Reduced O_2 concentration during CAM development—Its effect on physiological parameters of broiler embryos[1]

S. Druyan,*[2] E. Levi,* D. Shinder,* and T. Stern†

*Institute of Animal Science, Agricultural Research Organization (ARO), the Volcani Center, PO Box 6, Bet Dagan 50250, Israel; and †Department of Molecular Genetics, Weizmann Institute of Science, PO Box 26, Rehovot 76100, Israel

ABSTRACT Embryo development is a dynamic process, determined by both the genetic background of the organism and the environment in which it develops. Environmental alterations during an organism's embryogenesis may induce changes in the development of some physiological regulatory systems, thereby causing permanent phenotypic changes in the embryo. The present study aimed to assess the effect of 17% O_2 concentration during chorioallantoic membrane (CAM) development on a) CAM development, b) cardiovascular parameters, and c) embryo development postexposure and up to hatch. Two replicated trials, each with 840 fertile Cobb eggs, were conducted. At embryonic d 5 (E5) eggs were divided into 2 treatments: 1) control, and 2) 17% O_2 concentration for 12 h/d from E5 through E12 (12H). The 12H embryos exhibited a clear and significant increase in the vascular area of the CAM, which grew to 6.8% larger than that of the control. Hematocrit and hemoglobin levels, as measured on E13 and E14, increased in response to the hypoxic treatments, but these differences were not maintained subsequently. Heart rate and relative heart weight were not affected by hypoxic exposure, but eggshell temperature in the 12H treatment was higher than that of the control, indicating higher heat production, which is consistent with the elevated plasma concentrations of triiodothyronine and thyroxin and with the enhanced oxygen consumption and residual yolk intake rate that followed exposure to hypoxic conditions. These findings indicate that embryos adapted to hypoxic condition enhance angiogenesis processes, which subsequently increase their blood oxygen-carrying capacity, enabling the increase of oxygen consumption, which positively affects their growth development and maturation compared with the control embryos. Such alterations may affect posthatch performance and the ability of broilers cardiovascular system to meet elevated oxygen demand.

Key words: incubation, hypoxia, angiogenesis, metabolic rate, broiler

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INTRODUCTION

Recent decades have seen significant progress in genetic selection of fast-growing broiler chickens, as reported by Havenstein et al. (1994, 2003), who compared broilers of 1957 with those of 1991 and 2001. This progress has been accompanied by a significant increase in metabolic rate that appears even during embryo development (Hulet and Meijerhof, 2001; Druyan, 2010).

Embryo development is a dynamic process, determined by both the genetic background of the organism and the environment in which it develops. Environmental alterations during embryogenesis may induce changes in the development of some physiological regulatory systems, thereby causing permanent phenotypic changes in the embryo. It has been reported that mammalian neonates exhibited an unusual ability to withstand hypoxia, which was manifested as lowered preferred ambient temperature, and that could be regarded as a natural adaptation (Singer, 1999, 2004; Mortola, 1999). In ectothermic organisms, Bagatto (2005) showed that hypoxia delayed the onset of all cardiovascular responses during development of the zebra fish, but also shifted their onsets relative to the rest of the developmental process.

Oxygen demand of the chick embryo gradually increases as tissues grow and differentiate. Embryogenesis is supported by 3 gas-exchange organ systems: the yolk sac, the chorioallantoic membrane (**CAM**), and the lungs. From approximately embryonic d 5 (**E5**) the CAM starts to develop, and from E8, it becomes the primary means of oxygen uptake (Tullett and Deem-

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[1]Contribution No. 571/11 from the Agricultural Research Organization, the Volcani Center, Bet Dagan 50520, Israel.
[2]Corresponding author: druyan@agri.huji.ac.il

ing, 1982), implementing gas exchange until E19 (Rahn and Paganelli, 1974). The CAM is an extremely rich vascular network, connected to the embryonic circulation by the allantoic arteries and veins. Development of the branching patterns of the vascular structures of the CAM occurs mainly between E5 and E11, and CAM gas-exchange efficiency reflects the growth of the chick embryo (Vico et al., 1998; Ribatti et al., 2001). Differences in angiogenesis of the vascular structures of the CAM may have a major effect on the metabolism of the embryo and, subsequently, on the embryo growth (Verhoelst et al., 2011). Changes in an embryo's metabolic rate may be analyzed directly by measuring oxygen consumption and indirectly by the main metabolic hormones thyroxine ($\mathbf{T_4}$) and triiodothyronine ($\mathbf{T_3}$) (Druyan, 2010). This can also be correlated to eggshell temperature (Piestun et al., 2009).

As embryogenesis progresses toward hatch, the capacity for oxygen diffusion across the CAM must increase to match the gradually increasing O_2 demands (Tazawa, 1978). In light of restrictions imposed by the relatively fixed conductance of the shell and the limited diffusion capacity of the CAM, it is expected that hypoxemia would develop. The embryo offers several potential adaptive responses to hypoxic challenge. They include: alteration in cardiac output and redistribution of oxygenated blood from the periphery to vital organs; elevation of blood oxygen-carrying capacity by means of polycythemia and angiogenesis; and modification of hemoglobin molecules.

It is well documented that prolonged exposure to a hypoxic environment enhances vasculogenesis and angiogenesis in embryos and young animals (Temple and Metcalfe, 1970; Adair et al., 1990). Various levels of hypoxia or hyperoxia at different time points during embryogenesis were found to affect both anatomical and physiological morphogenesis (e.g., Mulder et al., 1998; Villamor et al., 2004), according to timing, intensity, and duration of exposure. Dusseau and Hutchins (1988) reported that exposing chick embryos to 15% O_2 from E7 through E14 induced an increase in vascularity, causing the vascular density index to increase by 34 to 41%. In contrast, eggs incubated in a hyperoxic environment exhibited diminished development of large chorioallantoic vessels (Flemister and Cunningham, 1940), which might affect the size of the yolk sac and the embryo (Allen, 1963).

Differing incubation conditions during the prenatal and early postnatal periods might account for differences in hatchability, embryonic development, and metabolism (Christensen et al., 1993; Lourens et al., 2007); they often result in subtle changes in chick hatching time and posthatch performance.

