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# Effects of Layer Breeder Age and Early Hypoxic Stimulation (ED 7-9) of the Chorioallantois Membrane on Eggshell Decalcification, Neovascularization of Heart Tissue, Mineralization and Morphometrics of Hatchlings

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## ABSTRACT

Oxygen concentration ($O_2$) during incubation is crucial for embryo development, and hypoxic conditions can influence phenotypic plasticity in poultry. Although low $O_2$ (hypoxia) can be detrimental, it may also promote adaptive responses. Breeder age, a known genetic determinant of egg quality and embryonic development, is likely to interact with $O_2$ levels during incubation, however, this relationship remains understudied in layer breeders. This study examined how layer breeder age and reduced oxygen ($O_2$) levels during early embryonic development affect various factors, including eggshell decalcification (Dcal-SHL), chorioallantoic membrane (CAM) weight, heart tissue vascularization, egg weight loss (EWL), eggshell temperature (EST), and calcium (Ca) and phosphorus (P) content in bone and blood and tibia and femur morphometrics. A total of 900 eggs from 33 and 50-week-old ISA brown layer breeders were incubated in a 2x3 factorial design with $O_2$ levels of 15%, 17% (hypoxic), and 21% (control). Oxygen was reduced for 1hr/day from embryonic days (ED) 7-9 using air-$N_2$ flushing. Results showed increased CAM weight and heart tissue vascularization under hypoxia, especially in older breeders (50 weeks). Hypoxic conditions (15% and 17% $O_2$) reduced embryo weight loss and eggshell temperature compared to controls during the post-exposure phase (ED 15-18). There was an interaction between breeder age and $O_2$ levels on mineral absorption, with reduced oxygen leading to lower Ca and P absorption in bones, higher eggshell P retention, and decreased tibia morphometrics (weight, length, diameter, and seedor index) in hatchlings. Additionally, CAM weight correlated negatively with Dcal-SHL Ca at 15% $O_2$. The study concluded that reduced oxygen during early embryonic development increases CAM weight, heart neovascularization, and Ca mobilization from eggshell to blood. However, older flocks exhibited reduced Ca transfer to bones, likely due to homeostatic imbalance.

**Keywords:** Breeder age, Bone mineralization, Eggshell quality, Hypoxia, Oxygen level

## INTRODUCTION

The poultry industry is a cornerstone for global food security, providing an essential source of animal protein through egg and meat production. As the demand for poultry products continues to rise, the industry must prioritize optimizing embryonic development and chick quality to sustain productivity and maintain the health of poultry flocks. A key aspect of this optimization involves understanding the factors that influence embryogenesis, particularly the genetic influence of breeder and hen age and the environmental conditions exposed to during incubation. Among these conditions, oxygen ($O_2$) levels in the incubator play a pivotal role in embryonic development and mineral absorption, which are critical for producing healthy chicks (Oviedo-Rondón et al., 2020; Tona et al., 2022; Yalçin and Oviedo-Rondón, 2023).

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Breeder age is a well-established genetic determinant of egg quality, embryonic development, and subsequent chick viability. As hens get older, they undergo significant physiological changes that affect their reproductive output, nutrient allocation, and eggshell quality (Park and Sohn, 2018). Typically, older hens produce larger eggs; however, these eggs may often have compromised eggshell integrity (Gu et al., 2021) and reduced calcium deposition (Al-Batshan et al., 1994). The decline in eggshell quality can adversely affect embryonic development, particularly bone strength in hatchlings (Kraus and Zita, 2019; Yenilmez and Atay, 2023; Varol Avcılar et al., 2024). During embryogenesis, changes in egg and eggshell traits like shell thickness and chemical properties need to be understood in order to make accurate assumptions about how embryos respond to different environmental conditions during incubation (Orłowski et al., 2019).

High mineral absorption from the eggshell during incubation is crucial for the developing embryo, as the eggshell is rich in calcium for bone mineralization. The genes responsible for mobilizing minerals from the egg during embryonic growth vary between the yolk sac and chorioallantoic membrane (CAM) in the embryo (Halgrain et al., 2021). Specialized genes in the chorioallantoic membrane facilitate the breakdown of the eggshell, allowing calcium to be absorbed into the bloodstream and transported to developing bones (Halgrain et al., 2022). Several other factors, including eggshell thickness, incubation conditions, and the embryo's metabolic activity, influence the efficiency of calcium absorption, directly impacting the embryo's skeletal development. Embryos with a greater ability to mobilize calcium from the eggshell would benefit from this pattern of embryonic calcium nutrition, which could enhance hatchling fitness by promoting growth (Stewart et al., 2019).

Oxygen concentration in the incubator during incubation is another critical environmental variable that directly influences embryonic development. Oxygen in the blood is essential for oxidative phosphorylation, the primary pathway for energy production in developing embryos (Almansa-Ordonez et al., 2020; May-Panloup et al., 2021; Deluao et al., 2022). Hypoxia, or low oxygen concentration in the blood can occur due to factors, such as high altitudes, inadequate ventilation, or suboptimal incubator settings. These conditions can significantly affect metabolic rates, thermoregulation, and overall growth. In response to hypoxia, embryos may exhibit adaptive changes in the chorioallantoic membrane, cardiovascular system, and mineral metabolism, which can alter bone formation and development (Druyan et al., 2012; Zhang et al., 2017; Haron et al., 2021). Hypoxic condition is also a vehicle for introducing phenotypic plasticity and adaptation in poultry, indicating that, although hypoxia may be detrimental, it has the potential to enhance embryo development (Hammarlund, 2020; Haron et al., 2021; Storz and Scott, 2021). Chan and Burggren (2005) reported that the timing and severity of hypoxia can significantly influence embryonic outcomes.

Despite the importance of oxygen during incubation, the interaction between breeder age and the level of oxygen concentration in the incubator remains poorly understood, especially concerning eggshell quality, mineral absorption, and bone morphometrics of hatchlings. Nangsuay et al. (2021) and several other researchers investigated the interaction between broiler breeder age and oxygen levels in the incubator, however, there is limited literature on the influence of oxygen levels during incubation on layer breeders and age effects. For example, older breeder hens tend to produce eggs with thinner shells, which may compromise calcium availability for embryonic bone development (Bain et al., 2011; Ahmed, 2016). The precise impact of varying oxygen levels on these processes is not fully elucidated, particularly concerning the role of the chorioallantoic membrane in facilitating gas exchange and mineral absorption under different oxygen tensions from the eggshell.

The incubation period of avian embryos is critical for their development, particularly during some specific windows when major physiological transformations occur. One such period is the early to mid-incubation phase, during which the chorioallantoic membrane develops and begins to play a major role in respiratory gas exchange and mineral absorption (Nowak-Sliwinska et al., 2014; Halgrain et al., 2022). The present study seeks to address these gaps by investigating the effects of layer breeder age and early reduced incubator oxygen (15% and 17% $O_2$) levels on chorioallantoic membrane development, eggshell decalcification, eggshell weight, embryo weight loss, eggshell temperature (EST), bone and blood Ca and P mineral absorptions, morphometrics of the chick's tibia and femur and neovascularization of the heart tissue. By integrating recent findings with correlative data, the present study aimed to provide a comprehensive understanding of the mechanisms underlying chorioallantoic membrane growth, eggshell decalcification, bone and blood mineralization, and skeletal development in chicks.

## MATERIALS AND METHODS

### Ethical approval

The current experimental protocols were approved in accordance with the guidelines of the Animal Ethics and Scientific Committee of the Regional Center of Excellence for Poultry Sciences at the University of Lomé (CERSA-UL), under approval number 008/2021/BC-BPA/FDS-UL.

### Experimental site and facilities

The study was conducted at the Regional Center of Excellence for Poultry Sciences, University of Lomé (CERSA-UL). Facilities used included a hatchery and a laboratory unit. Field experimentation and laboratory analysis of samples was carried out from February to March 2024. The incubators were situated in an environment of latitude 6°1′95″N, longitude 1°2′53″E and an elevation of 26m above sea level.

### Experimental design

A total of nine hundred (900) hatching eggs from ISA (Institut de Sélection Animale) Brown layer breeders aged 33 and 50 weeks with a respective average weight of 53.85 ± 2.40 g and 60.42 ± 2.02 g were incubated at three oxygen concentrations ($O_2$) levels that included 15%, 17% (experimental groups) and 21% (control group). From the total hatching eggs, 450 were allocated to each breeder age group. Each oxygen concentration level in a breeder age group had 150 hatching eggs which were subdivided into three replicates of 50 eggs on setting trays. On the embryonic day (ED) 7, 8, and 9, the 15% and 17% $O_2$ (experimental group) incubators were individually and sturdily flushed with an air-$N_2$ mixture (1hr/day) to maintain their respective $O_2$ levels. The oxygen concentration levels were continuously monitored with an $O_2$ gas detector (Model HFP-1201 BX, No. D6924, Xi'an Huafan Technology Co., Ltd., China) during flushing (Druyan et al., 2012; Zhang and Burggren, 2012). Following the 1-hour flushing on each day, experimental incubators were returned to normoxic incubation conditions as the controlled group.

### Storage and incubation conditions

Hatching eggs from ISA Brown layer breeders were collected from a commercial farm in Lomé, Togo. The eggs were stored at 18°C and 75% relative humidity for 4 days, pre-warmed at 24°C for 6 hours, and weighed individually before being subjected to the 2 x 3 incubation design in three PAS REFORM SmartPro Combi incubators (PasReform, Zeddam, Netherlands) with a holding capacity of 600 eggs. The eggs were kept warm for 18 days at a temperature of 37.7°C and a relative humidity level of 56% while being rotated every hour at a 90° angle. On the 18th day, eggs containing live embryos were identified through candling, weighed and transferred to the hatcher in baskets for the three-day hatching period (until day 21 of incubation).

### Pre-incubation egg quality measurement

Twenty-five (25) eggs were randomly selected from each breeder's age group (totaling 50) and examined for egg quality. Eggs selected for quality examination were excluded from the total experimental number.

