Hypoxic level and duration differentially affect embryonic organ system development of the chicken (*Gallus gallus*)

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ABSTRACT Hypoxia inhibits avian embryonic development, as well as increases embryonic mortality. However, the key organ systems affected by hypoxia, and their critical windows for development, are poorly understood. Consequently, chicken embryos were continuously exposed to 3 levels of oxygen (21, 15, or 13% O_2) throughout d 0 to 10, d 11 to 18, or d 0 to 18 of incubation, followed by morphometric and blood physiological measurements. Hypoxia occurring early during incubation (d 0 to 10) had larger effects on embryonic mortality and organ growth than hypoxia occurring at later stages (d 10 to 18). Growth of the heart and chorioallantoic membrane was stimulated by chronic hypoxia, whereas the lung, brain, eye, liver, stomach, beak, and toes showed no disruption. Sustained hypoxia from the beginning of incubation decreased blood hemoglobin, hematocrit, and red blood cell concentration of embryos at d 10, but the values among hypoxic and normoxic groups were not significantly different at d 18. Blood partial pressure of O_2 and partial pressure of CO_2 were dependent upon incubation O_2 level at a given day of development. These results indicated that either modest hypoxia (15% O_2) throughout development, or hypoxia at any level during the late stages (d 11 to 18), increased the heart and chorioallantoic membrane weight, which partly compensated for the detrimental effects of hypoxia on embryonic development. We conclude that the first half of embryonic development contained the critical windows for the detrimental effects of hypoxia, and the second half contained the critical windows for the compensatory response of hypoxia in key organs.

Key words: chicken, hypoxia, embryo, development

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INTRODUCTION

Hypoxia is a common fetal stressor that can induce increased mortality, decreased BW, as well as profound abnormalities in the developing cardiovascular and other systems, and even embryogenesis in the earliest developmental stage, all as a result of restricted oxygen delivery to somatic tissues (Dzialowski et al., 2002; Rouwet et al., 2002; Chan and Burggren, 2005; Wendler et al., 2007; Ghatpande et al., 2008). However, hypoxic stress may impose quite different effects on development, depending on the timing (duration and onset) of the hypoxic bout within incubation, because different organ systems have different critical windows during which environmental perturbation will exert maximal effects (see Burggren, 1999). There still remains some confusion and even disagreement in the literature as to specifically when and which organs in developing avian embryos are the most sensitive to hypoxia, as well as to the degree of effects produced by different levels of chronic hypoxia. In dispute is even such a fundamental observation of whether hypoxia stimulates or inhibits the development and growth of tissues and organs involved in gas exchange [e.g., heart, lungs, chorioallantoic membrane (**CAM**), and blood]. For example, Tintu et al. (2007) reported that chicken embryos exposed to 15% O_2 throughout embryonic development showed elevated hematocrit (**Hct**) and depressed total body, liver, and heart weights, as well as lower arterial blood partial pressure of O_2 ($\mathbf{P_{O2}}$) levels. However, other studies reported no significant effects of hypoxia (~15% O_2) on hemoglobin (**Hb**; Jalavisto et al., 1965; Dzialowski et al., 2002). Similarly, heart relative weight in chronic hypoxic exposure has variously been reported to increase (Rouwet et al., 2002; Lindgren and Altimiras, 2009), remain unchanged (Altimiras and Phu, 2000), or decrease (Ruijtenbeek et al., 2000). Upon a return to normoxia after a hypoxia bout part way through embryonic development, catch-

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up growth of embryonic body and organs has variously been reported (Miller et al., 2002) or not observed (Azzam and Mortola, 2007).

To attempt to clarify these conflicts in the literature, in the current study we test for dose-dependent and timing-dependent effects of sustained hypoxia on embryonic growth and organs development in the chicken (*Gallus gallus*). Specifically, we hypothesize that organs such as the heart that are involved in acquiring and transporting O_2 in the embryo will develop compensatory growth in response to modest hypoxia (especially if occurring outside of the critical window for that organ), but exhibit detrimental responses by severe hypoxia or hypoxia during the sensitive critical window.

Three levels of oxygen (21, 15, and 13% O_2) were used in the present study, based on the most prevalent exposures used in previous studies. Additionally, 3 different durations of hypoxic exposure were selected: d 0 to 10, d 11 to 18, and d 0 to 18. By measuring body and organ morphology and blood physiology of developing chicken embryos according to these experimental protocols for hypoxic incubation, we were able to assess the magnitude and timing of organ-specific effects and have determined those key organs that experienced altered developmental trajectories resulting variously in embryonic survival or death.

MATERIALS AND METHODS

Fertilized White Leghorn eggs were obtained from Texas A&M University (College Station) and shipped to the University of North Texas (Denton) where they were incubated in Hova-Bator incubators (Hova-Bator, G.Q.F. Manufacturing, Savannah, GA). All experimental procedures were approved by the University of North Texas Institutional Animal Care and Use Committee.

Incubation Protocols

Incubators were maintained a steady temperature of ~37.8°C and ~60% RH, and provided with 45° egg rotation once every 4 h. One incubator was set to normoxic conditions (21% O_2), whereas 2 others were held at either 15% O_2 or 13% O_2. The desired oxygen levels were achieved by flushing the incubators with a steady stream of the appropriate air-N_2 mixture. Oxygen levels within the incubators were continuously monitored by an oxygen sensor (PROOX110, BioPherix, New York, NY).

Seven groups of embryos were exposed to one of the following protocols: 1) continuous normoxia (21% O_2): 21% O_2 for the duration of incubation, serving as the control population; 2) continuous 15% hypoxia (15% O_2): 15% O_2 for the duration of incubation; 3) continuous 13% hypoxia (13% O_2): 13% O_2 for the duration of incubation; 4) early 15% hypoxia (early 15% O_2): 15% O_2 from d 0 to 10, and then 21% O_2 for the duration of incubation; 5) late 15% hypoxia (late 15% O_2): 21% O_2 from d 0 to 10, and then 15% O_2 for the duration of incubation; 6) early 13% hypoxia (early 13% O_2): 13% O_2 from d 0 to 10, and then 21% O_2 for the duration of incubation; and 7) late 13% hypoxia (late 13% O_2): 21% O_2 from d 0 to 10, and then 13% O_2 for the duration of incubation.

All eggs were weighed, individually numbered, and randomly divided into 1 of the 7 different above-mentioned groups. At d 10 of incubation, the eggs were weighed again, candled to verify embryonic viability, and then transferred to the appropriate incubator for late incubation condition. Mortality and egg weight loss (equivalent to water loss) were recorded and calculated.

