Hypoxia during incubation and its effects on broiler's embryonic development

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ABSTRACT In all vertebrates, hypoxia plays an important role in fetal development, driving vasculogenesis, angiogenesis, hematopoiesis, and chondrogenesis. Therefore, the ability to sense and respond to changes in the availability of oxygen ($\mathbf{O_2}$) is crucial for normal embryonic development as well as for developmental plasticity. Moderate levels of hypoxia trigger a regulated process which leads to adaptive responses. Regulation of angiogenesis by hypoxia is an important component of homeostatic control mechanisms that link the cardio-pulmonary-vascular O_2 supply to metabolic demands in local tissues. Hypoxia leads to the activation of genes that are important for cell and tissue adaptation to low O_2 conditions, such as hypoxia-inducible factor 1. Previous studies have shown a dose-response effect to hypoxia in chicken embryos, with lower and/or prolonged O_2 levels affecting multiple mechanisms and providing a spectrum of responses that facilitate the ability to maintain O_2 demand despite environmental hypoxia. In chicken embryos, mild to extreme hypoxia during embryogenesis improves chorioallantoic membrane and cardiovascular development, resulting in an increase in O_2 carrying capacity and leading to developmental plasticity that may affect post-hatch chick performance and improve adaptation to additional environmental stresses at suboptimal environmental conditions.

Key words: chicken, embryo, hypoxia, oxygen, embryogenesis, CAM

2021 Poultry Science 100:100951
https://doi.org/10.1016/j.psj.2020.12.048

INTRODUCTION

Hypoxia is a lifelong environmental challenge for species in a variety of ecological niches, including during embryogenesis in the uterus or eggs. Over time, it has acted as a strong selective force that has driven the evolution of physiological and cellular traits that enhance tolerance to low oxygen ($\mathbf{O_2}$) stress. These systems detect changes in environmental O_2 availability and respond by increasing the O_2 carrying capacity and supply to the tissues or by decreasing O_2 demand at the cellular and molecular level. More specifically, this adaptation is based on chemosensors that detect changes in O_2, carbon dioxide ($\mathbf{CO_2}$), and pH, which subsequently triggers changes in respiratory ventilation and cardiac output. These changes alter the rate of O_2 delivery and CO_2 clearance, directly impacting on cellular and systemic metabolism and the metabolic demands of tissues. Such adaptive responses are neurally and hormonally controlled at the medulla oblongata and the hypothalamus of the brain. Hypoxia is a normal part of fetal life in all vertebrates. It plays a requisite role in development, driving vasculogenesis, angiogenesis, hematopoiesis, and chondrogenesis. However, episodes of more severe hypoxia can lead to developmental abnormalities or embryonic death (Grabowski, 1966; Webster et al., 1996). Experimental studies have also implicated hypoxia during late organogenesis and the early fetal period as causing growth retardation and programming of adult-onset diseases such as hypertension (Peyronnet et al., 2002; Alexander et al., 2005; Eckardt et al., 2005; Alexander, 2006; Bourque et al., 2013; Rook et al., 2014). Therefore, the ability of an embryo to regulate its tissue O_2 requirements and responses to altered availability of O_2 is crucial for normal development as well as for developmental plasticity. This is achieved either through postnatal adaptation or acclimation or by ways of phenotypic or developmental plasticity. This review will elucidate the important conditions and mechanisms that drive physiological and cellular responses that enhance tolerance to low O_2 partial-pressure ($\mathbf{ppO_2}$) stress.

Received April 5, 2020.
Accepted December 15, 2020.

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Developmental Plasticity Responses to Hypoxia

Acclimation involves an array of autonomically controlled physiological mechanisms working in concert to enhance tolerance to environmental changes. Its expansion of the dynamic hypoxic range of an animal involves concerted procedures at all levels of the body, which induce a shift in the level of homeostasis so as to efficiently improve hypoxia tolerance under the new environmental conditions. In contrast, phenotypic plasticity is the phenomenon of a genotype producing different phenotypes in response to different environmental conditions (Ghalambor et al., 2007). Phenotypic plasticity in gas or ion exchange structures manifests as morphological changes in the lungs of birds and mammals at high altitude (Storz et al., 2010) or as a response to pulmonary artery hypertension (Sakao et al., 2010). Similarly, the gills of adult fish are rather morphologically plastic, and can be remodeled in response to environmental toxins or hypoxia (Sollid and Nilsson, 2006).

Phenotypic plasticity is also a phenomenon highly relevant to developmental physiology. The term developmental plasticity has been used to describe long-standing or permanent alterations of the developmental trajectories of physiological regulatory systems in immature animals (Spicer and Burggren, 2003; Carroll, 2003). These occur during "critical periods" described as developmental time windows devoted to structural and/or functional shaping of the physiological/neurological control systems. During the critical period, environmental changes can disrupt and alter the developmental trajectory, whereas the same event occurring outside of the window has little or no effect and, in some cases, even a negative effect. Studies of the avian embryo, a highly developed model of early cardiovascular development, have yielded protocols for determining the critical windows of cardiorespiratory development (Dzialowski et al., 2002; Chan and Burggren, 2005). Development of the chorioallantoic membrane (**CAM**), skeletal structures, and the eye have all shown variable timing and length of critical windows, as evident from their developmental vulnerability to chronic hypoxia (15% O_2) (Chan and Burggren, 2005).

