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Effect of breeder age and early hypoxic stimulation of the chorioallantoic membrane on vascularization, internal organ development, blood profile and chick organ histology

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Primary Audience: Embryologists and animal physiologists

SUMMARY

This study examines how the combination of layer breeder age and oxygen concentration in the incubator impacts Chorioallantoic **(CAM)** vascularization, embryo and chick organs, blood profiles, and organ histology at hatch. Nine hundred (900) eggs from 33 to 50 wk ISA breeders were incubated at different O_2 levels (15%, 17%, and 21%). Results showed significant interactions between breeder age and oxygen levels, affecting liver and heart weights, blood indices, and CAM vascularity. Hypoxic conditions led to adaptive changes in embryonic organs, with notable differences between breeder age groups, suggesting that mild hypoxia can influence compensatory growth, depending on exposure stages.

Key words: breeder age, chorioallantoic membrane, hypoxia, histology, layers, oxygen concentration

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DESCRIPTION OF PROBLEM

Breeder age significantly influences egg quality traits and subsequent embryonic development (Machado et al., 2020; Nasri et al., 2020; Zita et al., 2022). The composition of eggs at oviposition and oxygen concentration (O_2) during incubation are crucial factors affecting nutrient metabolism during avian embryo development (Wangensteen and Rahn, 1970; Wilson, 1997). Despite advancements in artificial incubation, aspects related to the gaseous environment are still under investigation to understand epigenetic effects and enhance process efficiency (Decuypere and Bruggeman, 2007; Druyan et al., 2018; Okur et al., 2022). Oxygen supports embryonic growth through yolk beta-oxidation and conductance through shell pores, notably in older breeders (Decuypere and Bruggeman, 2007; Druyan et al., 2018; Okur et al., 2022). Gas exchange rates

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vary during different incubation stages, influencing embryo development and organ formation, each with distinct critical windows (Burggren and Elmonoufy, 2017).

The influence of O_2 and carbon dioxide **(CO_2)** on embryonic development, particularly hypoxia, a condition characterized by a deficiency in oxygen supply to tissues, has significant effects on internal organ development (Decuypere and Bruggeman, 2007). Hypoxia regulates angiogenesis, influencing embryo metabolism, growth, and health (Carmeliet, 2003; Verhoelst et al., 2011). Altimiras and Phu (2000) and Sharma et al. (2006) emphasize the importance of a specific threshold of O_2 and its availability for initiating and sustaining early embryo development. Each hypoxic exposure or stimulation window tends to have an impact on embryo and organ growth and development. While mild hypoxia can improve gaseous diffusion capacity and mitigate detrimental effects on embryonic development, acute or sustained hypoxia during early development may negatively impact vital organ growth (Zhang and Burggren, 2012). However, a study suggested that chronic hypoxia during specific incubation periods may not affect embryo or organ weight at hatching (Miller et al., 2002). Hypoxia-induced changes and adaptations in embryos depend on the timing, intensity, and duration of exposure (Chan and Burggren, 2005; Stenmark et al., 2006; Storz et al., 2010; Burggren and Elmonoufy, 2017; Zhang et al., 2017; Storz and Cheviron, 2021). A variety of organs are affected by hypoxic conditions due to alterations in gene expression and physiological responses (Miot et al., 2012).

Hypoxic stimulation of the chorioallantoic membrane (**CAM**) is proposed to improve respiratory and cardiovascular development in embryos (Haron et al., 2021). Researchers have found that exposure to hypoxic environments can affect hemoglobin levels, indicating a physiological response (Huang et al., 2017). During development, this alteration in blood profile may be an adaptive response to reduced oxygen availability. Hypoxia also plays a role in the vascularization of the heart via its vasodilatory effects, once the coronary circulation is functional (Tomanek et al., 2003). This response is in line with the chick's body's attempt to increase oxygen supply or provide rapid support for angiogenesis under conditions of tissue damage especially to tissues of the heart and lungs, when reduced oxygen availability is present (Hsia et al., 2013). Pearce (2006) and Miller and Zachary (2017) stated that low oxygen levels may compensate for breeder aging, resulting in a more controlled and normal neovascular response in the heart and lung tissues of the chicks.

Hypoxia selectively affects Chorioallantoic membrane **(CAM)** development (Azzam and Mortola, 2007). Early-stage hypoxia affects the development of the CAM, enhancing its growth and vascularization (Druyan et al., 2012; Druyan and Levi, 2012; Druyan and Levi, 2012; Haron et al., 2021). Conflicting reports exist regarding CAM vascularization under hypoxia, with some studies showing no change (Burton and Palmer, 1992) or decreased effects (Burton and Palmer, 1992; Wagner-Amos and Seymour, 2003). While literature exists on these differences mostly for broilers, there is limited information on the effect on layer breeders, in terms of its breed and age. The developmental trajectory of embryos in broilers and layers is different in response to differences in gaseous exchange conditions during incubation. This research, therefore, investigates the effect of early hypoxic stimulation of two (2) layer breeder age eggs during embryogenesis on organ development, CAM vascularization, organ tissue histology and blood indices of chicks hatched as a consideration to adaptability to low oxygen during incubation.

MATERIALS AND METHODS

Experimental Site, Ethics and Facilities

This research was conducted at the University of Lomé at the Regional Center of Excellence for Poultry Science (**CERSA-UL**) hatchery, research farm and laboratory. All experimental procedures were approved by the Animal Ethics and Scientific Committee following the guidelines of the University of Lomé, CERSA (008/2021/BC-BPA/FDS-UL). The incubators used for the experiment were located at latitude 6°1′95″N and longitude 1°

2′53″E with an elevation of 26 m above sea level (Google. n.d., 2024).

Experimental Design

A total of 900 eggs were tested in a 2 × 3 factorial arrangement of 2 breeder flocks ages (33- and -50 wk) and 3 oxygen concentration **(O_2)** levels that include;

1. 15%, 17% O_2 (experimental groups) and
2. 21% O_2 (control group).

Each breeder age group had 450 eggs and in each group of O_2 levels, 150 eggs were divided into 3 replicates of 50 eggs.

From embryonic day (**ED**) 7-9, a steady stream of air-N_2 mixture was used to flush the experimental incubators for only 1 hr/d to reduce the O_2 levels to 15% and 17% for the experimental groups. An O_2 gas detector (Model HFP-1201 BX, No. D6924, Xi'an Huafan Technology Co., Ltd.) was used to continuously monitor the O_2 levels (Druyan et al., 2012; Zhang and Burggren, 2012) in the three (3) PAS REFORM (PasReform, Zeddam, Netherlands, SmartPro Combi model) incubators used. The experimental group incubators were returned to equivalent incubation condition as the control after the 1 h exposure period each day.