Therefore, the present study aimed to assess the effects of 17% O_2 concentration during CAM development on a) CAM development itself; b) cardiovascular parameters; and c) embryo development, postexposure and until hatch.

MATERIALS AND METHODS

Experimental Design

All of the procedures in this study were carried out in accordance with the accepted ethical and welfare standards of the Israel Ethics Committee (IL-161/08).

Two replicated trials were performed, each with 840 fertile Cobb-strain broiler chicken (*Gallus domesticus*) eggs with an average weight of 64.0 ± 2.5 g that were obtained from a breeder flock of hens during their optimal period of egg production (35 wk old). All eggs were numbered and weighed individually before incubation. They were incubated in a Danki medium-size incubator for 2,500 eggs (Danki ApS, Ikast, Denmark) under standard incubation conditions of 37.8°C and 56% RH, and they were turned once per hour. At E5, eggs were randomly divided into 2 treatments: 1) control, and 2) O_2 concentration of 17% for 12 h/d from E5 through E12 (designated as 12H). Exposure to 17% O_2 was accomplished by transferring eggs of treatment 12H from the control incubator into a medium-size incubator (Danki ApS) equipped with a model 2BGA-SP-MA O_2 and CO_2 control system (Emproco Ltd., Ashkelon, Israel) for 12 h. The 2BGA-SP-MA control system includes an infrared CO_2 detector with a sensitivity range of 0 to 5 ± 0.01% and an electrochemical cell for oxygen measurement that covered 0 to 25 ± 0.1%. In response to the CO_2 sensor readings, an electronically controlled pump infused ambient air into the incubator to maintain the CO_2 concentration at the standard level of 0.03%. The O_2 sensor activated an electronically controlled pump that infused N_2 into the incubator to maintain the desired oxygen concentration of 17 ± 0.2%.

On E19, all eggs were transferred into hatching trays. Although the experiment was terminated immediately after hatch, BW and body temperature were measured, and blood samples were drawn from the jugular vein of 10 randomly selected chicks approximately 2 h after hatch (Yahav et al., 2004) for further analysis. In light of first-trial results, the vascular area of the CAM was determined in the second trial.

Quantification of CAM Blood Vessels

The CAM is probably the organ most widely subjected to in vivo assay for studying angiogenesis (Staton et al., 2004). Verhoelst et al. (2011) concluded that although various methods have already been developed to quantify angiogenesis in the CAM, they are not suitable for assessing overall vascular development in the CAM of chicken embryos under differing incubation conditions.

The CAM blood vessels were quantified with the CAM-era, version 1.0 computer software (T. Stern, unpublished report), which had been designed specifically for analyzing areas of images of CAM blood vessels to

assess changes in the overall vascular area of the CAM of chicken embryos incubated under differing environmental conditions; i.e., hypoxic and standard O_2 levels.

Sample Preparation

Ten fertile eggs from each treatment were killed on E6, E7, E9, E12, and E13. The eggs were placed in an 80°C water bath for 2 min, and then opened from the midpoint of the air sac to its antipodal point at the sharp end, so that yolk, albumen, and embryo could be removed carefully. Subsequently, the eggshells were moved to dry cabinets that were kept at low RH of 21%.

Verhoelst et al. (2011) found local differences in angiogenesis across the egg, which reflected development of the CAM: the vascular network in the CAM was significantly more developed at the blunt end than at the sharp end, where it was the last to develop (Romanoff, 1960). As one spot would not represent the whole CAM, photographic images were taken from different samples along the middle section of the egg equation.

Measurements

Camera. A Nikon SMZ-U zoom 1:10 binocular microscope (Nikon Inc., Tokyo, Japan) set at magnification ×0.75, connected to a model 60800 digital camera, and using Magna Fire 2.1 software (Optronics, Goleta, CA), was used to take pictures of the framed part of the CAM (50 mm^2) (Supplemental Figure 1; available in the online version of this paper).

Image Processing. The images were processed by implementing algorithms in Matlab software (The Mathworks Inc., Natick, MA). Briefly, the "CAM-era" algorithm was implemented by means of a Laplacian of Gaussian (LoG) filter for image segmentation, followed by a Graph Theoretic approach for modeling the segmented plexus (Supplemental Figure 2; available in the online version of this paper). The software enables extraction of the hierarchy of blood vessels, general parameters of the entire plexus (fractional area of blood vessels, total number of vessels, etcetera), as well as a collection of parameters pertaining to each individual vessel (length, width, number of branches, etcetera).

The images were registered as red-green-blue, and converted to a grayscale space. To reduce blurring by artifacts generated by inherent microscopy limitations, Lucy-Richardson deconvolution (Biggs, 1997) has been applied with a Gaussian point spread function. Then, a Laplacian-of-Gaussian filter with a sensitivity threshold of zero was applied to the resulting image to find closed contours on the edges of blood vessels as the basis for separating vessels from background. Application of this procedure to an image enables calculation of all aforementioned whole-plexus parameters.

Embryo Measurements

Eggshell Temperature. From E13 eggshell temperature ($\mathbf{T_{egg}}$) of 15 eggs (n = 15) was measured daily with a ThermoScan type 6022 infrared thermometer (Braun, Kronberg, Germany) (Leksrisompong et al., 2007; Piestun et al., 2009).

Heart Rate. From E13 onward, the heart rate (**HR**) of 15 embryos from each treatment was measured daily with a Buddy digital egg monitor (Avitronics, Torquay, UK). Use of infrared transmitters and sensors enabled amplification of the cardiovascular signal of an embryo within the egg by as much as 20,000 times, which enabled detection of the actual heartbeat of the embryo as early as 12 d after incubation started (Druyan, 2010).

Oxygen Consumption. To measure O_2 consumption of the embryos during incubation, on each day from E13 onward, 5 eggs from each treatment were placed in a small cylindrical metabolic chamber, measuring 7 × 7 cm in diameter and height, which was placed in a water container maintained at 37.8°C. Oxygen consumption was measured according to Buffenstein and Yahav (1991) and calculated by using the standard temperature and pressure dry method. Briefly, dried air was pumped at 50 mL/min into the metabolic chamber, which was fitted with a flow meter scaled from 0 to 60.56 mL/min (Aalborg Instruments and Controls, Orangeburg, NY). Dried air from the metabolic chamber was measured for oxygen partial pressure with a model S-3A/I oxygen analyzer (Ametek, Pittsburgh, PA), and O_2 consumption was measured continuously for 15 min, in light of previous studies (Druyan, 2010).