Embryonic Hypoxic Responses

A continuous O_2 supply adequate to support the metabolic outputs of the tissue is essential for proper tissue function, development, and homeostasis. The oxygenic balance can be disturbed by rapid cellular division during embryonic development, tumor growth, or vasculature dysfunction due to vessel occlusion or rupture (Zimna and Kurpisz, 2015). In the case of hypoxia, the changes can affect both metabolism and O_2 transport, including alteration of cardiac output and redistribution of oxygenated blood from the periphery to vital organs (Mulder et al., 1998). In the case of increased cardiac output, there is an increase in the amount of O_2 delivered by the blood, which can be achieved by increasing the heart rate and/or stroke volume. Increased blood O_2 carrying capacity can be achieved by various means: polycythemia (Dusseau and Hutchins, 1988), modification of hemoglobin (Liu et al., 2009), increased vascularization, and angiogenesis (Dusseau and Hutchins, 1989), or combinations of these.

These adaptive responses result in more efficient consumption of residual O_2. Reduced O_2 consumption is a very common response of all classes of animals to prolonged hypoxic conditions. In mammals and birds, it is mostly found in the neonatal stage, and has been suggested to be a regulated process, rather than a passive response to the decrease in O_2 availability (Mortola, 2001).

Hypoxia and the Vascular System

It is widely accepted that blood vessels arise during development by 2 mechanisms, that is, vasculogenesis and angiogenesis (Risau, 1997). Vasculogenesis is an embryonic process that involves the de novo formation of endothelial cells and their organization into vascular channels. O_2 tension plays a crucial role in vasculogenesis (Semenza, 2014). During the first stage of embryogenesis, before development of the circulatory system, the O_2 pressure is relatively low and does not exceed 3% (Mitchell and Yochim, 1968). The developing embryo requires an increase in O_2 levels which leads to the formation of primary vessels, including the dorsal aorta and the early vascular network in the yolk sac (Risau and Lemmon, 1988). Angiogenesis entails new vessel formation from preexisting vessels, capillaries, and postcapillary venules (Ribatti et al., 2001a). The combination of these processes lays the foundation for networks of bona fide venules, veins, arteries, and arterioles (Maltepe and Celeste Simon, 1998).

The principal cells involved in these processes are endothelial cells, which line all blood vessels and constitute the entirety of capillaries. They escape their original location by breaking through the basement membrane, and then migrate toward an angiogenic stimulus. During migration, they proliferate and subsequently reorganize into 3-dimensional tubular structures (Auerbach et al., 2003). Different factors affect each stage of the processes. For example, intussusceptive angiogenesis will follow higher levels of shear stress, whereas sprouting is stimulated by angiogenic factors released from the tissue in response to hypoxia (Styp-Rekowska et al., 2011).

Molecular Mechanisms That Affect Vascular Development

The cellular response to hypoxia is largely dependent on changes in gene expression, which are mainly commanded by a unique family of transcription factors named hypoxia-inducible factors (**HIF**) (Bunn and Poyton, 1996; Semenza, 2001). HIF-1 is a widely expressed heterodimeric protein composed of the HIF-1α subunit bound to the aryl hydrocarbon nuclear translocator, both of which belong to the basic helix-loop-

helix PAS (per-ARNT-sim) family (Wang and Semenza, 1995; Safran and Kaelin, 2003). HIF-1α generally rapidly degrades in the presence of O_2, but as oxygen tension drops below 5% (35 mmHg), its degradation is slowed, and its DNA binding capacity steadily increases (Jiang et al., 1996). Following hypoxia-induced nuclear translocation and dimerization, HIF binds to the hypoxia-response element of target genes, which has the core consensus sequence 50-CGTG-30 (Rajakumar and Conrad, 2000).

HIF activity is an absolute requirement for the normal development of the heart, neural crest migration (Dunwoodie, 2009), vasculature, and hematopoiesis (Ramírez-Bergeron and Simon, 2004; Ramírez-Bergeron et al., 2006). Knockout mice lacking HIF-1 showed arrested development at gestational day 9 and died by gestational day 10.5 (Iyer et al., 1998), with abnormalities of the heart and cephalic blood vessels.

In chick embryos, HIF-1α and HIF-1β transcription levels were found to be high under normoxic conditions during the first days of embryogenesis. Druyan et al. (2007) documented the presence of HIF-1α, heme oxygenase, and hypoxia upregulated protein 1 (**HYOU1**), in chick hearts during in ovo development. All 3 genes were stimulated by hypoxia and expressed at embryonic E7. A significant decline in their expression was noted at E15, with a further decrease at E19, due to an increase in embryo oxygen consumption. These studies underscore the importance of a precise hypoxic window for the formation and development of vascular networks.

Cross talk between HIF-1 and proangiogenic factors is a fundamental element driving capillary formation under hypoxia. One of the earliest described angiogenic factors regulated by HIF-1 is vascular endothelial growth factor (**VEGF**) (Shweiki et al., 1992), and angiogenesis initiated by hypoxia and by HIF-1 is often VEGF dependent. VEGF is known to promote endothelial cell proliferation and migration, increase permeability of blood vessels and vascular remodeling, and drive sprouting angiogenesis (Fong, 2008; Fantin et al., 2013). Nanka et al. (2006) found that angiogenesis resulted from a cascade that involved tissue hypoxia, paralleled by increased expression of HIF-1 factors, followed by VEGF expression. The newly formed vessels were oriented toward the regions of increased VEGF expression.