Hatching eggs, storage and incubation conditions

The hatching eggs from ISA Brown layer breeders were collected and stored at 18˚C temperature **(T)** and 75% relative humidity **(RH)** for 4 d. Following storage, the eggs were prewarmed at 24˚C for 6 h before incubated. At egg setting, all groups of randomized hatching eggs were incubated in three (3) incubators calibrated to equivalent temperature of 37.7˚C, relative humidty of 56% and automated turning at a 90˚ angle every hour. On the 3rd hour of each day of embryo age (ED 7, 8 and 9) from the time of setting, the two (2) experimental group incubators were continuously flushed with air-N_2 for only 1 h. During the period of flushing, oxygen concentration was reduced in the various experimental incubators to 15% and 17%. The incubator's O_2 levels were continuously monitored and adjusted using the oxygen gas sensor. The experimental incubators were returned to calibrated equivalent condition as the control group after the 1 h oxygen reduction period. On d 18, eggs were candled to identify living embryos, which were then moved to hatching baskets for the 3-d hatching process.

Embryo and Chick Internal Organ Measurements

Following the ED 7-9 exposure to lowered oxygen concentrations, 6 embryos were sampled from each replicate at ED 11. Eggs were weighed and eggshells were broken in the airspace region for the embryo to be removed and weighed. The embryo was dissected for the weight of the heart and liver using a sensitive weighing scale (Ohaus STX8200 Scout). Weights obtained were used to calculate relative organ weights using the following equation:

$$\text{Relative embryo organ weight } (\%) = [(\text{organ weight})/(\text{embryo weight})] \times 100$$

At hatch, all chicks were weighed from each treatment group (data not shown). Nine (9) chicks from each treatment were humanely sacrificed by cervical dislocation and the fresh weight of the yolk sac, heart, liver and lungs was taken. Yolk-free chick weight was then estimated and used to calculate the relative organ weights using the equation:

$$\text{Relative chick organ weight}(\%) = [(\text{organ weight})/(\text{yolk free chick weight})] \times 100$$

Chorioallantoic Membrane Vessel Measurements

On ED 16, 1-2 mL of 10% formalin was injected through the airspace into three (3) eggs from each treatment group for retention of erythrocytes into vessels of the CAM. After

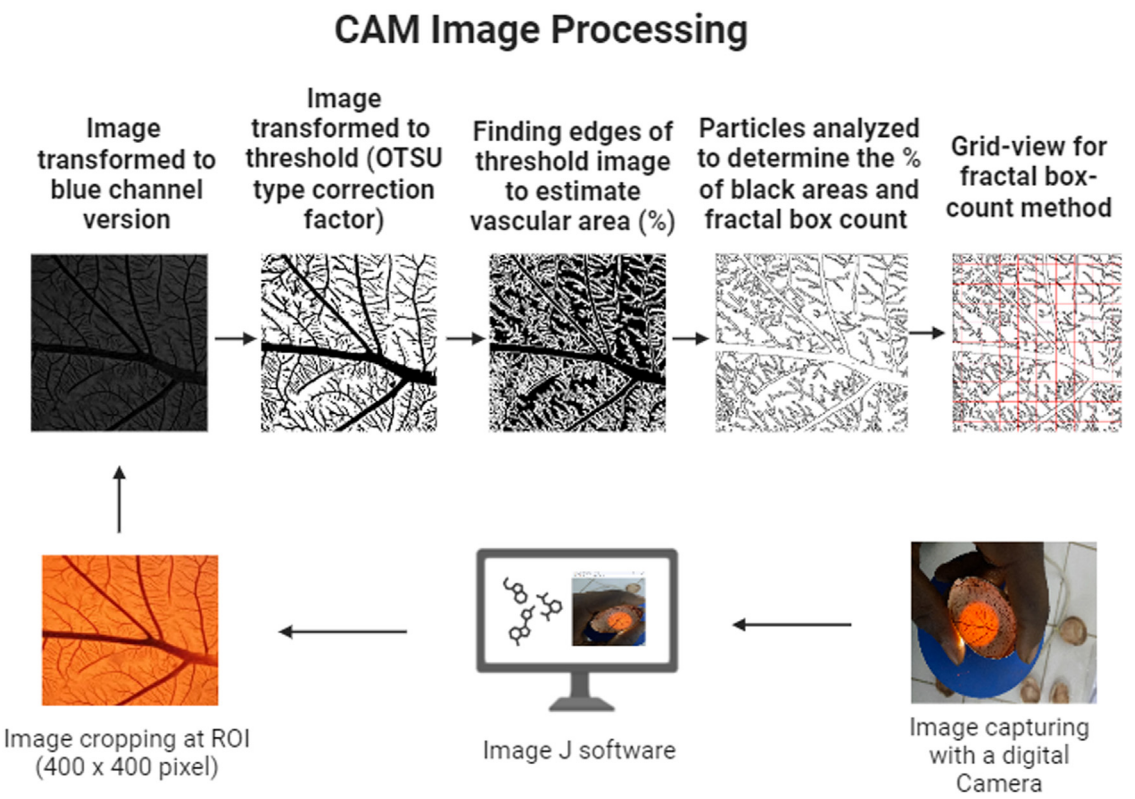

Figure 1. Image processing procedure using Image J software. Abbreviation: ROI, region of interest: The region of interest (ROI) is the subset area of the main initial image cropped to 400 × 400 pixels for analysis. The region was manually chosen based on clearly defined major and minor vessel branching.

24 h, the egg was cut into 2 longitudinal sections along the equatorial region using a Dremel drill with a round blade. The embryo, yolk and albumen were carefully removed from the eggshell with little disturbance to the adhered CAM on the eggshell. A beam of 100W halogen light in an all-closed box-like device with a conical opening with a diameter 3 cm on 1 side was passed through the eggshell from the outer part. Digital images were taken with a Canon (EOS Rebel T5i) camera with a focused lens (EF-S 18-55 mm) at 2 different spots in each half section of the longitudinally divided eggshell. A spot represents the circular region covered by the beam of light that passes through the conical-sided opening of the box. From each circular spot image, 2 regions of interest **(ROI)**, each 400 × 400 pixels were cropped for analysis. The ROI is the subset of the spotted image that was manually identified based on clear vessel branches. This procedure followed slight modification from Verhoelst et al. (2011) and Fernandes et al. (2017).

Eight cropped ROI images were obtained from each egg. A total of 24 images/treatment group were processed and analysed as described by Fernandes et al. (2017), using Image J software (version 1.54j, NIH, USA) for the vascular fraction (VF) (%), and fractal dimension (**FD**) of the vascular network of the CAM (Figure 1). The FD is the measure of the degree of branching of vascular network while the VF is determined as a measure of vessel density, which is in percentage (%) (Verhoelst et al., 2011; Fernandes et al., 2017).

Blood Sampling and Analysis

At hatch, blood samples were collected from the heart of nine (**9**) chicks from each group with a 27-G needle and 1 mL syringe into ethylene-diamine tetra-acetic acid (**EDTA**) and plain

gel tubes. The blood samples in the EDTA tubes were promptly analyzed using an automated hematoanalyzer (DH36, Dymind Biotechnology) for haematological parameters while blood stored in the gel tubes were centrifuged at 3000 rpm at 15 min to obtain serum samples which were stored at -20˚C until Triiodothyronine ($\mathbf{T_3}$), thyroxine ($\mathbf{T_4}$) and biochemical analysis. A volume of 100 mL of serum was used for T_4 and T_3 concentration determination in an automated VIDAS system, which is an enzyme-linked fluorescent assay (**ELFA**) technique. The antibodies, anti-T_3 and anti-T_4 of mutton, provided by VIDAS were used in the assay for the determination of the concentrations of T_3 and T_4, respectively. Serum biochemistry was analyzed by the colorimetric method using an automatic device for total protein (**TP**), albumin (**ALB**), total cholesterol (**TC**), triglycerides (**TG**) and high-density lipoprotein (**HDL**) cholesterol. low-density lipoprotein (**LDL**) cholesterol was estimated using the formula; LDL-chol = [TC] - [HDL-chol] - [TG]/5.

