et al., 2004). In addition, green light stimulation in incubation phase could up-regulate the gene expression of *MyoG* and *MyoD* in chicks, thus leading to the increase of cross-area of muscle fiber and weight of breast muscle 1 week after hatching (Zhang et al., 2012, 2014). Therefore, muscle growth-promoting effect of green light irradiation may be related to the regulation of growth-promoting hormone secretion and muscle growth factor gene expression.

In addition to green light, other colors of light during the embryonic period also positively affect chicken embryo development and posthatch muscle growth. White light stimulation during E14–E21 increased the proportion of breast muscle at hatching (Özkan et al., 2012b). Blue light stimulation during the incubation period had no effect on posthatch body weight and muscle mass of broiler chicks (Zhang et al., 2012), but another study showed that blue light stimulation during the embryonic period increased the average posthatch body weight of broiler chicks (Li et al., 2021), and the reason for this occurrence may be related to factors such as the type and size of embryonic eggs and the age of the breeder hens. Overall, it is demonstrated that light stimulation can promote muscle fiber development and muscle growth, but it may cause changes in muscle composition and impair muscle quality when broiler growth rate is too fast (Duclos et al., 2007). Continuous green light stimulation during incubation did not have any negative effect on muscle composition and meat quality of broilers at market age (Zhang et al., 2012).

2.4.3 Oxygen and Carbon Dioxide on Muscle Development

Generally, the concentration of oxygen in the incubator is maintained at 21% (De Smit et al., 2006), but the concentration of carbon dioxide in the incubator should be maintained within the range of approximately 0.1–0.5% (Buys et al., 1998). The process of hypoxia is a normal part of fetal life in all vertebrates, when the embryo is exposed to hypoxic stress, blood vessels in the tissues begin to grow and promote vascular development to meet the demand for oxygen to the embryo (Druyan and Levi, 2012). Mild hypoxia leads to a decrease in oxygen consumption in chicken embryos and affects hatching weight. Prolonged hypoxia can affect embryonic viability (Szdzuy et al., 2008) and can self-regulate through functions such as inhibition of tissue growth, and this adverse effect may manifest itself at later stages of hatching or growth, affecting embryonic growth and development, especially at the beginning and end of hatching (Decuypere et al., 2002; Lundy, 1969).

Subjecting chicken embryos to low oxygen conditions with 14% oxygen content from E0 to E10 reduced embryonic and hatching body weights (Miller et al., 2002). Incubation of embryonic eggs at high altitude conditions reduced broiler body weight on 14D and inhibited pre-growth, but did not affect final body weight (Hassanzadeh M, 2004). However, incubation of Dwarf chickens at an altitude of 2,900 m significantly reduced hatchability and shell weight. Increasing oxygen concentration to 27.5% during mid and late incubation phase improved this situation and increased broiler hatching weight (Zhang et al., 2008). In contrast, exposing embryos to a low oxygen (17%) environment at E5–E12 increased 7D broiler body weight, a positive effect that lasted until 28D, and increased breast muscle production (Druyan et al., 2018). This occurred probably because the low-oxygen environment stimulated the embryonic vasculature to develop in a good

direction and to better deliver nutrients to the pectoral muscle, (Hadad et al., 2014), thus promoting posthatch muscle development.

Carbon dioxide is an important gas during embryo development and bird egg incubation, and developing embryos require different levels of carbon dioxide at specific developmental stages and is an essential factor affecting embryo development (Mortola, 2009). Placing well-developed embryos in a CO_2 incubator at a concentration of 4% at E10–E18 reduced embryo weight, but had no effect on chick weight on the day of fledging or day-old chicks (Everaert et al., 2007). However, placing fertilized duck eggs in an incubator with 1% CO_2 content 10 days before hatching increased T3, T4 and corticosterone levels and body weight in plasma of Pekin ducks at embryonic stage and on the day of hatching, with positive effects on body weight lasting until market age (El-Hanoun et al., 2019). Thyroid hormones play an important regulatory role in maintaining chicken embryo development and normal development and can promote embryonic development (King et al., 1984). It was reported that 1% concentration of CO_2 may affect embryonic muscle fiber development and posthatch muscle growth by raising blood levels that may stimulate plasma corticosterone and T3, leading to the onset of hatching (De Smit et al., 2006), thereby increasing Pekin duck embryo and market-day weight.

2.4.4 Humidity on Muscle Development

Water release from the embryo to the external environment in the form of water vapor due to the electrical conductivity of the eggshell surface and the presence of stomata is inevitable and necessary throughout the incubation phase (Christensen et al., 2005). However, excessive water loss from embryonic eggs during incubation affects embryo development (Rahn and Ar, 1974), and improving this problem by changing the incubator humidity can positively affect embryo development and increase chick weight (Peebles and Brake, 1987; Swann and Brake, 1990). The optimal incubation humidity range has been reported to be quite wide, between 40 and 70% humidity (Lundy, 1969). Incubation of embryonated eggs at 53% or 63% humidity increased the hatching weight of broiler chicks compared to the low humidity group (43% humidity) (Bruzual et al., 2000). During incubation, the optimal weight of Pekin ducks on the day of hatching and at 21D was achieved only at 60, 65 and 70% of incubation humidity with increasing parental age (El-Hanoun et al., 2012). The incubation humidity of 63% increased the crude protein and crude fat content in the embryos while increasing the weight of the embryos at the later stages of hatching (Peebles et al., 2001). The increase in crude protein content in chicken embryos may be related to the acceleration of embryonic muscle development induced by the increased humidity. Although an increase in embryonic humidity can increase crude protein and crude fat content in chicken embryos, resulting in changes in the nutritional composition of chicken embryos, it is unclear whether changes in embryonic humidity can affect the nutritional composition of muscle in broiler chickens at market age and thus improve meat quality.

2.5 Other Factors on Muscle Development

2.5.1 Holding Time of Fertile Egg

Holding time of fertile egg before hatching is also an important factor to affect embryonic development (Pokhrel et al., 2018). Embryonic eggs can be maintained at a specific embryonic stage after leaving the mother, when the embryo temporarily stops significant metabolic activity or development (Fasenko, 2007), but embryonic eggs can only be preserved for a certain developmental period (Pokhrel et al., 2021). Pearl chick embryos stored for 5 days improved their hatching day and final body weight after hatching compared to the counterparts stored for 10 days (Kouame et al., 2021). Compared to 12 days of storage for embryonic egg, 5 days of storage increased the hatching weight of broilers, and this weight promotion effect lasted until 42D (Damaziak et al., 2018). Similarly, embryos stored for 4 days had higher body weights after hatching compared to embryos stored for 21 days (Dymond et al., 2013). Another study showed that the embryonic development of fertilized eggs stored for 14 d was found to be delayed by about 12.2 h compared to embryonic eggs that were not stored (Mather and Laughlin, 1977). Long-term storage of embryonic eggs can cause embryonic stress, resulting in embryonic necrosis and reduced cell numbers (Arora and Kosin., 1968; Bloom et al., 1998), which can cause serious damage to the embryo, as well as altering the albumin pH in the embryonic eggs (Karoui et al., 2006), affecting the recovery of the embryonic eggs and reducing the hatchability and chick quality (Hamidu et al., 2011; Akhlaghi et al., 2013). Therefore, with the increase of embryonic egg storage time, there is a developmental lag in the embryo during incubation, which makes the embryo development slower and thus affects the muscle fiber development, which is not conducive to muscle growth after hatching.

2.5.2 Short Periods of Incubation

After the fertilized egg leaves the mother, the development of the blastodermal cells is influenced by environmental factors, and embryos will sometimes stop developing when they are at 20.5°C (Edwards, 1902). Embryonic development is also affected when embryonic eggs are stored at constant temperature conditions (Özlü et al., 2018). Pre-incubation, temporary heating of the eggs before hatching, a method thought to mimic the natural conditions provided by birds for eggs before they start to hatch (Meijer and Siemers, 1993), allows embryos to form hypocotyls before hatching, reduces the negative effects of storage time and improves hatchability (Fasenko et al., 2001a; Fasenko et al., 2001b). It was found that embryonated eggs heated four times at 37.5°C at four-day intervals during 21 days of storage increased the body weight of broiler chicks before and after hatching compared to single heating for 6 h or 12 h during storage (Dymond et al., 2013). Embryonated eggs heated for 4 h at 30°C under 12 days of storage increased the body weight of broiler chicks at 21 D, and this positive effect on body weight could be sustained up to market age (Damaziak et al., 2018).

2.5.3 Egg Turning

In artificial incubation of domestic poultry eggs, the angle of egg turning is also crucial to achieve high hatching performance

(Deeming, 2009). Turning prevents inappropriate adhesion of the embryo to the inner shell membrane or of the allantoic sac to the yolk sac early in embryonic development (Cutchin et al., 2009). Stimulating vasodilation of the yolk sac membrane improves blood circulation and accelerates nutrient uptake into the yolk, thus promoting embryonic development (Deeming, 1989a,b). During egg incubation, a typical flip angle set at 45 degrees per hour can better promote embryonic development (Wilson, 1991). During gosling egg incubation, adjusting the rotation angle of the incubator to 70 degrees upregulated *GHRH*, *GH*, and *IGF-1* mRNA expression in E29 gosling embryos and also promoted *Pax7*, *MyoD*, and *MYF5* mRNA expression in the leg muscles, increasing embryo and leg muscle weight of E29 and increasing the cross-sectional area of leg muscle myofibrils and increasing at hatching weight. Therefore, increasing the rotation angle of the incubator was able to increase the number of myogenic cells and satellite cell number activity in muscles in the late incubation period, beneficial to embryonic muscle development.

2.5.4 Electromagnetic Fields

The increasing number of various types of radiofrequency electromagnetic fields used for wireless communication in the living environment requires a critical assessment of their potential health effects, and the chicken embryo has traditionally been considered an ideal model for studying the effects of environmental factors. Electromagnetic stimulation providing various parameters during incubation has no effect on chicken morphology. There is no effect on tissue or gene expression (Woelders et al., 2017). However, another study showed that microwave frequency electromagnetic fields produced stress on embryonic cells and increased Hsp70 protein levels without causing a significant temperature increase (Shallom et al., 2002). However, it is not clear whether electromagnetic fields have an effect on embryonic muscle fiber development and posthatch meat quality.

3 FUTURE DIRECTIONS AND PERSPECTIVES

Despite extensive research on myofiber development in avian embryos, it is unclear how environmental factors during incubation regulate myofiber development. Myofiber development is closely related to the myoblast dynamics including proliferation, differentiation and apoptosis. The role of incubation environment on the myoblast dynamics in developing embryos and early chicklings is worthy to be explored (Rozenboim et al., 2003; Zhang et al., 2012). The persisting or lasting effects of hatching environmental factors on muscle fibers transformation and meat quality on market age chickens also warrants further research. Furthermore, the synergistic or combinative effects of different incubation environmental factors on muscle development and meat quality are needed to investigated, which will lay the foundation for embryonic environmental regulation of muscle development and meat quality from theory to production.

It is worth noting that the modulation of incubation temperature has good industrial prospects on the alleviation of muscle disease in birds, as evidenced by the reduction of adverse effects of heat stress on meat quality. Muscle is high quality protein source, that is, popular with consumers worldwide, including some tropical regions. However, broilers kept under high temperature conditions may suffer from heat stress and have delayed muscle growth and adverse meat quality (Song and King, 2015; Gonzalez and Jackson, 2020). Compared with the normal incubation temperature, increasing the incubation temperature (38.8–39.5°C) could make broilers to be heat tolerant after shelling, thereby reducing susceptibility and severity to common myopathy and subsequently, improving the meat quality (Janisch et al., 2015; Morita et al., 2016; Clark et al., 2017). It is of value that an appropriate increase in incubation temperature could be applied in the poultry industrial hatching process and used to mitigate the negativity associated with high temperatures during muscle growth and development.

The cause of woody breast, white striping and spaghetti meat may be related to imbalance between the fast breast muscle growth rate and lagging cardiovascular systems development (Kawasaki et al., 2018; Zaboli et al., 2019). As hypertrophic breast muscle led to a reduced capillary density, this vascular hypoplasia may result in an impaired oxygen supply and metabolic waste product displacement from breast myofibers (Sihvo et al., 2018). This condition may exacerbate the occurrence of venous inflammation and become a major cause of muscle abnormalities (Papah et al., 2017). Although embryos in a hypoxic environment have faster cardiovascular systems development and better nutrient delivery to the pectoral muscle, whether moderate hypoxia can improve woody meat, white striping and spaghetti of pectoral muscle by promoting vascular development to the chest is unclear and needs to be further investigated.

4 CONCLUSION

Storage of fertile egg before incubation and incubation conditions affect embryonic muscle development and meat quality in poultry. During storage, embryos development can be stalled, and prolonged storage can lead to changes in the physical and chemical properties of embryonic components, affecting embryonic activity and muscle fiber development, which is detrimental to muscle growth and final meat quality, but short phase of incubation during egg storage can improve the ability of embryos to resume development and reduce the harmful effects of storage time on embryo development (Dymond et al., 2013). During incubation, changes in light, temperature and other condition parameters may alter myofiber development by regulation of hormones and growth factors as well myogenic regulatory factors, which may contribute to the improvement of final meat quality. Although, the precise actions of incubation environmental factors on myofiber development during incubation is still unclear, the muscle fiber metabolism and transformation is warranted for further research.

Nevertheless, the manipulation of environmental factors during the incubation phase is convenient and low-cost, which may avoid the stress of various regulatory approaches on birds after emergence, and is in line with the requirements and development of animal welfare. We have reasons to believe that the regulation of incubation conditions may benefit the poultry industry by stimulation of muscle fiber development and improvement of meat quality. Therefore, as more accurate and reliable studies become available, the role of embryonic environmental changes in regulating muscle fiber development and meat quality will play an increasingly important role in the meat poultry production industry.

AUTHOR CONTRIBUTIONS

G-HQ, H-JZ, and Y-HW conceived and designed the article. Y-HW and JL conducted the data search and wrote the manuscript. G-HQ and H-JZ assisted with the framework analysis and paper writing. JW, S-GW, and KQ provided continuous guidance for the manuscript preparation. All authors read and approved the final manuscript. All authors did not have any conflict of interest. and approved it for publication.