VEGF activity induces the expression of fms-related tyrosine kinase (Flt-1) and VEGF receptor 2 (**VEGFR2**), also known as kinase insert domain receptor (Terman et al., 1992; Yamaguchi et al., 1993). During vascularization of the developing retina, the leading endothelial cell expresses VEGFR2, which then prompts VEGF-A reactivity and guides migration of the cells in the sprout process (Gerhardt et al., 2003). VEGFR2 expression was found to be regulated by hypoxia independently of VEGF-A (Tuder et al., 1995; Brogi et al., 1996; Elvert et al., 2003).

In cases of disrupted O_2 balance, VEGF will bind its receptors and stimulate capillary outgrowth. At the same time, VEGF directly (by binding to hypoxia-response elements) or indirectly (cascade effect) (Pugh and Ratcliffe, 2003) enhances the expression of other proangiogenic factors such as placental growth factor and fibroblast growth factor (**FGF**). Hypoxia could suppress glycolysis in turn resulting in altered energy metabolism. Interestingly, glycolysis level and environmental pH recently were found to play a role in the regulation of FGF/WNT signaling, which is critical in axis elongation in the developing embryo (Sparrow et al., 2012; Oginuma et al., 2017).

In birds FGF2 was found to induce vasculogenesis in blastodisc-derived embryoid bodies of quail, and in dissociated epiblasts (Flamme and Risau, 1992; Krah et al., 1994). Nanka et al. (2008) found no hypoxia-induced changes in the protein expression of FGF2 in either the myocardium or other parts of the embryo. Aside from VEGF, other factors participate in angiogenesis, including platelet-derived growth factor, angiopoietins 1 and 2, and metalloproteinases (Papetti and Herman, 2002; Presta et al., 2005).

Chick Embryo CAM as an Experimental Model for the Study of Angiogenesis

Several in vivo and in vitro assays have been developed to study vasculogenesis and angiogenesis (Obeso and Auerbach, 1984; Madri et al., 1988; Pasqualini et al., 2000). Evidence has shown that the CAM model is best suited for the quantification of angiogenesis (Ribatti et al., 2001b; Ribatti and Tamma, 2019). The tissue composition and accessibility of the CAM for experimental manipulation make it an attractive preclinical in vivo model for drug screening and/or for studies of vascular growth (Nowak-Sliwinska et al., 2014).

The CAM is of physiological importance to the embryo because it functions as an external respiratory organ for gaseous exchange until hatching, and serves as a bladder into which waste products can be secreted (Hamilton, 1965). In chickens, the embryological origin of the CAM is the allantois, appearing at about 3.5 d of incubation (E3.5) as an evagination from the ventral wall of the endodermal hind gut. During E4, it pushes out of the embryo body into the extraembryonic coelom. Between E4 and E10, the allantoic vesicle enlarges very rapidly. The CAM is formed from a fusion of the mesodermal layer of the allantois with the adjacent mesodermal layer of the chorion. An extremely rich vascular network develops in the CAM, connected to the embryonic circulation by the allantoic arteries and veins (Patten, 1951).

Morphologically, on E4, all CAM vessels are undifferentiated capillaries characterized by a single layer of endothelial cells lacking a basal lamina. By E8, a thin-walled capillary appears, with its lumen fully wrapped by basal lamina. From E10 to E12, the mesodermal vessels are distinct arterioles and venules (Ausprunk et al., 1974).

Differences in angiogenesis of the vascular structures of the CAM may have a major impact on the metabolism

of the embryo and, subsequently, on its growth and health (Verhoelst et al., 2011). A variety of compounds have been reported to stimulate and inhibit angiogenesis in the CAM among them, growth factors, hormones, natural molecules, proangiogenic molecules, and gasses (Ribatti, 2016). An angiogenic response will cause stimulation in the form of an increased vessel density, while an antistatic compound will decrease density; the vessels become less dense and eventually disappear.

Hypoxia was found to have selective effects on CAM growth (Azzam and Mortola, 2007). While hypoxia depressed the CAM weight (Burton and Palmer, 1992) when applied chronically throughout the duration of incubation, hypoxic exposure in early or mid-development did not affect CAM weight, and late or continuous hypoxia markedly increased its weight (Chan and Burggren, 2005).