### Parameters determined

#### Egg quality measurement before setting

Prior to incubation, the characteristics of the eggs measured included egg weight (EW, g), egg length (L, mm), egg width (W, mm), eggshell weight (SW, g) and thickness (ST, mm), as well as yolk weight (YW, g). All weights were measured using an analytical scale balance, while L, W, and ST were measured using a digital Vernier caliper. Additional geometric qualities such relative eggshell weight (RSW, %), shape index (SI, %), geometric diameter (Dg, mm), egg volume (V, $cm^3$), elongation (Elong, mm), specific gravity (SSG, $g/cm^3$), eggshell surface area (SA, $cm^2$), eggshell sphericity (SP, %), eggshell volume (SV, $cm^3$), pore number (PN), eggshell density (SD, $cm^2$), eggshell weight/surface area (SW/SA) were estimated using the following formulas (Formula 1-11).

$RSW\ (\%) = [(SW)/(EW)] \times 100$ (Formula 1)

$SI\ (\%) = [(W/L)] \times 100$ - (Carter, 1968) (Formula 2)

$PN = 304W^{0.767}$ - (Rahn and Paganelli, 1989) (Formula 3)

$Dg = (L*W^2)^{1/3}$ - (Mohsenin, 2020) (Formula 4)

$EV\ (cm^3) = (0.6057 - 0.0018W) * LW^2$ - (Narushin, 2005) (Formula 5)

$SA\ (cm^2) = (3.155 - 0.0136L \pm 0.0115W) * LW$ - (Narushin, 2005) (Formula 6)

$SP\ (mm) = [(LW^2)^{1/3} / L] \times 100$ - (Severa et al., 2013; Kumbar et al., 2016) (Formula 7)

$SV = SV\ (cm^3) = ST \times S$ (Rahn and Paganelli, 1989) (Formula 8)

$SD\ (g/cm) = (SW / S \times ST)$ - (Rahn and Paganelli, 1989; Shafey, 2002) (Formula 9)

$Elong\ (mm) = L/W$ (Formula 10)

$SSG\ (g/cm^3) = (EW/V)$ - (Rahn and Paganelli, 1989) (Formula 11)

### Chorioallantoic membrane weight

On embryonic day (ED) 11, post-exposure, six embryonated eggs were taken from each of the three replicates of each factorial group. The eggs were weighed, and the shells were broken in the air space region to remove the embryo. The chorioallantoic membrane (CAM) was weighed to estimate the relative CAM (rCAM) weight using the formula (Formula 12).

$rCAM\ weight\ (\%) = [(CAM\ weight)/(EW)] \times 100$ (Formula 12)

where; rCAM = relative chorioallantoic membrane weight, CAM = Chorioallantoic membrane, EW = egg weight

**Egg weight loss**

Egg weight loss (EWL) was estimated by weighing 30 viable eggs at embryonic day (ED) 0 ($EW_{ED\ 0}$) and reweighed daily from $ED_6$ to $ED_{18}$. Egg weight loss was calculated using the Formula 13.

EWL (%) = [(WL)/($EW_{ED\ 0}$)] × 100, where; WL = $EW_{ED\ 0} - EW_{ED\ 6-18}$ (Formula 13)

where; EWL = egg weight loss; WL = weight loss; EW = egg weight

**Eggshell temperature**

An infrared thermometer (XS-IFT002B, Ganzhou Xianshun Technology Co. Ltd, China) was used to measure eggshell temperatures (EST) for a total of 15 eggs per treatment from ED 11 to ED 18, following the procedure described by Olojede et al. (2016). During exposure to air-$N_2$ from ED 7-9, EST was measured before exposure, immediately after exposure, and two hours after exposure.

**Blood sampling**

At hatch (day 21), nine blood samples were collected from the hearts of nine alive chicks per group using a 27-G needle and 1 mL syringe into plain gel tubes. Samples were centrifuged with a BIOBASE Plus spectrophotometer for 15 minutes at 4,000 rpm and serum was transferred into Eppendorf tubes and stored at -21°C for calcium (Ca), phosphorus (P), and magnesium (Mg) testing.

**Tibia and femur morphometric measurement**

Twelve (12) chicks were cervically dislocated their tibia and femur. The chicks were killed before the tibia and femur were removed and air-dried for 72 hours. Weight, length, and diameter (width at the midpoint and endpoint) were determined with a sensitive scale (Ohaus STX8200 Scout, China) and a digital Vernier caliper respectively. The relative weight of the tibia or femur weight, seedor index (SI), and robusticity index (RI) were calculated using the specific formulas (Formula 14-16) by Riesenfeld (1972) and Evaris et al. (2021).

Relative tibia or femur weight (%) = [(tibia or femur weight)/(yolk free chick weight)] × 100 (Formula 14)

$SI = \frac{weight\ of\ bone\ (g))}{length\ bone\ (cm)}$, SI = seedor index (Formula 15)

$RI = \frac{length\ of\ bone\ (mm)}{\sqrt[3]{weight\ of\ bone\ (g)}}$, RI = robuscity index (Formula 16)

**Determination of calcium and phosphorus in the eggshell, tibia femur bone, and blood of chicks**

To determine the calcium (Ca) and phosphorus (P) content in the eggshells and bones of day-old chicks, six samples from each treatment group were cleaned with alcohol and benzene for 96 hours and dried in an oven (Memmert Universal Oven U, Germany) at 105°C until a constant weight was achieved. The specimens were burned to ashes at a temperature of 550°C for a period of 6 hours in a muffle furnace (Nabertherm GmbH, Bahnhofstr 20, 28865 Lilienthal/Bremen, Germany). The Ca content was determined by titration with $KMnO_4$ in a 0.02 N EDTA solution from a red to blue endpoint (Okalebo et al., 2002; Song et al., 2022). Calcium in samples was estimated by Formula 17.

Ca (mg) = Titer value of EDTA x 0.4008 (Formula 17)

$Ca\ (\%) = \frac{mg\ Ca}{\text{Sample wt x volume}}$ x 100

The phosphorus concentrations were measured on the Spectronic 20 spectrophotometer to give absorbance measurements at a wavelength of 420 nm. The observed absorbance was used to determine the P content from the standard curve (Okalebo et al., 2002). The percentage of P was calculated as Formula 18.

P content (g) in 100 g sample (P %) = $\frac{C\ x\ df\ x\ 100}{1\ 000\ 000} = \frac{C\ x\ 1000\ x\ 100}{1\ 000\ 000} = \frac{C}{10}$, (Formula 18)

where

C = concentration of P (µg/ml) as read from the standard curve; *df* = dilution factor, which is 100 *10 = 1000.

Magnesium (Mg), Ca, and P in the blood sample were determined using the enzyme-linked fluorescent assay (ELFA) method on VIDAS®. Glucose was determined at hatch using an ACCU-CHEK Active Glucometer.

**Heart tissue sampling**

After the period of hypoxic exposure, three heart tissues dissected from the embryos were individually fixed in 10% buffered formalin at ED 11. After undergoing alcohol (100, 96, 80, and 70%) and xylol treatments, a 5 µm thick portion was sliced from the paraffin-embedded blocks. The specimens were additionally treated with xylene to remove paraffin and then dyed with Hematoxylin and Eosin, following Al-Sabawy et al. (2021). A 100um microphotograph (100x magnification) image was taken under a light microscope (Thermo Fisher Scientific, Massachusetts, USA) and neo-vascularization in the tissue of the histopathological images was graded by a histopathologist using the scale of Okur et al. (2022). Four positives (++++) were very high (ectatic vessels with high congestion), three positives (+++) were high (vessels seat of moderate congestion) and two positives (++) were normal (normal tissues).

**Statistical analysis**

Egg weight and eggshell characteristics measured before incubation were analyzed using a one-way ANOVA with the general linear model (GLM).

Yijk = µ + Ai + eijk, (Formula 19)

where Yijk represented the measured variable, µ was the overall mean, Ai was the main effect of breeder age (33 or 50 weeks), and eijk was the random residual error.

Other data were analyzed using a completely randomized design with a 2 x 3 factorial arrangement and a two-way ANOVA according to the following formula:

Yijk = µ + Ai + Oj + $AO_2$ij + eijk, (Formula 20)

where Yijk was the measured variable, µ was the overall mean, Ai was the main effect of breeder age (33 or 50 weeks), $O_2$j was the effect of oxygen concentration levels (15%, 17%, or 21%), $AO_2$ij was the interaction

between breeder age and oxygen concentration, and eijk was the random residual error. All statistical analyses were performed using Minitab Statistical Software, version 21.2 (Minitab, LLC, NY, US, 2021). Data for egg weight loss was transformed using the square root of the arcsine before analysis. Correlation analysis was conducted between Ca and P content in decalcified eggshells, blood, bones and the morphometries of the tibia and femur. The Tukey post-hoc test was used to compare means, with significance set at $p < 0.05$. The results were presented as the mean ± the Standard Deviation (SD). GraphPad Prism, version 9.5.1 (2023) was used for charts and graphs.

## RESULTS

### Fresh egg weight and eggshell characteristics

Tables 1 and 2 present the results for egg weight, external geometry and eggshell Ca and P content from 33 and 50 weeks breeder flocks. In Table 1, eggs from 50 weeks flocks are significantly ($p < 0.001$) heavier by 6.57 grams than those from 33 weeks flocks. Egg length and width were significantly ($p = 0.01$, 0.008 respectively) greater in 50 weeks flocks compared to the 33 weeks breeder flocks. The calcium content was significantly higher ($p = 0.023$) in 33 weeks eggshells than in the 50 weeks eggshells. Table 2 shows that geometric diameter (Dg), egg volume (V), eggshell volume (SV), and pore number (PN) were significantly ($p < 0.001$) greater in 50 weeks flocks than in the 33 weeks breeder flocks. However, specific gravity (SSG) was significantly higher ($p < 0.001$) in 33 weeks than in the 50 weeks breeder flocks. However, phosphorus levels in the eggshell, relative eggshell weight, thickness, sphericity, shape index, eggshell density, and the ratio of eggshell weight to surface area were not significantly ($p > 0.05$) affected by the 33 and 50 weeks ages of breeder flocks used in the present study.

### Chorioallantoic membrane and eggshell weight

The chorioallantoic membrane (CAM) and eggshell weights on embryonic day (ED) 11 following air-$N_2$ exposure are shown in Figure 1. Chorioallantoic membrane weight was significantly influenced ($p < 0.001$) by the interaction between breeder age and oxygen ($O_2$) levels in the incubator, with 50 weeks breeders having higher weights than the 33 weeks flocks. Chorioallantoic (CAM) weight was also significantly ($p < 0.001$) higher at 15% and 17% compared to 21% incubator oxygen level. No significant ($p > 0.05$) main effect of breeder age was observed on CAM weight. Figure 1b shows no interaction or main effects ($p > 0.05$) of breeder age or incubator oxygen level on eggshell weight on ED 11 post-exposure.