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Post-Hatch Performance of Broilers Following Hypoxic Exposure During Incubation Under Suboptimal Environmental Temperature

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The modern broiler is selected to exploit its full genetic potential, to sustain a rapid growth rate, and to lower the feed conversion rate (FCR). Recently reported reductions in FCR have been associated with augmented tissue formation at the expense of physiological functions such as thermoregulation. In turn, modern broilers exhibit a relatively low capability to balance energy expenditure under suboptimal ambient temperature. Hypoxic conditions at late incubation stages play a role in reforming metabolic plasticity. This work examined the effect of exposure to 12-h hypoxia (12H; 17% O_2) for three consecutive days (from E16 through E18), or continuous hypoxia exposure for 48 h (48H), from E16 through E17, as compared to standard incubation (21% O_2) on post-hatch performance of broilers maintained under suboptimal ambient temperatures (cold, hot, and diurnal cyclic ambient temperature). 12H chicks kept under hot ambient temperature had significantly lower body temperature (Tb) as compared to the control chicks. On day 42, both 12H and 48H chicks grown in the cyclic temperature room had significantly lower Tbs than controls. In parallel, from week 4, onward, 12H chicks had a significantly lower FCR than controls, and the 48H chicks demonstrated a lower FCR from week 5 and on. 12H and 48H broilers maintained under diurnal cyclic ambient temperature, exhibited significantly greater relative breast muscle weight, and a similar pattern was found in hypoxic broilers raised under standard and hot ambient temperatures. Hypoxic manipulation affects and create an adaptive bias in allocating metabolic energy between maintenance and growth, thus resulting in improved broiler performance, thermoregulation, and rearing under suboptimal environmental temperature.

Keywords: embryonic development, hypoxia, incubation, broiler performance, thermoregulation, metabolism

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1 INTRODUCTION

Since the 1950s, commercial genetic selection programs have led to dramatic improvements in broiler production traits. Genetic selection for performance traits resulted in considerable enhancement in daily feed consumption, elevated metabolic rate (Tickle et al., 2018), and consequently elevated internal heat production. These, in turn, translated to rapid growth, high feed conversion efficiency, and higher meat production characteristics (Havenstein et al., 1994; Zuidhof et al., 2014). While highly desirable for efficient production traits, such developments

logically necessitate parallel increases in the size of the cardiovascular and respiratory systems, as well as enhancements in their functional efficiency (Druyan 2008). However, insufficient development of these critical systems has led to a relatively low capability to adequately maintain dynamic steady-state mechanisms in the body, that should balance energy expenditure and body water balance under suboptimal environmental conditions (Yahav, 2009). The phenomenon is highlighted by the limited broiler energy budget (Tickle et al., 2018), which provides minimal allowance for increasing resting metabolic rate (RMR) to support the energetic resources available for growth when thermoregulatory costs are increased. All this leads to insufficient maintenance of the dynamic steady-state of thermoregulation processes, resulting in the enhancement of body temperature (Tb) fluctuations.

Broilers have developed certain responses in order to cope with environmental stress. The direct responses stimulated by environmental conditions have been characterized as acclimation/acclimatization (Yahav et al., 1997; Horowitz, 1998). While, adaptive, maladaptive or neutral with regard to an individual's fitness (Ghalambor et al., 2007) ability of a phenotype to be modified by the environment (Bradshaw, 1965), is characterized as a phenotypic plasticity. Phenotypic plasticity may involve short-term, reversible changes within an individual or developmental plasticity, which involves irreversible changes that result from developmental processes (Piersma and Drent, 2003). More specifically, plasticity underlies changes in age-dependent susceptibility of an embryo or juvenile animal to environmental stressors, due to changes in the development of their physiological regulatory systems (Spicer and Burggren, 2003). Environmental changes during critical developmental time windows, e.g., structural and/or functional shaping of the control of physiological/neurological systems, can disrupt and alter the developmental trajectory. However, the same condition outside of the critical window has little or no effect, and, in some cases, may have a negative effect (Carroll, 2003; Spicer and Burggren, 2003).

Hypoxia during broiler embryo incubation has been found to trigger adaptation of the embryonic cardiovascular system to the altered environment, with elevations in blood parameters, such as hematocrit, hemoglobin (Haron et al., 2017), and heart rate (Tomi et al., 2019). The actual effects of hypoxia on embryo development depend on the critical period of exposure, hypoxia level, and duration of hypoxic exposure. Hypoxia during embryonic development was reported to affect growth and metabolism of embryos, contingent on the hypoxia regimen (Haron et al., 2022). Amaral-Silva et al. (2017) showed that 15% O_2 during the last third of the incubation led to lower hatchling body mass. Haron et al. (2022) reported that while exposure for 48 h to 17% O_2 during the plateau period was associated with lower body weight at hatch, embryos subjected to cycling exposure of 12 h–21/17% O_2 between E16 and E18, had similar body weights at hatch to those of chicks incubated under standard conditions. The intermediate cycling hypoxia protocol enables embryos to adequately adapt to the shortage of oxygen and compensate for the gap that developed after the first exposure window before hatching (Haron et al., 2017, 2022). Moderate hypoxic exposure during the plateau period seems to trigger metabolic adaptation. The combination of lower Tb at hatch and lower plasma thyroid hormone concentrations in hypoxic manipulate hatchlings suggest that metabolic plasticity (Haron et al., 2017, 2022) and/or decreased heat production (Piestun et al., 2008a; Collin et al., 2011) underlie the responses to hypoxic exposure.

This study evaluated the effects of exposure of broiler embryos to a moderate O_2 concentration (17%) during the plateau phase of embryonic development on, growth rate and feed conversion of broilers maintained under suboptimal environmental (ambient temperature) conditions up to the age of poultry marketing.

2 MATERIALS AND METHODS

2.1 Experimental Design

2.1.1 Egg Origin and Incubation

Cobb (500) strain broiler chicken eggs (n = 900) with an average weight of 62.0 ± 2.5 g, were obtained from a breeder flock of hens during their optimal period of egg production (35 weeks old). Eggs were individually numbered and weighed and then incubated in a 2,500-egg incubator (Danki ApS, Ikast, Denmark) under standard incubation conditions of 37.8°C and 56% relative humidity (RH), with turning once per hour (ended on E18). The incubator was located 31 m above sea level, with 20.9% O_2 in the air. At E16_0 after candling, fertile eggs were randomly assigned to one of three treatment groups (300 eggs per treatment):

1) O_2 concentration of 17% for 12 h per day (h/d) from E16 through E18 (designated as 12H). The eggs were exposed at three time point: E16_0 to E16_12, E17_0 to E17_12, and E18_0 to E18_12.
2) Continuous exposure to 17% O_2, from E16 through E17, a total of 48 h (designated as 48H).
3) Control—O_2 concentration of 21%.

Exposure to 17% O_2 was accomplished by transferring eggs from both hypoxia treatment groups to an incubator with 17% O_2 equipped with a Model 2BGA-SP-MA O_2 and CO_2 Control System (Emproco Ltd., Ashkelon, Israel) the incubation conditions of 37.8°C and 56% relative humidity (RH), with turning once per hour were kept. The O_2 sensor activated an electronically controlled pump that infused N_2 into the incubator to maintain the oxygen concentration at 17% ± 0.2%, while the CO_2 level was 0.03 ± 0.01, as previously described by (Druyan et al., 2012).

On E19, all eggs from all treatment groups were transferred to hatching trays. In all three incubation treatments hatching stood on 95%.

2.1.2 Rearing and Growing Period

At hatch, 80 male chicks from each incubation treatment were selected, individually tagged and weighed. Chicks from each group were divided into groups of 10, and raised together in battery cages until the age of 14 days. The birds were maintained

under the recommended temperature regime according to broiler management guide (https://www.cobb-vantress.com/assets/Cobb-Files/045bdc8f45/Broiler-Guide-2021-min.pdf). At the age of 14 days, 20 chicks from each treatment group, were subjected to one of four thermal condition, in one of four controlled-environment rooms):

1) Cold ambient temperature, starting from 27°C on day 14 with a reduction of 1°C a day to constant 16°C at day 23 onwards.
2) Standard ambient temperature (control), starting from 27°C on day 14 with a reduction of 0.5°C a day to a constant 24°C at day 19 onwards.
3) Hot ambient temperature, starting from 27°C on day 14 with an increase of 0.7°C a day to constant 32°C at day 21 onwards.
4) Diurnal cyclic ambient temperature, starting from 27°C on day 14, with an increase of 0.7°C a day to constant 32°C at day 21 onwards during daytime (12 h). During night time, from 27°C on day 14 with a reduction of 0.5°C a day to a constant 24°C at day 19 onwards. Leading to environmental temperature of 32°C daytime and 24°C nighttime from day 21 onwards.

In all four environmental conditions, RH was 55% and light: dark cycles of 20:4 h were implemented. Water and feed in mash form were available for *ad libitum* consumption, with diet designed to meet the breeder recommendation for broilers. The diet consisted of a "pre-starter" (d 0–10d), "starter" (11–21d), grower (21–28d), and finisher (28d to marketing 42d) feed, with respective contents of crude protein (%) and energy (cal/kg ME) of: 22 and 3,035, 21.5 and 3,100, 20 and 3,180, and 19 and 3,250.

The chicks were distributed in the rooms randomly, with one chick per cage. Each chick was weighed every week, and weekly food consumption was calculated for each individual chick; FCR (kg of feed consumed/kg of live body weight) was calculated for each chick in each treatment. At the end of the experiment, at the age of 42 day, the chickens were individually weighed, and the feed was removed 12 h before slaughter. Breast muscle, abdominal fat pad, heart, and liver were removed and weighed and their weights were calculated relative to their live body weight.

2.2 Measurements

2.2.1 Body Weight

Chicks were weighed on a weekly basis using a Sartorius Signum SIWADCP-V14 scale (capacity ± readability 35 kg ± 1 g). Weight gain was calculated as the difference between the current body weight of the chick and the body weight at the previous measurement.

2.2.2 Feed Intake

Feed was weighed on a weekly basis, using a Sartorius Signum SIWADCP-V14 scale. Feed consumption per chick was calculated by subtraction of out feeder weight at the end of the week from the in feeder weight at the beginning of the week in order to calculate chick's weekly feed consumption.

2.2.3 Feed Conversion Rate

FCR was calculated by dividing each chick's feed consumption by its weight gain during the period (per week or for the entire growth phase).

2.2.4 Body Temperature Measurements

Broiler Tb was measured at weekly intervals using a digital thermometer (Super Speed Digital Thermometer; Procare Measure Technology Co., San Chung City, Taipei, Taiwan) with ±0.1°C accuracy, that was inserted 1.5 cm into the cloaca. Temperature was measured for 10 chicks per treatment.

2.2.5 Organ Weight Following Slaughter

At 42 d of age, chickens were individually weighed and the feed was removed for 12 h prior to slaughter. Breast muscle, abdominal fat pad heart, and liver were removed and weighed, and their relative weights calculated based on live body weight.

2.3 Statistical Methods

Due to the high ascites mortality and morbidity, performance of broilers from all three incubation treatments kept under cold temperature was negatively affected, with less than 10 birds per incubation treatment. Data of broilers kept under cold temperature was amiss from the statistical analysis (only standard, hot, and cycling effects were tested).

Individual growth performance, feed consumption, FCR, and slaughter data were statistically processed using two-way ANOVA, according to the model:

$$Y = \mu + treatment + \text{temp} + \text{treatment Xtemp} + e$$

with treatment (Control, 12H and 48H) and ambient temperature (standard, hot, and cycling) as the main fixed effects, and their interaction (treatment × temperature).

No significant interactions were found (treatment × temperature) the data is given in **Supplementary Tables S1–S5**.

In order to study how hypoxic incubation advantageous broilers performance under each sub-optimal condition, individual growth performance, feed consumption, FCR, and slaughter data were statistically processed within each ambient temperature using one-way ANOVA, according to the model:

$$Y = \mu + treatment + e$$

with treatment (Control, 12H and 48H) as the main fixed effect.

Values that differed (at a level of $p \leq 0.05$) were considered statistically significant. In addition, the Tukey test was conducted to compare the averages of the treatment effect.

2.4 Ethics Approval

All the procedures in this study were carried out in accordance with the accepted ethical and welfare standards of the Israel Ethics Committee (IL-581/15).

TABLE 1 | Body weight (g) of broilers exposed to different hypoxia regimes during embryonic development and then raised under different environmental ambient temperatures (analyzed within each ambient temperature).

Age (d)	Ambient temperature								
	Standard—23°C			Hot—32°C			Diurnal cyclic—24°C–32°C		
	Con	12H	48H	Con	12H	48H	Con	12H	48H
0	44.0 ± 0.4	44.2 ± 0.4	44.5 ± 0.4	43.8 ± 0.4	44.2 ± 0.4	43.5 ± 0.4	44.3 ± 0.4	44.5 ± 0.4	44.4 ± 0.4
7	203.7 ± 2.8	200.5 ± 2.9	198.9 ± 3.0	197.6 ± 2.7	198.3 ± 2.8	199.0 ± 2.9	200.9 ± 2.7	199.3 ± 2.7	205.0 ± 2.8
14	554.0 ± 7.5	552.9 ± 7.5	550.4 ± 7.9	556.3 ± 7.3	556.8 ± 7.5	552.0 ± 7.7	556.4 ± 7.3	553.2 ± 7.3	551.2 ± 7.5
21	1039.6 ± 15.3	1038.7 ± 15.7	1044.1 ± 16.7	1050.0 ± 14.9	1046.1 ± 15.3	1030.7 ± 15.7	1089.3 ± 14.9	1075.0 ± 14.9	1061.8 ± 15.3
28	1753.8 ± 28.2	1749.5 ± 28.2	1726.3 ± 29.9	1505.3 ± 26.8	1567.2 ± 27.3	1556.9 ± 28.2	1743.5 ± 26.8	1742.1 ± 27.5	1743.1 ± 27.5
35	2609.1 ± 48.9	2535.6 ± 50.2	2547.7 ± 55.3	1961.72 ± 46.3	2037.9 ± 48.8	2038.3 ± 50.2	2383.8 ± 46.3	2474.4 ± 48.8	2455.7 ± 48.8
42	3283.9 ± 62.5	3285.0 ± 70.9	3355.0 ± 76.6	2221.1 ± 59.3	2318.8 ± 62.5	2364.5 ± 64.4	2833.5* ± 59.3	3049.3* ± 66.3	3009.9* ± 62.5

Means ± SE are presented. n = *20 for each incubation treatment group under each environmental condition.*
**On each day, Different letters indicate significant differences* ($p \leq 0.05$) *across incubation treatments within each ambient temperature.*

TABLE 2 | Weight gain (g/d) of broilers that were exposed to different hypoxia regimes during embryonic development and then raised under different environmental ambient temperatures (analyzed within each ambient temperature).