Blood Oxygen Transport in the Chick Embryo

Development of the avian embryo is supported by 3 gas-exchange organ systems: the yolk sac, CAM, and the lungs. During the first week of embryonic development, the vascularized portion of the yolk sac is the principal gas-exchange organ (Baumann and Meuer, 1992). O_2 is carried by primitive red blood cells (**RBC**) generated in primary yolk sac erythropoiesis (Brown and Ingram, 1974; Moorman et al., 1987). These primitive RBC lack a stem cell compartment and their final number is determined by the number of cells initially committed to the erythroid pathway. The cells contain embryonic hemoglobins, which have a higher O_2 affinity than adult hemoglobins; however, their main function is not O_2 transport but, rather, the creation and maintenance of an adequate O_2 pressure gradient inside the embryo (Baumann and Meuer, 1992). The undifferentiated nature of the primitive RBC, their limited number, and the low hemoglobin concentration in the blood, in combination with the developing embryo's metabolic demands, may cause tissue hypoxia (Druyan et al., 2007). At approximately E8, the allantoic sac develops and fuses with the chorion to create the CAM (Wangensteen and Rahn, 1970). This highly vascularized structure, in conjunction with the porosity of the eggshell, enables diffusion of O_2 and CO_2 between the environment and the blood (Tullett and Deeming, 1982). As incubation and embryo development progress into the third week, O_2 consumption increases; however, the O_2 supply is restricted by the fixed conductance of the shell and the limited diffusion capacity of the CAM. Thus, hypoxic conditions develop, leading to hypoxia-regulated gene expression (Druyan et al., 2007). Subsequently, a complex series of changes in the total number of circulating blood cells (Tazawa, 1980), their type, and hemoglobin type (Chapman and Tobin, 1979), as well as changes in the concentration of RBC metabolites that affect the O_2 affinity of hemoglobin (Baumann et al., 1983), lead to a continuous increase in blood O_2 affinity in the embryo (Bartels et al., 1966). Toward the end of incubation, at approximately E19, the embryo pierces the air sac membrane with its beak ("internal pipping") and after approximately 24 h (Dawes, 1981; Burton and Tullett, 1985), it begins to rupture the eggshell ("external pipping"). Once the embryo internally pips the air sac, the respiratory system shifts from diffusive gas exchange through the CAM to convective gas exchange via the lungs (Ar et al., 1980). During this period, hypoxia and hypercapnia gradually develop due to the decline in the allantois gas exchange. The CAM O_2 exchange becomes the primary issue rather than CO_2, owing to the fact that the CAM resistance to CO_2 flow is about half of that to O_2 (Piiper et al., 1980). Fifteen hours before hatching, there is a decline in allantois gas exchange and regression of the CAM (Visschedijk, 1968) and any additional metabolic requirement by the embryo must be met exclusively by the lungs, thus limiting the O_2 supply (Ar and Rahn, 1985; Menna and Mortola, 2002).

This mismatch between the increasing metabolic rate of the embryo and the reduction in the gas-transfer capabilities of the CAM is considered a key parameter in the timing of the hatching process (Pettit and Whittow, 1982a). As the embryo develops, the restrictions imposed by the fixed gas conductance of the shell and the limited diffusion capacity of the CAM result in increasing diffusion gradients for O_2 and CO_2 (Pettit and Whittow, 1982b). Eventually, the embryo oxygen demands exceed the CAM diffusion capacities, inducing hypoxia and acidosis. The time of external pipping in the chicken is subject to the level of O_2 and CO_2 in the air space (Visschedijk, 1968).

Hypoxic Environment and the Chick Embryo

Hypoxia is a known stressor affecting normal ontogeny. In a comprehensive series of experiments, Grabowski (1964, 1966) studied the effects of hypoxia on the early developing chick embryo, and demonstrated characteristic dose and duration responses, as well as differential gestational stage sensitivity to hypoxia. Severe hypoxia invariably induced embryonic death. Moderate hypoxia was less fatal, with approximately equal percentages of normal, malformed, and dead embryos.

The embryo is quite capable of recovering from an acute brief hypoxic episode, even if severe (Mortola, 2011); however, the chances of surviving prolonged hypoxia are slim especially at the beginning and end of incubation (Lundy, 1969). During sustained hypoxia, the erythropoietic response of the avian embryo is very modest, if at all present (Mortola, 2009). The reduction in O_2 consumption during prolonged hypoxia is a result of both the reduction in the cost of tissue maintenance and of the energy saved due to reduced body growth. The O_2 consumption of hypoxic embryos is lower than that of controls, which is contradictory to cases of cold exposure, which merely decreases embryo growth,

without affecting body weight (Mortola and Cooney, 2008).

As long as the hypoxia level is not severe, the reduction of O_2 consumption is not caused by a limitation in O_2 availability; rather, it is a regulated process that comprises suppression of functions such as thermogenesis or tissue growth in the embryo (Rohlicek et al., 1998).

Various studies have exposed the chick embryo to different hypoxia levels and durations, at various developmental periods, with the aim of determining the critical exposure windows. Chicken hatchlings had lower O_2 consumption when exposed to chronic hypoxia during the middle phase of embryonic incubation (days 6–12), but not when chronically exposed from day 1 to 6 or 12 to 18 (Dzialowski et al., 2002). Embryos exposed to 15% O_2 hypoxia for a period of 6 d on embryonic days E1 to E6, E6 to E12, and E12 to E18 were developmentally retarded and smaller compared to controls (Chan and Burggren, 2005). Changes in normal chick embryo growth depend not only on the timing of hypoxia, but also on its severity, with lower O_2 levels more significantly disrupting growth and size (Zhang and Burggren, 2012). Mild hypoxia (15% O_2) is probably the most commonly tested level of hypoxia, because it represents a significant hypoxic challenge to the embryo without inducing excessively high mortality (Dzialowski et al., 2002; Chan and Burggren, 2005; Zhang and Burggren, 2012). During internal pipping, mild hypoxia caused a decline in O_2 consumption and affected the weight at hatching, but otherwise it had little morphological effect on chicken embryos, whereas severe hypoxia (10% O_2) affected embryo viability (Szdzuy et al., 2008). Responses to both levels of hypoxia increased during the external pipping phase (Menna and Mortola, 2003). Hatched ducklings that had spent the last 24 h of incubation in severe hypoxia (10% O_2) showed a rise in muscle (+50%) and heart (+69%) lipid peroxidation, increased susceptibility of RBC to free radicals, marked overexpression of avUCP mRNA (+105%), and a rise in mitochondrial adenine nucleotide translocator content (+54%) (Rey et al., 2010).