Measurement Protocols

Embryos were sampled at d 6, 8, 10, 12, 14, 16, and 18. On the measurement day, eggs were candled to locate major chorioallantoic veins. A small portion of the shell and outer membrane over the vessel was removed, and 50 to 200 μL of blood was withdrawn using a heparinized 31-gauge needle and syringe. Blood pH, P_{O2}, and partial pressure of CO_2 ($\mathbf{P_{CO2}}$) in mmHg were measured with a Radiometer ABL5 blood gas meter. Hemoglobin in grams per deciliter, Hct in milliliters per 100 mL, and red blood cell concentration ([**RBC**]) in $\times 10^{12}$/L were measured with a Coulter counter analyzer (Beckman Coulter AcT10), and the values of Hct and [RBC] were calculated from equations developed by Tazawa et al. (2011) for chicken embryos: $Hct_{Cal} = 0.983 + 0.905 \times Hct_{Measured}$, and $[RBC]_{Cal} = 0.07 + 1.05 \times [RBC]_{Measured}$.

Following blood sampling, embryos were killed by decapitation and BW was determined with a precision scale (Zennex, the Netherlands) to the nearest 0.01 g. Embryonic BW did not include the yolk sac. Weight measurements were made on the following organs once dissected free from the embryo: CAM, heart (ventricles with attached atria), lungs, brain, eyes, liver, and stomach. Organs were placed individually in separate plastic weighing boats. They were then gently blotted with KimWipes (Kimberly Clark, Dallas, TX) until all visible free liquid on the organ surfaces was eliminated, and weighed. As indicators of skeletal growth, length of the third toe (from phalanx 1 to 4 inclusive) and length of the beak (from its tip to the beginning of the eye socket) were measured by use of a fine caliper.

Statistical Analyses

Embryonic mortality was analyzed using a logistic regression model. Goodness of fit was determined with the Homer-Lemeshow goodness-of-fit test. Differences between embryo weight and CAM weight were examined using analysis of covariance (**ANCOVA**), and initial egg weight was used as a covariate for embryo weight. When significant differences were observed with the ANCOVA, a Bonferroni post hoc test assessed differences among groups. A 2-way ANOVA was used to

Table 1. Egg weight, water loss, and mortality during incubation

Item	21% O_2	15% O_2	13% O_2	Early 15% O_2	Early 13% O_2	Late 15% O_2	Late 13% O_2
Number of fertile eggs	166	202	213	185	196	159	165
Initial egg weight (g)	61.05 ± 0.41[a]	60.96 ± 0.47[a]	60.78 ± 0.45[a]	60.92 ± 0.40[a]	61.12 ± 0.52[a]	61.11 ± 0.43[a]	61.23 ± 0.47[a]
d 0 to 10 water loss (%)	4.781 ± 0.22[a]	4.55 ± 0.24[a]	4.45 ± 0.31[a]	4.67 ± 0.29[a]	4.63 ± 0.20[a]	4.75 ± 0.19[a]	4.57 ± 0.19[a]
d 0 to 10 mortality (%)	4.8[a]	15.8[b]	37.1[c]	18.9[c]	35.7[c]	5.7[a]	6.1[a]
d 11 to 18 mortality (%)	2.4[a]	12.9[b]	18.3[c]	10.3[b]	14.8[bc]	3.8[a]	3.6[a]

[a–c]Values within a row without a common letter in their superscripts differ at $P < 0.05$.

examine the effect of group, age, and their interactions on organs, Hb, Hct, blood gases, and so on. A significance level of 0.05 was used for all tests. All statistical tests were performed with SAS 8.2 using the PROC MIXED function. Data are expressed as mean ± 1 SE.

RESULTS

Mortality and Egg Weight Loss

At the start of incubation, the weight of eggs used for measurements was not significantly different among groups ($P > 0.05$; Table 1). No significant difference was observed in egg water loss (as evident from egg weight decrease) during the first 10 d of incubation ($P > 0.05$).

Mortality during d 0 to 10 of embryonic development was highest in the 13% O_2 group, including the early 13% O_2 group (Table 1). Mortality was moderate in 15% O_2 (including the early O_2 15% group), and lowest in 21% O_2 (including the late 15% O_2 and late 13% O_2 groups). The differences in mortality among the 3 groups were all significant ($P < 0.05$). The mortality during d 11 to 18 was higher in 13% O_2, early 13% O_2, 15% O_2, and early 15% O_2 groups compared with the 21% O_2, late 15% O_2, and late 13% O_2 groups. Arranging the groups according to mortality throughout d 0 to 18, in descending order, yields 13% O_2 (55.4%), early 13% O_2 (50.5%), early 15% O_2 (29.2%), 15% O_2 (28.7%), late 13% O_2 (9.7%), late 15% O_2 (9.4%), and 21% O_2 (7.2%).

Embryo Growth

The BW of the embryos growing in hypoxia, collected every 2 d from d 8 to 18, were significantly lower than in control (Figure 1 and Supplemental Table 1, available online at http://ps.fass.org/). At d 6, embryos were very small, and whereas the weight of the hypoxic groups was less than that of the control, the variation was relatively high and the differences were not significant ($P > 0.05$). At d 8, embryos of 15% O_2 and 13% O_2 weighed significantly less than control embryos whether expressed as absolute weight (1.06 ± 0.04 g and 1.03 ± 0.04 g vs. 1.20 ± 0.04 g, respectively) or as a percentage of egg weight (1.75 ± 0.06% and 1.70 ± 0.05% vs. 1.97 ± 0.07%, respectively). After d 8, except for the late 15% O_2 group at all measurement days and late 13% O_2 at d 12 and 14, embryo weight decreased significantly in all hypoxic groups compared with controls (21% O_2; Figure 1).

Loss of embryo weight was greater in 13% O_2 than in 15% O_2. At both O_2 levels, however, BW loss was greatest during d 0 to 18 and d 0 to 10, whereas less BW loss occurred for hypoxic exposure during d 10 to 18. In both early 15% O_2 and early 13% O_2, although the later stages of incubation occurred under normoxic conditions, embryo growth was nonetheless decreased significantly at d 18 to levels similar to those in the population exposed to continuous hypoxia. Embryo weights of the late 15% O_2 were not significantly different from controls at all measurement days. Collectively, these data suggest that the 0- to 10-d period contained the critical window for long-lasting reduction of embryo weight.