Age (d)	Ambient temperature								
	Standard—23°C			Hot—32°C			Diurnal cyclic—24°C–32°C		
	Con	12H	48H	Con	12H	48H	Con	12H	48H
7	22.8 ± 0.4	22.3 ± 0.4	22.1 ± 0.4	22.0 ± 0.4	22.0 ± 0.4	22.1 ± 0.4	22.4 ± 0.4	22.1 ± 0.4	22.9 ± 0.5
14	50.0 ± 0.8	50.4 ± 0.8	50.3 ± 0.9	51.2 ± 0.9	51.2 ± 0.9	51.0 ± 0.8	50.8 ± 0.8	50.6 ± 0.8	49.5 ± 0.8
21	69.4 ± 1.6	69.4 ± 1.6	70.5 ± 1.7	70.5 ± 1.9	69.9 ± 1.9	70.5 ± 1.9	76.1 ± 1.5	74.5 ± 1.5	72.9 ± 1.6
28	100.5 ± 3.1	101.5 ± 3.1	97.5 ± 3.3	65.0* ± 2.9	74.4* ± 3.0	72.6* ± 3.0	93.5 ± 2.9	95.7 ± 3.0	97.3 ± 3.0
35	112.2 ± 4.6	111.1 ± 4.7	113.3 ± 5.2	65.2 ± 4.4	67.8 ± 4.6	67.9 ± 4.7	91.5* ± 4.4	104.4* ± 4.6	103.3* ± 4.6
42	96.3 ± 4.7	99.7 ± 4.7	100.2 ± 5.7	37.0 ± 4.4	40.1 ± 4.7	46.6 ± 4.8	64.2* ± 4.4	83.5* ± 5.0	79.2* ± 4.7

Means ± SE are presented. n = *20 for each incubation treatment group under each environmental condition.*
**On each day, different letters indicate significant differences* ($p \leq 0.05$) *across incubation treatments within each ambient temperature.*

3 RESULTS

3.1 Mortality During the Rearing Period

Mortality during rearing did not exceed 5% in groups exposed to control, hot or diurnal cyclic thermal condition in all three incubation treatments. In contrast, under cold thermal conditions, mortality was significantly higher ($p\chi 2 \leq 0.001$) than in the other three groups, mainly due to manifestation of ascites syndrome, with 48%, 45%, and 39% mortality in the control, 12H and 48H chickens, respectively. Total percentage of ascites was 69%, 55%, and 50% in the control, 12H and 48H chickens, respectively. Due to the high ascites mortality and morbidity, data of broilers kept under cold temperature was amiss from the performances statistical analysis. No significant differences in mortality or ascites rates were observed across incubation treatments subgroups within each of the four thermal condition groups.

3.2 Body Weight and Growth During the Rearing Period

Table 1 shows the mean body weights and **Table 2** shows the mean daily growth rate per week (end of each week) of chickens from different incubation treatments during their rearing under standard, hot or diurnal cyclic ambient temperature (starting from 14 days of age).

The main effect on broiler body weights (**Supplementary Table S1**) and growth rate (**Supplementary Table S2**) was the thermal condition; from the age of 21d onward, there were significant differences in the two parameters between the rearing conditions groups. The weights of birds from all three incubation group were significantly heavier when reared under standard ambient temperature, while birds that were maintained under a hot ambient temperature had a significantly lower body weight than expected on day 42 (**Supplementary Table S1**). Significant effect of incubation condition on broilers body weight and growth was found at the last week of growth at day 42, with higher body weight and growth rate of the hypoxic incubated chicks as compare to control (**Supplementary Tables S1, S2**).

In general, body weights and growth of all broilers from the three incubation treatments within each rearing subgroups was similar, regardless of the ambient temperature conditions. The exception, was found at day 42 for broilers raised under Hot and Diurnal cyclic temperature condition. In both ambient conditions, chicks exposed to either the 12H or 48H schedule of hypoxia during incubation had higher body weights as compared to the control group 42d broilers. This difference in body weight was significant for

TABLE 3 | Body temperature (°C) of broilers exposed to different hypoxia regimes during embryonic development and then raised under different environmental ambient temperatures (analyzed within each ambient temperature).

Age (d)	Ambient temperature								
	Standard—23°C			Hot—32°C			Diurnal cyclic—24°C–32°C		
	Con	12H	48H	Con	12H	48H	Con	12H	48H
0	40.5 ± 0.1	40.5 ± 0.1	40.5 ± 0.1	40.6 ± 0.1	40.5 ± 0.1	40.5 ± 0.1	40.6 ± 0.1	40.4 ± 0.1	40.1 ± 0.1
7	41.4 ± 0.1	41.3 ± 0.1	41.3 ± 0.1	41.3 ± 0.1	41.2 ± 0.1	41.4 ± 0.1	41.4 ± 0.1	41.3 ± 0.1	41.3 ± 0.1
14	41.4 ± 0.1	41.3 ± 0.1	41.4 ± 0.1	41.4 ± 0.1	41.3 ± 0.1	41.3 ± 0.1	41.3 ± 0.1	41.2 ± 0.1	41.3 ± 0.1
21	41.3 ± 0.1	41.3 ± 0.1	41.2 ± 0.1	41.8* ± 0.1	41.6* ± 0.1	41.7* ± 0.1	41.5 ± 0.1	41.4 ± 0.1	41.4 ± 0.1
28	41.5 ± 0.1	41.4 ± 0.1	41.4 ± 0.1	42.4 ± 0.1	42.2 ± 0.1	42.2 ± 0.1	41.6 ± 0.1	41.5 ± 0.1	41.6 ± 0.1
35	41.5 ± 0.1	41.5 ± 0.1	41.5 ± 0.1	42.6 ± 0.1	42.4 ± 0.1	42.4 ± 0.1	41.8 ± 0.1	41.6 ± 0.1	41.7 ± 0.1
42	41.5 ± 0.1	41.4 ± 0.1	41.5 ± 0.1	42.5 ± 0.1	42.3 ± 0.1	42.4 ± 0.1	41.5* ± 0.1	41.3* ± 0.1	41.2* ± 0.01

Means ± SE are presented. n = *20 for each incubation treatment group under each environmental condition.*
**On each day, different letters indicate significant differences* (p ≤ *0.05) across incubation treatments within each ambient temperature.*

broiler kept under Diurnal cyclic temperature condition (3049.3 ± 62.6 and 3009.9 ± 59.1 vs. 2833.5 ± 56.0 g for the 12H, 48H and control birds, respectively) (**Table 1**). As with body weight, ambient temperature had the most significant effect on broiler growth (**Supplementary Table S2**). During the entire period, within all environmental conditions, weight gain of the broilers was similar, except for broilers maintained under diurnal cyclic temperature, where 35d and 42d broilers from both hypoxia treatment groups had significantly higher growth compared to the control group broilers (**Table 2**), similar but non-significant pattern was found under standard and hot ambient temperature.

3.3 Body Temperature During the Rearing Period

When all birds were raised under standard brooding conditions (hatch to 14 days), Tb of chickens from the three incubation treatment groups was similar. Exposure to different ambient temperatures thereafter, affected chicken Tb, with a marked increase in Tb among chickens maintained under hot environmental conditions (35°C) (**Supplementary Table S3**). From day 21 onward, significant effect was also found to incubation conditions, with standard broilers Tb higher to significantly higher from 12H to 48H broilers Tb. Within each of the environments, the pattern of difference in Tb between standard to hypoxic broilers was maintained (**Table 3**). While under standard ambient temperature, the differences between standard and hypoxic broiler Tb wasn't constant, under suboptimal ambient conditions, Tb of 12H and 48H chickens was lower throughout the growth period compared to control. This difference in Tb across incubation groups was significant between 21-day-old 12H and control broilers maintained under a hot ambient temperature (41.6°C ± 0.1°C, 41.7°C ± 0.1°C, and 41.8°C ± 0.1°C for the 12H, 48H, and control broilers, respectively). On Day 42 in the diurnal cyclic ambient temperature, both hypoxia-treated groups demonstrated significantly lower Tb compared the control (41.3 ± 0.1 and 41.2°C ± 0.1°C vs. 41.5°C ± 0.1°C for the 12H, 48H, and control embryos, respectively; **Table 3**).

3.4 Feed Intake and Feed Conversion Rate During Exposure to Different Ambient Temperatures

The different ambient temperatures had a significant effect on broiler feed consumption, with the highest consumption during the entire brooding period (14–42 days) measured under standard ambient temperature and the lowest under hot condition (**Supplementary Table S4**). Despite the absence of a significant difference in feed intake among incubation treatment groups grown post-hatch under different environments, there was a trend of lower feed intake among 12H broilers as compared to control broilers when reared under standard, or diurnal cyclic ambient temperatures (**Table 4**).

Succeeding the effect of ambient temperatures on food intake and growth, a significant effect was also found on FCR of the broilers (**Supplementary Table S5**).

From week 4 and onward, the slight difference in growth combined with the trend in feed intake of broilers from the different incubation treatments, resulted in significant lower FCR of 12H broiler as compare to control with 48H broilers rank in the middle (1.80 ± 0.02 vs. 1.81 ± 0.02 and 1.85 ± 0.02 for the 12H, 48H, and control broilers, respectively; **Supplementary Table S5**).

A comparison between incubation treatments within each ambient temperature found that while the difference in FCR was found between groups of broilers in all three ambient temperature, it was highly significant when the broilers were raised under diurnal cyclic ambient temperature.

Under diurnal cyclic ambient temperature, significant lower FCR (**Table 5**) was found for both hypoxic broilers groups as compared to the control from week 4 onward. The total FCR (14–42d) of the hypoxia-exposed broilers was significantly lower and more efficient compared to that of the control broilers (1.73 ± 0.03 vs. 1.78 ± 0.02 and 1.85 ± 0.02 for the 12H, 48H, and control broilers, respectively).

3.5 Organ Weight Following Slaughter

Slaughter took place on day 43, after 12 h without feeding. Environmental ambient temperature significantly affected broilers performance with a significant reduction in meat yield

TABLE 4 | Feed intake (kg per week) of broilers exposed to different hypoxia regimes during embryonic development and then raised under different environmental ambient temperatures (analyzed within each ambient temperature).

Age (wk)	Ambient temperature								
	Standard—23°C			Hot—32°C			Diurnal cyclic—24°C–32°C		
	Con	12H	48H	Con	12H	48H	Con	12H	48H
3 weeks	720 ± 16.6	723 ± 17.6	702 ± 18.1	687 ± 16.2	685 ± 17.1	678 ± 17.1	769 ± 16.2	765 ± 17.1	737 ± 17.1
4 weeks	1132 ± 24.3	1109 ± 27.5	1175 ± 27.5	941 ± 24.3	967 ± 24.3	1001 ± 24.3	1100* ±23.6	1038* ± 24.3	1078* ± 23.6
5 weeks	1444 ± 52.3	1418 ± 59.3	1463 ± 64.1	963 ± 49.6	994 ± 50.9	977 ± 52.3	1236 ± 50.9	1216 ± 53.8	1299 ± 50.9
6 weeks	1406 ± 43.1	1354 ± 48.8	1425 ± 52.7	829 ± 43.1	784 ± 45.7	811 ± 45.7	1256 ± 45.7	1277 ± 48.8	1295 ± 44.3
Total:3–6 weeks	4709 ± 94.6	4599 ± 111.3	4871 ± 121.0	3499 ± 100.3	3493 ± 107.3	3542 ± 100.3	4401 ± 100.3	4388 ± 111.3	4387 ± 100.3

Means ± SE are presented. n = *20 for each treatment incubation group under each environmental condition.*
**On each day, different letters indicate significant differences (p ≤ 0.05) across incubation treatments within each ambient temperature.*

TABLE 5 | Feed-conversion ratios (FCR) of broilers that were exposed to different hypoxia regimes during embryonic development and then raised under different environmental ambient temperatures (analyzed within each ambient temperature).