Histological Analysis

Heart, lung and liver tissues dissected from the set of chicks were fixed in 10% buffered formalin. Thick sections of 5 μm were cut from the paraffin-embedded blocks after a series of alcohol (100, 96, 80, and 70%) and xylol treatments, deparaffinized in xylol and stained with Hematoxylin and Eosin. A 100um microphotograph image was taken under a light microscope (Thermo Fisher Scientific, Massachusetts) after examination. As used by Okur et al. (2022), a similar grading score was emplored by a histhopathogist in grading the neo-vascularization in heart, lungs and liver tissues. The neo-vascularization grading score include; ++++ = Very high (ectatic vessels with high congestion); +++ = high (vessels seat of moderate congestion); ++ = Normal (normal tissues).

Statistical Analysis

The data were processed with the statistical software package GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). The factors: breeder age (A_b) and oxygen concentration **(O_2)** levels were used in a 2 × 3 factorial arrangement. The statistical analyses of the results were performed using the 2-way analysis of variance (**ANOVA**) model. All data obtained for the analysis were first transformed using the arc sinus data transformation rule and then tested using the Shapiro-Wilk test for normality (Levene's test for homogeneity of variance). The 2-way ANOVA designs used followed the general linear model (GLM) procedure which is as follows:

$$\mathbf{Yijk} = \boldsymbol{\mu} + \mathbf{Ai} + \mathbf{O_2j} + \mathbf{AO_2ij} + \mathbf{eijk};$$

Where**Yijk** is the Dependent Variable

$\boldsymbol{\mu}$ is the overall mean,

Ai is the effect of age (i= 33- and -50 wk),

O_2j is the effect of the O_2 levels (j = 15%, 17% and 21%) in the experiment,

AO_2ij is the effect of the interaction between A_b and O_2, and

eijk is the random error term.

The post-hoc Tukey test was used to separate and compare the means of each parameter in relation to the effect on A_b, O_2 level and the interaction (A_b * O_2) between the 2 factors. Means were compared and separated at a significant level of 5% *($P < 0.05$)*.

RESULTS AND DISCUSSION

Relative Organ Weights

Table 1 summarizes the impact of breeder age **(A_b)** and oxygen concentration **(O_2)** level on embryo and chick organ weight taken at ED 11 afterexposure and d 1 respectively. The relative weight of organs of the embryo was with regards to embryo weight while those of the chicks were with regards to yolk-free chick weight (data not shown for embryo weight and yolk-free chick weight). No significant *($P > 0.05$)* changes were noted in relative heart weight at ED 11, but the relative liver weight was affected by O_2 levels, with the 21% O_2 group having significantly ($P = 0.003$) lower weight than low O_2 levels. Interactively, chicks from the 50 wk breeders incubated at 21% O_2 level exhibit superior relative heart weight ($P < 0.001$) compared to 15% and 17% low O_2 levels of the same breeder age. Additionally, the 33 wk breeders had relatively higher heart weights

Table 1. Effect of breeder age and reduced oxygen concentration level from ED 7- 9 on embryo and chick relative internal organ weights at ED11 and hatch.

Parameters	Rel. embryo heart weight (ED11) (%)	Rel. embryo liver weight (ED11) (%)	Rel. DOC heart weight (%)	Rel. DOC liver weight (%)	Rel DOC lung weight (%)
Breeder age (A_b)					
33 wk	5.77	9.86	0.95[a]	2.74	0.65
50 wk	7.01	15.85	0.91[b]	2.83	0.60
SEM[1]	0.540	1.240	0.017	0.041	0.048
Oxygen level (O_2)					
15%	6.45	14.16[a]	0.91[b]	2.77[a]	0.63
17%	6.86	14.04[a]	0.88[b]	2.64[b]	0.69
21%	5.87	10.37[b]	1.00[a]	2.95[a]	0.56
SEM[1]	0.660	1.510	0.021	0.059	0.051
Interaction (A_b * O_2)					
33 wk * 15%	5.32	9.17	0.96[ab]	2.41[b]	0.71
33 wk * 17%	6.23	10.08	0.95[ab]	2.73[b]	0.75
33 wk * 21%	5.76	10.33	0.95[ab]	3.09[a]	0.49
50 wk * 15%	7.57	19.14	0.86[b]	3.14[a]	0.54
50 wk * 17%	7.49	18.00	0.81[bc]	2.54[b]	0.62
50 wk * 21%	5.98	10.40	1.06[a]	2.80[b]	0.64
SEM[1]	0.930	2.140	0.030	0.083	0.083
P-value[2]					
A_b	0.118	0.160	0.106	0.242	0.465
O_2	0.573	0.003	<0.001	0.003	0.342
A_b * O_2	0.560	0.076	<0.001	<0.001	0.123

Abbreviations: Rel., relative; DOC, day-old chick; ED, embryonic day.

[a-d]Means within the same column with different superscripts are significant at $P < 0.05$.

[1]SEM, pooled standard error of means.

[2]P: probability value.

($P = 0.013$) than the 50 wk breeders. The main effect of O_2 levels on relative heart weight reveals significantly higher weight ($P < 0.001$) at 21% O_2 compared to 15% and 17% low O_2 levels. The interactive effect of A_b and O_2 levels significantly influenced relative liver weight, with 33 wk breeder embryos at 21% O_2 and 50 wk breeder eggs at 15% O_2 having heavier weights ($P < 0.001$) than the other interactive groups. The main effect of O_2 levels showed a significantly decreased relative liver weight ($P = 0.002$) for 17% O_2 compared to other precedent O_2 levels. No significant ($P > 0.05$) main or interactive effect was observed for relative lung weight across treatment groups.

The effect of breeder age **(A_b)** and oxygen **(O_2)** levels on hypoxia-induced embryogenesis and internal organ development was investigated. The current research is consistent with previous studies that under hypoxic conditions of 17% and 15% O_2 levels, relative embryo weight is decreased (result not shown). Literature presents conflicting results on the effects of hypoxia on embryo heart weight. A hypoxic environment may increase relative heart weight (Ben-Gigi et al., 2021; Lindgren and Altimiras, 2011), cause no change (Altimiras and Phu, 2000; Druyan et al., 2012) or decrease (Ruijtenbeek et al., 2000; Itani et al., 2016). Druyan et al. (2012) showed that 17% of O_2 hypoxia did not affect the relative heart weight of embryos, but 15% of O_2 did. In the study by Haron et al. (2021), neither the heart nor the liver weights were affected by low O_2 levels. The interaction between A_b and O_2 levels indicates a change in the function of the liver as an oxygen elicitor on ED 11. The metabolic pathways in older breeder flocks may be different from those in younger breeder flocks, explaining the differences in organ weights (Nangsuay et al., 2021).