CAM Development

The CAM weight in 15% O_2 (0.08 ± 0.01 g, n = 10) and 13% O_2 (0.07 ± 0.01 g, n = 11) groups was not sig-

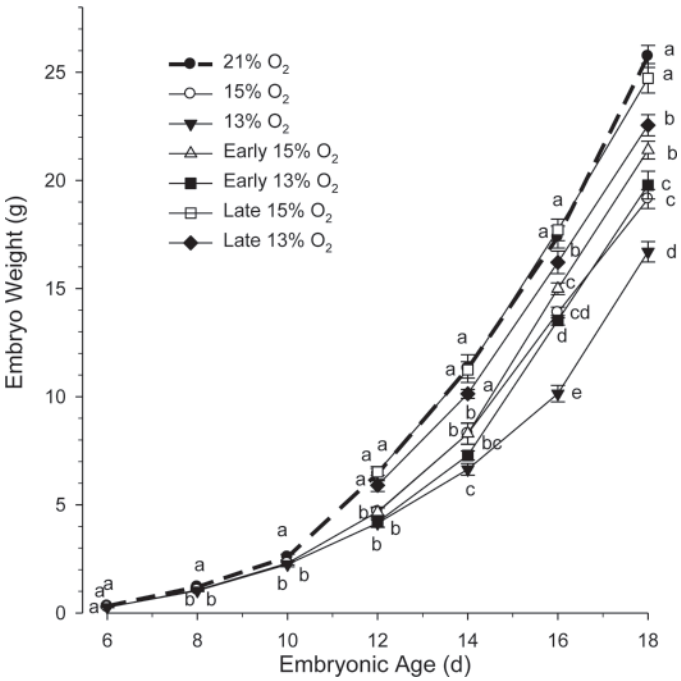

Figure 1. Trajectories of embryonic development in normoxia and hypoxia at d 6 to 18 during development. Mean values ± 1 SE are presented. Significant differences at a given day of development are indicated by different letter (a–e) designations. The heavy dashed line indicates control group.

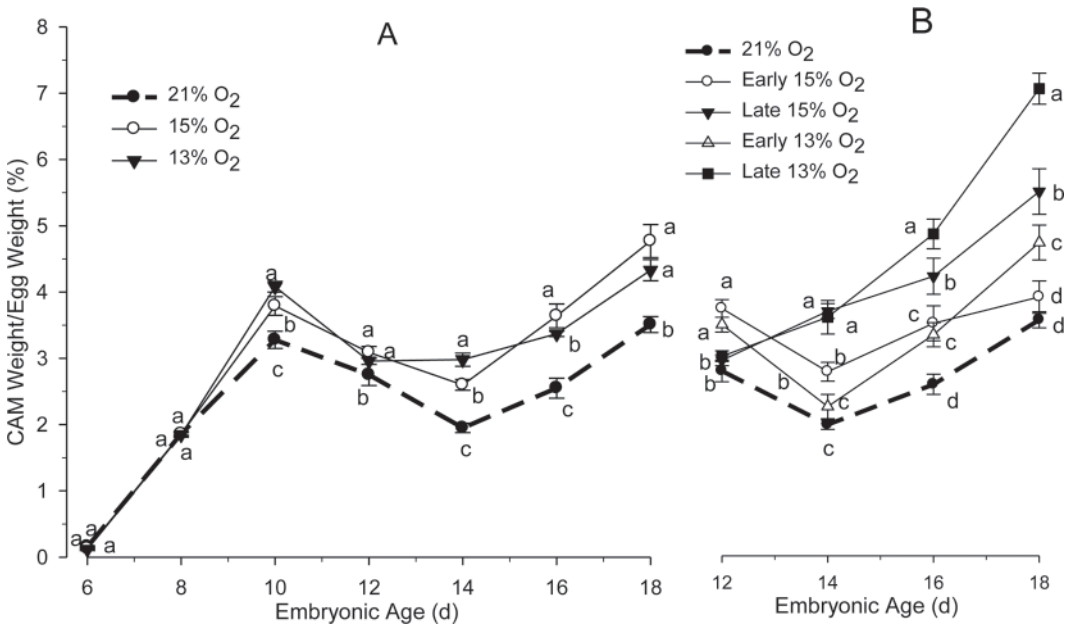

Figure 2. Chorioallantoic membrane (CAM) weight as a percentage of egg weight at different embryonic ages. Panel A compares among 21% O_2, 15% O_2, and 13% O_2. Panel B compares among 21% O_2, early 15% O_2, late 15% O_2, early 13% O_2, and late 13% O_2. Significant differences at a given day of development are indicated by different letter (a–d) designations. The plotting convention used in this figure is used for subsequent figures unless otherwise noted. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

nificantly different from that of control embryos (0.10 ± 0.02, n = 8) at d 6 of incubation. The CAM weight was similarly unaffected by hypoxia at d 8: 1.14 ± 0.04 g (n = 8) for 15% O_2, 1.12 ± 0.03 g (n = 9) for 13% O_2, and 1.13 ± 0.04 g (n = 8) for 21% O_2 (Supplemental Table 1, available online at http://ps.fass.org/). However, after d 10 of embryonic development, hypoxia (either 15% O_2 and 13% O_2) resulted in significant CAM growth (Figure 2A). Thus, hypoxic exposure after d 10 stimulated CAM growth to a greater extent than hypoxic exposure during either the first 10 d or during continuous hypoxia (Figure 2B).

Heart Development

During control embryonic development, heart mass growth was disproportionately higher than embryo growth before d 10, but after this point in development heart growth was relatively slower than that of the embryonic body (Figure 3). Thus, the highest relative heart weight (heart weight/embryo weight) peaked at d 10 of development. However, the peak heart weight/embryo weight was delayed to d 12 in both hypoxic groups (13% and 15% O_2; Figure 3A). From d 12 of development, relative heart weight was higher in all hypoxic groups compared with controls at the same measurement age. However, the influence was larger in the 2 early hypoxia groups than in the 2 late hypoxia groups (Figure 3B).

When compared at the same embryo weight, heart weight was heavier in hypoxic (15% and 13% O_2) incubation than in normoxic (21% O_2) incubation within a given weight (Figure 4).

Development of Brain, Lung, and Other Organs

During normal embryonic development, organ development rates were highly disproportional to embryonic body growth. Some organ development slowed in later stages, so the relative weight (BW-specific weight) of these organs decreased [e.g., brain, eye, lung, and length of beak and third toe (Figure 5)]. However, other organs, such as liver and stomach, developed rapidly at later developmental stages, so that the relative weight increased with embryonic development time (Figure 6).

After d 14, hypoxic incubation increased the relative weight of those organs with a late peaking of relative weight (brain, eye, lung, and length of beak and third toe) compared with the normoxic embryos (e.g., brain, Figure 5). However, for those organs increasing their relative weight with development (liver and stomach), the corresponding values in hypoxia were significantly lower than in the control (e.g., liver in Figure 6). Thus, hypoxic incubation delayed both the overall development of the embryo as well as that of specific internal organs.