Age (week)	Ambient temperature								
	Standard—23°C			Hot—32°C			Diurnal cyclic—24°C–32°C		
	Con	12H	48H	Con	12H	48H	Con	12H	48H
3 weeks	1.49 ± 0.02	1.49 ± 0.02	1.45 ± 0.03	1.40 ± 0.02	1.38 ± 0.02	1.38 ± 0.02	1.45 ± 0.02	1.46 ± 0.02	1.43 ± 0.02
4 weeks	1.62 ± 0.04	1.55 ± 0.04	1.61 ± 0.05	1.96* ± 0.04	1.81* ± 0.05	1.98* ± 0.04	1.64* ± 0.04	1.55* ± 0.03	1.58* ± 0.03
5 weeks	1.70 ± 0.09	1.74 ± 0.10	1.70 ± 0.11	2.33 ± 0.08	2.27 ± 0.09	2.22 ± 0.09	1.97* ± 0.08	1.78* ± 0.04	1.84* ± 0.04
6 weeks	2.11 ± 0.20	1.94 ± 0.22	2.05 ± 0.24	3.54 ± 0.20	2.69 ± 0.21	2.62 ± 0.21	2.49* ± 0.08	2.10* ± 0.08	2.24* ± 0.07
Total: 3–6 weeks	1.73 ± 0.03	1.70 ± 0.03	1.72 ± 0.04	1.98 ± 0.03	1.96 ± 0.03	1.93 ± 0.03	1.86* ± 0.03	1.73* ± 0.03	1.78*,b ± 0.02

Means ± SE are presented. n = *20 for each treatment incubation group under each environmental condition.*
**On each day, different letters indicate significant differences (p ≤ 0.05) across incubation treatments within each ambient temperature.*

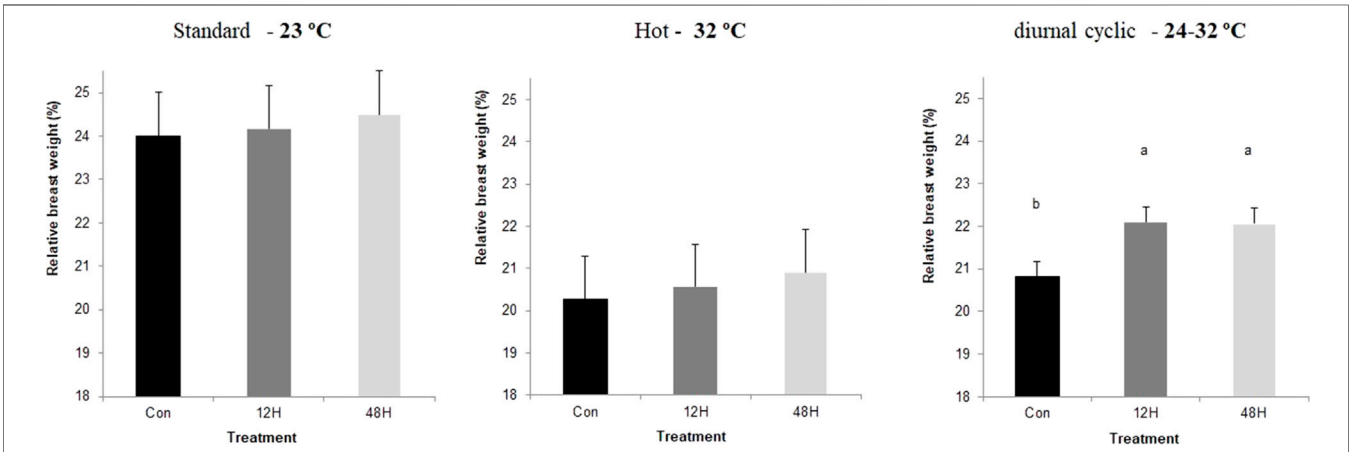

FIGURE 1 | Relative breast weight (%final body weight at 42 day), of broilers that were exposed to different hypoxia regimes (12H and 48H) during embryonic development and then raised under different environmental ambient temperatures (cold, standard, hot, and diurnal cyclic ambient temperature).

and breast relative weight, when broilers from all incubation treatments were kept under suboptimal ambient temperatures, with broilers kept under hot ambient temperature exhibiting the lowest relative breast weight (**Figure 1**). Despite the absence of a significant difference in relative breast weight among incubation treatment groups, there was a trend of a higher relative breast weight among broilers of both hypoxia-incubated groups later exposed to standard and hot ambient temperatures. This trend was significant in hypoxic incubated broilers maintained under diurnal cyclic ambient temperature. Under hot ambient temperature, relative abdominal fat pad was heavier, as expected, with broilers from both hypoxic incubation treatments exhibiting lower relative abdominal fat weight compared to control broilers. A similar trend was also found under standard and diurnal cyclic ambient temperatures as well (**Figure 2**). No difference in relative heart weight or relative liver

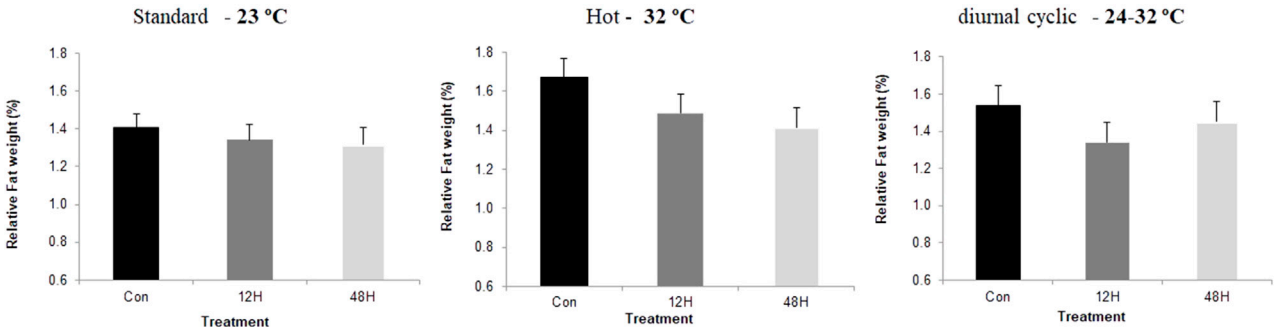

FIGURE 2 | Relative abdominal fat pad (%final body weight at 42 day), of broilers that were exposed to different hypoxia regimes (12H and 48H) during embryonic development and then raised under different environmental ambient temperatures (cold, standard, hot, and diurnal cyclic ambient temperature).

weight was found between broilers from all three-incubation group under the different ambient conditions.

4 DISCUSSION

Developmental changes induced by environmental conditions during incubation may impact post-hatch growth and metabolism (Shinder et al., 2007; Yahav and Brake, 2014). During chicken embryonic development, several critical windows associated with sensitivity to hypoxia (Druyan et al., 2012; Haron et al., 2017; Ben-Gigi et al., 2021), control of ventilation (Ferner and Mortola, 2009), thermal manipulation (Piestun et al., 2009a, 2011), and metabolic rate (Tazawa et al., 2004) have been defined. Hypoxia has been demonstrated an environmental incubation condition that can affect embryonic development, cardiovascular development, metabolism, O_2 demand, and the available energy for post-hatch growth and development (Haron et al., 2021). The latter is based on the assumption that environmental factors have a strong influence on determination of the "set-point" for physiological control systems during "critical developmental phases" (Dörner, 1974; Shinder et al., 2011).

Recent studies by Haron et al. (2017, 2021, 2022) demonstrated that hypoxic exposure during the plateau stage led to alterations in metabolism and cardiovascular system of the developing embryo, which resulted in more efficient energy utilization. These prior studies concluded that moderate hypoxic exposure from E16 to E18 elicited a long-lasting effect on the energy-balance axis, which suggested a metabolic plasticity and/or decreased heat production (Haron et al., 2022). The combination of lower Tb at hatch and lower plasma thyroid hormone concentrations in the 12H broilers suggested a long-lasting effect of hypoxia on post-hatching thermotolerance (Haron et al., 2022).

This work examined the effect of exposure to 12-h hypoxia (17% O_2) for three consecutive days (from E16 through E18) as compared to continuous hypoxia exposure for 48 h, from E16 to E17, on post-hatch performance of broilers maintained under suboptimal environmental temperatures (cold, hot, and diurnal cyclic ambient temperature). The technique of coping with sub-optimal conditions might emphasize advantageous traits of the hypoxic incubated broilers that are not evident under standard conditions.

While rearing the broiler under hot or diurnal cyclic ambient temperature caused redundant mortality, raising the broilers under cold ambient temperature led to ascites syndrome (Druyan, 2012). Total percentages of ascites were 69%, 55%, and 50% in the control, 12H and 48H chickens, respectively. Druyan et al. (2007, 2008) hypothesized that the tendency to develop the syndrome is associated with high growth rate, only because the latter increases oxygen demand in the genetically susceptible individuals above a threshold reflecting their lower inherent oxygen supply capacity. Druyan et al. (2012); Druyan and Levi, 2012) concluded that embryos adapted to hypoxic condition have an increased oxygen consumption capacity, which enables their growth, development, and maturation to proceed as well as those of control embryos. Although hypoxia exposure reduced the manifestation of ascites in the hypoxia treatment groups compared to control, the difference was not significant.

In this study, all chicks exhibited similar performance in the early stages of growth, regardless of their incubation treatments. The first significant difference between broilers from the three-incubation treatments was observed on day 21, 7 days after introduction to the new ambient temperature; 12H chicks had significantly lower Tb as compared to the control chicks raised in the hot room, similar pattern was also found under diurnal cyclic temperature. This difference in Tb signifying 12H broilers ability to maintain the Tb in the thermo-neutral zone, despite the hot environment. Amaral-Silva et al. (2017) reported that despite the fact that 10-day-old hypoxia-incubated chicks had higher oxygen consumption than controls, animals of both groups had a similar Tb. They suggested that hypoxic incubation may have also decreased heat conservation/increased heat loss, in comparison to control animals at this age.

Energy consumption, in general, and in the domestic fowl, in particular, is divided between maintenance and production. In endotherms, the ability to maintain Tb depends on daily energy requirement for maintenance. Therefore, lowering the demands for maintenance, while preserving an approximately

constant total energy consumption, allows for increased allocation of energy toward production. An alternative beneficial metabolic response may consist of a combination of reduced maintenance energy demands coupled with an overall reduction in energy consumption. Intermittent thermal manipulation during broiler embryo development and maturation of the thyroid axis has been shown to have a long-lasting effect on energy balance of broilers subjected to hot environments, improving thermo-tolerance and performance parameters (Piestun et al., 2011).

Without an interaction between incubation conditions and ambient temperature, it appear that hypoxic incubation had affected broilers metabolism in terms of internal heat production (lower body temperature), energy utilization and in terms of FCR. Raising the hypoxic manipulated broilers under sub-optimal environmental conditions helped emphasize the long lasting effect it had on metabolic plasticity. From all tested environmental conditions it appears that, raising the broilers under diurnal cyclic ambient temperature exposed the most the effect of hypoxic incubation on broilers performance, whereas when raised under other ambient temperatures the pattern was present but no significant differences were found between treatments.

On the last day of the growth trial (day 42), broilers were at their peak body weight and with maximum energy demand in the tissues. By this time point, both 12H and 48H chicks grown in the cyclic temperature room had significantly lower Tbs than controls. In parallel, from week 4, 1 week after introduction to the cyclic temperature, until week 6, 12H chicks had a significantly lower FCR than controls, and the 48H chicks demonstrated a lower FCR from week 5 and onward. Taken together, despite their lower Tbs and lower feed intake, hypoxia-exposed chicks maintained their growth rate, while the control chicks failed to maintain a low Tb, indicating higher energy expenditure on maintenance.

Another effect that can be related to reduce requirements for maintenance energy was the relative size of the breast muscle, which was used in this study as a central indicator organ for growth and development. When broilers from both hypoxic groups were maintained under diurnal cyclic ambient temperature, they exhibited significantly greater relative breast muscle weight, and a similar pattern was found in broilers raised under standard and hot ambient temperatures. This increase was associated with reduced abdominal fat, suggesting better energy channeling towards growth rather than accumulation of fat (Piestun et al., 2013b, 2013a). Similar results were reported by Druyan et al. (2018), who suggested that hypoxia exposure during incubation has an angiogenic effect on breast muscle, likely leading to improved nutrient delivery to the breast, and to subsequent utilization of for tissue growth rather than for fat accumulation.

5 CONCLUSION

Hypoxic conditions during the incubation period may improve post-hatch chick growth, metabolism, and health. Mild hypoxia exposure during chick embryogenesis causes alterations in metabolism and cardiovascular system, improving chorioallantoic membrane (CAM) and cardiovascular development, with a subsequent improvement in O_2-carrying capacity.

Once hypoxic conditions return to normal, development of exposed embryos not only progresses, but follows a unique developmental trajectory, demonstrating developmental plasticity that has an effect on post-hatch chick performance and enables improved adaptation to additional environmental stress, such as suboptimal environmental conditions. This outcome is of economic and practical relevance for the commercial broiler rearing phase, where a shift from the natural thermal zone to either the lower or upper critical level can compromise productivity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All the procedures in this study were carried out, reviewed and approved in accordance with the accepted ethical and welfare standards of the Israel Ethics Committee (IL-581/15).

AUTHOR CONTRIBUTIONS

AH and SD designed the research studies. AH, DS, MR, and SD carried out the experimental studies. SD and AH analyzed the data and performed statistical analysis. AH and SD wrote the paper. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.934676/full#supplementary-material

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Storage temperature dictates the ability of chicken embryos to successfully resume development by regulating expression of blastulation and gastrulation genes

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The avian embryo has a remarkable ability that allows it to suspend its development during blastulation for a long time at low temperatures, and to resume normal development when incubated. This ability is used by poultry hatcheries to store eggs prior to incubation. We have previously found that this ability correlates with the temperature during storage; embryos recover much better following prolonged storage at 12°C rather than at 18°C. However, the molecular and cellular mechanisms underlying these differences are poorly understood. To successfully resume development following storage, the embryo has to shift from the blastulation phase to gastrulation. Several genes are known to partake in the blastulation-to-gastrulation transition under normal conditions, such as the pluripotency-related genes *Inhibitor of DNA Binding 2 (ID2) and NANOG* that are expressed during blastulation, and the gastrulation-regulating genes *NODAL* and *Brachyury (TBXT)*. However, their expression and activity following storage is unknown. To elucidate the molecular mechanisms that initiate the ability to successfully transit from blastulation to gastrulation following storage, embryos were stored for 28 days at 12°C or 18°C, and were assessed either prior to incubation, 12, or 18 h of incubation at 37.8°C. Immediately following storage at 18°C group showed remarkable impaired morphology compared to the blastoderm of the 12°C group and of non-stored control embryos. Concurrently with these, expression of *ID2* and *NANOG* was maintained following storage at 12°C similar to the control group, but was significantly reduced upon storage at 18°C. Nevertheless, when the 18°C-stored embryos were incubated, the morphology and the reduced genes were reverted to resemble those of the 12°C group. At variance, key gastrulation genes, *NODAL* and its downstream effector *Brachyury* (*TBXT*), which were similarly expressed in the control and the 12°C group, were not restored in the 18°C embryos following incubation. Notably, ectopic administration of Activin rescued *NODAL* and *TBXT* expression in the 18°C group, indicating that these embryos maintain the

potential to initiate. Collectively, this study suggests a temperature-dependent mechanisms that direct the transition from blastulation to gastrulation. These mechanisms promote a successful developmental resumption following prolonged storage at low temperatures.

KEYWORDS

Egg storage conditions, Embryonic survival, Resumption phase, Prolong egg storage, Brachyury (TBX), Nanog, ID2

Introduction

Avian embryos undergoing blastulation have a unique ability to arrest their development and remain viable at low temperature for a long time. This ability allows to store eggs prior to incubation (Cai et al., 2019; Pokhrel et al., 2021b). Highly critical for embryonic survival, the storage temperature affects the ability of embryos to successfully resume development and hatch by the end of incubation (Fasenko, 2007). Extending storage duration, without affecting hatchability and chick quality may contribute to poultry hatcheries in planning eggs storage and conditions. Following prolonged time, of up to 28 days, embryos stored at 12°C have better chances to successfully resume development, than at 18°C (Pokhrel et al., 2018; Pokhrel et al., 2021b). Thus, the ability to recover from storage during the first hours of incubation is a manifestation of processes occurring during storage. However, the cellular and molecular mechanisms involved in embryonic recovery following storage are poorly understood.