Short durations of hypoxic exposure during different time windows exert diverse effects on embryo viability depending upon the timing. For example, several studies in chicken embryos have indicated that, with respect to viability, the most hypoxia-sensitive period is early in incubation (Hoper and Jahn, 1995; Miller et al., 2002; Sharma et al., 2006), with tolerance to acute hypoxia increasing with further development of the embryo (Taylor and Kreutziger, 1965, 1969; Taylor et al., 1971). Zhang and Burggren (2012) reported higher mortality during E0 to E10 (37.1% for 13% O_2 and 15.8% for 15% O_2) than during E11 to E18 (18.5% for 13% O_2 and 12.7% for 15% O_2) during hypoxic incubation.

As hypoxia exerts a profound effect on vascular development, gas diffusion and transport capacity of blood are expected to be more efficient in embryos that develop under hypoxic conditions (Mulder et al., 1998; Liu et al., 2009). Adair et al. (1988) showed that intermittent hypoxia was 90% as effective as continuous hypoxia, at the same level, in decreasing structural vascular resistance; the vascular system adapted its structure to meet almost the maximal O_2 needs of the tissues. Burton and Palmer (1992) reported that chronic hypoxia applied throughout the duration of incubation depressed CAM weight. Nanka et al. (2008) reported that exposure to 16% O_2 from E2 to E10 resulted in a 10% survival rate and coronary artery abnormalities at E10.

Hypoxic Manipulation During CAM Development (E5–E12)

Phenotypic plasticity of broiler physiology enhances the cardiovascular system's ability to satisfy O_2 demands during growth, without impacting genetic selection, namely hatchability, and post-hatch growth performance and quality.

With regard to environmental incubation conditions, low ppO_2 (hypoxia) can affect embryonic development, cardiovascular development, metabolism, O_2 demand, and the available energy for post-hatch growth and development (Haron et al., 2017; Druyan et al., 2018). The latter is based on the assumption that environmental factors have a strong influence on determination of the "set-point" for physiological control systems during "critical developmental phases" (DOrner, 1974; Shinder et al., 2011).

Druyan et al. (2012) demonstrated the vascular response of CAM to reduced environmental O_2 concentrations (Figures 1 and 2). The CAM vascular area was significantly increased following exposure to 17% ppO_2 for 12 h/d from E5 through E12, with a deviation from control eggs already seen after the first 12-h exposure and a 5.7% difference between the 2 groups measured on E13 (Figures 1 and 2). Hypoxia-influenced angiogenesis processes in the CAM occurred mostly during the first 2 d of exposure, with passive CAM tissue responses to hypoxic stimulation measured from E7 onward. The observed adaptation of the chick embryo vascular system during the 12-h normoxic period was in agreement with the findings of Adair et al. (1988), who found that the vascular system reacted at an accelerated rate during the gaps between hypoxic events. Druyan and Levi (2012) found that the most prominent hypoxic influence on gene expression was an increased expression of HIF-1α, at 12 and 24 h after hypoxic exposure. VEGF-A is involved in many processes that are related to the functioning of endothelial cells and to vascular remodeling, above and beyond its role in sprouting angiogenesis (Bates and Harper, 2002; Stratmann et al., 2009).

In the chicken, the following variants of VEGF have been identified: VEGF-A-122, VEGF-A-146, VEGF-A-166, and VEGF-A-190, where each differs in its molecular weight and biochemical properties (Robinson and Stringer, 2001). While both VEGF-A-122 and VEGF-A-166 were found to be expressed in the chick embryo CAM (during E7–E14), only VEGF-A-166 has been

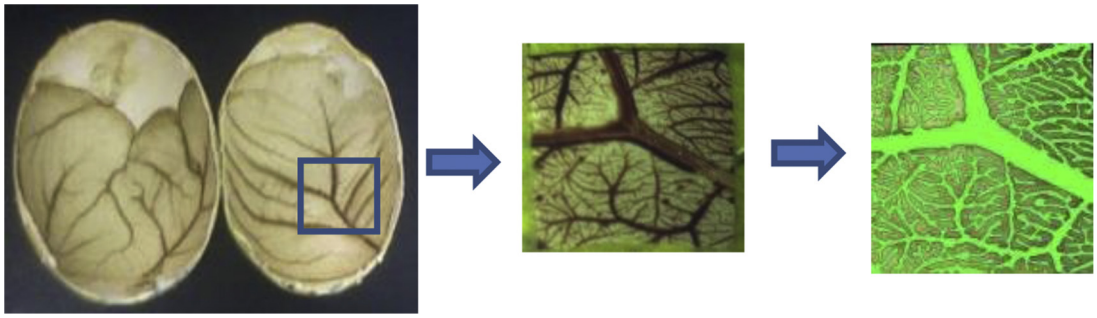

Figure 1. CAM blood vessel area (mm^2) quantification. Following hypoxic exposure, the CAM overall vascular area was quantified in chicken embryos incubated under differing environmental conditions (hypoxic and standard O_2 levels) with the "CAM-era" algorithm as describe by Druyan et al., 2012. Abbreviation: CAM, chorioallantoic membrane.