When examined at same embryo weights, the developmental trajectories of brain, eye, lung, liver, and stomach weight and length of beak and third toe in normoxia and 15 and 13% hypoxia overlapped or crossed each other (e.g., brain in Figure 7), which meant that in hypoxia the weight of these organs, different from the heart, was appropriate for embryonic body size.

Blood Physiology

[RBC], Hb, and Hct. The [RBC], Hb, and Hct were depressed by chronic hypoxia (13% O_2 and 15% O_2)

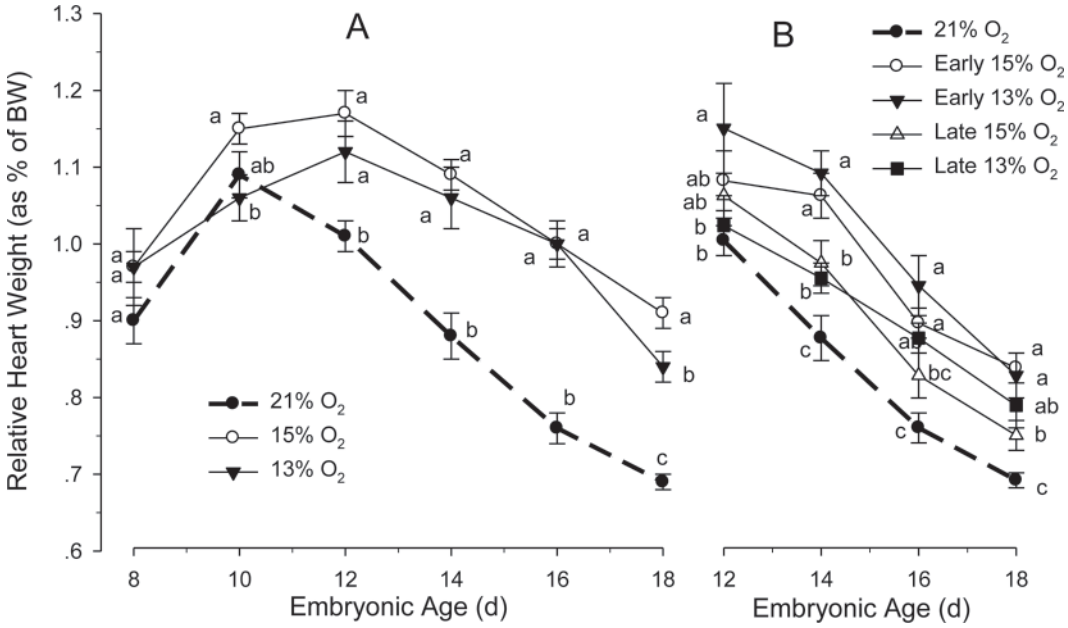

Figure 3. Heart weight/BW ratio at different days of embryonic development. Panel A compares 21% O_2, 15% O_2, and 13% O_2. Panel B compares 21% O_2, early 15% O_2, late 15% O_2, early 13% O_2, and late 13% O_2. Significant differences at a given day of development are indicated by different letter (a–c) designations. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

measured at d 10 of embryonic development, but the effects were not significant at later stages ($P > 0.05$; Figure 8A). However, both late 15% O_2 and late 13% O_2 almost had no significant effect ($P > 0.05$) on [RBC], Hb, or Hct (Figure 8B; Supplemental Table 2, available online at http://ps.fass.org/).

Blood Gases and pH. Blood P_{O2} was dependent on O_2 incubation level; there was a trend of decreasing in a dose-dependent fashion (Figure 9), except for the 15% O_2 group at d 10. The P_{O2} was lower in both hypoxic groups during the first 10 d when compared with the control group (Figure 9).

Blood P_{CO2} and [HCO_3^-] were dependent on the incubation O_2 level and increased with developing time. Interestingly, hypoxic incubation lowered these values in a dose-dependent manner at any given embryonic age, with the differences increasing with advancing development (Figure 10).

Blood pH decreased as development progressed in all populations. But at same measurement age, the differences of pH values between hypoxic groups and control were not large, though statistically different between some groups (Figure 11).

DISCUSSION

Embryonic Developmental Trajectory

Generally, a fertilized egg follows a predictable, largely genetically dictated developmental trajectory under normal (control) environmental conditions, resulting in a predictable hatchling phenotype (e.g., Burggren and Fritsche, 1995; Burggren, 1999). Normal anatomical development during avian development reveals that structure and function do not progress linearly, nor do all organs progress increase embryonic complexity at similar rates (Chan and Burggren, 2005). In the present study, embryonic body growth was very slow during the first 10 d of incubation when some key vital organs (e.g., CAM, heart, brain, eyes, and so on) were forming, but accelerated after d 10 of incubation when the general body form and most internal organs all increased markedly in weight and size. These results are consistent with previous literature that reported that the embryo reaches less than 10% of the ultimate weight and less

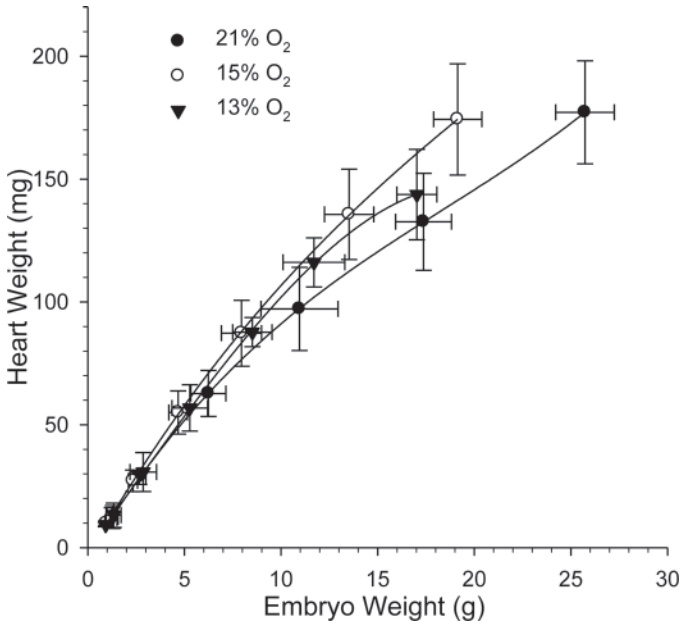

Figure 4. The weight of heart at different embryonic BW. Horizontal and vertical error bars represent mean SD of embryo's weight and organ weight, respectively.