To successfully resume development, the first steps of embryogenesis at the beginning of incubation are the completion of the blastulation process (Eyal-Giladi and Kochav, 1976; Kochav et al., 1980) and the transitioning to gastrulation, which is manifested by the formation of the Primitive Streak (PS), which occur in stages 2–3 H&H (Hamburger and Hamilton., 1951; Vasiev et al., 2010; Parfitt and Shen, 2014). Relying on cellular remodeling, these events require that the embryonic cytoarchitecture should be preserved during storage. Notably, embryos stored for 28 days at 12°C, maintain their cytoarchitectural structure, similarly to freshly laid embryos, around stage X EG&K (Eyal-Giladi and Kochav, 1976; Pokhrel et al., 2017; Pokhrel et al., 2018). This is facilitated by cell cycle arrest at the G2/M transition, which is regulated by the checkpoint kinase, WEE1 (Pokhrel et al., 2022). In contrast, embryos stored for 28 days at 18°C undergo substantiate cellular remodeling, including overall tissue thickening, formation of deep recesses at the dorsal aspect of the central disc (Area Pellucida, AP), and cells clustering at the ventral side of the AP. These maladaptive changes correlate with poor embryonic survivability and hatchability (Pokhrel et al., 2018).

Several signaling pathways are involved in the blastulation-to-gastrulation transition (Parfitt and Shen, 2014). For example, in blastulating embryos, the Bone Morphogenetic Protein 4 (*BMP4*) signaling pathway is active in the surrounding ring of the embryo, known as the Area Opaca (AO), as well as in the AP, and in cells polyingressing from the epiblast (Streit et al., 1998). In mouse embryonic stem cells, *BMP4* was found to regulate pluripotency by elevating the expression of its downstream effectors—Inhibitor of DNA Binding 2 (*ID2*), and the homeobox transcription factor *NANOG* (Hollnagel et al., 1999; Ying et al., 2003; Qi et al., 2004; Morikawa et al., 2016). Notably, mouse embryonic stem cells and chick blastoderm share similar gene regulatory networks in regulating pluripotency (Parfitt and Shen, 2014; Jean et al., 2015). *Chordin* and *Noggin*, two main BMP4 protein antagonists (Streit et al., 1998), are also expressed during blastulation and gastrulation at the posterior border of the AP (Koller's sickle region) and at the PS, respectively (Bertocchini and Stern 2008; Vasiev et al., 2010). Hence, *BMP4* activity becomes restricted upon gastrulation initiation (Morgani and Hadjantonakis, 2020).

Concomitant with reduced *BMP4* expression in the onset of gastrulation, *NANOG* expression is also downregulated in the PS (Lavial et al., 2007), whereas, the signaling protein NODAL–which regulates PS formation and mesodermal induction, becomes apparent along the PS (Zimmerman et al., 1996; Bertocchini and Stern, 2002; Umulis et al., 2009; Pereira et al., 2012). Highlighting its evolutionary conserved role in mesoderm induction, *NODAL* is also the earliest marker expressed in presumptive mesoderm in *Xenopus* (Agius et al., 2000). Recently, *NODAL* expression was shown to be induced by Activin, through the same TGFβ receptor subtypes as NODAL protein, in human gastruloids model (Pauklin and Vallier, 2015; Liu et al., 2022). Essential for PS formation and extension, in chick and mouse (Lawson et al., 2001; Yanagawa et al., 2011; Pauklin and Vallier, 2015), *NODAL* expression in the PS succeeds the expression of the mesodermal marker gene, the T-box transcription factor *Brachyury* (*TBXT*) (Agius et al., 2000; Brennan et al., 2001; Takenaga et al., 2007). Moreover, two central BMP4 protein inhibitors, *Noggin* and *Chordin*, become upregulated at the anterior part of the PS where they are necessary for neural lineage commitment (Streit et al., 1998; Bachiller et al., 2000). Collectively, these findings suggest that while *BMP4* and its downstream pluripotency gene targets *NANOG* and *ID2*, are expressed and active during blastulation, they are down-regulated upon the onset of gastrulation, whereas genes which regulate PS formation and lineage-commitment, such as *NODAL*, *TBXT*, *Noggin* and *Chordin* are becoming upregulated. Nevertheless, it is

unknown whether these molecular processes are affected by different storage conditions, which in turn, may impact the ability to successfully resume development.

This study aimed at investigating the initial molecular events of recovery in embryos exiting a prolonged storage at lower or higher temperature. Effects on blastoderm morphology and expression of key genes involved in pluripotency and differentiation were examined during the first hours of incubation at 37.8°C, referred hereinafter as the resumption phase.

Animals, materials and methods

Chick embryos

Gallus gallus domesticus freshly laid eggs from Ross (308) broiler breed were purchased from a commercial breeder. The flock age was 30–34 weeks. The eggs were laid in automatic nests, automatically collected, and delivered to the lab within 2.5–3 h from laying. The eggs were stored for 28 days in cooler incubator (VELP SCIENTIFICA, SN 265959, Italy) set at maintained temperature of either 12°C (28 days/12°C group) or 18°C (28days/18°C group) and in 70–80% relative humidity (RH). The temperature and RH were monitored using data logger (U-Sensor Plus, PN: 100475, United States). Freshly laid eggs were used as control, and were incubated upon delivery. Following storage, embryos were isolated as previously described (Pokhrel et al., 2018) either before incubation (fresh, 28 days/12°C, and 28 days/18°C groups), after 12 h of incubation at 37.8°C in 56% RH (fresh + 12 h, 28 days/12°C + 12 h, and 28 days/18°C + 12 h groups), or after 18 h of incubation (fresh +18 h, 28days/12°C + 18 h, and 28 days/18°C + 18 h groups). The incubator used in all experiments was Masalles model 65-I (Spain), equipped with data logger. Collectively, embryos isolated from the 9 treatment groups were collected for whole mount RNA *in situ* hybridization (WMISH) or for real time PCR.

For visualizing the morphological changes that occur during the resumption phase, 28 days/18°C stored eggs were windowed using fine scissors, and about 10 µl of 1% Fast Green dye was injected through the Vitelline membrane ventrally (underneath) to the embryo, using fine borosilicate glass tube, without rupturing the blastoderm. The blastoderm were visualized and pictured using dissecting microscope (Nikon, Model SMZ800, United States). For the time-course experiments, pictures were taken at time 0, and 3, 8, and 16 h of incubation later. During incubation periods, the windowed eggshells were sealed with parafilm.

Whole mount *in situ* hybridization

Embryos were fixated in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, Bioprep, Cat #PBSX10-1L, Israel) for overnight, as previously described (Weisinger et al., 2010, 2012; Kayam et al., 2013) and underwent WMISH analysis. At least 4 embryos per group were used for WMISH analysis. In brief, fixated embryos were washed three times with PBS and dehydrated in a series of 50%, 75%, 90% and 100% methanol (Biolab, Catalog No. 001368052100, Israel), each step for 20 min, at room temperature (RT). Embryos were stored in 100% methanol for at least 24 h at -20°C, and pooled to ensure the same hybridization conditions for all groups. The embryos were rehydrated in a series of methanol- 100%, 90%, 75%, 50% and washed twice with PBS- each step for 20 min at RT, and treated with deoxycholic acid (DOC) solution for 8 min for tissue permeabilization. DOC solution consists of 1% NP-40 (Merch, Cat No. 492016, Germany), 1% SDS (Hylabs, REF. BP716/500D, Israel), 0.5% DOC (Sigma, D-6750, United States), 0.5% Tris HCl (pH 8), 0.5% EDTA (pH 8), and 0.15 M NaCl. The embryos were washed twice with PBS containing 2% Tween20 (Amresco, CAS. 9,005–64–5, United States) (PBST), and fixated in 4% PFA for 20 min at 4°C. Embryos were washed twice with PBST (5 min each) and incubated at 70°C with pre-hybridization buffer for 2 h, which was replaced with pre-hybridization buffer containing 1–2 µg of dig-labeled RNA probe. RNA probes for *NANOG*, *ID2*, *TBXT*, *NODAL* and *Noggin* genes were prepared according to Roche Applied Science's protocol (Roche Applied Science, Germany), and their forward and reverse sequences are provided in Table 1. Samples were hybridized with dig-labeled RNA probes for overnight at 70°C. Following hybridization, the samples were washed with solution X (25% formamide, Sigma-Aldrich, Cat No. F9037, United States; 0.2% SSC, pH 4.5; 0.1% SDS; in DDW) for 30 min, 4 times at 68°C. For detection reaction, the embryos were equilibrated in MABT (1M Maleic acid buffer pH 7.5, Roche, REF. 33813900, Germany; containing 2% Tween] at RT for 10 min for 4 times, which was followed by 2 h blocking (10% Normal goat serum (NGS), 1% BBR, Roche, REF. 11096176001, Germany, in MABT). Anti-DIG antibody (1: 1,000 diluted in blocking solution, Roche, REF. 11093274910, Germany) was added, prior to an overnight incubation at 4°C. Washing of the Ab was done with MABT for 1 h, 3 times at RT, followed by equilibration with NTMT (0.1M NaCl; 0.1 M Tris-HCl, pH 9.5; 0.05 M $MgCl_2$; 2% Tween, in DDW) twice for 10 min at RT. Coloration reaction was done with NBP/BCIP mix (BCIP, 1:1,000 diluted in NTMT, Promega, REF. 28526902, United States, and NBP 1:1,000 diluted in NTMT, Promega, REF. 28116602, United States). Color development in samples was periodically monitored and the reaction was stopped by washing the samples in NTMT (X1 time for 5 min) and TBST (X3 times, each for 10 min). Stained embryos were re-fixated in 4% PFA overnight at 4°C, washed in PBS for 3 times, and imaged for microscopy analysis (Nikon, Model SMZ800, United States).

TABLE 1 List of primers used for making the template for the RNA probes.

Genes	Forward primer	Reverse primer	Melting temperature (°C)(forward)	Melting temperature (°C)(reverse)	Product size	References
NANOG Full name: Nanog homeobox Gene ID: 100272166 https://www.ncbi.nlm.nih.gov/gene/100272166	CAGCAGCAGACCTCTCCT TGAC	CCAAAGAAG CCCTCA TCCTCC	63.8	64.4	595	(GEISHA, Darnell et al. (2007); Lavial et al. (2007)
ID2 Full name: Inhibitor of DNA binding 2, HLH protein Gene ID: 395852 https://www.ncbi.nlm.nih.gov/gene/395852	CCTTTCGGAGCACAACCT	GAGCGCTTT GCTGTC ACTC	58.8	59.9	354	(Lorda-Diez et al., 2009)
NODAL Full name: Nodal growth differentiation factor Gene ID: 395205 https://www.ncbi.nlm.nih.gov/gene/395205	CGGCTGGGCAGTGTT CAAC	GCACCTGGC TGGGCTTGT AGAG	65.2	67.6	529	GEISHA (Darnell et al., 2007)
TBXT Full name: T-box transcription factor T Gene ID: 395782 https://www.ncbi.nlm.nih.gov/gene/395782	TTCATCGCTGTGACG GCGTA	AGGGAG GACCAATTG TCATG	66.1	59.8	432	Stuhlmiller and García-Castro, (2012)

cDNA preparation and real time-PCR analysis

Embryos were pooled at five biological repeats per group, and stored in RNA save solution (Biological industries, REF 018911A, Israel) for RNA extraction (as described in Pokhrel et al., 2022). Embryo tissues were homogenized in Bio-Tri RNA extraction solution (Bio-Lab, Catalog No. 009010233100, Israel) using a pestle (United States Scientific, REF 1415–5,390, United States) and a motor (Kimble, Catalog No. 7495400000, United States) and incubated for 5 min at RT. 1-Bromo-3-chloropropane (Sigma, B9673-200ML, United States) was added to the sample (at a ratio of 1:10 to the added volume of Bio-Tri RNA solution), vortexed vigorously, and the samples were incubated at RT for 10 min. Samples were then centrifuged (Eppendorf, SN 5409IM308416, Model 5427 R, Germany) at 4°C for 30 min at 14000 rpm. The aqueous upper layer was collected and equal volume of chilled iso-propanol (Gadot, CAS 67–63–0, Israel) was added and mixed. For mRNA precipitation the samples were stored overnight at -20°C, followed by centrifugation at 14000 rpm for 30 min at 4 °C. The RNA pellet was washed with 70% and 100% ethanol, dried at RT, resuspended in DEPC water (Biological Industries, 01-852-1A, Israel), and concentration was measured using Nanodrop (ThermoFisher Scientific, Model NanoDrop One C, United States). For cDNA synthesis, 1 µg of RNA was used according to the protocol of Promega cDNA synthesis kit (Promega, REF 017319, United States). For Real-time PCR 1 µl of cDNA was added to SYBR Green PCR Master Mix Kit (REF-4309155, Applied biosystems by Thermo Fisher Scientific, Inchinnan, United Kingdom) as previously described (Pokhrel et al., 2022), in the Applied biosystems Real-Time PCR Detection System (SN 2720011,007, Applied biosystems stepOnePlus Real-Time PCR System, Singapore). Samples were loaded in duplicates. Primers for the *NANOG*, *ID2*, *NODAL*, *TBXT* and *GAPDH* (as a normalizing gene) were designed for the qRT-PCR analysis according to sequence information from the NCBI database using Primer3 Input software (version V. 0.4.0) (Koressaar and Remm, 2007). Primer sequences are given in Table 2. The relative gene expression value was calculated using the $2^{-\Delta\Delta CT}$ method. First, the obtained Ct value of each gene in each sample was normalized by the Ct value of *GAPDH* of the respective samples. The *GAPDH*-normalized expression value of the *NANOG*, *ID2*, *GATA4*, *NODAL* and *TBXT* genes in the different samples was again normalized with the *GAPDH*-normalized expression value of respective target genes of the fresh control samples. The obtained value was converted using the $2^{-\Delta\Delta CT}$ formula. Thus, the obtained gene expression level is presented in fold change, which is relative to the control fresh embryos. Statistical analysis was done as described below.