Vascularization in CAM

In Figure 2, the fractal dimension (**FD**) and vascular fraction (**VF**) of blood vessels in the CAM were compared for eggs incubated under

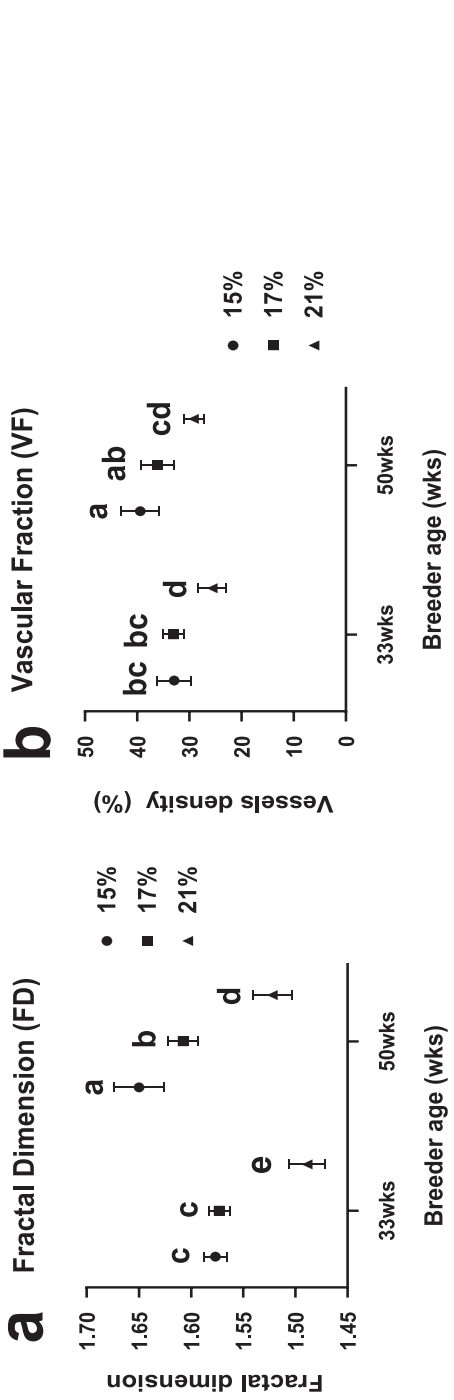

Figure 2. Effect of breeder age and reduced oxygen concentration levels from ED 7-9 on CAM vascularization parameters. [a-e] Letters on each error bar means significance at $P < 0.05$.

normoxic conditions of 21% and hypoxic conditions of 15% and 17% O_2 levels. The results for the FD (the degree of branching of the vessels, Fig 2a) showed there was an interaction ($P = 0.001$) between A_b and O_2 levels and likewise for the individual main effect of A_b and the different O_2 levels of incubation ($P < 0.001$). Eggs from 50 wk breeders had higher FD under 15% and 17% O_2 levels compared to the 33 wk. As showed in Fig 2b, no significant interaction ($P = 0.156$) was found for the VF (degree of vessel density, %) in CAM, however, an increase ($P < 0.001$) was observed for exposure to 15 and 17% O_2 levels (hypoxic) as compared to the 21% (normoxic) condition, and also for 50 wk compared to 33 wk breeder ages.

The CAM was seen to cover a large area with increased vessel branching for embryos exposed to 15 and 17% O_2 levels (hypoxia) than normoxic incubations at both breeder ages. This agrees with Druyan et al. (2012) who observed a significant increase in the vessel density of the CAM of embryos that were incubated in environments with lower O_2 concentrations from 5 to 12 d. However, the higher effect in 50 wk breeders indicated that older breeders were more receptive to hypoxic stimulation, probably due to large surface area and thickness of eggshell. This is an adaptive response that is geared towards phenotypic plasticity in embryos. According to Druyan et al. (2012), embryos that develop under hypoxic conditions are expected to have improved gas diffusion and blood transport abilities, which can be attributed to increased CAM vascularization, and, therefore, a greater supply of oxygen to the embryo. A stimulating effect of hypercapnia and systemic acidosis on angiogenesis, as described by Everaert et al. (2008) lowers the pH of egg albumen. As a result, the embryo is forced to adapt by altering their cardiac output and redistributing oxygenated blood to vital organs, including the brain, heart, and adrenal glands for growth (Mulder et al., 1998). These findings suggest that the age of the breeder hens and the oxygen levels during incubation can significantly impact the development of the embryonic vasculature (Lin et al., 2008; Yalçın et al., 2012; Almeida et al., 2016). Older breeders may produce eggs with a greater capacity for vascular branching under hypoxic

conditions, potentially enhancing the embryo's ability to adapt to low oxygen environments (Fasenko et al., 1999). Further research is needed to clarify the underlying mechanisms and the implications for embryonic and post-hatch development.

Haematological Profile

Table 2 outlines the effect of A_b and O_2 levels on the haematological profile of hatchlings. The interactive effects indicate that chicks hatched from 33 wk * 15% to 50 wk * 17% O_2 level exhibited significantly higher ($P = 0.001$) white blood cell (**WBC**) counts than their precedent counterparts. Lymphocyte (**LYMP**) count from 33 wk * 21% to 50 wk * 15% O_2 level was also statistically ($P = 0.005$) higher in percentages compared to chicks hatched from 33 wk * 15% to 33 wk * 17% O_2 level. Granulocyte (**GRAN**) counts were statistically ($P < 0.001$) higher in eggs from 33 wk * 15% and 33 wk * 17% O_2 levels and 50 wk * 21% O_2 had markedly higher levels compared to other interaction groups. Haemoglobin (**HGB**) concentration interactively significantly ($P = 0.020$) increased in this order; 33 wk * 21% ≤ 33 wk * 17% < 33 wk * 15% < 50 wk * 21% < 50 wk * 17% < 50 wk * 15% O_2 level. Likewise, Hematocrit (**HCT**) levels also took a significant ($P = 0.031$) increasing order as follows; 33 wk * 21% ≤ 33 wk * 17% = 33 wk * 15% ≤ 50 wk * 21% = 50 wk * 17% = 50 wk * 15% O_2 level. Mean corpuscular volume (**MCV**) was significantly greater ($P = 0.040$) in the 50 wk * 15% and 50 wk * 21% O_2 levels compared to the 33 wk * 15%, 17%, and 21% O_2 levels. Mean cell haemoglobin concentration (**MCHC**) was significantly higher ($P = 0.006$) in the 33 wk * 15% and 50 wk * 17% O_2 levels compared to all the other interaction groups. A significantly higher main effect of O_2 level is observed for WBC count for 15% O_2 level being higher compared to other counterparts which were not different among each other. LYMP was also influenced *($P = 0.019$)* by the O_2 level. Neither A_b nor O_2 levels, interactively or individually ($P > 0.05$) affected the blood proportions of mean cell haemoglobin (**MCH**) and platelets (**PLT**).