Blood Parameters. Each day from E13 through E19, approximately 0.5 mL of blood was drawn from the allantoic vein of 10 embryos per treatment into a heparinized syringe. After hatch, blood was sampled from the jugular vein. Whole blood samples were used to determine hematocrit and hemoglobin concentration, and plasma samples were used to determine plasma T_4 and T_3 concentrations.

Blood for hematocrit measurements was drawn into heparinized microcapillary tubes and centrifuged in a microliter centrifuge (Hettich, Tuttlingen, Germany) for 8 min at 4,000 × g at 4°C.

Hemoglobin concentration in whole blood samples was determined calorimetrically with a Hemoglobin Reagent Set (catalog no. H7504; Pointe Scientific, Canton, MI), according to the manufacturer's instructions.

Plasma samples were radio-immunoassayed for total T_4 and T_3 concentrations with a Coat-A-Count Canine T_4 RIA Kit (Diagnostic Products Corporation, Los Angeles, CA) and an RIA-gnost T_3 Kit (CIS Bio International, Gif sur Yvette, France), respectively. The intra-assay and inter-assay CV of the T_4 assay were 5.0 and 7.5%, respectively, and those of the T_3 assay were 7.8 and 8.2%, respectively.

Egg, Yolk, Embryo, Liver, Breast Muscle, and Heart Weights. Each day from the initiation of the second half of incubation (E13) until hatch, 10 eggs per treatment were killed. Yolk-free embryo weights were recorded with a Type E154 analytical scale (Gibertini, Novate, Italy), accurate to ± 0.1 mg, and were used to calculate relative embryo weight (percentage):

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

The yolk was separated from the embryo, amniotic fluid, and albumen, and the yolk was weighed. The yolk weight was used to calculate relative yolk weight (percentage):

$$\text{Relative yolk weight (\%)} = [(\text{yolk weight})/(\text{initial egg weight})] \times 100.$$

Liver, breast muscle, and heart were dissected and weighed, and their weights were used to calculate the relative organ weights, i.e., the ratio (percentage) of organ weight to embryo BW:

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

The resulting relative embryo weights and relative yolk weights were corrected by analysis of covariance with their initial egg weights.

Hatching Time. Every 2 h from 460 through 504 h of incubation, the eggs were screened for hatching, and chick emergence from each individual egg was recorded. These data were used to calculate hatching duration from the first egg to 100% hatch of viable embryos. The total incubation duration was calculated as the time between setting and emergence. Hatching percentage was calculated from numbers of hatched chicks and of fertile eggs.

Statistical Analysis. Although T_{egg} and HR were recorded as repeated measurements, they were analyzed in a single dependent-data file with the same GLM that was used to analyze the rest of the data. Comparison of incubation data between embryonic days of development within each incubation treatment (control or 12H) revealed significantly differing variances. The incubation data were subjected to ANOVA within each development day according to the following model:

$$Y = \mu + Treatment + Trial + Treatment \times Trial + e,$$

with *Treatment* (control or 12H) and *Trial* (1 or 2) as the main fixed effects, and where μ is the mean and e is random error. Interaction between *Treatment* and *Trial* was also included.

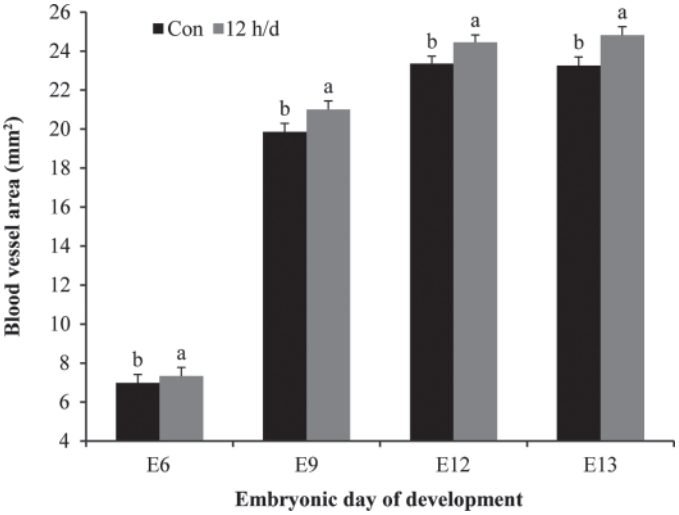

Figure 1. Chorioallantoic membrane blood vessel area of control (Con) and embryos incubated under 17% O_2 concentration for 12 h/d (n = 10) from E5 through E12. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

No significant differences between trials or significant interaction between treatment and trial were found; results are presented for the treatment least squares means and include both trials together. These statistical analyses were conducted with the JMP software of the SAS Institute (2005).

RESULTS

CAM Blood Vessels Quantification

The effects on the CAM vascular area of exposing embryos to an O_2 concentration of 17% for 12 h are depicted in Figure 1. Exposure for 12 h/d caused a significant increase in vascular area as early as E6, i.e., 1 d after beginning the exposure. From E7 onwards, the CAM vascular areas of the 12H and control embryos differed significantly, with the former being greater by 6.8, 5.3, 6.1, and 5.7% on days E7, E9, E12, and E13, respectively.

Embryo HR, Eggshell Temperature, and Oxygen Consumption

Figure 2 illustrates the changes in HR of the 2 incubation treatments between E13 and E19; there were no differences through E18. On E19, before internal pipping, an increase in HR was demonstrated in both treatments.

In both treatments, T_{egg} was above the incubation temperature (Figure 3), and T_{egg} of 12H embryos was significantly higher than that of the controls from E13 through E19.