In vivo embryo treatment

Heparin acrylic beads (Sigma, Catalog No. H5263, United States) were soaked with 2 µl of recombinant Human Activin protein (Pepro Tech, Catalog No.1201450UG, Israel; 100 ng/µl; prepared by adding sterile 0.1% bovine serum albumin solution, Biological Industries, REF 030101B, Israel) or with PBS as control for 2 h at 4°C in ice. Embryos stored at 18°C for 28 days were incubated at 37.8°C for 12 h. A small

TABLE 2 List of primers used for qRT-PCR.

Genes	Forward primer	Reverse primer	Melting temperature (°C)(forward)	Melting temperature (°C)(reverse)	Product size
GAPDH Full name: Glyceraldehyde-3-phosphate dehydrogenase Gene ID: 374193 https://www.ncbi.nlm.nih.gov/gene/374193	ACTGTCAAGGCT GAGAACGG	ACCTGCATC TGCCCATTTGA	60.4	63.9	98
NANOG Full name: Nanog homeobox Gene ID: 100272166 https://www.ncbi.nlm.nih.gov/gene/100272166	CTCTGGGGCTCA CCTACAAG	AGCCCTGGT GAAATGTAGGG	59.9	61.3	167
ID2 Full name: Inhibitor of DNA binding 2, HLH protein Gene ID: 395852 https://www.ncbi.nlm.nih.gov/gene/395852	CTGACCACGCTC AACACAG	TGCTGTCAC TCGCCATTAGT	59	59.5	82
NODAL Full name: Nodal growth differentiation factor Gene ID: 395205 https://www.ncbi.nlm.nih.gov/gene/395205	GTCCTGCTGCTC GTCTTCTC	CCTCTGCCTCTC CTTCCTG	60.3	60.1	150
TBXT Full name: T-box transcription factor T Gene ID: 395782 https://www.ncbi.nlm.nih.gov/gene/395782	AACTCCTCTGCC TGCCTTC	GTGCTGTTACTC ACGGACCA	59.5	59.8	155

window in the eggshell was opened through the air sac for *in vivo* transplantation of the Activin protein-soaked heparin beads. The beads were injected by a micropipette between the vitelline membrane and epiblast region. Following transplantation, the eggshell was sealed using leucoplast tape (BSN medical GmbH, REF 72668–02, Germany) and the embryos were reset for 6 h of incubation. Following incubation, the embryos were isolated, fixed overnight in 4% PFA and processed for RNA WMISH, as described above.

Statistical analysis

Fold change expression levels of gene in different groups from at least three experiments were log2 transformed for normalization and analyzed by one-way ANOVA statistical tool (Tukey's multiple comparison test). Data are presented as mean ± standard error of mean (SEM) that was calculated as the standard deviation divided by the square root of the count of samples. Statistical significance was determined at $p < 0.05$. Statistical calculations were done using Microsoft Excel and GraphPad Prism 6 software.

Results

Epiblast cells re-organize their morphology during the resumption phase

We have previously described the morphological changes occurring following prolong storage, notably the thickening of the embryonic tissue and formation of deep recesses in the 28 days/18°C stored embryo (Pokhrel et al., 2018, 2022). Thus, to analyze the blastoderm morphological changes during the resumption phase of the 28 days/18°C group, embryos were incubated and in a time-course experiment, were imaged following 0, 3, 8, and 16 h (Figures 1A–D). In agreement with our previous findings, we found that following storage, the blastoderm displayed overt formed recesses in the dorsal epiblast (Figure 1A, blue arrowheads). However, within the first hours of incubation, the embryos gradually rearranged their cytoarchitecture, leading to the sequential disappearance of the deep recesses until they were no longer visible by 16 h of incubation (Figures 1B–D, blue arrowheads).

Expression of the pluripotency-related genes during the resumption phase

To investigate the changes in expression of pluripotency-related genes during the resumption phase, embryos of the 28 days/12°C and 28 days/18°C groups were analyzed before incubation or after 12 h of incubation, and compared to freshly-laid embryos which served as control (Figures 2, 3). The spatio-temporal expression pattern and levels of the examined genes were analyzed using WMISH and qRT-PCR. The expression of two genes, which are expressed during blastulation was analyzed–*NODAL* and *ID2*, are the results that are presented in Figures 2, 3, respectively.

NANOG

Broadly expressed in early embryonic cells, the pluripotency-associated homeobox transcription factor, *NANOG* is downregulated during gastrulation with the onset of mesodermal lineage commitment. Accordingly, *NANOG* expression in the control group was found throughout the epiblast during

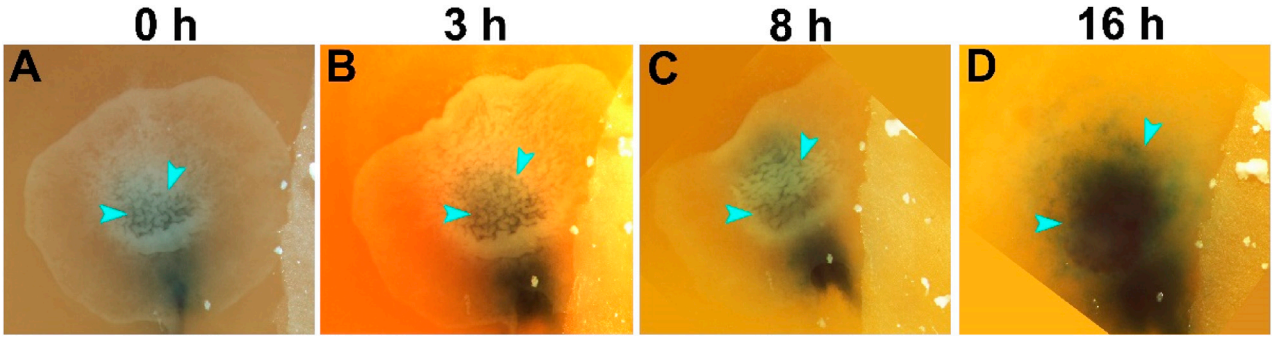

FIGURE 1
Morphological changes in embryos during the resumption phase. Embryos were stored for 28 days at 18°C (n = 22 embryos). The cellular changes during the resumption phase were demonstrated in a series of time-course images, at the beginning of incubation at 37.8°C, at time 0 **(A)**, and 3, 8, 16 h later (**B–D**, respectively). The dark color underneath the blastoderm is Fast Green dye, injected to highlight the contours of the embryos. While at the first hours of incubation after storage, the embryos are thicker with deep recesses in the epiblast (**A–C**, arrowheads), these recesses gradually disappear such that within 16 h of incubation, the recesses are no longer visible and the embryo becomes transparent (**D**, arrowheads).

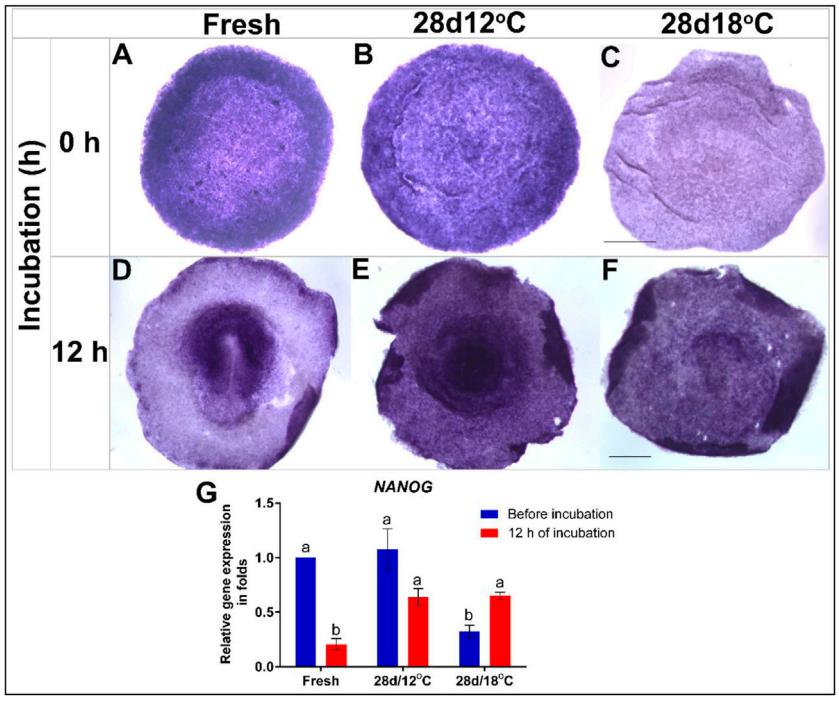

FIGURE 2
Expression of *NANOG* gene in embryos following storage, and following 12 h of incubation in the resumption phase. **(A–C)** WMISH analysis of *NANOG* expression in freshly-laid embryo (XI EG&K) or in embryos stored for 28 days at 12°C (XII EG&K), or 18°C (XIII EG&K). **(D–F)** WMISH analysis of *NANOG* expression following 12 h of incubation of the same embryonic groups. **(G)** Quantification of *NANOG* gene expression in 6 different embryo groups using RT-real-time PCR. *GAPDH* was used for normalization of *NANOG* gene expression. One-way ANOVA statistical analysis of 6 different embryo groups-different connecting letters denote that the expression of *NANOG* is significantly different between groups; F value = 16.51; Fresh, a vs. b: $p < 0.0001$; 28 days/18°C, a vs. b: $p = 0.0357$; Fresh (before incubation) vs. 28days/18°C (before incubation), a vs. b: $p = 0.0008$; 28 days/12°C (before incubation) vs. 28 days/18°C (before incubation), a vs. b: $p = 0.0007$; Fresh (after incubation) vs. 28 days/12°C (after incubation), a vs. b: $p = 0.0023$; Fresh (after incubation) vs. 28 days/18°C (after incubation), a vs. b: $p = 0.0018$). Bar = 1 mm.

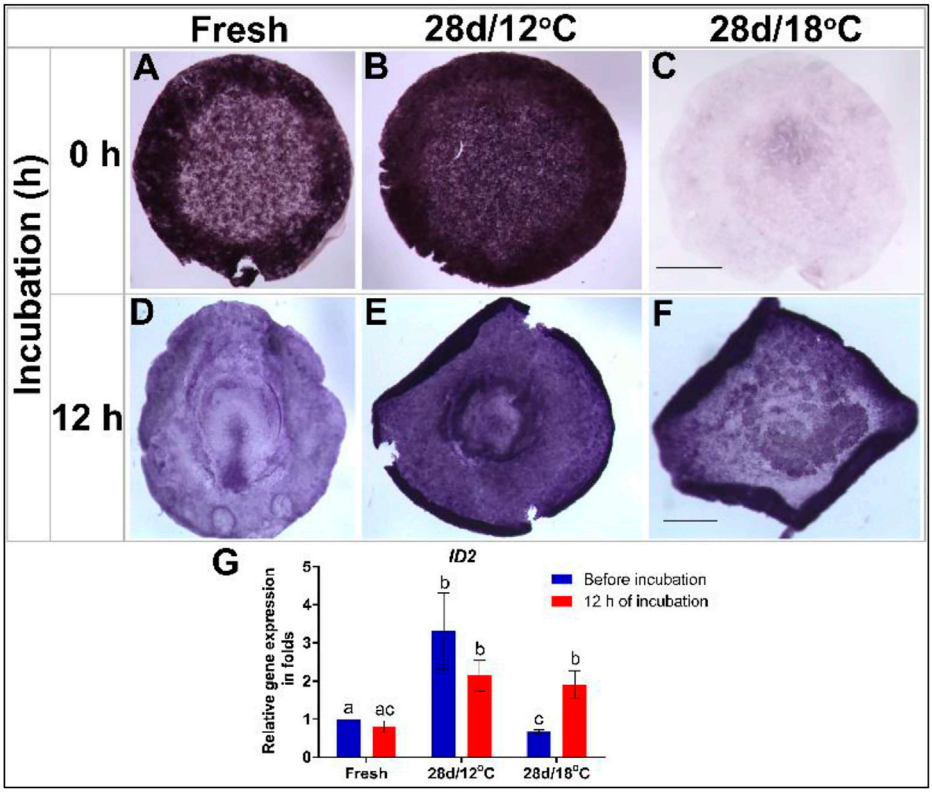

FIGURE 3
Expression of *ID2* gene in embryos following storage, and following 12 h of incubation in the resumption phase. **(A–C)** *ID2* gene expression in freshly laid embryo (X EG&K), embryo stored for 28 days at 12°C (XII EG&K), and embryo stored for 28 days at 18°C, respectively. **(D–F)** *ID2* gene expression during 12 h of resumption phase of embryos. **(G)** Quantification of *ID2* gene expression in 6 different embryo groups using real-time PCR. *GAPDH* was used for gene expression normalization and the determined *ID2* expression in the groups is represented as a fold of change relative to the fresh control. One-way ANOVA analysis of 6 different embryo groups-different connecting letters denote that the expression of *ID2* is significantly different between groups; F value = 11.83; Fresh (before incubation) vs. 28 days/12°C (before incubation), a vs. b: $p = 0.0061$; Fresh (before incubation) vs. 28 days/18°C (before incubation), a vs. c: $p = 0.0079$; 28 days/18°C, b vs. c: $p = 0.0032$; 28 days/12°C (before incubation) vs. 28 days/18°C (before incubation), b vs. c: $p = 0.0002$. Bar = 1 mm.

blastulation but as the embryos progress to gastrulation stage, and the PS has formed, *NANOG* expression decreased (Figures 2A,D,G). Similarity to control embryos, *NANOG* mRNA was also broadly expressed in the epiblast of the 28 days/12°C group following storage (Figures 2B,G), and following 12 h of incubation, *NANOG* expression level remained unchanged (Figures 2E,G). This is at variance from the control group which displayed lower *NANOG* expression at this stage (Figures 2D,G). This variation may possibly result from a developmental delay in the 28 days/12°C group following the prolong storage.

In contrast, *NANOG* expression was significantly reduced in the 28 days/18°C group at the end of storage (Figures 2C,G; fresh vs. 28 days/18°C, $p = 0.0008$; 28 days/12°C vs. 28 days/18°C, $p = 0.0007$). Interestingly, following 12 h of incubation of the 28 days/18°C group, *NANOG* expression got significantly upregulated (Figures 2F,G, $p = 0.03$), and became comparable with the 28 days/12°C groups (Figure 2G), suggesting that as far as *NANOG* expression, these embryos can regain their pluripotency state.

