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Respiratory Physiology & Neurobiology 145 (2005) 251–263
RESPIRATORY PHYSIOLOGY & NEUROBIOLOGY
www.elsevier.com/locate/resphysiol

Hypoxic incubation creates differential morphological effects during specific developmental critical windows in the embryo of the chicken (*Gallus gallus*)

Tammy Chan*, Warren Burggren

Department of Biological Sciences, University of North Texas, P.O. Box 305189, Denton, TX 76203-5189, USA

Accepted 17 September 2004

Abstract

Hypoxia inhibits vertebrate development, but the magnitude and timing of organ-specific effects are poorly understood. Chick embryos were exposed continuously to hypoxia (15% O_2) throughout Days 1–6, 6–12, 12–18 or Days 1–18 of development, followed by morphometric measurements of major organ systems. Early hypoxic exposure reduced eye mass and beak length when measured in middle development. Liver, brain, heart, kidneys, stomach, intestines and skeletal long bones were not affected by hypoxia at any developmental stage. The chorioallantoic membrane (CAM) mass was unchanged by hypoxic exposure in early or mid-development, but CAM mass on Day 18 increased strikingly (40 and 60% in late and continuous populations, respectively) in response to hypoxic exposure. The increase in CAM mass presumably enhances oxygen delivery, thus minimizing the detrimental effects of hypoxia on development and growth. Hypoxic exposure at key critical windows in development thus results in differential effects on organ development, some of which can subsequently be repaired through additional incubation (yolk mass, eye mass, beak length).

Keywords: Heart; Development; Chick; Chronic hypoxia; Organogenesis

1. Introduction

The oxygen dependency of embryonic growth and development has been probed for decades by incubating embryos under various conditions of hypoxia and hyperoxia. Chronic hypoxic exposure at key points during development can significantly impact both anatomical and physiological ontogeny (see recent studies by Corona and Warburton (2000), Dzialowski et al. (2002), Rouwet et al. (2002), Crossley et al. (2003a) for an entry into the older literature). Yet there also remains some degree of confusion and disagreement in the literature as to when in development avian embryos are sensitive to hypoxia, as well as to the degree of the effects and whether they are reversible by return

* Corresponding author. Present address: Interdepartmental Biological Sciences, Northwestern University, Hogan Hall 2-106, 2205 Tech Drive, Evanston, IL 60208-3500, USA. Tel.: +1 847 477 0651; fax: +1 847 467 1380.
E-mail address: t-chan@northwestern.edu (T. Chan).

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doi:10.1016/j.resp.2004.09.005

to normoxia. Some studies have focused on only a few tissues, while others have more broadly surveyed embryonic growth. There are even contradictions in the literature about whether hypoxia stimulates or inhibits organ growth, especially of key structures involved in gas exchange and nutrient transport—e.g. the chorioallantoic membrane (CAM) and the area vasculosa (vasculature covering the yolk). In part, this has arisen from the lack of an "industry standard" for magnitude of hypoxic exposure or its duration, with studies defining hypoxia as values ranging from 10% to 15% O_2 for anywhere from a few days or hours to the entire incubation period.

In this study, we test the hypothesis that chronic hypoxic exposure during development causes *differential* effects on tissue development and growth in the chicken *Gallus gallus*. Specifically, we anticipate that organs most O_2-sensitive (e.g. brain, eyes) will be inhibited by hypoxic incubation, while organs involved in acquiring and transporting O_2 either in the embryo or adult may be stimulated.

2. Materials

2.1. Embryos and protocol

White Leghorn eggs were transported from the Texas A&M Poultry Center to the University of North Texas and stored for a maximum of 2 weeks before incubation. Up to 42 eggs were placed in one of two closed incubators (G.F.Q. Manufacturing). The incubator rotated eggs every hour. All incubators were maintained at $37 \pm 1\,^{\circ}C$ and relative humidity of $56 \pm 1\%$ for the duration of incubation (6, 12, or 18 days).