shown to stimulate the fusion of endothelial cells that precedes the formation of larger vessels in quail hearts (Yue and Tomanek, 2001). VEGF-A-166 tends to be a major player in the establishment of the larger vessels, whereas VEGF-A-122 is associated with organization of the capillaries (Baum et al., 2010).

Furthermore, in 12 and 24 h from the beginning of hypoxic exposure, the gene expression pattern of VEGFα2 in the CAM of hypoxic embryos was higher than that of the controls (Druyan and Levi, 2012). An increase in FGF2 gene expression at E6, 24 h after the beginning of the first hypoxic exposure, was also recorded (Figure 3).

The angiogenic response and the increase in CAM vascular area likely improve the O_2 diffusion capacity through the proliferation of additional gas-exchange vessels and the extra surface area they provide, which can diminish the detrimental effects of hypoxia on development and growth (Monge and León-Velarde, 1991; Zamudio, 2003).

Hypoxia serves as a potent driver of erythropoiesis in chicken embryos. Baumann and Meuer (1992) found that hypoxia caused changes in hematocrit and hemoglobin levels to occur earlier. The effect of exposure to 17% O_2 during CAM development (E5–E12) on both hematocrit and hemoglobin concentrations persisted for 2 d post-exposure, with levels that were high in comparison to embryos incubated at 21% O_2. However, from E14 onward, their levels were similar to those measured in control eggs, until hatch, when hematocrit concentrations were significantly higher in hypoxia-treated eggs as compared to controls (Druyan et al., 2012). The enhanced vascularization and increased O_2 carrying capacity resulting from the hypoxic exposure likely assist in maintaining normal levels of blood O_2 transport in the chicken embryo. Hypoxia also imposes changes in O_2 transport by increasing the heart rate and stroke volume, which leads, in turn, to increased blood circulation (Ar et al., 1991).

Literature contains conflicting information about the effect of hypoxia on relative heart weight. Exposure to hypoxia was found to increase the heart mass of domestic chicken and Canadian goose hatchlings (Snyder et al., 1982; Lindgren and Altimiras, 2009), which led to ventricular hypertrophy (Burggren and Keller, 1998), decrease heart relative weight (Ruijtenbeek et al., 2000), or cause no change (Altimiras and Phu, 2000; Druyan et al., 2012) (Table 1). The minor observed cardiovascular response to hypoxia suggests

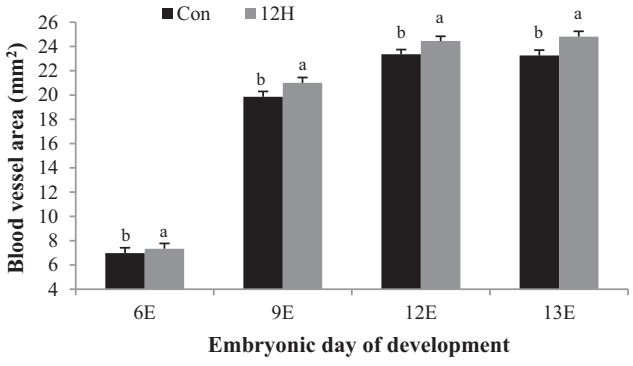

Figure 2. CAM blood vessel area (mm^2) of control and 12H embryos (n = 10) incubated under differing O_2 regimes from E5 through E12. On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments (Druyan et al., 2012). Abbreviations: CAM, chorioallantoic membrane; E, embryonic day.

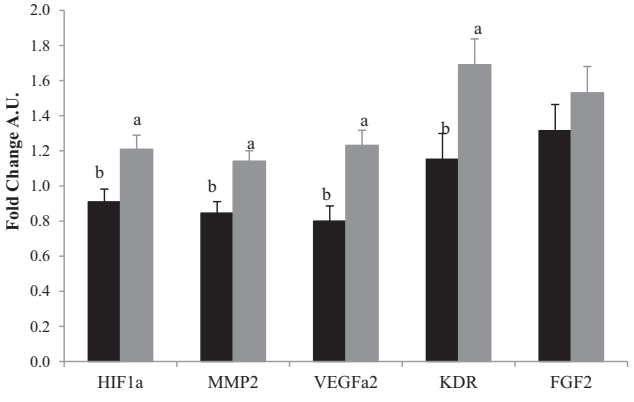

Figure 3. HIF-1α, MMP2, VEGFα2, FGF2, and KDR folds change in CAM of E5.5 normoxic (black) and hypoxic (gray) embryos, relative to control embryos on E5. For each gene, different letters indicate significant differences ($P \leq 0.05$) among treatments (Druyan and Levi, 2012). Abbreviations: CAM, chorioallantoic membrane; E, embryonic day; FGF, fibroblast growth factor; HIF, hypoxia-inducible factors; KDR, kinase insert domain receptor; MMP, metalloproteinases; VEGF, vascular endothelial growth factor.