### Egg weight loss

Figure 2 illustrates egg weight loss (EWL) during incubation. A significant interaction between breeder age and incubator oxygen levels was observed for EWL only at ED 10 ($p = 0.046$). However, breeder age consistently had a significant effect on EWL throughout the post-exposure incubation period up to ED 18 ($p < 0.001$), as 50 weeks breeders lost more weight compared to the 33 weeks breeder flocks. Egg weight loss under 15% $O_2$ level during early incubation was significantly different from the 17% and 21% (control group), specifically on ED 15, ED 16, ED 17, and ED 18, with p-values of 0.025, 0.002, 0.016, and < 0.001, respectively. Reduced oxygen levels (15% and 17%) led to less weight loss compared to the 21% $O_2$ (control group) across both breeder ages.

### Eggshell temperature during and post-air-$N_2$ exposure period

Eggshell temperature (EST) measured before, immediately after and 2 hours after exposure during the oxygen reduction period is shown in Table 3. The interaction effect between breeder age and oxygen level on EST was not significant before exposure on embryonic days (ED) 7 to 9. However, a significant interaction was observed after exposure to ED 9 ($p = 0.011$). Eggshell temperature was higher at 21% oxygen compared to 15% and 17%, with 33 weeks breeders showing a more pronounced response on ED 8 ($p = 0.009$) and ED 9 ($p < 0.001$) compared to 50 weeks breeders. Two hours after exposure (2hrAE) on ED 9, EST remained significantly elevated at 21% ($p = 0.05$) compared to 15% but was not significantly different from the 17% incubator oxygen level. No main effect of breeder age was observed during the entire period and embryonic days of exposure. Figure 3 shows the post-exposure EST of incubated eggs from 33 and 50 weeks breeder flocks exposed to 15%, 17%, and 21% oxygen levels. No interaction effect ($p > 0.05$) between breeder age and oxygen levels was observed during the entire post-exposure incubation period until hatching. However, oxygen levels had a significantly decreased effect on ED 15 ($p = 0.010$), ED 16 ($p = 0.006$), ED 17 ($p < 0.001$), and ED 18 ($p = 0.005$) compared to 21% (control group). The main effect of breeder age was also noted, with 33 week breeder flocks recording higher EST at ED 17 ($p = 0.034$) and ED 18 ($p = 0.036$) compared to the 50 weeks breeder flocks. Calcium and phosphorus levels in decalcified eggshell, bone, and blood of chicks

Table 4 shows the calcium (Ca) and phosphorus (P) levels in the bone, and blood of chicks at hatch and decalcified eggshells from 33 and 50 weeks of breeder eggs. The current findings indicated that there was a significant ($p < 0.05$) interaction between breeder age and oxygen levels in an incubator on decalcified eggshells (Dcal-SHL) P, bone calcium (BNE-Ca), bone phosphorus (BNE-P), serum calcium (SER-Ca), serum phosphorus (SER-P), serum magnesium (SER-Mg) and plasma glucose (GLU). Breeder age had a significant effect ($p < 0.001$), as chicks hatched from 50 weeks breeder flocks had lower amounts of Ca in Dcal-SHL, lower BNE-P, higher SER-Ca and SER-P and lower GLU ($p = 0.003$) levels compared to 33 weeks flocks. Reduced oxygen

levels by 15 and 17% in the incubator resulted in a significantly ($p < 0.001$) lower BNE-Ca and P absorption and a slightly high but unclear significant trend for P retention in Dcal-SHL ($p = 0.03$) and SER-Mg ($p < 0.001$) compared to 21% incubator oxygen level.

**Tibia and femur morphometrics**

Tables 5 and 6 show the effect of breeder age and oxygen levels on the tibia and femur morphometrics of chicks at hatch. Table 5 indicates significant interaction effects ($p < 0.05$) of breeder age and oxygen levels on absolute and relative tibia weight, tibia length, diameter, robusticity, and seedor index. Embryos exposed to 15% and 17% oxygen levels in the incubator hatched into chicks which showed significantly ($p < 0.05$) reduced tibia absolute and relative weights, shorter lengths, diameters, and seedor index in comparison to embryos exposed to 21% oxygen levels. The main significant effect ($p = 0.015$) of breeder age was observed on only tibia robuscity as 33 weeks breeders were noted to be higher than 50 weeks breeders. All other tibia morphometrics were not affected by breeder age.

Table 6 shows a significant interaction effect ($p < 0.05$) of breeder age and oxygen levels on absolute and relative femur weight, femur robusticity, and seedor index. No main effect ($p > 0.05$) of oxygen levels (i.e. 15%, 17% and 21%) was observed on femur morphometry. Only absolute femur weight ($p = 0.025$) and the femur seedor index ($p = 0.039$) differed significantly by breeder age, with the 50-week breeder flocks recording heavier weight compared to the 33-week flock. Other femur morphometric parameters, including relative tibia weight, tibia length, diameter, and robusticity were not affected ($p > 0.05$) by the age of the layer breeders.

**Correlation between calcium and phosphorus retained in decalcified eggshell, chorioallantoic membrane weight, calcium and phosphorus mobilized into the blood and bone, and tibia and femur morphometry under different oxygen concentration levels in the incubator**

Tables 7 and 8 show correlations between calcium (Ca) and phosphorus (P) levels in decalcified eggshells, chick bone, serum (blood), CAM weights, and hatchling tibia and femur morphometrics under different oxygen levels, including 15% (below diagonal in Table 8), 17% (above diagonal in Table 8), and 21% (Table 7).

Under 21% oxygen level, a moderate positive correlation existed between decalcified eggshell calcium (Dcal SHL-Ca) and tibia robusticity (TB-rob; $r = 0.57$, $p = 0.05$), and between decalcified eggshell phosphorus (Dcal SHL-P) and tibia diameter TB-dm; $r = 0.63$, $p = 0.03$). Bone calcium (BNE-Ca) positively correlated with serum phosphorus (SER-P) and tibia seedor index (TB-SI; $0.58 \leq r \leq 0.62$, $p < 0.049$). Bone phosphorus (BNE-P) correlated positively with (TB-rob; $r = 0.58$, $p = 0.048$) and femur robuscity (FM-rob; $r = 0.72$, $p = 0.009$). Serum calcium (SER-Ca) was positively correlated with the femur seedor index (FM-SI; $r = 0.59$, $p = 0.042$). A negative correlation was observed between Dcal SHL-P and both SER-Ca and SER-P ($-0.75 \leq r \leq -0.58$, $p < 0.05$). Serum phosphorus showed a strong negative association with TB-rob and FM-rob ($-0.79 \leq r \leq -0.71$, $p \leq 0.02$), while it exhibited a positive correlation with relative tibia weight (rTB-wgt), TB-SI, and FM-SI ($0.62 \leq r \leq 0.79$, $p \leq 0.01$). There was a negative correlation between SER-Ca and FM-rob ($r = -0.69$, $p < 0.014$). Additionally, a moderately weak negative correlation was found between BNE-P and SER-P, TB-SI, and FM-SI ($-0.61 \leq r \leq -0.58$, $p < 0.05$). Chorioallantoic weight (CAM-wgt) was moderately positively correlated with Dcal SHL-P ($r = 0.70$, $p = 0.025$) and moderately negatively correlated with BNE-Ca ($r = -0.66$, $p = 0.036$).

At 15% $O_2$, strong positive correlations were observed between Dcal SHL-Ca and both BNE-Ca and BNE-P ($0.72 \leq r \leq 0.82$, $p < 0.01$). Decalcified eggshell phosphorus (Dcal SHL-P) had moderate to strong positive correlations with CAM-wgt and FM-SI ($0.64 \leq r \leq 0.89$, $p \leq 0.02$). In contrast, Dcal SHL-P had strong negative correlations with BNE-Ca and BNE-P ($-0.96 \leq r \leq -0.80$, $p < 0.01$), while BNE-P showed a moderately negative relationship with FM-SI ($-0.58 \leq r \leq 0.57$, $p \leq 0.05$). Chorioallantoic membrane weight (CAM-wgt) had a strong negative correlation with Dcal SHL-Ca and BNE-P ($-0.88 \leq r \leq 0.81$, $p \leq 0.005$), but a strong positive correlation with Dcal SHL-P ($r = 0.89$, $p = 0.001$).

At 17% oxygen level, a moderately strong negative correlation was observed between Dcal SHL-Ca and BNE-P ($r = -0.61$, $p = 0.04$). Decalcified eggshell phosphorus (Dcal SHL-P) showed very strong negative associations with BNE-P and SER-P ($-0.95 \leq r \leq -0.84$, $p \leq 0.001$). Bone phosphorus (BNE-P) had a moderate positive relationship with SER-Ca ($r = 0.63$, $p = 0.03$) and a very strong positive association with SER-P ($r = 0.93$, $p < 0.001$). Finally, CAM-wgt showed a moderate positive correlation with FM-SI ($r = 0.662$, $p = 0.037$) and a moderate negative correlation with FM-rob ($r = -0.62$, $p = 0.05$).

**Heart tissue histology**

According to Table 9 and Figure 4, the result is presented for the graded level of neo-vascularization in the heart tissue of an embryo at ED 11. The heart tissue from embryos incubated under hypoxic conditions of 15% and 17% oxygen levels for 50 weeks breeder flocks compared to the 33 weeks group and the control for 50 weeks were graded to be very high (ectatic vessels with high congestion, "++++") beyond normal tissue development. For the control (21%) and 17% $O_2$ level for the 33-week breeders, compared to the 50-week groups and 15% oxygen level for 33 weeks, vessel dilation was graded as high (vessels seat with moderate congestion, "+++").