Oxygen consumption gradually increased as embryogenesis progressed and leveled out on days E16 and E17

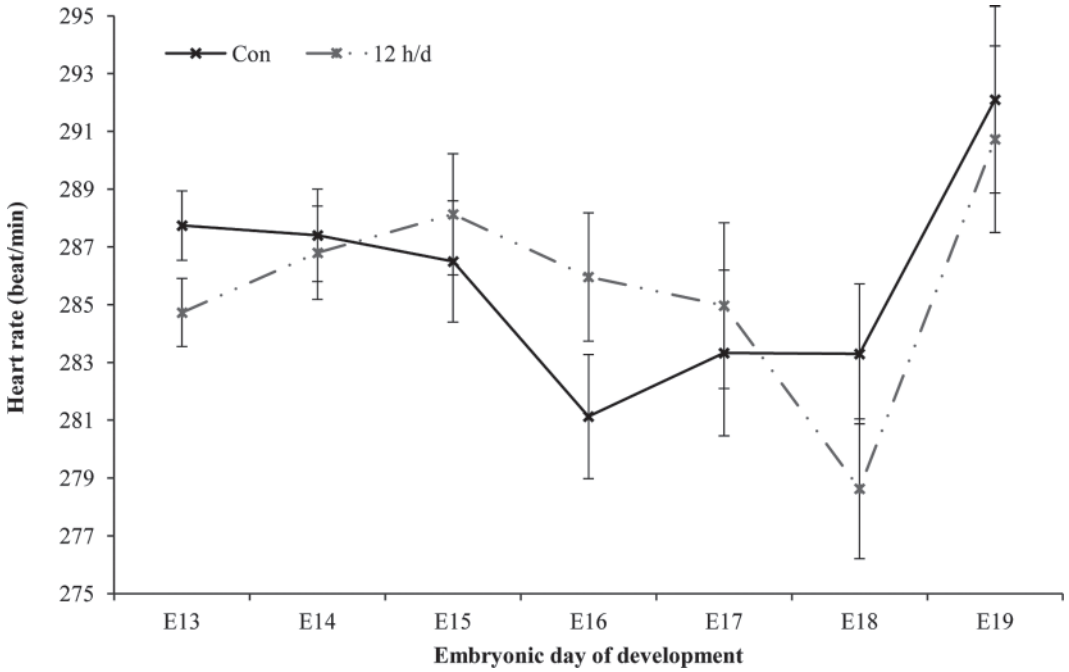

Figure 2. Heart rate of control (Con) and broiler embryos (n = 15) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through E19.

of the 12H and control treatments, respectively (Figure 4). Significantly higher O_2 consumption was observed in 12H than in control embryos on E14 and E16 (E14, $P = 0.01$; E16, $P = 0.046$); it was only higher on the other embryogenesis days. On E18 and E19, O_2 consumption was similar in both treatments, at approximately 0.34 mL/gh.

Blood Parameters and Hormones

The 12H embryos exhibited significantly higher hematocrit values than controls (Figure 5) on E13 ($P = 0.00009$), E14 ($P = 0.023$), and at hatch ($P = 0.042$).

Hemoglobin concentrations generally tended to be similar in the 2 treatments (Figure 6). On E13 and

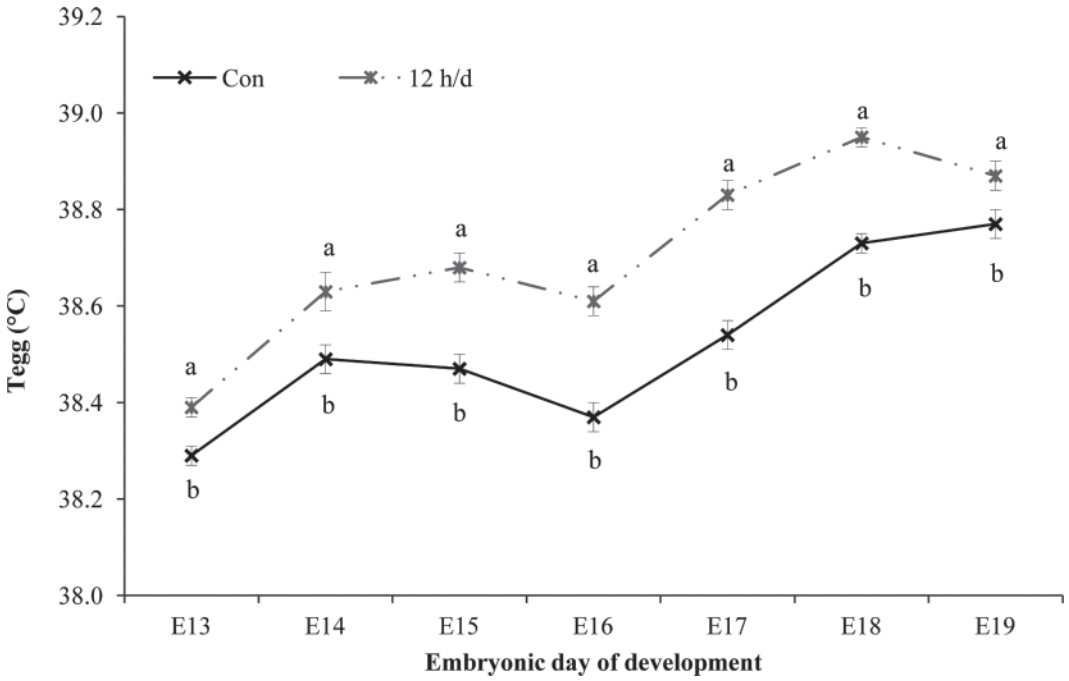

Figure 3. Eggshell temperature (Tegg; °C) of control (Con) and embryos (n = 15) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through E19. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