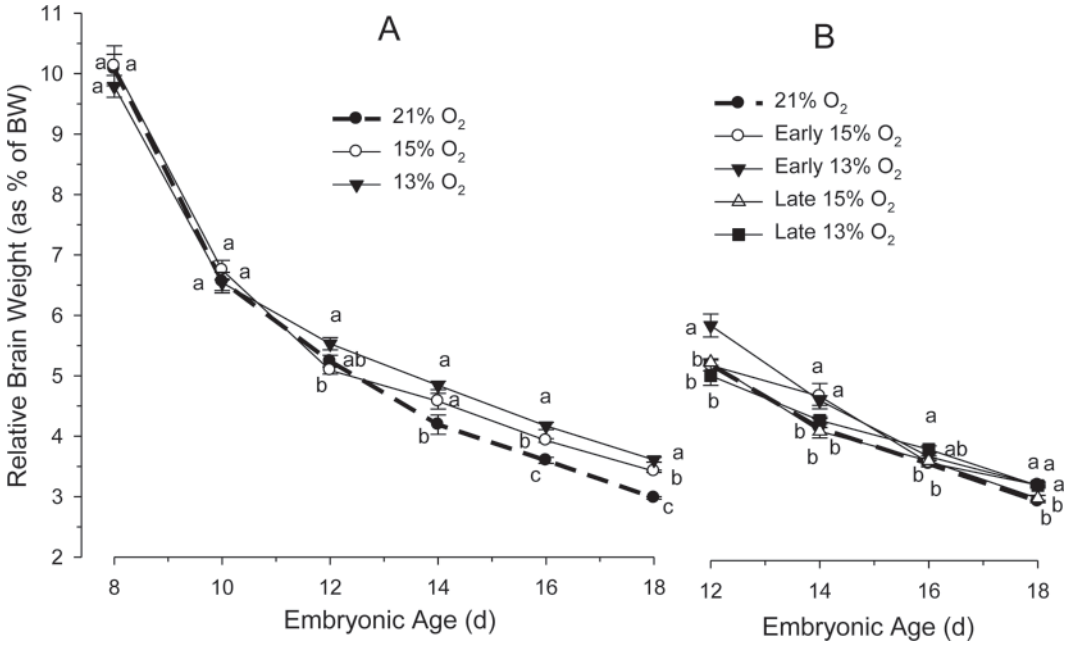

Figure 5. Brain weight/BW ratio at different days of embryonic development. Panel A compares 21% O_2, 15% O_2, and 13% O_2. Panel B compares 21% O_2, early 15% O_2, late 15% O_2, early 13% O_2, and late 13% O_2. Significant differences at a given day of development are indicated by different letter (a–c) designations. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

than 16% of the maximum size by the middle of incubation, with embryonic weight then increasing about 10-fold during the second half of embryonic development (Ar et al., 1987).

Abiotic or biotic environmental perturbations may influence genetically programmed developmental trajectory of an embryo forcing the embryo developing along a new developmental trajectory (Burggren, 1999). Hypoxia is a common abiotic stressor that slows embryonic growth, resulting in an altered developmental trajectory. Importantly, however, different levels of hypoxia or hypoxic exposure occurring at different times or for different durations also differentially affect the embryo's developmental trajectory. In the present study, hypoxia during the first 10 d restrained embryonic BW very little up to d 10, but influenced the survivability of the embryo. In fact, as much mortality occurred as during late stages in groups returned to

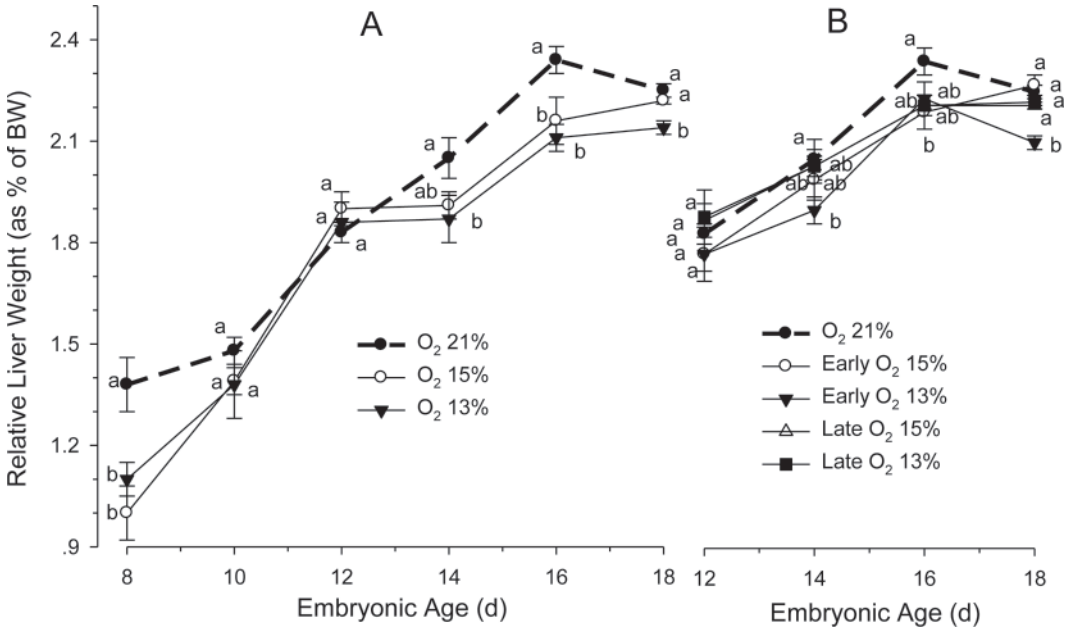

Figure 6. Liver weight/BW ratio at different days of embryonic development. Panel A compares 21% O_2, 15% O_2, and 13% O_2. Panel B compares 21% O_2, early 15% O_2, late 15% O_2, early 13% O_2, and late 13% O_2. Significant differences at a given day of development are indicated by different letter (a,b) designations. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

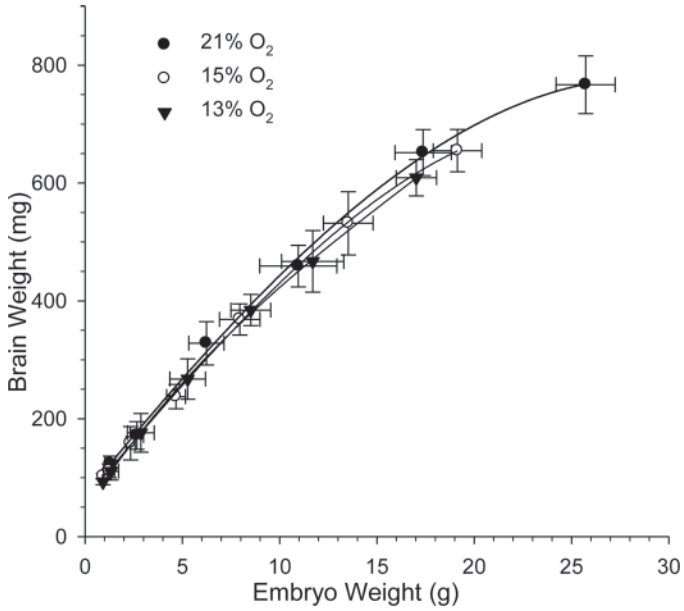

Figure 7. The weights of brain at different embryonic BW. Horizontal and vertical error bars represent mean SD of embryo's weight and organ weight, respectively.