**Table 1.** Fresh egg weight and shell qualities of 33 and 50 weeks ISA brown layer breeders into early hypoxic stimulation

| Parameters | Egg weight (g) | Egg length (mm) | Egg width (mm) | Eggshell weight (%) | Shell thickness (mm) | Eggshell Ca (% DM) | Eggshell P (% DM) |
|---|---|---|---|---|---|---|---|
| 33 weeks | 53.85±2.40$^{b}$ | 36.96±1.41$^{b}$ | 27.36±0.98$^{b}$ | 10.14±0.80 | 0.37±0.05 | 31.32±2.81$^{a}$ | 0.39±0.04 |
| 50 weeks | 60.42±2.02$^{a}$ | 40.32±0.84$^{a}$ | 28.85±0.52$^{a}$ | 9.82±0.52 | 0.40±0.05 | 29.93±3.22$^{b}$ | 0.41±0.03 |
| P-value | < 0.001 | 0.001 | 0.008 | 0.418 | 0.314 | 0.023 | 0.232 |

$^{a,b}$: Values within the same column followed by different subscripts differ significantly ($p < 0.05$). All results are presented as mean ± SD, Ca: Calcium; P: Phosphorus

**Table 2.** Eggshell geometry of 33 and 50 weeks ISA brown layer breeders in early hypoxic stimulation

| Parameters | Geometric diameter (mm) | Egg volume ($cm^3$) | Elongation (mm) | Specific gravity ($g/cm^3$) | Eggshell SA ($cm^2$) | Eggshell sphericity (%) | Eggshell volume ($cm^3$) | Pore number | Shape index (%) | Eggshell density ($cm^2$) | Eggshell wgt/SA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 weeks | 30.23±0.68$^{b}$ | 15.39±0.99$^{b}$ | 1.35±0.08 | 3.50±0.08$^{a}$ | 26.92±26.92$^{b}$ | 81.89±3.37 | 0.99±0.13$^{b}$ | 6465.90±220.90$^{b}$ | 74.14±4.57 | 5.59±0.55 | 2.00±0.02 |
| 50 weeks | 32.25±0.54$^{a}$ | 18.59±0.91$^{a}$ | 1.40±0.03 | 3.25±0.05$^{b}$ | 30.48±0.92$^{a}$ | 80.00±1.00 | 1.21±0.16$^{a}$ | 7062.90±181.30$^{a}$ | 71.56±1.33 | 4.96±4.96 | 1.98±0.01 |
| P-value | < 0.001 | < 0.001 | 0.236 | < 0.001 | < 0.001 | 0.217 | 0.022 | < 0.001 | 0.213 | 0.126 | 0.113 |

$^{a,b}$: Values within the same column followed by different subscripts differ significantly ($p < 0.05$); All results are presented as mean ± SD, wgt: Weight; SA: Surface area

**Table 3.** Eggshell temperature of 33 and 50 weeks ISA brown layer breeders taken before, immediately after, and two hours after exposure to early hypoxic stimulation (ED 7-9)

| | | Before Exposure | | | After Exposure | | | 2 Hours After Exposure | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ED-7 | ED-8 | ED-9 | ED-7 | ED-8 | ED-9 | ED-7 | ED-8 | ED-9 |
| Breeder age (A) | 33 weeks | 36.81±0.10 | 36.94±0.05 | 36.96±0.05 | 36.75±0.13 | 36.75±0.17 | 36.80±0.18 | 36.79±0.15 | 36.87±0.25 | 36.95±0.06 |
| | 50 weeks | 36.80±0.04 | 36.91±0.02 | 37.99±0.11 | 36.68±0.20 | 36.71±0.22 | 36.83±0.14 | 36.71±0.20 | 36.77±0.11 | 36.94±0.09 |
| Oxygen level ($O_2$) | 15% | 36.78±0.04 | 36.91±0.03 | 36.98±0.09 | 36.64±0.14 | 36.63±0.24$^{b}$ | 36.75±0.13$^{b}$ | 36.75±0.15 | 36.77±0.31 | 36.89±0.03$^{b}$ |
| | 17% | 36.80±0.08 | 36.93±0.02 | 36.96±0.11 | 36.70±0.22 | 36.66±0.07$^{b}$ | 36.71±0.10$^{b}$ | 36.69±0.25 | 36.77±0.12 | 36.96±0.08$^{ab}$ |
| | 21% | 36.82±0.11 | 36.94±0.07 | 36.99±0.07 | 36.81±0.11 | 36.92±0.09$^{a}$ | 36.99±0.07$^{a}$ | 36.81±0.11 | 36.92±0.09 | 36.99±0.07$^{a}$ |
| Interaction (A * $O_2$) | 33 weeks * 15% | 36.81±0.04 | 36.92±0.02 | 36.95±0.02 | 36.68±0.13 | 36.62±0.02 | 36.70±0.17$^{ab}$ | 36.77±0.23 | 36.79±0.48 | 36.90±0.02 |
| | 33 weeks * 17% | 36.78±0.12 | 36.93±0.03 | 36.93±0.07 | 36.73±0.10 | 36.66±0.05 | 36.71±0.16$^{ab}$ | 36.78±0.07 | 36.84±0.04 | 36.97±0.08 |
| | 33 weeks * 21% | 36.83±0.16 | 36.97±0.09 | 36.99±0.03 | 36.83±0.16 | 36.97±0.09 | 36.99±0.03$^{a}$ | 36.83±0.16 | 36.97±0.09 | 36.99±0.03 |
| | 50 weeks * 15% | 36.76±0.03 | 36.91±0.04 | 37.00±0.13 | 36.59±0.16 | 36.63±0.38 | 36.80±0.05$^{ab}$ | 36.73±0.06 | 36.75±0.10 | 36.88±0.05 |
| | 50 weeks * 17% | 36.83±0.03 | 36.92±0.02 | 36.99±0.14 | 36.67±0.32 | 36.65±0.10 | 36.69±0.01$^{b}$ | 36.60±0.36 | 36.71±0.14 | 36.96±0.10 |
| | 50 weeks * 21% | 36.80±0.05 | 36.91±0.02 | 36.98±0.10 | 36.80±0.05 | 36.86±0.04 | 36.98±0.10$^{ab}$ | 36.80±0.05 | 36.71±0.04 | 36.98±0.10 |
| P-value | A | 0.786 | 0.154 | 0.383 | 0.430 | 0.704 | 0.766 | 0.337 | 0.321 | 0.795 |
| | $O_2$ | 0.808 | 0.587 | 0.894 | 0.186 | 0.009 | < 0.001 | 0.504 | 0.371 | 0.052 |
| | A * $O_2$ | 0.887 | 0.455 | 0.926 | 0.583 | 0.104 | 0.011 | 0.741 | 0.710 | 0.375 |

$^{ab}$: Values within the same column followed by different subscript letters differ significantly ($p < 0.05$); All results are presented as mean ± SD; ED: Embryonic day

**Table 4.** Calcium and phosphorus levels in both tibia and femur bone, blood serum of chicks at hatch, and decalcified eggshell of 33 and 50 weeks ISA brown layer breeder eggs incubated in different incubator oxygen concentration levels (ED 7-9)

| Parameters | Dcal SHL-Ca (% DM) | Dcal SHL-P (% DM) | Bone Ca (% DM) | Bone P (% DM) | Ser Ca (mmol/L) | Ser P (mmol/L) | Ser Mg (mmol/L) | GLU (mmol/L) |
|---|---|---|---|---|---|---|---|---|
| Breeder age (A) | | | | | | | | |
| 33 weeks | 26.24±2.34$^{a}$ | 0.33±0.0314 | 10.73±0.39 | 8.70±1.44$^{a}$ | 11.68±0.10$^{b}$ | 7.20±0.67$^{b}$ | 3.74±0.04 | 197.53±12.64$^{a}$ |
| 50 weeks | 22.01±3.12$^{b}$ | 0.33±0.0219 | 10.51±0.91 | 7.86±1.32$^{b}$ | 11.80±0.10$^{a}$ | 8.39±0.68$^{a}$ | 3.73±0.02 | 180.00±19.18$^{b}$ |
| Oxygen level ($O_2$) | | | | | | | | |
| 15% | 24.24±3.27 | 0.33±0.0293$^{ab}$ | 10.09±0.46$^{b}$ | 7.92±1.16$^{b}$ | 11.75±0.09 | 7.87±0.98 | 3.71±0.02$^{b}$ | 193.10±11.81 |
| 17% | 22.55±3.18 | 0.35±0.0259$^{a}$ | 10.35±0.46$^{b}$ | 7.00±0.11$^{c}$ | 11.77±0.14 | 8.16±0.96 | 3.76±0.04$^{a}$ | 180.65±22.32 |
| 21% | 25.59±3.48 | 0.31±0.0068$^{b}$ | 11.42±0.21$^{a}$ | 9.92±0.46$^{a}$ | 11.70±0.12 | 7.34±0.57 | 3.73±0.01$^{b}$ | 192.54±17.94 |
| Interaction (A * $O_2$) | | | | | | | | |
| 33 weeks * 15% | 26.75±0.32$^{a}$ | 0.30±0.0028$^{e}$ | 10.43±0.11$^{b}$ | 9.00±0.02$^{c}$ | 11.70±0.11$^{ab}$ | 7.38±1.02$^{bc}$ | 3.70± 0.01$^{b}$ | 203.58±3.32$^{a}$ |
| 33 weeks * 17% | 24.68±0.16$^{ab}$ | 0.37±0.0023$^{a}$ | 10.50±0.01$^{b}$ | 6.90±0.01$^{d}$ | 11.70±0.09$^{ab}$ | 7.33±0.51$^{bc}$ | 3.79±0.04$^{a}$ | 196.25±19.75$^{a}$ |
| 33 weeks * 21% | 27.29±3.72$^{a}$ | 0.31±0.0073$^{cd}$ | 11.24±0.12$^{a}$ | 10.21±0.52$^{a}$ | 11.64±0.12$^{b}$ | 6.88±0.24$^{c}$ | 3.73±0.01$^{b}$ | 192.75±8.31$^{a}$ |
| 50 weeks * 15% | 21.73±2.89$^{b}$ | 0.36±0.0025$^{b}$ | 9.74±0.41$^{c}$ | 6.85±0.40$^{d}$ | 11.80±0.01$^{ab}$ | 8.37±0.70$^{ab}$ | 3.72±0.02$^{b}$ | 182.61±5.67$^{ab}$ |
| 50 weeks * 17% | 20.43±3.37$^{b}$ | 0.32±0.0060$^{c}$ | 10.20±0.63$^{bc}$ | 7.10±0.03$^{d}$ | 11.84±0.15$^{a}$ | 8.98±0.36$^{a}$ | 3.74±0.01$^{b}$ | 165.06±11.04$^{b}$ |
| 50 weeks * 21% | 23.88±2.42$^{ab}$ | 0.31±0.0052$^{de}$ | 11.60±0.09$^{a}$ | 9.64±0.01$^{b}$ | 11.75±0.10$^{ab}$ | 7.81±0.37$^{bc}$ | 3.73±0.01$^{b}$ | 192.33±25.30$^{a}$ |
| P-value | | | | | | | | |
| A | < 0.001 | 0.968 | 0.371 | < 0.001 | 0.002 | < 0.001 | 0.303 | 0.003 |
| $O_2$ | 0.096 | 0.003 | < 0.001 | < 0.001 | 0.284 | 0.077 | < 0.001 | 0.172 |
| A * $O_2$ | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.034 | < 0.001 | < 0.001 | 0.002 |

**$^{a,b,c}$:** Values within the same column followed by different subscript letters differ significantly ($p < 0.05$); All results are presented as mean ± SD; ED: Embryonic day; Dcal SHL-Ca: Decalcified eggshell calcium; Dcal SHL-P: Decalcified eggshell phosphorus; Ser Ca: Serum calcium; Ser P: Serum phosphorus, Ser Mg: Serum magnesium; GLU: Glucose.