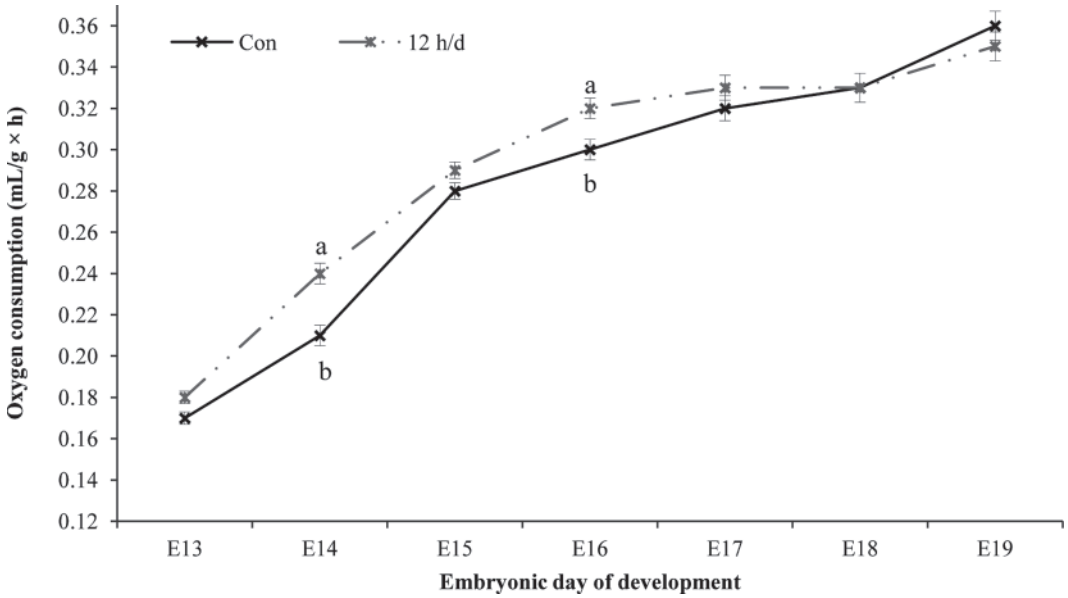

Figure 4. Oxygen consumption of control (Con) and embryos (n = 5) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through E19. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

E14, the hemoglobin concentration of the 12H embryos was significantly higher than that of the controls ($P = 0.043$ and $P = 0.035$, respectively).

In both treatments, plasma T_4 concentration increased during embryogenesis (Figure 7). During most of the period when plasma T_4 concentration was monitored (E13 onwards), it tended to be higher in the 12H embryos than in the control ones. After hatch, plasma T_4 concentrations were similar in both treatments.

Plasma T_3 concentration increased slightly from E13 throughE18 (Figure 8), and in both treatments, it increased dramatically on E19 and then declined. During most of the measurements, plasma T_3 concentration of the hypoxia-exposed embryos was higher to significantly higher than that of the control ones.

Embryo Development: Relative Weights of Body, Yolk, Breast Muscle, Liver, and Heart

Relative embryo weight (percentage of initial egg weight) is presented in Figure 9; it increased gradually as embryogenesis progressed. No differences were

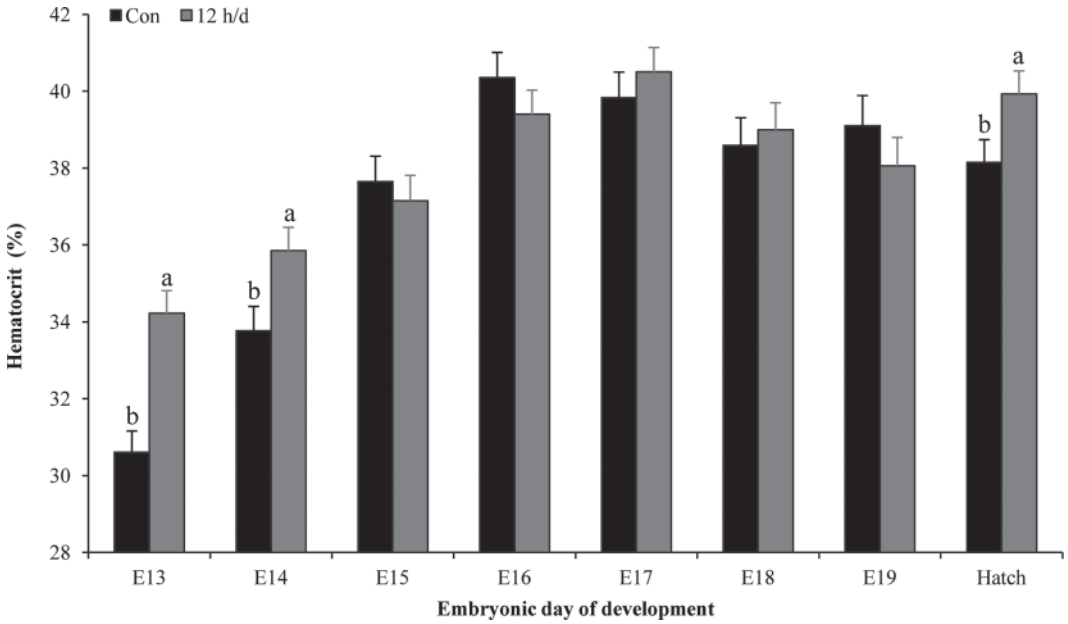

Figure 5. Hematocrit values (%) of control (Con) and embryos (n = 10) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through E19. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

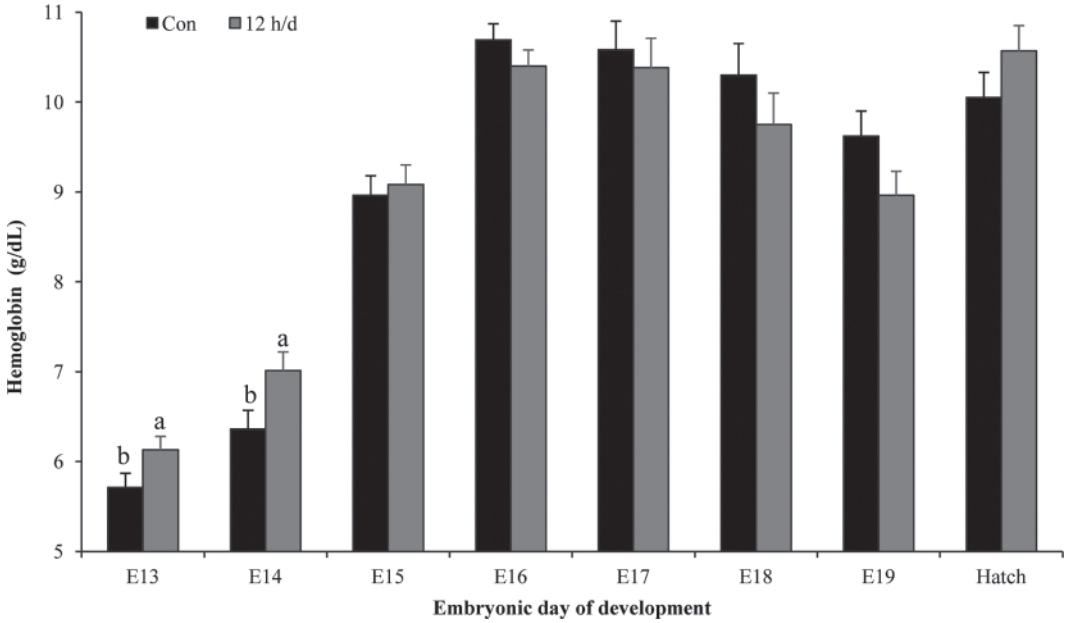

Figure 6. Hemoglobin concentration of control (Con) and embryos (n = 10) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through E19. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