Table 1. Hematological and heart values of normoxic and hypoxic (17% O_2) embryos incubated under different O_2 regimes from E5 through E12, but measured under standard incubation conditions from E13 through hatch.

	Hematocrit (%)		Hemoglobin concentration (g/dL)		Heart rate (bit/min)		Relative heart weight (%)	
Embryo age	Normoxia	17% O_2	Normoxia	17% O_2	Normoxia	17% O_2	Normoxia	17% O_2
E13	30.6[b]	34.3[a]	5.7[b]	6.2[a]	288	285	1.03	1.08
E14	33.8[b]	35.9[a]	6.4[b]	7.1[a]	287	287	1.01	1.00
E15	37.6	37.2	8.9	9.1	287	288	0.94	0.95
E16	40.4	39.4	10.7	10.4	281.	286	0.90	0.91
E17	39.8	40.5	10.6	10.4	283	285	0.82	0.82
E18	38.6	39.0	10.3	9.8	283	279	0.76	0.74
E19	39.1	38.1	9.6	9.0	292	291	0.68	0.69
Hatch	38.2[b]	39.9[a]	10.1	10.6			0.72	0.72

[a,b]On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.
Abbreviation: E, embryonic day.
(Adapted from Druyan et al., 2012).

that changes in angiogenesis are sufficient to meet the developing embryos' O_2 demands following exposure to hypoxia, so that only slight changes, if any, in the cardiovascular system are needed. As long as the hypoxic conditions are mild, the cardiovascular system can efficiently recruit RBC and increase the hemoglobin concentration to meet the broiler's O_2 demands.

Following exposure to mild hypoxic conditions (17% O_2), hypoxic embryos converted more residual yolk after they were shifted back to normal O_2 conditions (Molenaar et al., 2010; Druyan et al., 2012) (Table 2). Embryos exposed to hypoxia at early stages demonstrated catch-up growth when normoxic conditions were restored, as shown by the higher utilization of yolk and hatch weight similar to that of normoxic chicks (Metcalfe et al., 1981; Miller et al., 2002; Druyan, 2010).

These findings indicate the developmental plasticity of the chick embryo cardiovascular system. As long as the hypoxia level is not severe, and is set at the right timing and critical window length, it can ensure sufficient O_2 supply to the embryo. Such alterations may affect post-hatch performance and the ability of the cardiovascular system to meet O_2 demands under suboptimal conditions.

Effect of Hypoxia Manipulation During CAM Development on Post-Hatch Performance

Developmental changes induced by environmental conditions during incubation may impact post-hatch growth and metabolism (Shinder et al., 2007; Yahav and Brake, 2014). During the embryonic development of chickens few critical windows have been broadly defined: for sensitivity to hypoxia (Druyan et al., 2012; Haron et al., 2017), for control of ventilation (Ferner and Mortola, 2009), for thermal manipulation (Piestun et al., 2008a,b, 2011), and for metabolic rate (Tazawa et al., 2004). It has been shown that incubation at an altitude of 2,000 m above sea level (chronic hypoxic conditions) led to slower initial growth, resulting in a significantly lower body weight up to 14 d post-hatch, but with no significant effecst on body weight in the advanced stages (Hassanzadeh et al., 2004). Chronic hypoxia also impacted growth and feed conversion ratio, and slightly affected the survival of broilers (Huang et al., 2017) and of lowland chickens incubated at high altitudes (Hao et al., 2014). In contrast, Druyan et al. (2018) found no adverse effect on broiler post-hatch growth, following exposure to mild hypoxia of 15 or

Table 2. O_2 consumption (mL/gh), and embryo and yolk relative weight (%) values of normoxic and hypoxic (17% O_2) embryos incubated under different O_2 regimes from E5 through E12, but measured under standard incubation conditions from E13 through hatch.

	O_2 consumption (mL/ gh)		Relative embryo weight (%)		Relative Yolk weight(%)	
Embryo age	Normoxia	17% O_2	Normoxia	17% O_2	Normoxia	17% O_2
E13	0.17	0.18	15.01	15.48	23.87[a]	20.75[b]
E14	0.21	0.24	20.49	20.75	22.1[a]	20.23[b]
E15	0.28	0.29	26.02	25.69	20.92	20.17
E16	0.30	0.32	30.58	30.14	19.89[a]	18.3[b]
E17	0.32	0.33	36.93	36.53	18.22	16.55
E18	0.33	0.33	42.99	43.58	19.23	18.31
E19	0.36	0.35	52.55	51.04	16.07	16.19
Hatch			60.12	59.96	11.95	11.89

[a,b]On each day of incubation, different letters indicate significant differences ($P \leq 0.05$) among treatments.
Abbreviation: E, embryonic day.
(Adapted from Druyan et al., 2012)