**Table 5.** Effects of breeder age and incubator oxygen concentration levels on tibia morphometry of chicks from 33 and 50 weeks ISA brown layer breeders in early hypoxic stimulation (ED 7- 9)

| Parameters | Absolute weight (g) | Relative weight (%) | Length (mm) | Diameter (mm) | Robusticity (mm/g) | Seedor index (g/mm) |
|---|---|---|---|---|---|---|
| Breeder age (A) | | | | | | |
| 33 weeks | 0.05±0.0069 | 0.17±0.03 | 25.15±1.02 | 2.53±0.22 | 6.86±0.23$^{a}$ | 0.020±0.0023 |
| 50 weeks | 0.05±0.0108 | 0.16±0.03 | 24.92±1.28 | 2.55±0.22 | 6.63±0.30$^{b}$ | 0.022±0.0037 |
| Oxygen level ($O_2$) | | | | | | |
| 15% | 0.05±0.0075$^{b}$ | 0.15±0.03$^{b}$ | 24.26±0.97$^{b}$ | 2.41±0.16$^{b}$ | 6.73±0.21 | 0.020±0.0025$^{b}$ |
| 17% | 0.05±0.0069$^{b}$ | 0.17±0.03$^{ab}$ | 25.21±0.98$^{ab}$ | 2.49±0.17$^{b}$ | 6.85±0.28 | 0.020±0.0024$^{ab}$ |
| 21% | 0.06±0.0100$^{a}$ | 0.19±0.02$^{a}$ | 25.64±1.10$^{a}$ | 2.71±0.19$^{a}$ | 6.66±0.34 | 0.023±0.0036$^{a}$ |
| Interaction (A * $O_2$) | | | | | | |
| 33 weeks * 15% | 0.05±0.0075$^{b}$ | 0.16±0.04$^{ab}$ | 24.63±0.83$^{ab}$ | 2.37±0.10$^{b}$ | 6.79±0.23$^{ab}$ | 0.020±0.0025$^{b}$ |
| 33 weeks * 17% | 0.05±0.0078$^{b}$ | 0.18±0.03$^{ab}$ | 25.56±1.30$^{ab}$ | 2.51±0.15$^{ab}$ | 6.91±0.30$^{a}$ | 0.020±0.0026$^{b}$ |
| 33 weeks * 21% | 0.05±0.0063$^{b}$ | 0.18±0.02$^{ab}$ | 25.27±0.80$^{ab}$ | 2.71±0.23$^{a}$ | 6.88±0.15$^{ab}$ | 0.020±0.0020$^{b}$ |
| 50 weeks * 15% | 0.05±0.0082$^{b}$ | 0.14±0.02$^{b}$ | 23.89±1.02$^{b}$ | 2.44±0.20$^{ab}$ | 6.67±0.18$^{ab}$ | 0.019±0.0026$^{b}$ |
| 50 weeks * 17% | 0.05±0.0065$^{b}$ | 0.15±0.02$^{b}$ | 24.86±0.35$^{ab}$ | 2.47±0.19$^{ab}$ | 6.80±0.27$^{ab}$ | 0.020±0.0025$^{b}$ |
| 50 weeks * 21% | 0.07±0.0049$^{a}$ | 0.20±0.01$^{a}$ | 26.01±1.30$^{a}$ | 2.72±0.16$^{a}$ | 6.44±0.34$^{b}$ | 0.025±0.0023$^{a}$ |
| P-value | | | | | | |
| A | 0.168 | 0.446 | 0.553 | 0.825 | 0.015 | 0.078 |
| $O_2$ | 0.010 | 0.008 | 0.007 | < 0.001 | 0.238 | 0.027 |
| A * $O_2$ | < 0.001 | 0.002 | 0.015 | 0.008 | 0.035 | 0.001 |

$^{a,b}$: Values within the same column followed by different subscript letters differ significantly ($p < 0.05$); All results are presented as mean ± SD; Rel: Relative; ED: Embryonic day

**Table 6**. Effects of breeder age and incubator oxygen concentration levels on femur morphometry of chicks from 33 and 50 weeks ISA brown layer breeders in early hypoxic stimulation (ED 7-9)

| Parameters | Absolute Weight (g) | Relative Weight (%) | Length (mm) | Diameter (mm) | Robusticity (mm/g) | Seedor index (g/mm) |
|---|---|---|---|---|---|---|
| Breeder age (A) | | | | | | |
| 33 weeks | 0.03±0.006$^{b}$ | 0.11±0.03 | 17.60±0.63 | 2.33±0.19 | 5.59±0.33 | 0.018±0.003$^{b}$ |
| 50 weeks | 0.04±0.007$^{a}$ | 0.12±0.03 | 18.08±0.88 | 2.27±0.14 | 5.46±0.25 | 0.020±0.003$^{a}$ |
| Oxygen level ($O_2$) | | | | | | |
| 15% | 0.03±0.005 | 0.11±0.02 | 17.87±0.80 | 2.29±0.10 | 5.50±0.24 | 0.019±0.002 |
| 17% | 0.04±0.007 | 0.12±0.03 | 17.70±0.67 | 2.28±0.22 | 5.47±0.30 | 0.020±0.004 |
| 21% | 0.03±0.009 | 0.11±0.03 | 17.94±0.92 | 2.34±0.16 | 5.61±0.34 | 0.019±0.004 |
| Interaction (A * $O_2$) | | | | | | |
| 33 weeks * 15% | 0.03±0.004$^{ab}$ | 0.11±0.01$^{b}$ | 17.66±0.22 | 2.28±0.10 | 5.60±0.25$^{b}$ | 0.02±0.002$^{ab}$ |
| 33 weeks * 17% | 0.04±0.008$^{ab}$ | 0.13±0.03$^{a}$ | 17.59±0.75 | 2.32±0.28 | 5.36±0.25$^{c}$ | 0.02±0.004$^{ab}$ |
| 33 weeks * 21% | 0.03±0.004$^{b}$ | 0.10±0.01$^{bc}$ | 17.54±0.85 | 2.41±0.14 | 5.82±0.33$^{a}$ | 0.02±0.002$^{b}$ |
| 50 weeks * 15% | 0.04±0.004$^{ab}$ | 0.12±0.02$^{ab}$ | 18.08±1.13 | 2.31±0.12 | 5.39±0.19$^{c}$ | 0.02±0.001$^{ab}$ |
| 50 weeks * 17% | 0.03±0.008$^{ab}$ | 0.11±0.03$^{b}$ | 17.80±0.64 | 2.24±0.16 | 5.58±0.33$^{b}$ | 0.02±0.004$^{ab}$ |
| 50 weeks * 21% | 0.04±0.009$^{a}$ | 0.12±0.03$^{ab}$ | 18.35±0.86 | 2.27±0.16 | 5.41±0.19$^{bc}$ | 0.02±0.004$^{a}$ |
| P-value | | | | | | |
| A | 0.025 | 0.675 | 0.064 | 0.243 | 0.172 | 0.039 |
| $O_2$ | 0.932 | 0.763 | 0.752 | 0.676 | 0.463 | 0.812 |
| A * $O_2$ | 0.039 | 0.045 | 0.457 | 0.595 | 0.044 | 0.031 |

**$^{abc}$:** Values within the same column followed by different subscript letters differ significantly ($p < 0.05$). All results are presented as mean ± SD; ED: Embryonic day

**Table 7**. Correlation between calcium and phosphorus in decalcified eggshell after incubation, calcium, and phosphorus absorbed by chick's tibia and femur bone, blood serum, glucose and tibia and femur morphology of chicks hatched from 21% (control) oxygen concentration level of 33 and 50 weeks ISA brown layer breeders