Chick blood profile measurements showed an interaction between breeder age and O_2 levels on WBC, HGB, HCT, MCV and MCHC. The current finding agrees on HGB and HCT with several studies that embryos exposed to hypoxia during early or late development had elevated HGB concentrations and HCT (Dzialowski et al., 2002; Ruijtenbeek et al., 2000; Chan and Burggren, 2005; Haron et al., 2021, 2022). The higher interaction effect observed for 50 wk breeders compared to 33 wk breeders suggests an increase in oxygen-carrying capacity for older breeders than younger ones. Other studies have also reported that neither RBC count nor PCV or HGB values were affected by high O_2 and CO_2 (Maxwell et al., 1987; Tong et al., 2015; Okur et al., 2022). In avian embryos, the RBC transport oxygen and CO_2 through direct diffusion (Mueller et al., 2022). Tazawa et al. (2012) attributed the increase in HCT (ED 11-ED 19) to increased MCV and not likely due to O_2 transport. This could be true for older breeders but not for younger breeders. An increase in blood O_2-carrying capacity can be achieved by various means: polycythemia (Dusseau and Hutchins, 1988), modification of HGB (Liu et al., 2009), increased vascularization and angiogenesis (Dusseau and Hutchins, 1988; Zhang et al., 2017) or combinations of any of the above factors. Hypoxia during incubation is a known developmental stressor for an embryo (Haron et al., 2021). The combined effect on LYMP and GRAN counts serves as evidence of the impact of A_b and reduced O_2 levels on the immunity and developmental stress response of chicks during hatching. On the contrary, Beker et al. (1995) reported no effect of low O_2 on leukocytes.

Biochemical Profile

The effects of A_b and O_2 levels on serum biochemical indices are presented in Table 3. The results indicated that neither A_b nor O_2 levels, individually or interactively, had a significant ($P > 0.05$) effect on total protein and albumen concentrations ($P > 0.05$). Triglyceride concentrations showed a statistical increase ($P = 0.046$) in the 50 wk breeders incubated at 15%, 17%, and 21% O_2 levels compared to those at 33 wk, irrespective of O_2 levels. The

Table 2. Effect of breeder age and reduced oxygen concentration level from ED 7- 9 on the haematological indices of chicks at hatch.

Parameters	WBC (10^9/L)	LYMP (%)	GRAN (%)	RBC (10^12/L)	HGB (g/L)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/L)	PLT (10^9/L)
Breeder age (A_b)										
33 wk	65.11	0.90[b]	0.02[a]	2.18[b]	135.33[b]	0.28[b]	127.66[b]	61.99	485.78	2.72
50 wk	69.54	0.91[a]	0.01[b]	2.62[a]	163.89[a]	0.34[a]	131.62[a]	62.85	477.50	1.94
SEM[1]	1.550	0.002	0.001	0.051	3.460	0.007	0.730	0.570	3.560	0.290
Oxygen level (O_2)										
15%	72.04[a]	0.91[ab]	0.02	2.46	155.50	0.32	129.47	63.22	488.67	2.42
17%	67.95[ab]	0.90[b]	0.02	2.41	149.50	0.31	128.75	62.09	482.25	2.17
21%	61.98[b]	0.91[a]	0.02	2.32	143.83	0.30	130.70	61.95	474.00	2.42
SEM[1]	1.900	0.003	0.001	0.062	4.240	0.009	0.900	0.700	4.360	0.360
Interaction (A_b * O_2)										
33 wk * 15%	76.00[a]	0.89[b]	0.02[a]	2.27	143.67[c]	0.28[b]	125.57[b]	63.50	505.33[a]	3.33
33 wk * 17%	60.90[b]	0.90[b]	0.02[a]	2.16	133.00[d]	0.28[b]	128.10[ab]	61.43	480.00[bc]	2.33
33 wk * 21%	58.43[b]	0.92[a]	0.01[b]	2.12	129.33[de]	0.27[bc]	129.30[ab]	61.03	472.00[c]	2.50
50 wk * 15%	68.07[ab]	0.92[a]	0.01[b]	2.65	167.33[a]	0.35[a]	133.37[a]	62.93	472.00[c]	1.50
50 wk * 17%	75.00[a]	0.91[ab]	0.01[b]	2.65	166.00[a]	0.34[a]	129.40[ab]	62.75	484.50[b]	2.00
50 wk * 21%	65.54[ab]	0.91[ab]	0.02[a]	2.51	158.33[b]	0.33[ab]	132.10[a]	62.87	476.00[bc]	2.33
SEM[1]	2.690	0.004	0.002	0.088	5.990	0.012	1.270	0.990	6.160	0.510
P-value[2]										
A_b	0.053	0.024	0.013	<0.001	<0.001	<0.001	0.001	0.295	0.110	0.069
O_2	0.003	0.019	0.219	0.272	0.168	0.425	0.314	0.385	0.074	0.850
A_b * O_2	0.001	0.005	<0.001	0.806	0.020	0.031	0.040	0.452	0.006	0.209

Abbreviations: WBC, white blood cell; LYMP, lymphocyte; GRAN, granulocyte; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet.

[a-e]Means within the same column with different superscripts are significant at $P < 0.05$.

[1]SEM, pooled standard error of means.

[2]P: probability value.

Table 3. Effect of breeder age and reduced oxygen concentration level from ED 7- 9 on the serum biochemical indices of chicks at hatch.

Parameters	TP (g/l)	ALB (g/l)	TG (g/l)	HDL (g/l)	TC (g/l)	LDL (g/l)
Breeder age (A_b)						
33 wk	28.79	9.77	0.81[b]	1.26	3.97[a]	2.53
50 wk	28.03	9.73	1.26[a]	1.04	3.60[b]	2.31
SEM[1]	1.360	0.580	0.061	0.100	0.120	0.120
Oxygen level (O_2)						
15%	28.33	9.70	1.03	1.10[ab]	3.91[a]	2.58
17%	26.05	9.48	1.12	0.94[b]	3.33[b]	2.17
21%	30.85	10.07	0.96	1.41[a]	4.12[a]	2.52
SEM[1]	1.670	0.712	0.074	0.130	0.150	0.150
Interaction (A_b * O_2)						
33 wk * 15%	27.20	9.10	0.73[b]	1.01[b]	4.26	3.07[a]
33 wk * 17%	27.47	10.33	0.83[b]	0.99[b]	3.33	2.18[bc]
33 wk * 21%	31.70	9.87	0.88[b]	1.78[a]	4.32	2.36[bc]
50 wk * 15%	29.47	10.30	1.33[a]	1.20[ab]	3.55	2.08[c]
50 wk * 17%	24.63	8.63	1.41[a]	0.89[b]	3.34	2.16[bc]
50 wk * 21%	30.00	10.27	1.04[ab]	1.03[ab]	3.91	2.67[b]
SEM[1]	2.360	1.010	0.110	0.180	0.210	0.210
P-value[2]						
A_b	0.697	0.968	<0.001	0.143	0.038	0.193
O_2	0.143	0.843	0.319	0.041	0.002	0.130
A_b * O_2	0.532	0.344	0.046	0.036	0.248	0.011

Abbreviations: TP, total protein; ALB; albumen; TG, triglycerides; HDL, high-density lipoprotein; TC, total cholesterol; LDL, low-density lipoprotein.

[a-b]Means within the same column with different superscripts are significant at $P < 0.05$.

[1]SEM, pooled standard error of means.