ID2

Essential for regulating the self-renewal characteristics and pluripotency of embryonic stem cells, the expression levels of the transcription factor *ID2* were similarly tested in embryos following 28 days of storage, before and after 12 h incubation and compared to freshly laid control embryos (Figure 3). Analysis of *ID2* expression pattern in non-incubated control embryos revealed its expression in the AO, AP, and polyingressing cells of the blastoderm (Figure 3A). Following incubation, *ID2* became restricted to the AP and PS (Figures 3D,G). A similar expression pattern of *ID2* was evident in different regions of the 28 days/12°C embryos prior to incubation (Figure 3B). However, *ID2* mRNA levels were significantly higher in this group compared to the control (Figures 3B,G, fresh vs. 28 days/12°C, $p = 0.0061$) and remained high after 12 h incubation (Figures 3E,G). At variance, a marked reduction in *ID2* expression was found throughout the blastoderm of embryos of the 28 days/18°C

TABLE 3 Summary results of expression of genes in embryos before and after incubation. Embryos were stored for 28 days at 18°C (28 days/18°C) or 12°C (28 days/18°C) and incubated at 37.8°C for 12 h for *NANOG* and *ID2*, and for 18 h for *NODAL* and *TBXT*. Freshly laid eggs, before and after incubation were used as controls. The embryos were isolated, fixed and stained by WMISH with *NANOG*, *ID2*, *NODAL*, and *Brachyury (TBXT)* probes. (–) indicates downregulated expression of the genes, whereas (+) indicates maintained or restored expression. The percentage of embryos in each groups and their count is given. Specific spatial expression in the Koller's sickle (KS), Area Opaca (AO), Area pellucida (AP), or the Primitive streak (PS), is noted.

Genes	Incubation	Fresh (control)	28 days/12°C	28 days/18°C
NANOG	Before incubation	+ (100%, n = 6)	+ (100%, n = 6)	– (100%, n = 6)
	After incubation (12 h)	+ (100%, PS, n = 10)	+ (100%, AP, AO, n = 6)	+ (100%, AP, AO, n = 9)
ID2	Before incubation	+ (100%, n = 6)	+ (100%, AP, AO, n = 6)	– (100%, n = 6)
	After incubation (12 h)	+ (100%, PS, n = 13)	+ (91%, AP; 9%, PS, n = 11)	+ (100%, AP, AO, n = 7)
NODAL	Before incubation	+ (100%, KS, n = 9)	+ (100%, KS, n = 6)	– (92%, n = 13)
	After incubation (18 h)	+ (100%, PS, n = 7)	+ (100%, PS, n = 6)	– (75%, n = 4)
TBXT (Brachyury)	Before incubation	– (100%, n = 7)	– (100%, n = 6)	– (100%, n = 6)
	After incubation (18 h)	+ (100%, PS, n = 12)	+ (100%, PS, n = 6)	– (83.3%, n = 12)

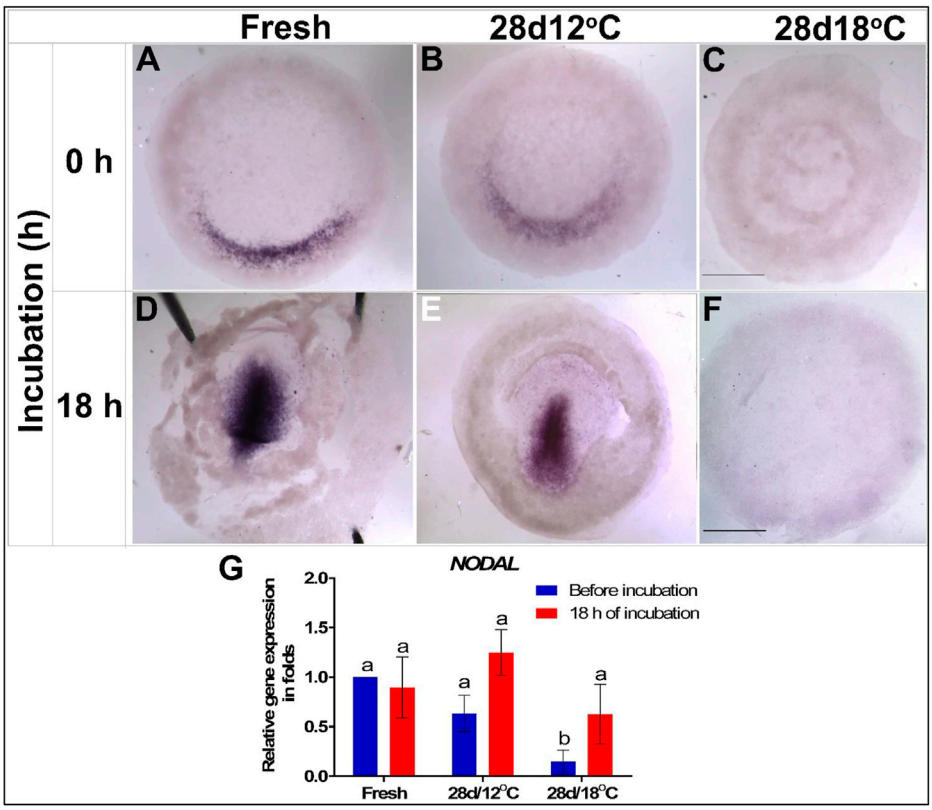

FIGURE 4
Expression of *NODAL* gene in embryos following storage, and following 18 h of incubation in the resumption phase. **(A–C)** *NODAL* gene expression in freshly laid (X EG&K), 28 days/12°C (XII EG&K), and 28 days/18°C (1 H&H) stored embryos, respectively. **(D–F)** Expression pattern of *NODAL* following 18 h of incubation, in freshly laid (3 H&H), 28 days/12°C (3 H&H), and 28 days/18°C (1 H&H) stored embryos, respectively. **(G)** Quantification of *NODAL* gene expression in 6 different embryo groups using real-time PCR. *GAPDH* was used for gene expression normalization and the determined *NODAL* expression in the groups is represented as a fold of change relative to the fresh control. One-way ANOVA analysis of 6 different embryo groups-different connecting letters denote that the expression of *NODAL* is significantly different between groups; F value = 7.913; Fresh (before incubation) vs. 28days/18°C (before incubation), a vs. b: p = 0.001; 28 days/12°C (before incubation) vs. 28 days/18°C (before incubation), a vs. b: p = 0.0098; 28 days/18°C (before incubation vs. after incubation), a vs. b: p = 0.025. Bar = 1 mm.

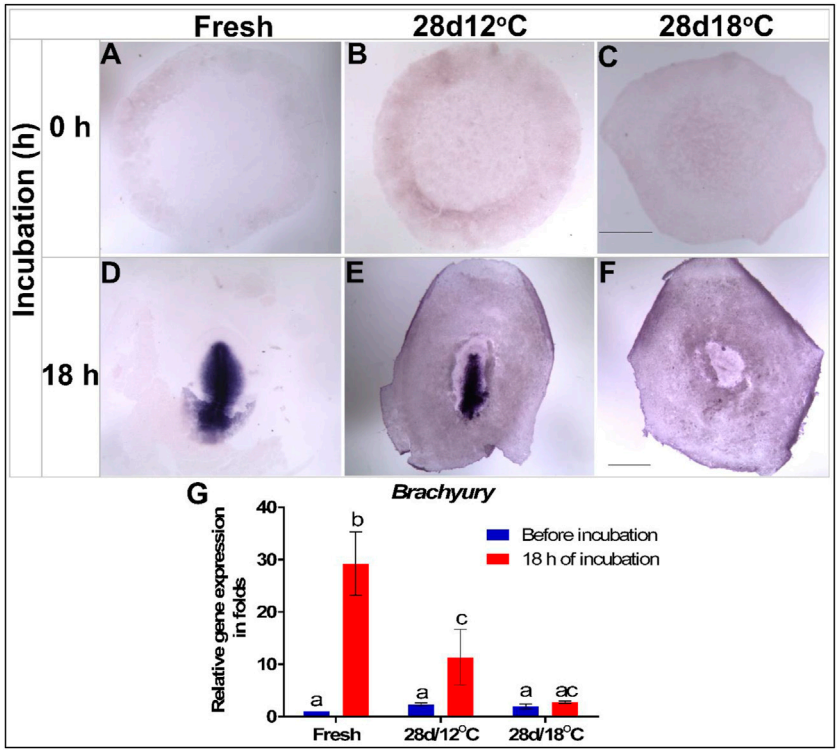

FIGURE 5
Expression of *Brachyury (TBXT)* gene in embryos following storage, and following 18 h of incubation in the resumption phase. **(A–C)** *TBXT* gene expression in freshly laid (X EG&K), 28 days/12°C (XI EG&K), and 28days/12°C (XIII EG&K) stored embryos, respectively. **(D–F)** Expression pattern of *TBXT* following 18 h of incubation, in freshly laid (3 H&H), 28 days/12°C (3 + H&H), and 28 days/12°C (1 H&H) stored embryos, respectively. **(G)** Quantification of *TBXT* gene expression in 6 different embryo groups using real-time PCR. *GAPDH* was used for gene expression normalization and the *TBXT* expression in the groups is represented as a fold of change relative to the fresh control. One-way ANOVA analysis of 6 different embryo groups; F value = 23.64; different connecting letters denote that the expression of *TBXT* is significantly different between groups; Fresh, a vs. b: $p < 0.0001$; 28 days/12°C, a vs. c: $p = 0.007$. Bar = 1 mm.

group prior to their incubation (Figures 3C,G; fresh vs. 28 days/18°C, $p = 0.0079$). Nevertheless, *ID2* expression was regained following 12 h of incubation (Figures 3F,G; 28 days/18°C before vs. after incubation, $p = 0.0032$) and became comparable to the 28 days/12°C group.

Collectively, these results demonstrate that expression of key pluripotent genes persist following storage at 12°C while gets down-regulated following storage at 18°C. However, at the resumption phase the affected embryos retain the ability to upregulate the expression of these genes, which may allow their normal initiation of development (Table 3).

Early mesodermal inducing genes are down-regulated at the resumption phase following prolonged storage at 18°C

To investigate the effects of storage on the expression of markers that regulate gastrulation and specification of the mesoderm lineage, we studied the expression pattern of *NODAL* and its downstream effector *TBXT*. In the chicken embryo, *NODAL* expression begins at the posterior side of the embryo, in a crescent-shape structure in the Kollers' sickle, and upon gastrulation, it gets upregulated within the PS (Chapman et al., 2007; Figures 4A,D, respectively). The expression pattern and levels of *NODAL* mRNA were tested in embryos of the 28 days/12°C and 28 days/18°C groups either before incubation or after 18 h of incubation, which correspond to gastrulation initiation (Figure 4). The results show that both, the non-stored control and the 28 days/12°C embryonic groups, express *NODAL* in the Kollers' sickle in a similar manner, prior to incubation, and following 18 h of incubation *NODAL* is expressed along the PS (Figures 4A,B,D,E). In contrast, in the 28 days/18°C group, *NODAL* expression was absent before and after incubation (Figures 4C,F). qRT-PCR quantification of *NODAL* expression levels confirmed its downregulation in 28 days/18°C embryos compared with fresh (Figure 4G; $p = 0.001$) and 28 days/12°C embryos ($p = 0.009$). However, following 18 h of incubation, expression of *NODAL* mRNA was marginally restored in 28 days/18°C embryos (Figure 4G), but not to an extent that

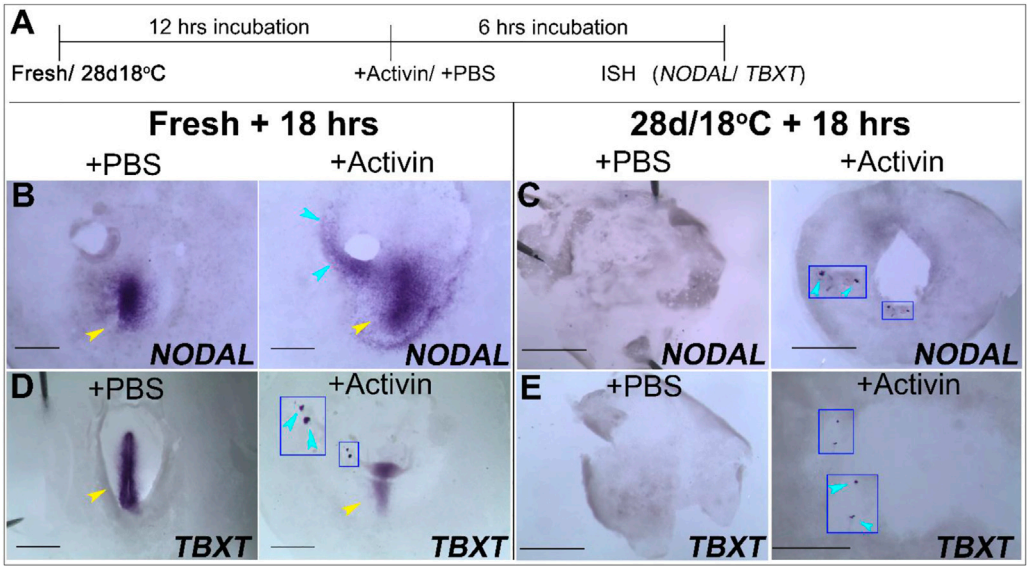

FIGURE 6
Ectopic application Activin induce the expression of *NODAL* and *TBXT* in fresh and 28 days/18°C embryonic groups. **(A)** Schematic illustration of the experimental design. Freshly laid embryos or 28 days/18°C stored embryos were incubated for 12 h at 37.8°C and then treated with PBS- or Activin-soaked bead for 6 h. Embryos were analyzed for *NODAL* and *TBXT* expression by WMISH. **(B,D)** WMISH analysis of *NODAL* and *TBXT* expression in fresh embryos following treatment with PBS or Activin protein. While the mock control fresh embryo treated with PBS-soaked beads expressed *NODAL* and *TBXT* gene in PS region only (yellow arrowhead, n = 4/4; 4/4, respectively), transplanting Activin-soaked beads in embryos, induced *NODAL* and *TBXT* expression near the transplanted beads (blue arrowhead, n = 4/4; 3/4, respectively, inset in **D** shows induced *TBXT* gene expression in high magnification view) as well as its normal expression in PS region (yellow arrowhead). (**C**,**E**) WMISH analysis of *NODAL* and *TBXT* expression in 28 days/18°C embryos following treatment with PBS or Activin protein. While in control embryos no *NODAL* and *TBXT* expression was induced (n = 4/4; 3/3, respectively), in Activin-treated embryos *NODAL* and *TBXT* expression were evident in the vicinity of the implanted beads (**C**,**E**, n = 5/5; 5/7, respectively; insets in **C**,**E** and blue arrowheads show induced *NODAL* and *TBXT* gene expression in high magnification view). Bar = 1 mm.

was noticeable by WMISH staining (Figure 4F). These result may suggest that 28 days/18°C embryos are impaired in their ability to induce *NODAL*, or are greatly delayed in development and therefore may induce *NODAL* expression at a later time point, during PS formation.