found between the control and the 12H embryos from E13 (9.84 ± 0.18 and 10.06 ± 0.19 g for control and 12H embryos, respectively) onwards until hatch, either in relative embryo weight (60.12 ± 0.36 g or 59.96 ± 0.36% for control and 12H embryos, respectively) or in free embryo weight (38.56 ± 0.23 and 38.45 ± 0.22 g for control and 12H embryos, respectively).

Relative yolk weight (percentage of initial egg weight) decreased gradually as development progressed (Figure 10); it tended to be lower in 12H embryos than in controls from E13 with a 2.12 g difference between treatments (15.62 ± 0.36 and 13.50 ± 0.37 g for control and 12H embryos, respectively) through E18 (12.67 ± 0.37 and 12.70 ± 0.35 g for control and 12H embryos, respectively). On E13, E14, and E16, the difference in relative embryo weight between control and 12H embryos was found to be significant ($P = 0.0003$, 0.0088, and 0.05, respectively). Upon hatch, yolk rela-

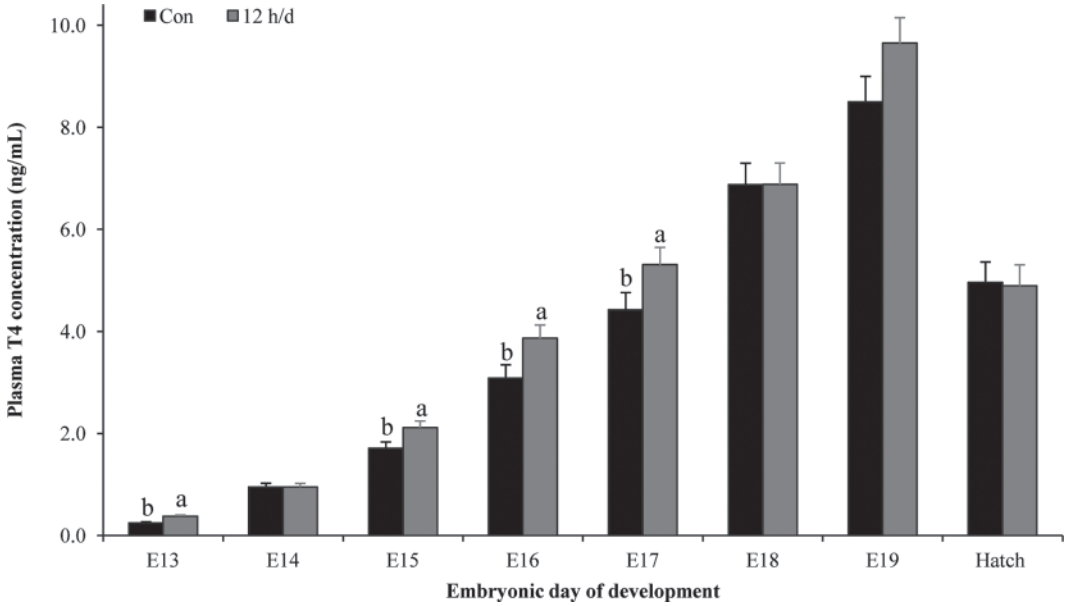

Figure 7. Plasma thyroxin (T_4) concentrations of control (Con) and embryos (n = 10) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through hatch. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

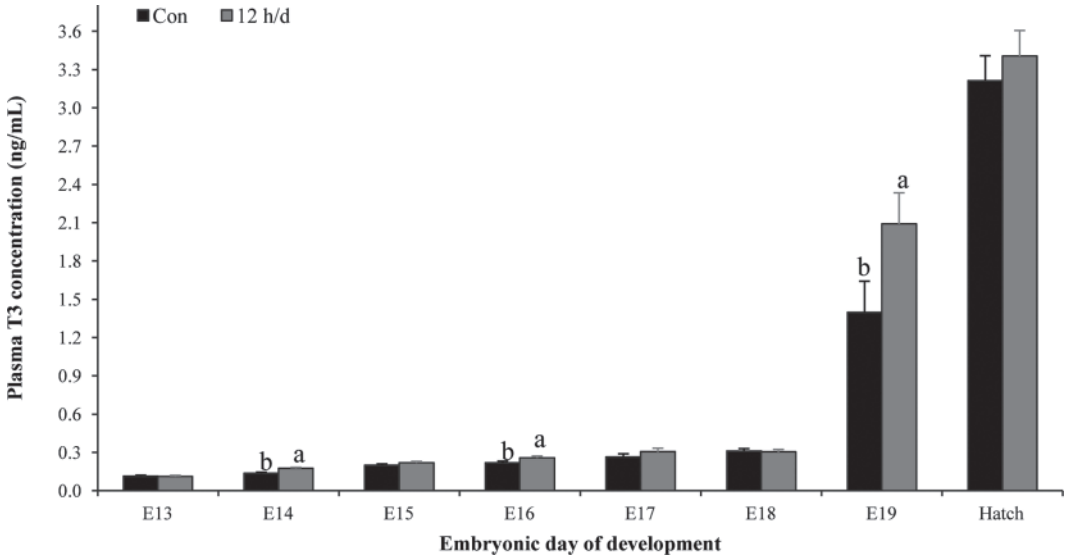

Figure 8. Plasma triiodothyronine (T_3) concentrations of control (Con) and embryos (n = 10) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through hatch. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.

tive weight (11.95 ± 0.33 and 11.89 ± 0.34% for control and 12H embryos, respectively) and yolk weight (7.65 ± 0.19 and 7.66 ± 0.19 g for control and 12H embryos, respectively) of 12H hatchlings were similar to that of the controls.