control levels after d 10 (early 13% O_2 and early 15% O_2). After d 10, the effects of either sustained hypoxia level were both decreased body growth and increased mortality. The groups that were returned to normoxia after d 10 (early 13% O_2 and early 15% O_2) grew more slowly and had higher mortality during the stage than the groups of late 15% O_2 and late 13% O_2. Collectively, these data reveal that hypoxia during the first 10 d of incubation has larger effects on an embryo's development than hypoxia occurring at d 10 to 18.

Hypoxic Levels and Durations

A variety of levels of hypoxic exposure have been used to study the hypoxic effects on avian development [e.g., 10% by Höper and Jahn (1995), 12% by Adair et al. (1987), 14% by Miller et al. (2002), and 15% by Chan and Burggren (2005)]. Fifteen percent oxygen is probably the most commonly used level because it represents a significant hypoxic challenge to the embryo without unduly high mortalities (Dzialowski et al., 2002; Chan and Burggren, 2005). Whether there is a hypoxic dose-response for various types of anatomical and physiological effects, however, has not been previously determined and can only be inferred from several different studies. The present study used 2 O_2 levels (15 and 13%) and 3 stages (d 0 to 18, d 0 to 10, and d 11 to 18) of hypoxic exposure. Our results showed that dose-dependent effects occurred in mortality, BW, blood gases, organ weight (brain, eyes, lung, liver, stomach), and the length of the beak and toe. Paradoxically, however, for CAM and heart weight, 15% hypoxia induced larger increases than the more severe 13% hypoxia. It is possible that tissue remodeling and hyperplastic/hypertrophic growth can be supported in mild hypoxia (15% O_2), but that a more severe hypoxia (13%) compromises that ability. Whereas additional studies of these differential effects are warranted, perhaps development of these tissues is stimulated at moderate hypoxia but actually inhibited at the more severe O_2 level.

The avian embryo and its organs have different critical windows for hypoxic responses, and within any given window, hypoxic exposure will typically exert maximal effects (Burggren, 1999). Early studies of hypoxic effects on chicken embryos revealed high sensitivity to O_2 deprivation during early incubation, with a higher

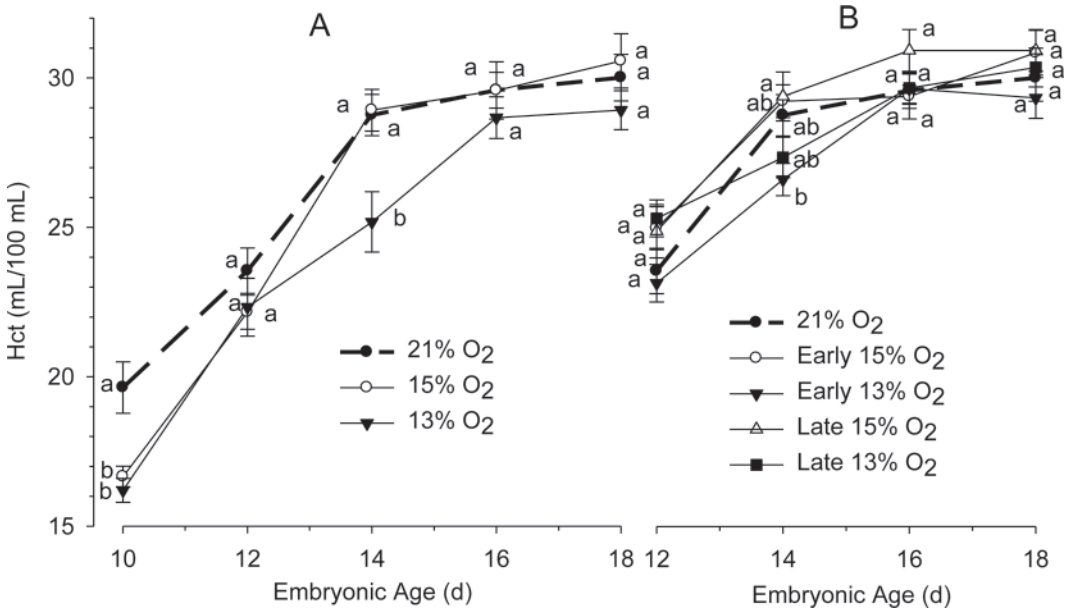

Figure 8. Blood hematocrit (Hct) at different embryonic ages. Panel A compares 21% O_2, 15% O_2, and 13% O_2. Panel B compares 21% O_2, early 15% O_2, late 15% O_2, early 13% O_2, and late 13% O_2. Significant differences at a given day of development are indicated by different letter (a,b) designations. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

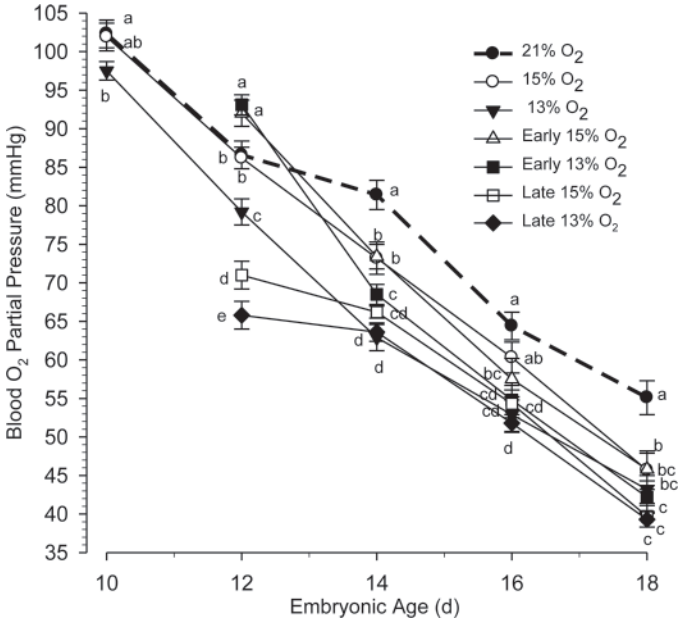

Figure 9. Blood O_2 partial pressure at different ages of embryonic development. Significant differences at a given day of development are indicated by different letter (a–e) designations. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