| Parameters | Dcal SHL-Ca | Dcal SHL-P | BNE-Ca | BNE-P | SER-Ca | SER-P | rTB-wgt | TB-leng | TB-dm | TB-rob | TB-SI | rFM-wgt | FM-leng | FM-dm | FM-rob | FM-SI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dcal SHL-P | 0.22 | | | | | | | | | | | | | | | |
| BNE-Ca | -0.41 | -0.51 | | | | | | | | | | | | | | |
| BNE-P | 0.54 | 0.21 | -0.51 | | | | | | | | | | | | | |
| SER-Ca | -0.43 | -0.75[b] | 0.27 | -0.30 | | | | | | | | | | | | |
| SER-P | -0.52 | -0.58[a] | 0.58[a] | -0.58[a] | 0.82 | | | | | | | | | | | |
| rTB-wgt | -0.48 | 0.06 | 0.46 | -0.45 | 0.14 | 0.62[a] | | | | | | | | | | |
| TB-leng | 0.12 | 0.11 | 0.43 | -0.10 | -0.37 | 0.03 | 0.37 | | | | | | | | | |
| TB-dm | -0.22 | 0.63[a] | -0.22 | -0.51 | -0.22 | -0.02 | 0.22 | -0.02 | | | | | | | | |
| TB-rob | 0.57[a] | 0.28 | -0.41 | 0.58[a] | -0.53 | -0.79[b] | -0.78 | 0.19 | -0.16 | | | | | | | |
| TB-SI | -0.49 | -0.26 | 0.62[a] | -0.58[a] | 0.36 | 0.79[b] | 0.92 | 0.27 | 0.08 | -0.89 | | | | | | |
| rFM-wgt | -0.25 | -0.06 | 0.25 | -0.44 | 0.50 | 0.57 | 0.17 | 0.00 | 0.46 | -0.22 | 0.17 | | | | | |
| FM-leng | 0.11 | 0.22 | 0.18 | -0.10 | 0.21 | 0.46 | 0.38 | 0.17 | 0.39 | -0.27 | 0.31 | 0.79 | | | | |
| FM-dm | 0.07 | -0.07 | -0.39 | 0.30 | 0.17 | -0.23 | -0.52 | -0.90 | -0.20 | 0.06 | -0.46 | -0.10 | -0.19 | | | |
| FM-rob | 0.47 | 0.55 | -0.47 | 0.72[b] | -0.69[b] | -0.71[b] | -0.14 | -0.08 | -0.23 | 0.33 | -0.33 | -0.71 | -0.28 | 0.28 | | |
| FM-SI | -0.34 | -0.29 | 0.47 | -0.61[a] | 0.59[a] | 0.74[b] | 0.29 | 0.16 | 0.35 | -0.35 | 0.38 | 0.93 | 0.70 | -0.29 | -0.87 | |
| CAM-wgt | -0.14 | 0.70[a] | -0.66[a] | 0.32 | -0.32 | -0.44 | -0.03 | -0.22 | 0.44 | 0.17 | -0.29 | -0.16 | -0.11 | 0.11 | 0.46 | -0.40 |

[a]: $p \leq 0.05$; [b]: $p \leq 0.01$; [c]: $p \leq 0.001$; Dcal SHL-Ca: Retained eggshell calcium; Dcal SHL-P: Retained eggshell phosphorus; BNE-Ca: Absorbed bone calcium; BNE-P: Absorbed bone phosphorus; SER-Ca: Absorbed serum calcium; SER-P: Absorbed serum phosphorus; rTB-wgt: Relative tibia weight; TB-leng: Tibia length; TB-dm: Tibia dm; TB-rob: Tibia robusticity; TB-SI: Tibia seedor index; rFM-wgt: Relative femur weight; FM-rob: Femur robusticity; CAM-wgt: CAM weight. No correlation within minerals or tibia or femur morphometrics was considered.

**Table 8.** Correlation between calcium and phosphorus remains in decalcified eggshell after incubation, calcium and phosphorus absorbed by chick's tibia and femur bone and blood serum at hatch, glucose and tibia and femur morphology of chicks in early 15% (below diagonal) and 17% (above diagonal) hypoxic stimulation of 33 and 50 weeks ISA brown layer breeders

| Parameters | Dcal SHL-Ca | Dcal SHL-P | BNE-Ca | BNE-P | SER-Ca | SER-P | rTB-wgt | TB-leng | TB-dm | TB-rob | TB-SI | rFM-wgt | FM-leng | FM-dm | FM-rob | FM-SI | CAM-wgt |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dcal SHL-Ca | | 0.75 | 0.37 | -0.61[a] | -0.15 | -0.52 | 0.41 | 0.29 | 0.06 | 0.05 | 0.08 | 0.12 | -0.32 | -0.09 | 0.01 | -0.09 | -0.07 |
| Dcal SHL-P | -0.80 | | 0.29 | -0.95[c] | -0.45 | -0.84[c] | 0.56[a] | 0.34 | 0.10 | 0.12 | 0.09 | 0.34 | -0.25 | 0.15 | -0.29 | 0.13 | -0.23 |
| BNE-Ca | 0.72[b] | -0.80[b] | | -0.41 | -0.43 | -0.42 | -0.01 | 0.26 | -0.24 | 0.47 | -0.36 | 0.41 | 0.34 | -0.05 | -0.39 | 0.43 | 0.50 |
| BNE-P | 0.82[c] | -0.96[c] | 0.84 | | 0.63[a] | 0.94[c] | -0.44 | -0.38 | -0.03 | -0.33 | 0.09 | -0.38 | 0.16 | -0.21 | 0.37 | -0.22 | 0.13 |
| SER-Ca | -0.41 | 0.57 | -0.42 | -0.55 | | 0.75 | 0.02 | -0.42 | 0.15 | -0.53 | 0.29 | 0.06 | 0.04 | 0.12 | -0.11 | 0.12 | 0.34 |
| SER-P | -0.47 | 0.49 | -0.27 | -0.55 | 0.84 | | -0.41 | -0.54 | -0.16 | -0.42 | 0.10 | -0.33 | 0.01 | -0.10 | 0.28 | -0.19 | 0.05 |
| rTB-wgt | 0.41 | -0.43 | 0.45 | 0.36 | -0.53 | -0.25 | | 0.51 | 0.14 | -0.48 | 0.80 | 0.39 | -0.06 | -0.32 | -0.35 | 0.25 | -0.01 |
| TB-leng | 0.39 | -0.43 | 0.52 | 0.38 | -0.51 | -0.11 | 0.80 | | 0.32 | 0.33 | 0.26 | -0.14 | -0.15 | -0.54 | 0.08 | -0.19 | 0.07 |
| TB-dm | -0.08 | 0.23 | -0.36 | -0.37 | -0.08 | 0.20 | 0.17 | 0.41 | | 0.03 | 0.16 | -0.08 | 0.22 | 0.24 | 0.14 | -0.12 | 0.24 |
| TB-rob | 0.02 | -0.26 | 0.24 | 0.36 | -0.06 | -0.13 | -0.51 | -0.07 | -0.25 | | -0.83 | -0.14 | -0.06 | 0.14 | 0.01 | -0.10 | 0.18 |
| TB-SI | 0.24 | -0.09 | 0.16 | -0.02 | -0.30 | 0.01 | 0.88 | 0.71 | 0.46 | -0.75 | | 0.05 | -0.05 | -0.43 | 0.02 | -0.01 | -0.16 |
| rFM-wgt | -0.31 | 0.34 | -0.29 | -0.23 | <0.001 | -0.30 | -0.13 | -0.47 | -0.60 | -0.22 | -0.17 | | 0.64 | 0.31 | -0.89 | 0.96 | 0.53 |
| FM-leng | -0.49 | 0.31 | -0.41 | -0.25 | 0.28 | -0.08 | -0.65 | -0.83 | -0.57 | 0.29 | -0.76 | 0.55 | | 0.16 | -0.43 | 0.71 | 0.61 |
| FM-dm | -0.11 | 0.19 | 0.05 | 0.01 | -0.19 | -0.43 | 0.06 | -0.10 | -0.58 | 0.02 | -0.08 | 0.83 | 0.31 | | -0.42 | 0.35 | 0.28 |
| FM-rob | 0.11 | -0.44 | 0.24 | 0.42 | <0.01 | -0.07 | -0.19 | -0.17 | -0.25 | 0.54 | -0.49 | -0.40 | 0.40 | -0.38 | | -0.92 | -0.62[a] |
| FM-SI | -0.41 | 0.64[c] | -0.51 | -0.57[a] | 0.17 | -0.03 | -0.27 | -0.43 | -0.18 | -0.35 | -0.04 | 0.82 | 0.30 | 0.63 | -0.76 | | 0.66[a] |
| CAM-wgt | -0.88[c] | 0.89[c] | -0.52 | -0.81[b] | 0.48 | 0.51 | -0.40 | -0.31 | -0.01 | 0.03 | -0.22 | 0.29 | 0.38 | 0.26 | -0.21 | 0.46 | |

[a]: $p \leq 0.05$; [b]: $p \leq 0.01$; [c]: $p \leq 0.001$; Dcal SHL-Ca: Retained eggshell calcium; Dcal SHL-P: Retained eggshell phosphorus; BNE-Ca: Absorbed bone calcium; BNE-P: Absorbed bone phosphorus; SER-Ca: Absorbed serum calcium; SER-P: Absorbed serum phosphorus; rTB-wgt: Relative tibia weight; TB-leng: Tibia length; TB-dm: Tibia dm; TB-rob: Tibia robusticity; TB-SI: Tibia seedor index; rFM-wgt: Relative femur weight; FM-rob: Femur robusticity; CAM-wgt: CAM weight No correlation within minerals or tibia or femur morphometrics was considered.

**Table 9.** Neo-vascularization in heart tissue of embryos from 33 and 50 weeks ISA layer breeder at ED 11 after early hypoxic stimulation

| Groups | | Heart tissue |
|---|---|---|
| **Breeder age ($A_b$)** | **Oxygen level ($O_2$)** | **Neo-vascularization score** |
| 33 weeks | 15% | ++++ |
| | 17% | +++ |
| | 21% | +++ |
| 50 weeks | 15% | ++++ |
| | 17% | ++++ |
| | 21% | +++ |

Breeder age and oxygen level (A * $O_2$): 33 weeks * 15% $O_2$ (33 weeks breeder eggs incubated in 15% $O_2$); 33 weeks * 17% $O_2$ (33 weeks breeder eggs incubated in 17% $O_2$), 33 weeks * 21% $O_2$ (33 weeks breeder eggs incubated in 21% $O_2$), 50 weeks * 15% $O_2$ (50 weeks breeder eggs incubated in 15% $O_2$), 50 weeks * 17% $O_2$ (50 weeks breeder eggs incubated in 17% $O_2$), 50 weeks * 21% $O_2$ (50 weeks breeder eggs incubated in 21% $O_2$). Neo-vascularization grading; ++++: Very high (ectatic vessels with high congestion); +++: high (vessels seat of moderate congestion); ++: Normal (normal tissues).