[2]P: probability value.

interactive effect on high-density lipoprotein was significantly higher ($P = 0.036$) in the 33 wk * 21%, 50 wk * 15, 21% O_2 level compared to the other groups. However, low density lipoprotein was markedly superior ($P = 0.011$) in the order; 33 wk * 15% > 50 wk * 21% ≥ 33 wk * 17% = 33 wk * 21%, 50 wk * 17% < 50 wk * 15% O_2 level compared to the other interaction groups. No interactive ($P > 0.05$) effect on total cholesterol concentration was found, nevertheless, the main effects of O_2 levels influenced ($P = 0.002$) its concentrations with 15%, and 21% O_2 levels being different from 17% O_2 levels but not each other. The breeder age effect showed a significantly lower ($P = 0.038$) concentration of total cholesterol observed in the 50 compared to the 33 wk breeder group.

During incubation, lipid metabolism plays an important role in the growth of the embryo. Our findings agree with Okasha et al., 2021 that there is a decreased TC but disagree with the high TG reported in our results for chicks hatched from 50 wk breeder flocks. The higher level of TG observed in the 50 wk breeder flock can be attributed to yolk size and the level of nutrients in the yolks as a result of the age of the breeder flocks. High absorption of this yolk sac is evident in 50 wk breeders exposed to hypoxic conditions. The lower TC and higher TG levels in the 50 wk breeder compared to the 33 wk group suggest age-related differences in cholesterol metabolism, and in our case, slightly impacted by hypoxic condition. In recent times, Jiang et al. (2023) reported a significant increase in TG and lipoprotein lipase in the liver under hypoxic stress for 6 h in golden pompano. Environmental conditions of hypoxia and normoxia substantially influence plasma TC, TG and HDL (Olanrewaju et al., 2010; Okasha et al., 2021) . According to Nangsuay et al. (2013), albumin from an old breeder flock contains relatively fewer proteins than albumin from a young breeder flock. This result is not clear in our finding and the main reason could be due to differences in breeds and the age of breeders used in both researches. Unlike our study which was in layers, Nangsuay et al. (2013)'s study was on broilers.

Table 4. Effect of breeder age and reduced oxygen concentration from ED 7- 9 on the thyroid hormone indices of chicks at hatch.

Parameters	T_3 (ng/ml)	T_4 (ng/ml)	T_3/T_4
Breeder age (A_b)			
33 wk	1.16	8.52	0.14
50 wk	1.24	8.49	0.15
SEM[1]	0.072	0.470	0.009
Oxygen level (O_2)			
15%	1.37	9.97[a]	0.14[ab]
17%	1.13	7.12[b]	0.17[a]
21%	1.10	8.43[ab]	0.13[b]
SEM[1]	0.088	0.570	0.011
Interaction (A_b * O_2)			
33 wk * 15%	1.63[a]	10.95[a]	0.15[ab]
33 wk * 17%	0.99[b]	7.49[b]	0.14[ab]
33 wk * 21%	0.86[b]	7.11[b]	0.12[b]
50 wk * 15%	1.10[ab]	8.99[ab]	0.13[b]
50 wk * 17%	1.27[ab]	6.74[b]	0.20[a]
50 wk * 21%	1.34[ab]	9.75[ab]	0.14[ab]
SEM[1]	0.130	0.810	0.015
P-value[2]			
A_b	0.460	0.967	0.170
O_2	0.079	0.005	0.039
A_b * O_2	0.001	0.021	0.026

Abbreviations: T_3, triiodothyronine; T_4, thyroxine

[a-b]Means within the same column with different superscripts are significant at $P < 0.05$.

[1]SEM, pooled standard error of means.

[2]P: probability value.

Hormonal Profile

Table 4 shows the effect of A_b and O_2 levels on the hormonal profile of hatchling chicks. T_3 concentration was significantly higher ($P = 0.001$) in the 33 wk * 15%, 50 wk * 15%, 50 wk * 21% O_2 levels compared to the other O_2 levels. A similar trend was observed for T_4 concentration, with a statistically different level ($P = 0.021$) noted in the 33 wk breeders compared to the 50 wk breeders incubated at 17% O_2 level. Furthermore, a significantly higher ($P = 0.026$) ratio of T_3 and T_4 was observed in the 50 wk breeders incubated at 17% O_2 level compared to its 15% O_2 level and the 33 wk * 21% O_2 level.

Thyroid hormones are vital for the maturation of essential organ structures, behavioural development (McNabb and Darras, 2015), pipping and hatching (Tullett, 2009) because they ensure a process of transition from allantoic to pulmonary respiration and also play a part in the length of the incubation process (Dewil et al., 1996). The current finding for T_3, T_4 and T_3/T_4 levels of chicks reveals a combined influence of A_b and O_2 levels on T_4 level and breeder eggs hatched from 15% O_2 than other interactive counterparts. The T_4 level was elevated at 15% O_2 compared with 17% but not different from the 21% O_2 level during incubation. Şahan et al (2011) reported a higher T_3, T_4 and T_3/T_4 ratio for high-altitude (hypoxic) hatched chicks than low-altitude (normoxic) chicks. In partial agreement with Hassanzadeh et al. (2004), no effect of both high and low altitudes on T_3, but breeder age and O_2 levels interacted to obtain higher levels for hypoxic for both 33 and 50 wk breeder eggs. Bahadoran et al. (2010) found higher plasma T_3 concentrations with a lower T_3/T_4 ratio for high-attitude hatched chicks. Hypoxia due to high altitude during early embryogenesis may change the endocrine functions of the embryo, enhance growth, or shorten the hatching process of chickens (Bahadoran et al., 2010). The interactive significant impact of high T_4 over T_3 and T_3/T_4 ratio for stimulated hypoxic conditions could indicate that embryos from different breeder ages may have different metabolic mechanisms or may require different levels of energy necessary for pipping and hatching.

Histological Analysis

The histological formation of neo-vascularization in the heart, lungs and liver is presented in Table 5 and Figures 3 to 5. The current study shows that with the exception of the liver tissue of 33 wk * 17% and 21% O_2 level and lungs at 17% O_2 level having normal tissue development, all the other tissues of both breeder ages had a high (vessels seat moderate congestion, "+++") to a very high (ectatic vessels with high congestion, "++++"). Very high congestion was seen in the tissue of the lungs and liver of 50 wk embryos exposed to 15% and 17% O_2 levels. The same is also observed for the heart and lungs of 33 wk breeder age. High and moderate congestion was seen in the heart tissue under 17% O_2 level for 33 wk breeders while under 15% and 17% O_2 level for 50 wk breeders and in the 15% and 21% O_2 of the lungs.

Neovascularization is the formation of new blood vessels irrespective of the type or size of

Table 5. Neo-vascularization in internal organ tissue of chicks of 33 and 50 wk breeder age exposed to reduced oxygen concentration levels from ED 7- 9.