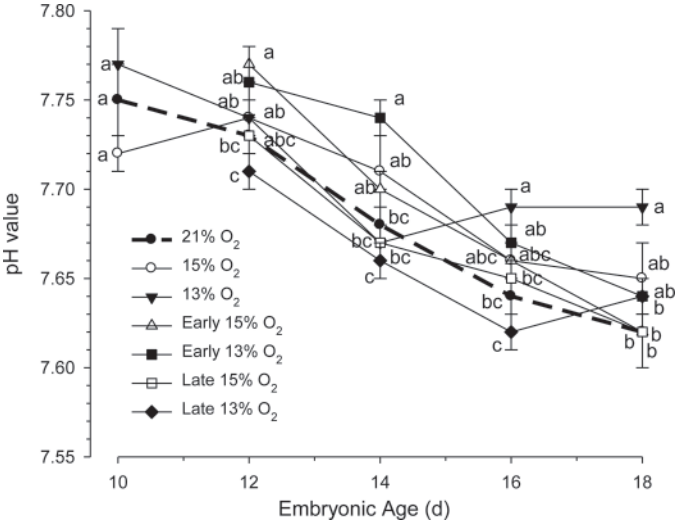

Figure 11. Blood pH values at different ages of embryonic development. Significant differences at a given day of development are indicated by different letter (a–c) designations. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

tolerance to acute hypoxia increasing with further development of the embryo (Taylor et al., 1956; Taylor and Kreutziger, 1965, 1966). More recent studies also showed high hypoxic sensitivity of embryonic metabolism and growth during early development in hypoxia. Collectively, these data suggest that there is a discrete threshold of O_2 availability that is required to both initiate and sustain early embryo development (Altimiras and Phu, 2000; Sharma et al., 2006), and that O_2 available during early incubation is important for hatchability (Zhang et al., 2008). However, some studies reported that chronic hypoxia (14% O_2) during the first 10 d of incubation had no effect on the weight of the embryo and its organs at the time of hatching (Miller et al., 2002), that the middle third of embryogenesis was critical for the effect of long-term hypoxia on metabolism (Dzialowski et al., 2002) or that the last week of incubation was sensitive for ventilatory response to hypoxia (Ferner and Mortola, 2009). In the present study, we observed that early embryos were quite sensitive to hypoxia. Mortality was higher during d 0 to 10 (37.1% for 13% O_2 and 15.8% for 15% O_2) than during d 11 to 18 (18.5% for 13% O_2 and 12.7% for 15% O_2) in hypoxic incubation. Embryo weight loss at d 18 of development in the early hypoxia group (early 15% O_2 or early 13% O_2) was higher than corresponding late hypoxia groups (late 15% O_2 or late 13% O_2). Thus, the detrimental effects of hypoxia on vital organs during early stages appear to be permanent, which could restrain embryonic development even when normoxia resumes at a later stage. Burggren et al. (2000) reported there is a vigorous aerobic metabolism by d 3 in avian embryos, and consequences of hypoxic exposure are to be expected. In the present study, almost all fertilized eggs exposed to early hypoxia begin to develop during the first 2 d of incubation, but the first peak of embryo death occurred during d 3 to 6. In addition, the incomplete structural development of the chorioallantoic membrane in younger embryos limits O_2 availability, aggravating tissue hypoxia (Mortola and Besterman, 2007). Prolonged hypoxia in early fetal development results in a thin, disorganized ventricular myocardium, likely to further reduce cardiac output. This likely creates a positive feedback spiral leading to death due to an early developmental version of congestive heart failure (Ream et al., 2008).

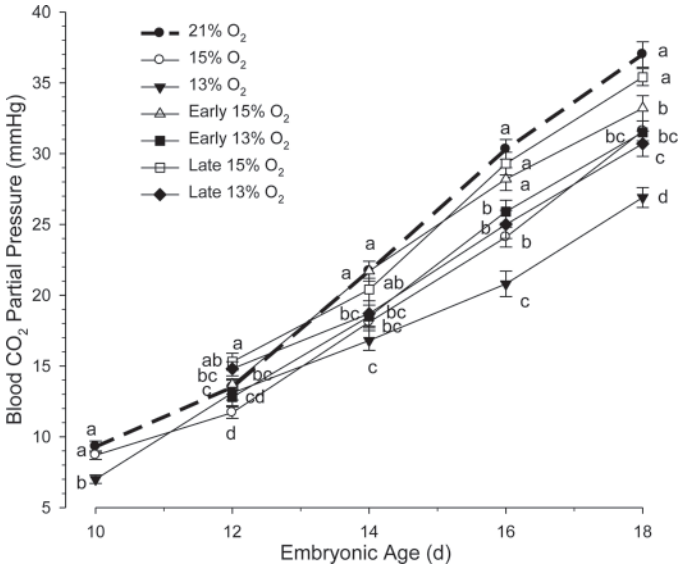

Figure 10. Blood CO_2 partial pressure at different ages of embryonic development. Significant differences at a given day of development are indicated by different letter (a–d) designations. Mean values ± 1 SE are presented. The heavy dashed line indicates control group.

Hypoxia and Internal Embryonic Organs

The internal organs of hypoxic embryos are smaller than controls. However, not all such organs are equally affected. Normalization of embryonic BW (organ weight/BW) revealed that some organs have different relative weights than in normoxic controls. Complex influences of hypoxia on internal organ growth may result from several different causes. Redistribution of cardiac output toward some key organs may be one of them (Mulder et al., 1998, 2002). Additionally, tissue- and organ-specific differences in metabolic requirements may alter the relative hypoxic sensitivity (Azzam and Mortola, 2007). Importantly, however, the relative weight of individual organs was not constant during embryonic development. The increase or decrease of organ relative weight [e.g., brain (Figure 5) and liver (Figure 6) compared with controls at any given age] might be due to the smaller embryo produced by hypoxia. Weight of organs such as the brain were different when comparing hypoxic and normoxic embryos at given chronological ages, whereas the lung, eye, liver, stomach, and length of beak and third toe were not different when compared at comparable BW in this study. These finding indicate no selective hypoxic effects on growth of these organs. Consequently, any differences in relative weights at any given age are most likely the result of an overall reduction in growth rate.