To further explore this possibility, the mRNA expression pattern and levels of the downstream effector of *NODAL–TBXT* (Pauklin and Vallier, 2015), was similarly tested (Figures 5A–G). The first expression of the *TBXT* in control embryos was noticeable following 18 h of incubation in the PS of the early gastrulating embryo (Figures 5D,E). As in the case of *NODAL*, *TBXT* expression in the 28 days/12°C embryos was comparable to that of the control group before and after incubation (Figures 5B,E). However, in accordance with the low expression of *NODAL* in the 28days/18°C embryos during the resumption phase (Figures 4F,G), WMISH showed that *TBXT* expression was also missing in this group (Figures 5C,F). Quantification of *TBXT* gene expression showed that the fresh and 28 days/12°C embryos significantly up regulated *TBXT* expression after 18 h of incubation (Figure 5G; fresh before vs. after incubation, $p < 0.0001$; 28 days/12°C before vs. after incubation, $p = 0.007$), whereas in 28 days/18°C embryos, the expression level of *TBXT* before and after incubation remained lower (Figure 5G; 28 days/18°C, $p = 0.9348$; Table 3).

Activin ectopic administration rescues *NODAL* and *TBXT* expression

The observation that the PS markers *NODAL* and *TBXT* are not upregulated in the 28 days/18°C stored embryos following 18 h of incubation at 37.8°C (Figures 4, 5; Table 3), correlates with their poor embryonic viability and hatchability (Pokhrel et al., 2018). In order to determine whether these embryos can restore the expression of these genes and enter gastrulation, a rescue experiment was designed using ectopic administration of Activin that induces *NODAL* expression. Embryos that were stored at 28 days/18°C and incubated for 12 h were implanted with acrylic heparin beads (Korn and Cramer, 2007; Mohammed and Sweetman, 2016) soaked with PBS or recombinant Activin, and re-incubated for additional 6 h, to allow them to progress into gastrulation (Figure 6A). Freshly laid embryos were also

incubated with PBS or Activin-soaked beads for the same duration to serve as mock controls and positive controls, respectively (Figures 6A,B,D). Embryos of all groups were then examined for *NODAL* and *TBXT* expression by WMISH (Figure 6A).

Our results show that exogenous application of Activin in control freshly laid embryos was able to induce ectopic expression of *NODAL* and *TBXT* in the PS region and near the grafted beads, in contrast to the PBS-added embryos (Figures 6B,D). Similarly, addition of Activin to the 28 days/18°C embryos resulted in upregulation of *NODAL* and *TBXT* in the vicinity of the transplanted beads, as opposed to their absence in the PBS-mock group (Figures 6C,E; see also Figures 4F, 5F). These results suggest that while prolonged storage at 18°C prevents the upregulation of gastrulation-related genes, the ability to enter gastrulation persists in the embryos upon receiving the instructive exogenous signals.

Discussion

The ability to suspend embryogenesis during blastulation allows the avian embryo to withstand prolong developmental arrest when kept at low temperatures, and resume normal development at appropriate incubation conditions thereafter (Brake et al., 1997; Reijrink et al., 2008). This arrest occurs at a critical developmental stage of the transition from blastulation to gastrulation, when the foundations for all the embryonic germ layers are set. The effects of prolong developmental arrest was previously studied in turkey breeding eggs, which were stored for up to 27 days, and the developmental stage following 8 days of incubation was evaluated. Embryos that were stored for 5 days or less were significantly more advanced developmentally than the embryos from eggs stored for longer (Bakst et al., 2016) highlighting the potential deleterious effects of storage conditions, and the importance of understanding the phenomenon for better hatcheries managerial decisions. We have previously shown the deleterious effects of prolonged storage at 18°C compared with 12°C, on embryonic survivability and hatchability. At 18°C, only about 17% of embryos which were stored at 18°C for 28 days were able to survive and hatch (Pokhrel et al., 2018). The surviving hatchlings highlight the robustness of some embryos to endure severe environmental conditions, however, the mechanisms which promote these abilities are poorly understood. This study investigated the cellular and molecular characteristics of embryos exiting storage and categorized the transition period from storage to exiting storage as the resumption phase. Broadly, the storage period can be divided into 2 phases, the first—the initiation phase, which begins following laying and up to 7 days, during which embryonic survival improves (Brake et al., 1997), and the second—the extension phase, which extends beyond 7 days of storage and may deteriorate survivability depending on environmental conditions. Notably, as the extension phase is highly sensitive to the temperature (Pokhrel et al., 2018), a successful resumption phase, which marks the transition between storage and the first hours of incubation, is critical for allowing successful developmental progression and hatching.

The effects of environmental conditions during storage were investigated for many years (Bakst and Akuffo, 2002), emphasizing that storage temperature and duration are the critical factors for later embryonic survivability (Reis et al., 1997; Gomez-de-Travecedo et al., 2014). These factors were shown to affect physical and biological properties of embryos within the eggs, and hatchability and chick quality post hatch (Brake et al., 1997; Reijrink et al., 2008; Hamidu et al., 2011; Özlü et al., 2021), indicating that changes in these properties perturb the ability to resume normal development. In agreement with these studies, our results suggest that embryos undergoing storage at lower or higher temperatures, evoke differently biological processes, which promote their ability to successfully resume development during the resumption phase. These include an adaptive cytoarchitectural changes and recovery of expression of genes associated with pluripotency, *NANOG* and *ID2* (Ying et al., 2003; Lavial et al., 2007; Pan and Thomson, 2007; Luo et al., 2012; Yu et al., 2020). Notably, BMP4 signaling was previously found to induce expression of *NANOG* and *ID2* in mouse embryonic stem cells and in chick blastoderm cells (Ying et al., 2003; Lavial et al., 2007; Pan and Thomson, 2007; Luo et al., 2012; Yu et al., 2020; Pokhrel et al., 2021a), raising the possibility that while BMP4 signaling is preserved during storage at 12°C, it is not maintained at 18°C, but can be reactivated after incubation of these embryos, leading to upregulation of *NANOG* and *ID2*. Notably, the expression of *NANOG* was broadly distributed in the AP region of the post-storage embryos, as opposed to previous studies which showed diminished expression of this gene, in the PS region (Lavial et al., 2007), suggesting a possible developmental delay in embryos following longer storage period at both storage temperatures. For instance, the developmental delay in the 28 days/18°C groups may be due to the recovery time required for embryos to rearrange the cytoarchitecture and restore the lost gene expression. However, activation of the BMP4 pathway as a recovery process might have accounted delay in formation of PS because downregulation of the BMP4 pathway is required for initiation of PS formation (Streit et al., 1998; Bertocchini and Stern, 2008; Vasiev et al., 2010; Supplementary Figure S1). Likewise, the delayed PS formation in the 28 days/12°C group could be due to a delay in the reactivation mechanisms associated for transition to gastrulation stages, perhaps due to the prolonged activation of the BMP4 pathway during storage. This delay in the initial developmental events during the resumption phase of prolong stored embryos at 12°C and 18°C may be responsible for the spread in the hatching time window from long-stored eggs, as observed previously (Tona et al., 2003). The changes in RNA

levels presented here require further validation in future studies at the protein level to fully confirm the involvement of the examined genes in the ability to resume development. However, the lack of available chicken-specific antibodies for these transcription factors prevent these assessments.

Interestingly, in contrast to the ability to restore the expression of *NANOG* and *ID2* after 12 h of resumption phase of the 28 days/18°C group, this did not hold for the initiation of PS formation and the restoration of mesoderm specification genes *NODAL* and *TBXT* even after 18 h of incubation. This finding varied from the ability of 28 days/12°C embryos to induce *NODAL* and *TBXT* expression in the PS region after the resumption phases, consistently with previous studies (Chapman et al., 2002; Mikawa et al., 2004). Notably, after incubation of some 28 days/12°C embryos, *NODAL* gene expression was restricted to the truncated region of PS, further highlighting that the onset of gastrulation is somewhat delayed in these embryos. Moreover, the finding that during the resumption phase of 28 days/18°C embryos, expression of some, but not all, examined genes can be restored suggests that the plasticity of these embryos to restore gene expression is limited to a few genes. Moreover, since the timing of *NODAL* gene expression in zebrafish embryos at stages from mid-blastula to mid-gastrula (van Boxtel et al., 2015) regulates the timing of mesendodermal induction and lineage commitment at later stages (Dougan et al., 2003; Weng and Stemple 2003; Fleming et al., 2013), the inability to restore these gastrulation-related signaling pathways following prolonged storage at 18°C may explain the broad embryonic mortality and the reduced hatching rate in these conditions (Pokhrel et al., 2018; 2021b). Finally, investigating the cellular and molecular dynamics of embryos during and after storage also revealed that embryos are viable during prolong storage at 18°C and restore development after incubation, however, they lack the molecular processes essential for differentiation and fail to develop upon incubation.

Notably, our findings regarding the association between storage temperature and the ability to induce gastrulation-related genes during the resumption phase is in agreement with other studies showing that modification of storage conditions can improve the successful resumption into development (Kgwatalala et al., 2013; Brady et al., 2022).

Hence, our data further highlight the interaction between developmental stage and the temperature during storage, and suggests that reactivation mechanisms are maintained during storage at low or high temperature to upregulate signaling pathways associated with pluripotency to facilitate successful resumption into development. Moreover, even the inability of the 18°C stored embryos to undergo gastrulation transition could be rescued by adding exogenous signals, further indicating that thermal manipulations can revert the negative effects of storage at high temperature on embryonic development.

The current study focused on previously characterizing genes which are known to be involved in maintaining pluripotency or differentiation in prolonged stored blastoderms. Other mechanisms are clearly involved in maintaining embryonic survivability. Recent studies have begun to uncover the molecular mechanisms that participate during storage by transcriptome profiling (Pokhrel et al., 2021a, 2022; Brady et al., 2022). These studies reported and highlighted the roles of cell-cycle, cellular death, metabolism, ubiquitination, and cytoskeleton-related mechanisms, which partake in embryonic survivability and enable embryos to successfully resume development. Along with these molecular mechanisms, the embryonic surrounding environment, namely the yolk and albumen properties, is known to be affected by storage condition, and affect embryonic survival. For instance longer periods of storage resulted in lower albumen weight and height, together with higher albumen pH, as opposed to freshly laid eggs (Scott and Silversidest, 2000; Silversides and Budgell, 2004). Identification of differences in additional molecular and physiological processes which relate to different storage conditions could be beneficial for the poultry industry to calibrate storage conditions and improve the levels of appropriate embryonic development and hatching.

Conclusion

Environment temperature plays a crucial role in avian embryonic development during storage and affects the ability of embryos to successfully resume development when incubated. Particularly, embryos that enter storage at lower temperatures, have higher chances to resume normal development than embryos which storage at higher temperatures. The overall decrease in metabolic activity at lower temperature may contribute to embryonic survivability, however, even at low storage temperature the embryo induces survival mechanisms and signaling pathways, which protect its morphology, cells viability, and pluripotency state, thereby allowing it to successfully resume normal development. Nevertheless, the molecular regulation that enables stored embryos at higher or lower temperatures, to resume development, is mostly unknown. In this study, we investigated how prolong storage at 12°C vs. 18°C affect the embryonic ability to resume development by examining their cellular and molecular dynamics following exit from storage. Our results show that embryos maintain the ability to express genes associated with pluripotency and differentiation during storage. These pathways are essential for the transition from blastulation to gastrulation during the successive development event at the time of exiting storage. Notably, embryos stored at lower or higher temperature display differences in expression of gastrulation-related genes, which could explain the divergence in resumption of development and thus the differences in embryonic survival at hatching following storage at different temperatures.

Collectively, this study suggests for the first time a temperature-dependent mechanism that enables the transition from blastulation-to-gastrulation stages in order to promote successful development following prolonged storage.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the animal study because this research involve chick embryos until 18 h of incubation. These embryonic stages are not regulated and require no approval.

Author contributions

NP-Experimental design, data acquisition and analysis, drafting the manuscript OG-Technical support DS-D—Experimental design and writing the manuscript YC-Experimental design and writing the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.960061/full#supplementary-material

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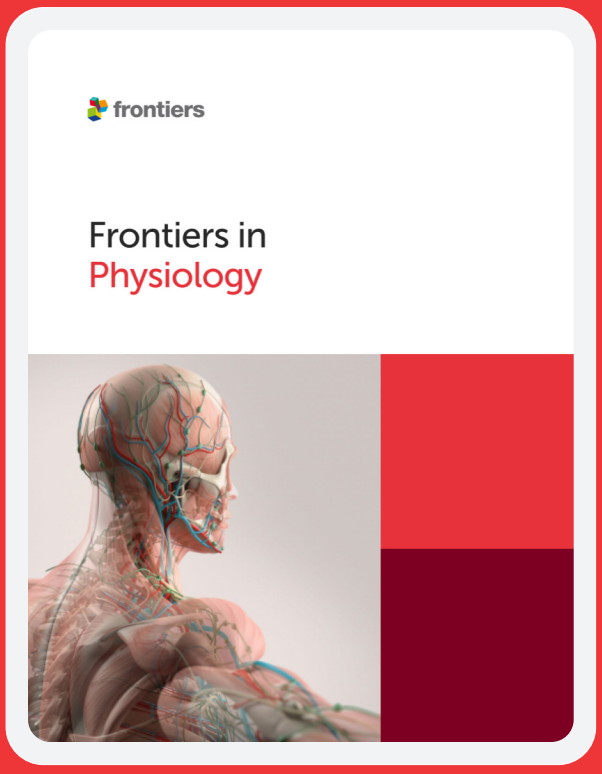

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