Groups		Neo-vascularization score		
Breeder age (A_b)	Oxygen level (O_2)	Heart	Lung	Liver
33 wk	15%	++++	++++	+++
	17%	+++	++	++
	21%	++++	++++	++
50 wk	15%	+++	++++	++++
	17%	+++	++++	++++
	21%	++++	++++	+++

Breeder age and Oxygen concentration (A_b * O_2): 33 wk * 15% O_2 (33 weeks breeder eggs incubated under 15% O_2); 33 wk * 17% O_2 (33 weeks breeder eggs incubated under 17% O_2), 33 wk * 21% O_2 (33 weeks breeder eggs incubated under 21% O_2), 50 wk * 15% O_2 (50weeks breeder eggs incubated under 15% O_2), 50 wk * 17% O_2 (50weeks breeder eggs incubated under 17% O_2), 50 wk * 21% O_2 (50 weeks breeder eggs incubated under 21% O_2).

Neo-vascularization grading; ++++ = Very high (ectatic vessels with high congestion); +++ = high (vessels seat of moderate congestion); ++ = Normal (normal tissues)

the organ. The neo-vascular differentiation in the tissue shows that the liver and lungs of older flocks may have a distinct response to hypoxia under 15% and 17% O_2 levels compared to the heart which might require a higher hypoxic level of 15% O_2 level to respond, regardless of the observed tissue congestion under 21% O_2 level. The varying levels of congestion in the heart, lungs and liver tissues of embryos suggest a highly intricate relationship between breeder age and O_2 levels in response to hepatic neovascularization. Adair et al. (1987) reported that sustained hypoxia in the embryo causes vasodilatation and decreases systemic vascular resistance. Literature is limited to explaining the above findings. Nonetheless, in some close research, Hao et al. (2014) noted a notable increase in miR-15a expression in embryonic lung tissue under low oxygen (hypoxic) conditions, emphasizing tissue-specific responses to hypoxia. This could confirm why the heart, lungs and liver have varying congestion under 15% and 17% O_2 levels. Hypoxia affects cardiac and vascular disease in chick embryos by adaptive transcriptional changes in the lungs and hearts of high-altitude animals (Salinas et al., 2009; Ge et al., 2021). By observing elevated plasma lactic acid levels in high-altitude embryos, Hassanzadeh et al. (2004) attributed the change in tissue to anaerobic metabolism of embryo organs caused by hypoxic conditions. Hypoxia plays a role in the vascularization of the heart via its vasodilatory effects, once the coronary circulation is functional (Tomanek et al., 2003). This response is in line with the chick's body's attempt to increase oxygen supply or provide rapid support for angiogenesis under conditions of tissue damage especially to tissues of the heart and lungs, when reduced oxygen availability is present (Hsia et al., 2013).

Low oxygen levels may have been compensated for by both breeder ages, resulting in a more controlled and normal neovascular response in the heart and lung tissues of the chicks (Pearce, 2006; Miller and Zachary, 2017). This observation could be true for younger breeders compared to older birds as observed in the current finding of 33 wk breeders at 17% O_2 level. The process occurring within tissues may be an adaptive response to oxygen deprivation or low O_2 levels and that triggers hypoxia-induced neovascularization (Michiels, 2004). Abdollahi et al. (2011) also discussed how micro-environmental conditions such as hypoxia regulate stem cell differentiation, and this could be pertinent to the neovascular response observed in heart and lung tissues. Hypoxia has been reported to influence the development (morphological and physiological) of chick embryos and their effects may depend on the timing of their application during incubation (Altimiras and Phu, 2000; Chan and Burggren, 2005; Onagbesan et al., 2007) since various organs develop and mature at different stages of embryo development.

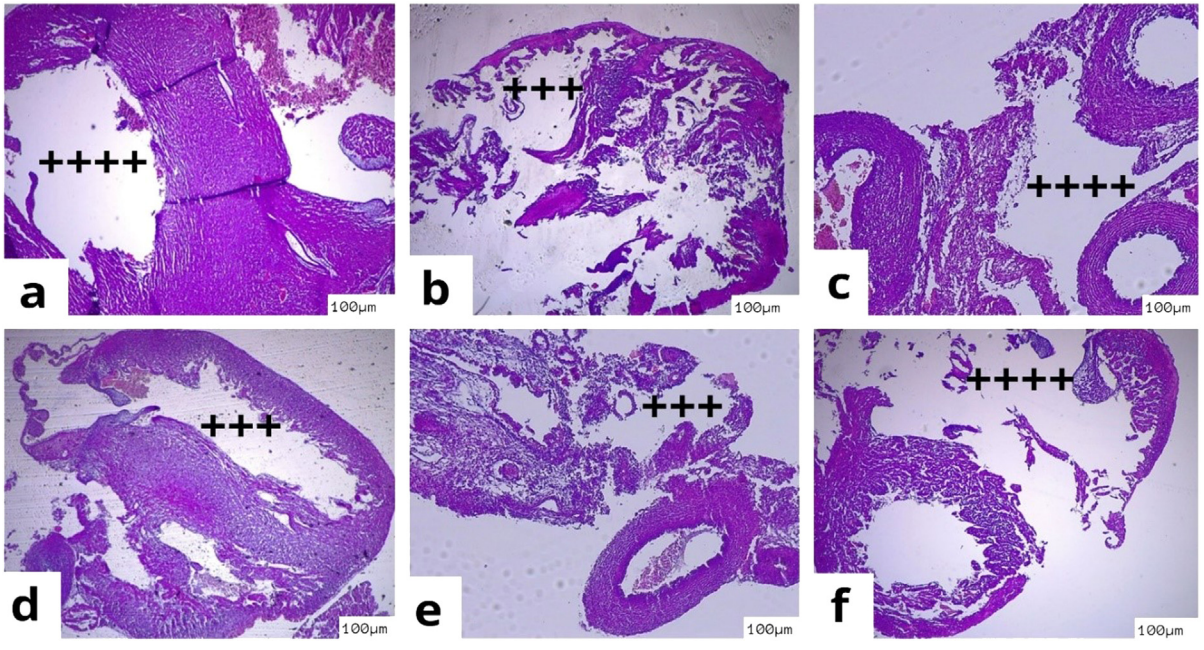

Figure 3. Histology of the heart of chicks showing neo-vascularization of embryos exposed to reduced oxygen concentration level from ED 7- 9. ^{abcdef}: Image of breeder age * oxygen concentration level (A_b * O_2); a: 33 wk * 15% O_2 (33 weeks breeder eggs incubated under 15% O_2); b: 33 wk * 17% O_2 (33 weeks breeder eggs incubated under 17% O_2), c: 33 wk * 21% O_2 (33 weeks breeder eggs incubated under 21% O_2), d: 50 wk * 15% O_2 (50 weeks breeder eggs incubated under 15% O_2), e: 50 wk * 17% O_2 (50 weeks breeder eggs incubated under 17% O_2), f: 50 wk * 21% O_2 (50 weeks breeder eggs incubated under 21% O_2). Neo-vascularization grading: ++++ = Very high (ectatic vessels with high congestion); +++ = high (vessels seat of moderate congestion); ++ = Normal (normal tissues).