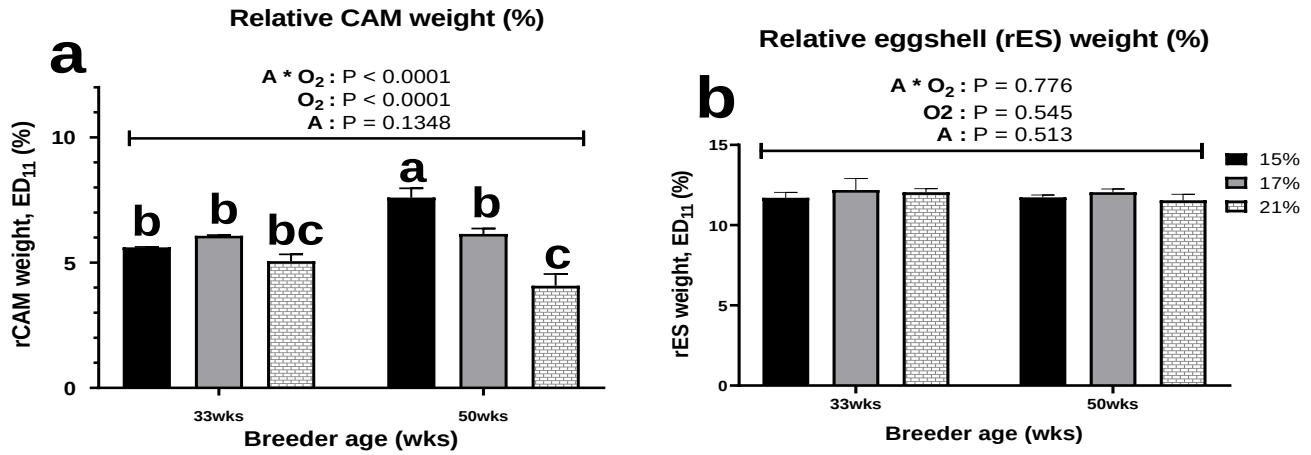


**Figure 1.** Chorioallantoic membrane (CAM) and eggshell (ES) weight at ED 11 of 33 and 50 weeks ISA brown layer breeder flocks exposed to early hypoxic stimulation (ED 7-9). [a,b,c]: Bar charts with different letters indicate significant differences ($p < 0.05$).

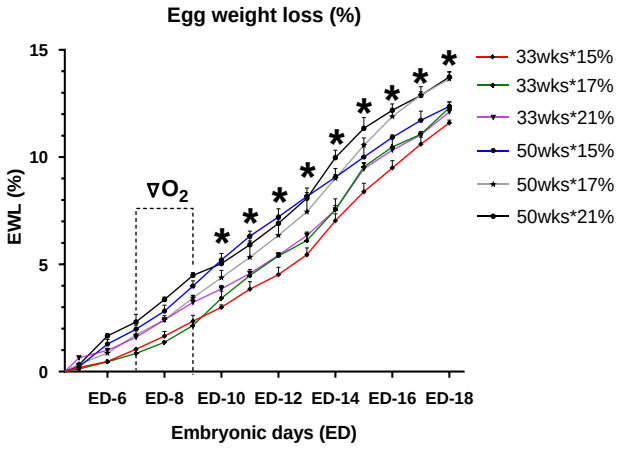


**Figure 2.** Egg weight loss of 33 and 50 weeks ISA brown layer breeder flocks exposed to early hypoxic stimulation (ED7-9). *: ED with asterisk symbol indicates significant differences ($p < 0.05$), $vO_2$: A period of hypoxic stimulation; ED: Embryonic day; EWL: Egg weight loss.

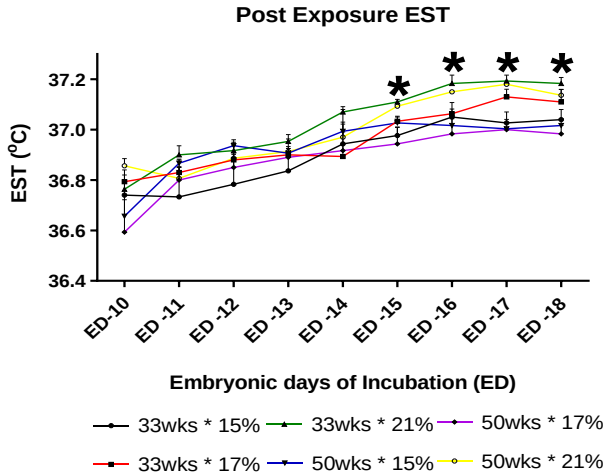


**Figure 3.** Post-exposure eggshell temperature (EST) of 33 and 50-week embryos exposed to early hypoxic stimulation (ED 7-9). *: An asterisk symbol indicates significant differences ($p < 0.05$); ED: Embryonic day.

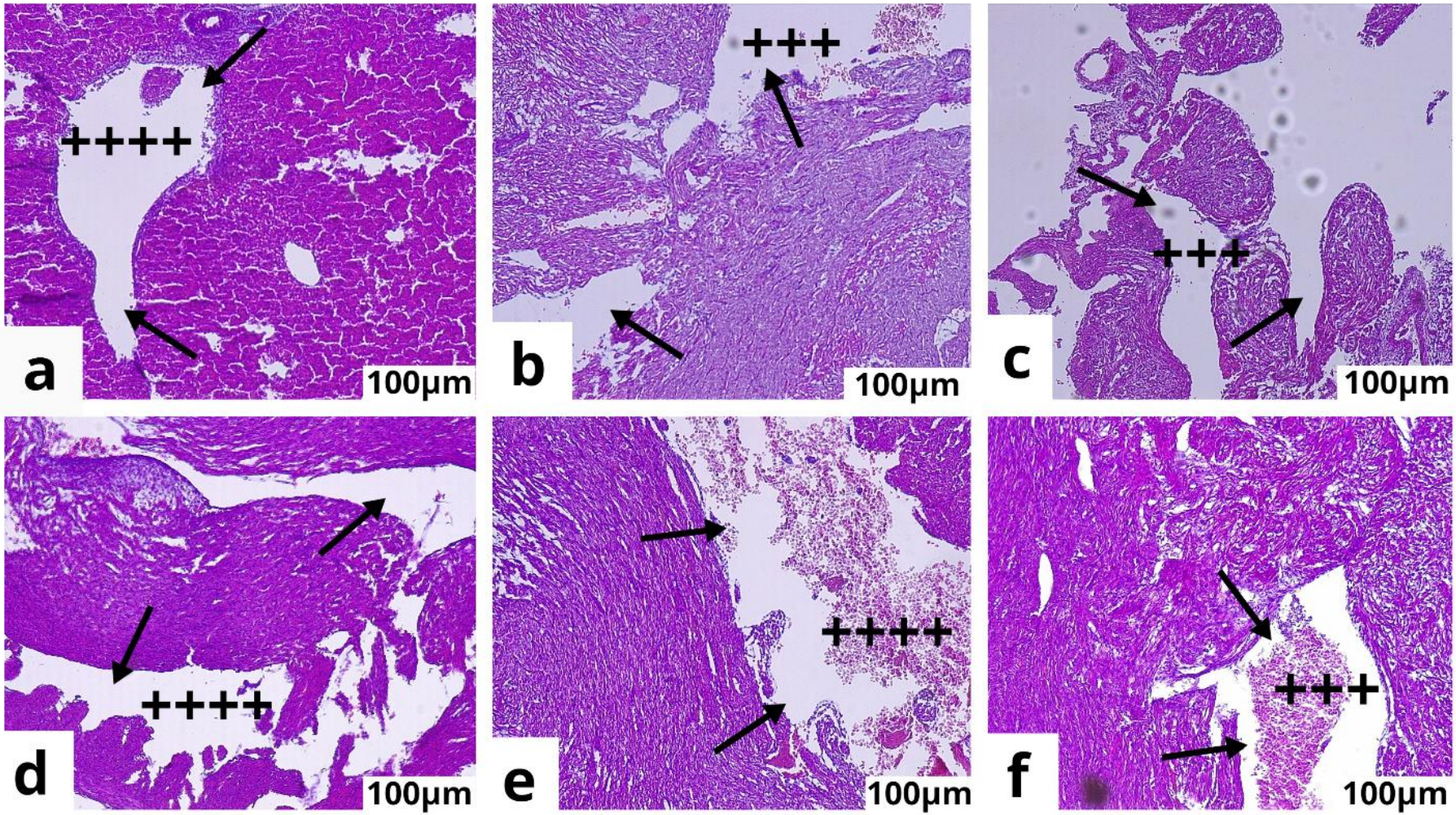


**Figure 4.** The heart tissue (100x magnification) showing neo-vascularization at ED11 of 33 and 50 weeks ISA breeder eggs in early hypoxic stimulation.
**abcdef**: Breeder age and Oxygen concentration (A * $O_2$); **a:** 33 weeks * 15% $O_2$ (33 weeks breeder eggs incubated in 5% $O_2$), **b:** 33 weeks * 17% $O_2$ (33 weeks breeder eggs incubated in 17% $O_2$), **c:** 33 weeks * 21% $O_2$ (33 weeks breeder eggs incubated in 21% $O_2$), **d:** 50 weeks * 15% $O_2$ (50 weeks breeder eggs incubated in 15% $O_2$), **e:** 50 weeks * 17% $O_2$ (50 weeks breeder eggs incubator 17% $O_2$), **f:** 50 weeks * 21% $O_2$ (50 weeks breeder eggs incubated in 21% $O_2$). Neo-vascularization grading: ++++: Very high (ectatic vessels with high congestion); +++: High (vessels seat of moderate congestion); ++: Normal (normal tissues).

## DISCUSSION

Eggshell quality is pivotal in embryonic development, influencing factors, such as gas exchange, moisture loss, and hatching success. The current study highlighted those eggs from older breeders (50 weeks) tended to be heavier with larger dimensions and more pores compared to those from younger breeders (33 weeks). The observation corroborated existing findings that egg size generally increases with breeder age due to alterations in the reproductive system and nutrient allocation (Park and Sohn, 2018). Interestingly, eggshells from younger breeders were found to have higher calcium content, suggesting a greater allocation of calcium to counterbalance the smaller egg sizes (Yair and Uni 2011; Santos et al., 2021). The current finding of higher Ca in younger breeders compared to older breeders aligned with studies indicating that older hens often produce eggs with thinner shells due to reduced calcium absorption and metabolism (Ahmed, 2016; Bain et al., 2016; Ketta and Tůmová, 2016). On the contrary, Dolgorukova et al. (2022) reported that older hens tend to deposit more calcium for embryonic development. Torres (2013) also indicated that differences in dietary calcium intake or physiological conditions between breeder ages could influence the amount of Ca deposited in the eggshell.