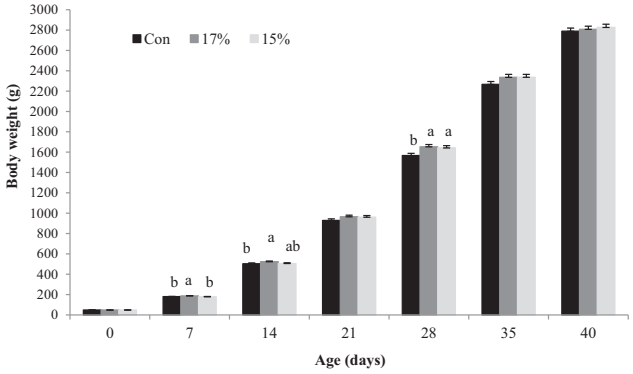

Figure 4. Effect of daily exposures to 17 or 15% O_2 for 12 h during the development of CAM (E5–E12) on individual weekly body weight from day of hatch until the end of the experiment (40 d). Abbreviations: CAM, chorioallantoic membrane; E, embryonic day. Adapted from Druyan et al., 2018.

17% O_2 during a short period of embryonic development; at the time of marketing, the hypoxia-treated broilers weighed as much as or more than the controls (Figure 4; Druyan et al., 2018). During the third and fourth weeks, hypoxic broilers exhibited an accelerated growth rate compared to controls. They also showed a feed intake similar to or lower than controls, which affected their food conversion rate efficiency (Figure 5).

Energy consumption is divided between maintenance and production. Therefore, lowering maintenance demands while maintaining a relatively constant total energy consumption likely increases the energy availability for production (Haron et al., 2017). An alternative response might consist of a reduction in the overall energy consumption deriving from a reduction in maintenance energy demand.

In terms of relative weights, Druyan et al. (2018) reported that the breast of hypoxic broilers (17%, E5–E12, 12 h/d) was larger, with reduced abdominal fat. Piestun et al. (2013) suggested that a reduction in relative abdominal fat weight following embryonic thermal manipulation is a result of allocation of energy from storage as fat accumulation to growth. This suggests that embryonic angiogenesis following hypoxic treatment leads to better vascularization, and presumably better nutrient delivery to the breast. As a result, the food was utilized for growing tissue rather than fat accumulation.

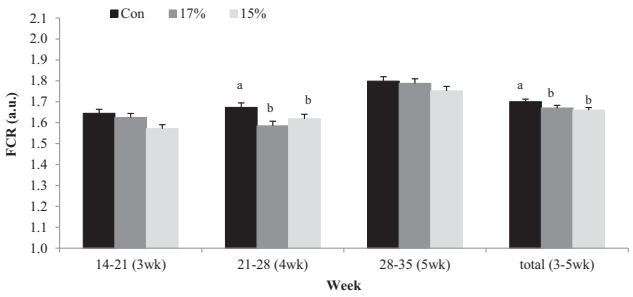

Figure 5. Effect of daily exposures to 17 or 15% O_2 for 12 h during the development of CAM (E5–E12) on individual weekly average food intake from the beginning of the third week until the end of the experiment (40 d). Abbreviations: CAM, chorioallantoic membrane; E, embryonic day; FCR, feed conversion ratio. Adapted from Druyan et al., 2018.

Hypoxic manipulation during embryonic development (E5–E12 12 h/d) was found to affect the broiler's ability to cope with a hot environment (Druyan et al., 2018). At day 35, 15% O_2, 17% O_2, and normoxia-incubated broilers were transferred to individual cages at a room temperature of 23°C, for 72 h for adaptation. At the end of the adjustment period, broilers were exposed to acute heat stress (elevation from 23°C–35°C over 30 min). Following 5 h of heat stress, the temperature was reduced back to 23°C over a 30-min period. The hypoxia-manipulated broilers (both 15 and 17% O_2) exhibited significantly lower body temperatures following the heat challenge and maintained their relative advantage for 5 h. Furthermore, although the difference was not significant, hypoxic broilers had lower mortality rates in response to heat stress than controls (Druyan et al., 2018). Taken together, moderate hypoxia (15 and 17% O_2) during CAM development can improve broiler performance post-hatch, via improved feed utilization efficiency and heat stress coping capacities. This can be adopted by commercial growers as a means of facilitating chickens in withstanding suboptimal environmental conditions and achieving their full growth potential.

CONCLUSION

During hypoxia, multiple mechanisms facilitate maintenance of the O_2 demand despite environmental hypoxia. Hypoxia is a normal part of fetal life in all vertebrates. It plays a requisite role in development, driving vasculogenesis/angiogenesis, hematopoiesis, and chondrogenesis. However, episodes of severe hypoxia can lead to developmental abnormalities or embryonic death. In chicken embryos, the CAM and the cardiovascular system play a crucial role in the adaptation to hypoxic conditions. Mild to extreme hypoxia during chick embryogenesis improves CAM and cardiovascular development, with a subsequent improvement in O_2 carrying capacity. Once hypoxic conditions return to normal, the development of exposed embryos not only progresses, but follows a novel developmental trajectory leading to developmental plasticity that may affect the post-hatch chick performance and provide for improved better adaptation to additional environmental stress, such as suboptimal environmental conditions. Harnessing mild hypoxia during embryogenesis to improve post-hatch performance must carefully consider the timing (critical period), the severity, and duration of ppO_2.

DISCLOSURES

The authors do not have any conflicts of interest to declare.

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