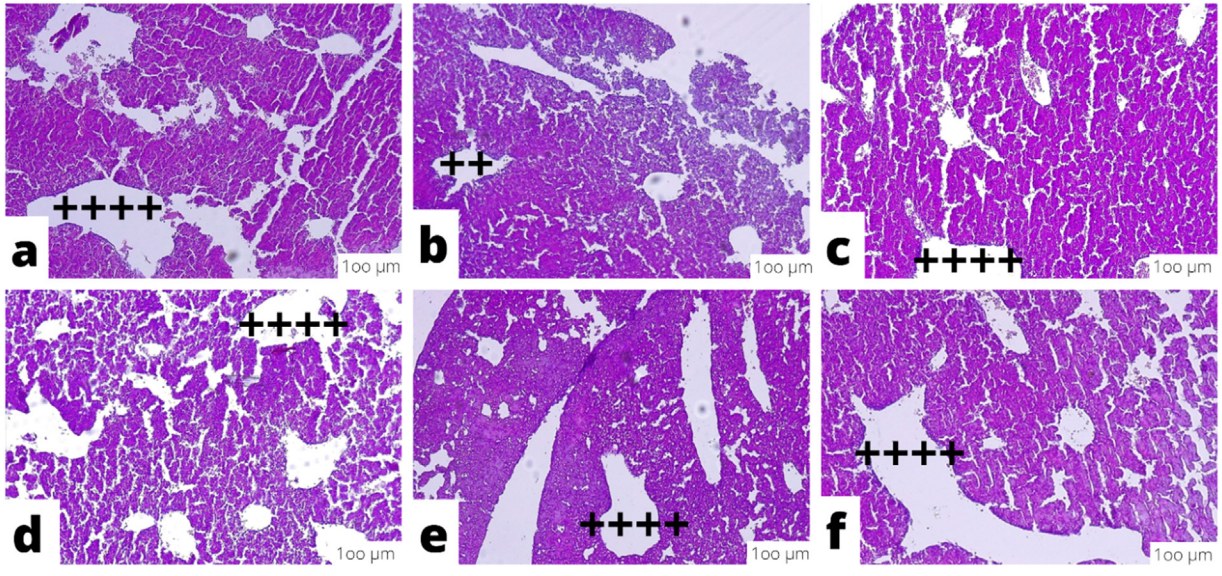

Figure 4. Histology of the lungs of chicks showing neo-vascularization of embryos exposed to reduced oxygen concentration level from ED 7- 9 ^{abcdef}: Image of breeder age * oxygen concentration level (A_b * O_2); a: 33 wk * 15% O_2 (33 weeks breeder eggs incubated under 15% O_2); b: 33 wk * 17% O_2 (33 weeks breeder eggs incubated under 17% O_2), c: 33 wk * 21% O_2 (33 weeks breeder eggs incubated under 21% O_2), d: 50 wk * 15% O_2 (50 weeks breeder eggs incubated under 15% O_2), e: 50 wk * 17% O_2 (50 weeks breeder eggs incubated under 17% O_2), f: 50 wk * 21% O_2 (50 weeks breeder eggs incubated under 21% O_2).

Neo-vascularization grading: ++++ = Very high (ectatic vessels with high congestion); +++ = high (vessels seat of moderate congestion); ++ = Normal (normal tissues).

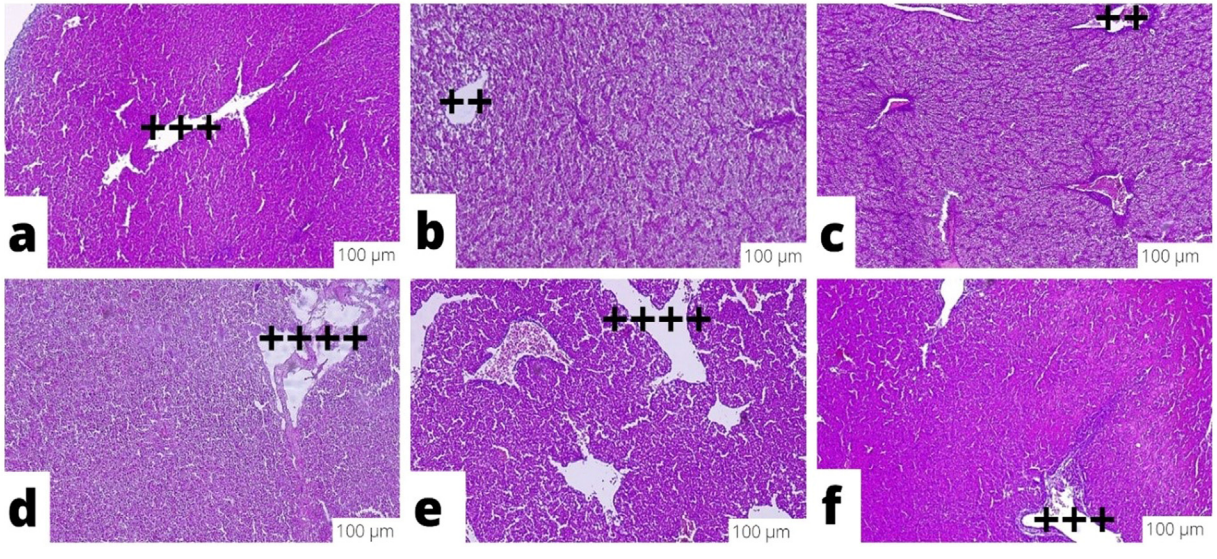

Figure 5. Histology of the liver of chicks showing neo-vascularization of embryos exposed to reduced oxygen concentration level from ED 7- 9. [abcdef]: Image of breeder age * oxygen concentration level (A_b * O_2); a: 33 wk * 15% O_2 (33 weeks breeder eggs incubated under 15% O_2); b: 33 wk * 17% O_2 (33 weeks breeder eggs incubated under 17% O_2), c: 33 wk * 21% O_2 (33 weeks breeder eggs incubated under 21% O_2), d: 50 wk * 15% O_2 (50 weeks breeder eggs incubated under 15% O_2), e: 50 wk * 17% O_2 (50 weeks breeder eggs incubated under 17% O_2), f: 50 wk * 21% O_2 (50 weeks breeder eggs incubated under 21% O_2).

Neo-vascularization grading: ++++ = Very high (ectatic vessels with high congestion); +++ = high (vessels seat of moderate congestion); ++ = Normal (normal tissues).

CONCLUSIONS AND APPLICATIONS

1. Early stimulation of embryos for 1 h daily from ED 7-9 (specifically on the 3rd hour of each day) with low O_2 (15% and 17%) during incubation did not affect the heart weight, however, a reduced growth rate was observed at hatch.
2. Liver weight increased in older breeders exposed to hypoxia compared to normoxia (control group).
3. Early mild hypoxic stimulation increases CAM vascularization, which is higher in 50 wk compared to 33 wk breeder flocks. Increased vascularization improves gas diffusion and blood transport abilities to embryonic organs as a form of an adaptive strategy.
4. In hypoxic-stimulated chicks at hatch, the combined effect of breeder age and O_2 level was associated with higher blood metabolite profiles indicating greater oxygen-carrying capacity of the blood metabolites.
5. High levels of ectatic vessel dilation and congestion are an adaptive response of the heart, lungs and liver to hypoxia.

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DISCLOSURES

We the authors (R K Agbehadzi, B Adjei-Mensah, P Sasu, A Bilalissi, C C Kpomasse, O N'nanle, J A Hamidu, K Tona) of the accompanying revised article, write to declare that there is no personal or professional conflict of interest with our work.

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