Most strikingly, however, heart weight curves examined as a function of embryo weight still showed hypertrophy in hypoxia, likely reflecting the key role played by the heart and the convective transport of O_2 it produces during all but early embryonic development. Accordingly, heart relative weight, generally viewed as a strong index of cardiac hypertrophy, is increased after chronic hypoxia exposure during incubation in chicken embryos (Miller et al., 2002; Rouwet et al., 2002; Wendler et al., 2007; Lindgren and Altimiras, 2009). In the embryonic heart, increases in stroke volume maintained over time, and are likely to induce ventricular hypertrophy, and thus higher overall heart weight (Harvey and Rosenthal, 1999). Cardiac output redistribution in response to hypoxemia also was reported during the last half of incubation in the chicken embryo (Mulder et al., 1998). Neural and endocrine components are thought to mediate these cardiovascular responses (Mulder et al., 1998).

The literature contains conflicting information about the effect of hypoxia on heart relative weight. Hypoxia had been reported to increase heart relative weight (Miller et al., 2002; Rouwet et al., 2002; Lindgren and Altimiras, 2009), cause no change (Altimiras and Phu, 2000) or decrease heart relative weight (Ruijtenbeek et al., 2000). Hypoxic incubation also affects other organs relative weight, including the brain (Stock and Metcalfe, 1987; Ruijtenbeek et al., 2000) and lung (Xu and Mortola, 1989). The sparing effects on these vital organs may be because of hypoxia-induced redistribution of cardiac output (Mulder et al., 1997). In the present study, only the heart was found to differentially be stimulated by hypoxia among the measured internal organs of the chicken embryo.

CAM

The CAM is of physiological importance to the embryo because it functions as an external respiratory organ for gaseous exchange until hatching, and it provides a bladder into which waste products can be delivered (Hamilton, 1965). In chickens, the CAM is formed on d 4 of incubation after the merging of the chorion and growing allantois with the branching of 2 chorioallantoic arteries and one vein in the mesodermal layer (DeFouw et al., 1989). The CAM is sensitive to hypoxic exposure during incubation, showing stimulated growth after 10 d (Melkonian et al., 2003). The CAM weight changes induced by hypoxia have been reported several times (e.g., Wagner-Amos and Seymour, 2003; Chan and Burggren, 2005). The CAM weight, unlike that of internal organs, increased both at specific developmental ages and as function of embryo BW. Hypoxia has selective effects on CAM growth (Azzam and Mortola, 2007). The increase in CAM weight presumably improves its O_2 diffusion capacity through the proliferation of additional gas exchange vessels and the extra surface area they provide, which would minimize the detrimental effects of hypoxia on development and growth (Monge and Le'on-Velarde, 1991; Zamudio, 2003).

The development of CAM is inversely related to ambient O_2 supplied during d 7 to 14 of incubation (Strick et al., 1991). Chan and Burggren (2005) reported that CAM weight was unaffected by hypoxic exposure in early or mid-development, with CAM weight markedly increasing only in late and continuous hypoxic populations. However, Burton and Palmer (1992) reported that chronic hypoxia depressed the CAM weight when applied chronically throughout the duration of incubation. In this study, all hypoxic groups (15% or 13% O_2, d 0 to 10, d 11 to 18, or d 0 to 18) increased CAM growth in late incubation. Furthermore, the increases in CAM weight induced by 15% hypoxia were a little larger than by 13% hypoxia, and the increases were larger by late hypoxia than by early hypoxia. When the hypoxic exposure occurred during d 11 to 18, the increases of CAM weight at late stage were larger by 13% O_2 than by 15% O_2 (Figure 2). These results indicate that when hypoxia was moderate and occurred in the critical window for the CAM response to hypoxia, growth induced by hypoxia would be maximal. Exposure to 15% O_2 was moderate for chicken embryo (Dzialowski et al., 2002) and the window of CAM sensitivity to hypoxia corresponds to incubation d 12 to 18 (Chan and Burggren, 2005). In addition, we propose that hypoxia during d 11 to 18 is a more effective stimulant of CAM growth than during d 0 to 10, 15%

O_2 throughout incubation was more effective than 13% O_2, and 13% O_2 during d 11 to 18 was more effective than 15% O_2.

Blood Physiology

The fundamental role of red blood cells is to transport oxygen and CO_2 in all but the youngest avian embryos, where direct diffusion accomplishes this task (Burggren et al., 2000; Burggren, 2005). Hypoxia serves as a potent erythropoietic agent in chicken embryos. Indeed, Hb concentration and Hct alterations can be induced by hypoxia well before hatching in bird embryos (Dragon and Baumann, 2003; Baumann and Dragon, 2005). The enhanced O_2 carrying capacity resulting from stimulated erythropoiesis likely assists in maintaining normal levels of blood O_2 transport in the chicken embryo. Hematocrit or Hb increases in response to hypoxia applied during late development stages in chicken embryos (Tazawa et al., 1971; Ruijtenbeek et al., 2000). Sustained hypoxia is reported to create a rather muted erythropoietic response of the avian embryo (Mortola, 2009). In the present study, we found that hypoxia during the first 10 d of incubation decreased Hct, and the 13% hypoxia also decreased [RBC] at d 10 of incubation compared with controls. In late incubation, especially at d 18, these values were not affected by hypoxia, indicating that hematological parameters were insensitive to modification by hypoxia in late embryonic development.

Blood gas values in chicken embryos depended upon both age and incubation O_2 level. As expected, arterial blood P_{O2} and P_{CO2} were lower in hypoxic embryos, and the values obtained from normoxic embryos were similar to previous reports (Tazawa et al., 1983; Copeland and Dzialowski, 2009). We propose that the decrease in blood P_{CO2} by hypoxia was related to reduced metabolism and growth resulting from hypoxic exposure during embryonic development.

Conclusion

Hypoxia had highly differential effects on embryonic development, depending on the level and timing of hypoxia. Hypoxia occurring early during incubation (first 10 d) has bigger effects on embryonic mortality and organs growth than hypoxia occurring at later stages (d 10 to 18).

The heart and CAM were the key organs that had stimulated growth in hypoxia during embryonic development. Either modest hypoxia or late-stage hypoxia increased their relative weight, likely helping to compensate for hypoxic effects on embryonic development. However, the compensatory response was reduced in severe hypoxia or early hypoxia, resulting in slower growth and greater mortality of embryos compared with modest or late-stage hypoxia during development. Hypoxia inhibits the growth of other organs, such as lung, brain, eye, liver, stomach, beak, and toes. Collectively, this pattern of reduced growth likely contributes to the overall decrease in embryonic BW.

Sustained hypoxia from beginning of incubation decreases blood Hct and [RBC] of embryos at d 10, but the values of Hct, [RBC], and Hb were similar at d 18 among hypoxic and normoxic groups. Blood P_{O2} and P_{CO2} were dependent upon incubation O_2 level at a given day of development.

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