No differences between the 2 incubation treatments were recorded in liver, breast muscle, or heart relative weights at any developmental stage (data not shown). At hatch, breast weight was 1.87 ± 0.04 and 1.89 ± 0.04 g, liver weight was 0.92 ± 0.02 and 0.89 ± 0.01 g, and heart weight was 0.274 ± 0.006 and 0.277 ± 0.006 g for control and 12H embryos, respectively.

Hatch

There were no significant differences between incubation treatments in hatchability (93.8 and 93.5%) for control and 12H embryos, respectively. Mean incubation duration of 12H embryos was shorter than that of controls: 482.0 and 483.8 h, respectively ($P = 0.0131$).

DISCUSSION

This study demonstrated some of the physiological and endocrinological changes that occur in broiler em-

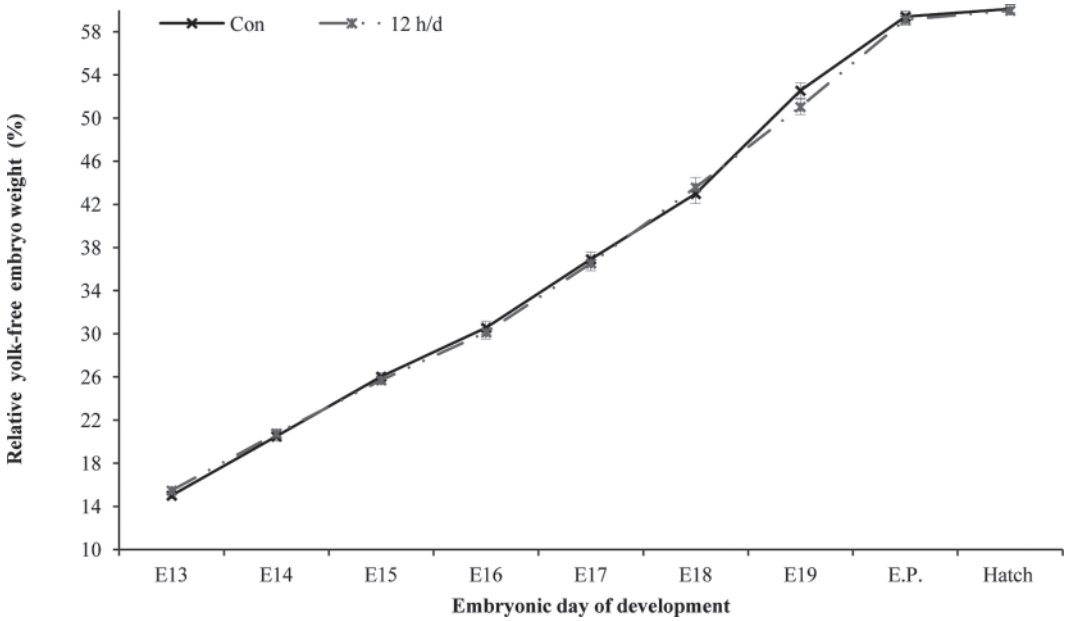

Figure 9. Yolk-free embryo relative weight (percentage of initial egg weight) of control (Con) and embryos (n = 10) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through hatch. E.P. = external pip.

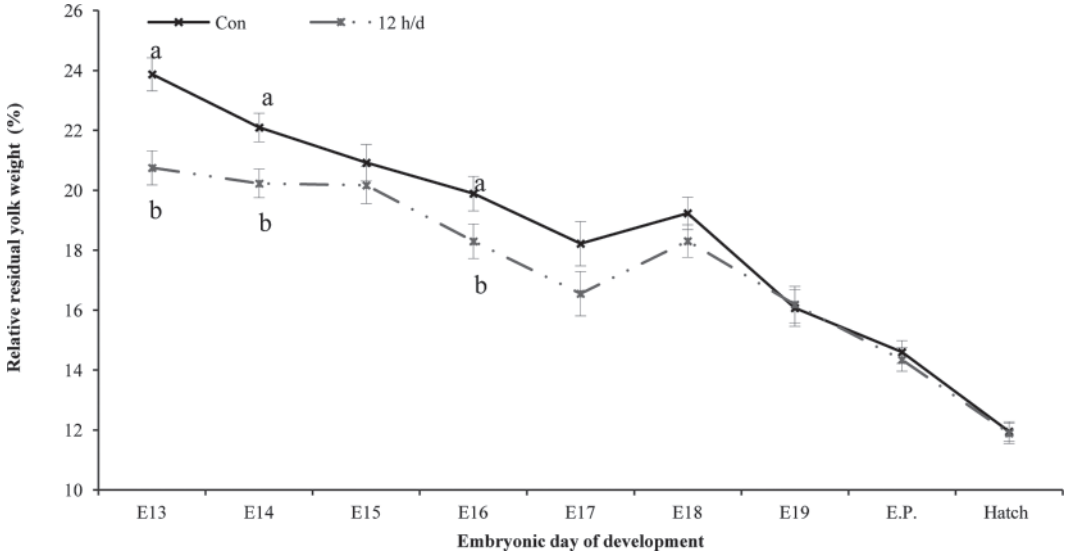

Figure 10. Residual yolk relative weight (percentage of initial egg weight) of control (Con) and embryos (n = 10) incubated under 17% oxygen from E5 through E12, but measured under standard incubation conditions from E13 through hatch. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments. E.P. = external pip.

bryos as a result of hypoxic exposure, that is, to 17% oxygen, for 12 h/d during CAM formation. In chicken embryos, general growth and metabolism are limited by oxygen availability. Alteration of oxygen partial pressure during embryogenesis was found to enhance or depress oxygen consumption (Tazawa et al., 1992) and posthatch growth rate (French and Tullett 1991). Bartels et al. (1966) observed that embryo blood oxygen-transport capacity and oxygen exchange capacity increased to meet increasing oxygen demands during embryogenesis under normal incubation conditions. This type of adaptation is especially important during the third week of development, when the CAM reaches its diffusion-limited capacity (Tazawa, 1980).