The chorioallantoic membrane (CAM) plays a critical role in gas exchange and calcium absorption during embryonic development. The current study observed an increase in CAM weight under reduced oxygen levels (15% and 17%), suggesting a compensatory mechanism to enhance gas exchange in a hypoxic environment (Zamudio, 2003; Nowak-Sliwinska et al., 2014; Zhang et al., 2020). The increase in chorioallantoic weight under reduced oxygen level in the present study agrees with the results of Druyan et al. (2012) and Haron et al. (2017; 2021) who found these increases as an adaptive response to growth under hypoxic stress. The increased CAM weight in older flocks may also be attributed to the larger dimension and pore distribution around the older eggs allowing enough gaseous exchange. The absence of a significant effect of breeder age on eggshell weight underscored that oxygen concentration levels during incubation are more crucial for CAM development than breeder age, although eggshell weight generally decreases under normal incubation conditions (Halgrain et al., 2021; 2022)

Unlike Nangsuay et al. (2021) who found an interaction between breeder flock age and oxygen concentration at ED18 of incubation for yolk-free body mass, the present study reported an interaction between breeder age and oxygen level on egg weight loss immediately after exposure to ED 10. A notably lesser egg weight loss under hypoxic conditions (15% and 17% $O_2$) through to ED 18, suggesting suppressed metabolic activity. The reduction in egg weight loss supports the idea that increased carbon dioxide ($CO_2$) which was an alternative to lower oxygen levels reduces metabolic rate, thereby decreasing water loss and conserving energy under suboptimal conditions (Bilalissi et al., 2022). Heavier eggs from older breeders typically exhibited greater weight loss during incubation (Lourens et al., 2006; Ahmed, 2016). Additionally, eggs from chickens adapted to high altitudes showed lower water loss when incubated under hypoxic conditions (Zhang et al., 2008).

Concurrently with embryo weight loss in the present study, the eggshell temperature (EST) under hypoxic conditions, especially from ED 15 to ED 18 was lower compared to the control group (21% oxygen level). The lower EST obtained from reduced oxygen levels (15% and 17% $O_2$) confirmed a state of lower metabolic rates and thermoregulation in embryos. Haron et al. (2017) reported a decrease in EST after returning hypoxic-treated embryos to normal conditions. Younger breeder (33 weeks) eggs demonstrated higher ESTs at later developmental stages (ED 17 and ED 18) and glucose levels of chicks at hatch compared to older (55 weeks) breeders' flocks. The current finding of an increased glucose level of chicks of the 33 weeks breeders compared to the 50 weeks breeders was consistent with the studies of Güz et al. (2020) and Nasri et al. (2020) who also reported similar results, suggested that the younger flocks had stronger thermoregulatory response to hypoxic stress during the time of pipping compared to the older flocks. In broilers, Nangsuay et al. (2021), reported no interaction between breeder age and oxygen concentration on embryonic heat production. However, in layers, the interaction between breeder age and incubator oxygen concentration on embryonic heat production may differ due to varying embryonic development trajectories (Tona et al., 2001; Hamidu et al., 2011).

Bone development in chicks is heavily dependent on calcium and phosphorus from the eggshell during incubation. The present study found significant interactions between breeder age and incubator oxygen level on bone mineral contents, with younger breeders and 21% controlled levels showing a higher Ca absorption by the bone. Reduced absorption of bone calcium and phosphorus under hypoxic conditions aligns with previous findings that suggested hypoxia impaired calcium

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metabolism and deposition in developing bones, leading to weaker skeletal structures (Chen et al., 2022; Wawrzyniak and Balawender, 2022). Older breeders exhibited lower bone phosphorus but retained higher serum calcium and phosphorus in chicks compared to the younger flocks. Ahmed (2016) attributed higher plasma Ca and P levels to greater eggshell calcium content in chicks from younger breeders. Some genes also play a critical role in Ca and P absorption during incubation. These genes involved in the mobilization of egg minerals during embryonic development are different between yolksac and CAM extraembryonic structures (Halgrain et al., 2021; 2022; 2023).

Hypoxic conditions (15% and 17% oxygen level) were associated with lower absolute and relative weights of the tibia and femur, along with shorter tibial lengths, diameters and seedor index, indicating that reduced oxygen during the early chorioallantoic development during embryogenesis can impair bone development during embryogenesis, likely due to decreased oxidative phosphorylation and resulting energy deficits in developing tissues (Oviedo-Rondón et al., 2008). The impact of oxidative stress on endochondral ossification could influence the rate and extent of bone mineralization in growing long bones (Glimcher, 2006). The interaction between breeder age and oxygen concentration was particularly evident in tibia than in femur morphometries. In chicks, the quality of bone development is largely determined by the quality of tibia development. The tibia bone was frequently observed for negative changes and was therefore examined in chicks for their skeletal growth, mineralization, and strength (Aguado et al., 2015). As Almeida Paz and Bruno (2006) indicated seedor density determines bone density. The current study showed lower seedor index values observed under hypoxic conditions (15% and 17%) compared to the control (21%) oxygen level groups, indicating a less dense bone of chicks possibly due to the less calcium availability.

Limited knowledge existed about the correlative impact of trace mineral bioavailability in eggshells and yolks and its effect on chick skeletal growth. The current study showed that calcium and phosphorus levels in decalcified eggshells, chick bones, and blood serum correlated with the tibia and femur morphometrics, confirming a dynamic relationship between mineral metabolism and skeletal development. Positive correlations between eggshell calcium and tibia robusticity, and between serum phosphorus and tibia seedor index, highlighted the importance of mineral absorption in supporting bone strength and structure. Conversely, negative correlations between serum calcium and femur robusticity under hypoxic conditions suggested that an impaired metabolism of Ca and P from the blood to the bone was possible and could likely cause a weak bone structure. Insufficient calcium intake and vitamin D deficiency are positively correlated with osteoporosis prevalence (Voulgaridou et al., 2023).

It was fascinating to report from the present research that there was a strong positive correlation between decalcified eggshell calcium and bone Ca and P under 15% oxygen level. The positive correlation between the amount of calcium remaining in the eggshell and those absorbed by the chick's bone reflects the importance of calcium transfer from the eggshell to the embryo, even under suboptimal conditions. Calcium mobilization from the eggshell was positively correlated with the number of mammillary tips (Karlsson and Lilja, 2008). The decrease in Ca and P levels in the eggshell under 15% hypoxic condition was likely due to increased CAM weight and vascularization. The decrease in eggshell Ca and increased CAM weight and vascularization under the 15% hypoxic conditions seemed to be more evident in older breeder flocks compared to the younger breeder flocks (Agbehadzi et al., 2024). An increase in CAM weight strongly correlated with a decrease in Ca retained in decalcified eggshell but unfortunately, calcium was not ionized into the chick bone. Increasing CAM weight potentially increases vascularization which may have resulted in more Ca absorption from the eggshell into the blood during embryogenesis. Sys et al. (2013) found that $Ca^{2+}$ was transported to the embryonic circulation by chorionic epithelial cells at 100 nanomoles per hour for one square centimeter of CAM surface. The inability of Ca to be ionized from the blood into the bone before hatch time confirms the report that many calcium signaling pathways may be altered by hypoxia, especially when it is chronic (Pearce, 2006; Quan et al., 2021). Besides, during embryo development, incubating eggs at 39°C also decreased the accessibility of blood-ionized Ca to bone mineralization (Sgavioli et al., 2016). The mechanism for this occurrence under different incubation conditions needs to be further investigated.

The high level of vessel dilation observed in the heart tissue of embryos incubated under hypoxic conditions suggests an adaptive response to increase $O_2$ supply to embryonic tissues (Hsia et al., 2013; Ramanlal and Gupta, 2023; Agbehadzi et al., 2024). Vascular dilation occurs due to the activation of adenosine monophosphate-activated protein kinase in vascular endothelial cells (Rodríguez et al., 2021). The direct role of increased

vasodilation on mineral transfer from the eggshell to bone tissue is unknown. However, the current study's findings assumed that the adaptive response causes an increased rate of Ca transport from the eggshell into the blood through the adaptive regulatory supply of oxygen.

## CONCLUSION

Eggs from older breeders (50 weeks) were heavier, had larger external dimensions, and exhibited a more porous distribution compared to those from younger breeders (33 weeks). However, eggshells from younger breeders contained more calcium, indicating age-related differences in calcium allocation. The reduction in oxygen levels, particularly under hypoxic conditions (15% and 17%) oxygen level notably affected the quality of eggs from different breeder ages. This effect led to compensatory mechanisms of the embryo, such as increased chorioallantoic membrane (CAM) weight, increased neo-vascularization in heart tissue, reduced metabolic activity, lower egg weight loss, and altered eggshell temperature (EST) during late incubation. Early hypoxic stimulations resulted in lower absolute and relative weights of the tibia and femur, shorter tibial lengths, diameter, and seedor index, and impaired calcium and phosphorus absorption from the blood into the bone, especially in older breeders. The study also identified strong negative correlations between CAM weight and Ca in decalcified eggshells under 15% oxygen level, indicating that increased vascularization had the potential to increase Ca absorption from the eggshell into the blood. However minimal Ca was ionized from the blood into the bone of chicks, particularly for the 50-week breeders. Future studies could investigate the molecular mechanisms behind the role of oxygen-sensing pathways in regulating mineral absorption from the blood into the bone under different environmental conditions.

## DECLARATIONS

### Authors' contributions

Richard Koblah Agbehadzi designed, executed, analyzed data, validated results, drafted, and edited the manuscript. Prince Sasu, Benjamin Adjei-Mensah, Hezouwe Tchilabalo Meteyake, Nideou Dassidi, Yaah Aimee Emmanuelle Kouame, and Achiamaa Asafu-adjaye Koranteng supported in reviewing and editing the manuscript. Jacob Alhassan Hamidu and Kokou Tona conceptualized, affirmed experimental design, validated results, and edited the manuscript. All authors approved the final version before publication.

### Ethical consideration

The authors used original analyzed data obtained from the present study to write the article and submitted only to this journal. The content of the article is checked for plagiarism before submission to the journal.

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### Availability of data and materials

All the data generated on the field and analyzed during this study are available from the corresponding author upon reasonable request.

### Competing interests

The authors declare no competing interests.

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