Changes in gas diffusion and transport capacity of the blood are expected to be more efficient for embryos that develop under hypoxic conditions. Faced with hypoxic challenge, the embryo has several potential adaptive responses, including alteration in cardiac output and redistribution of oxygenated blood from the periphery to vital organs, such as the brain, heart, and adrenal gland (Mulder et al., 1998). Other measures include increasing blood oxygen-carrying capacity by means of polycythemia (Dusseau and Hutchins, 1988), modification of hemoglobin (Liu et al., 2009), increasing vascularization (Dusseau and Hutchins, 1989), or any combination of these. The present study directly demonstrated the vascular response of the CAM following exposure to reduced environmental oxygen concentration, as described by Dusseau and Hutchins (1988). Yael et al. (2007) found an association between expressions of the matrix metalloproteinase gene (which is involved in angiogenesis) in the CAM and in the whole embryo, following exposure to elevated incubation temperatures. This finding suggested that by assessing changes in the overall vascular development of the CAM, one also could evaluate changes that showed similar trends in the chick embryo blood system.

Eggs incubated in a 17%-oxygen atmosphere for 12 h/d from E5 through E12 exhibited a clear and significant increase in the vascular area of the CAM, which was already evident within 12 h after the first and second hypoxic exposures; eventually this area grew to 6.8% greater than that of the control, and a 5.7% difference was maintained until E13.

The main reason for terminating measurements of the area of CAM blood vessels on E13 was based on previous findings of Dusseau and Hutchins (1988), who suggested that by E10, the time point at which CAM mitogenic activity is highest (Ausprunk et al., 1974), the angiogenic response was approaching its maximum rate, and that subsequently the CAM tissue responded passively to continued hypoxic stimulation. In general, during incubation, a gap of 32 to 36 h opened between the fastest- and slowest-developing broiler embryos, and this was reflected in our present decision to extend hypoxic stimulation through E12.

Baumann and Meuer (1992) found that hypoxia caused changes in hematocrit and hemoglobin levels to occur earlier. In the present study, both parameters, as measured on E13 and E14, increased in response to the hypoxic treatments, but no differences between their values in the hypoxic and control treatments were maintained thereafter, except at hatch, when hematocrit of the treated embryos was significantly higher than that of the controls. It can be concluded, therefore, that the effect of hypoxia on these parameters persisted through 2 d after exposure, and thereafter the cardiovascular system could supply the oxygen needs of the embryo efficiently. Exposure to hypoxia was found to increase heart mass of domestic chicken and Canadian goose hatchlings (Black and Snyder, 1980; Snyder

et al., 1982), which led to ventricular hypertrophy and increased heart mass (Burggren and Keller, 1997). Furthermore, hypoxia caused increased HR, stroke volume, or both, which led, in turn, to increased blood circulation, and thereby imposed changes in oxygen transport (Ar et al., 1991). However, in the present study, only HR and relative heart weight were measured, and neither was affected by hypoxic exposure. Collectively, the cardiovascular response to hypoxia may indicate that changes in angiogenesis may be sufficient to meet the embryos' oxygen demands following exposure to hypoxia, so that only slight changes, if any, in the cardiovascular system are needed. It seems that only under suboptimal environmental conditions will the cardiovascular system efficiently recruit red blood cells and increase hemoglobin concentration to meet the broiler's oxygen demands.

The thermoregulatory response is mediated mainly by the level of metabolism induced or permitted by the thyroid hormones axis (T4, T3), which affects body temperature. Eggshell temperature is well known to be an accurate reflection of the embryo's body temperature (Leksrisompong et al., 2007). The temperature of the embryo is considered to be affected by air temperature and the exchange of heat between the egg and its microenvironment (Van Brecht et al., 2005), which depends on the embryo's levels of heat production and heat loss, whereas heat loss in commercial incubators is directly related to convection (Van Brecht et al., 2003). In the present study, T_{egg} in the 12H treatment was higher than that in the control, indicating higher heat production, which is consistent with the elevated plasma T_3 and T_4 concentrations and with the enhanced oxygen consumption that followed exposure to hypoxic conditions.

van Golde et al. (1998) showed that as early as the middle of the incubation period, and well before metabolic demand exceeded the limitation of oxygen diffusion capacity of the eggshell, O_2 availability could be a limiting factor for growth. At E13 in the present study, the embryos' atmosphere was shifted back to the regular 20.94% O_2, and consequently, the 12H embryos that had enhanced hematocrit and hemoglobin concentration could have been enriched with O_2, which would have affected their oxygen consumption rate and residual yolk intake rate. In this study, yolk relative weight of 12H embryos was significantly lower on E13, E14, and E16 than that of controls. These results were consistent with the findings of Molenaar et al. (2010), who reported that embryos incubated under low-O_2 conditions converted more residual yolk after they were shifted back to normal O_2 conditions.

Although many studies, for example, Chan and Burggren (2005), had found that hypoxic exposure during incubation could affect embryo weight, our present results indicate that a daily 12-h exposure to 17% O_2 affected embryo growth to a lesser extent than previously indicated: 12H and control embryos had similar relative weights from E13 through hatch. These findings suggest that embryos that adapted to a hypoxic condition increased their oxygen consumption capacity and thereby enabled their growth, development, and maturation to proceed as well as those of control embryos.

It has been demonstrated in the chicken that the time of external pipping advances in response to decreased oxygen content and also to increased CO_2 content in the air sac (Visschedijk, 1968; Everaert et al., 2010). In the present study, 12H embryos had shorter incubation times by 1 to 2 h than controls, which suggests that as a result of their higher metabolic rate, the former embryos reached their oxygen diffusion-capacity limitation earlier than the controls.

The actual effects of hypoxia on embryo development depend on the critical period, hypoxia level, and duration of exposure. In the present study, the hypoxic embryos' metabolism increased following exposure to 17% O_2 for 12 h/d during CAM development. Possible explanations include increased oxygen-carrying capacity that enabled these embryos to use more yolk and thereby to grow as well as embryos incubated under standard conditions. The present findings indicate the possibility of enhanced angiogenesis, which would facilitate a sufficient supply of oxygen to the embryo, with fewer changes, if any, in hemoglobin level and hematocrit, parameters that reflect oxygen-carrying capacity. Such alterations may affect posthatch performance and the ability of a broiler's cardiovascular system to meet elevated oxygen demand. However, further research is needed that should focus on fine-tuning the exposure.

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