Ambient O_2 concentration was maintained at either 21% (normoxia) or 15% (hypoxia). (While past studies have used 10% O_2 acutely, chronic exposure to values well below 15% O_2 have often been reported as lethal to chicken embryos, see Section 4). Incubators were ventilated with appropriate gas mixtures produced by a Silent Air X6 Air Pump (Penn-Plax). O_2 concentration in each incubator was monitored with a Qubit S102 flow-through oxygen sensor and recorded with PowerLab/8S (AD Instruments) and Chart software (v.4.2).

Five groups of embryos were exposed to one of the following protocols:

(1) *Normoxia*: 20.95% O_2 for the duration of incubation.
(2) *Early Hypoxia*: 15% O_2 from Day 1 to Day 6, then 20.95% O_2 for duration of incubation.
(3) *Middle Hypoxia*: 20.95% O_2 from Day 1 to Day 6, then 15% O_2 for Days 6–12, then 20.95% O_2 for duration of incubation.
(4) *Late Hypoxia*: 20.95% O_2 from Day 1 to Day 12, then 15% O_2 for duration of incubation.
(5) *Continuous Hypoxia*: 15% O_2 for the duration of incubation.

To allow for repeated sampling and mortality, the following numbers of eggs were placed in each group at the beginning of incubation: normoxia, 133; early, 42; middle, 42; late, 42; continuous, 130. At each measurement stage (Days 6, 12 and 18), subsets of eggs (indicated by the *n* values in Tables 2–4) were removed from the original group and measured.Embryos in the sampled eggs were sacrificed by 30–60 min of exposure to halothane vapor in a closed container. Using a Wild M3Z microscope, an approximately 2 cm × 2 cm region of the eggshell above the air cell was removed with forceps, taking care not to damage any underlying structures. Embryos were removed from the egg with intact extraembryonic tissues—i.e. chorioallantoic membrane (CAM) and yolk—and placed in a plastic weighing boat. All albumin was carefully separated and removed from the egg and extraembryonic structures, which were weighed. (All masses in this study were determined to the nearest milligram with an Ohaus Explorer scale.)

All subsequent dissections and measurements were performed under 6.5×–16× magnification. Fine-tipped forceps were used to separate the CAM from the embryo. The yolk was then separated from the embryo and CAM, which were then carefully washed with 0.9% NaCl, gently blotted with KimWipes® until all visible free liquid on their surfaces was removed, and then weighed. The separated yolk was separately weighed.

2.2. Determination of organ mass or length

An incision was made from sternum to anus on the ventral side of the embryo with surgical scissors to provide access to the embryo's internal organs. For dissection of Day 6 embryos, 0.1 ml of highly dilute methy-

lene blue dye was applied into the opened body cavity to enhance visibility of organs. Dye was not used in the dissections of Days 12 and 18 embryos.

Mass measurements were made on the following organs: CAM, heart (ventricles with attached atria), lungs, brain, eyes, kidneys, liver, stomach, and combined small and large intestines. Each organ was dissected free from the embryo with standard straight surgical forceps and micro-surgery curved-tip forceps (Fine Science Tools). Organs were placed in separate plastic weighing boats and gently blotted with KimWipes® until all visible free liquid on the organ surfaces was removed. The separated organs were then washed with 0.9% NaCl, blotted again as described above, and weighed.

Length measurements (to the nearest 0.1 mm) were made with Manostat calipers on the following organs/structures: beak, femur, forelimb, hindlimb. The humerus, radius, and ulna were then dissected free from the forelimb, and their lengths measured. Similarly, the femur, tibia, and tarsus were carefully separated out of the hindlimb and lengths measured.

2.3. Statistical analyses

Data were analyzed using a two way ANCOVA (SAS v8.2) with treatment (O_2 level) and developmental stage (Days 6, 12 or 18) as covariables. A significance level of 0.05 was used. Where significance was established, appropriate tests for normality (e.g. Shapiro-Wilk) were followed by post-hoc multiple comparison tests (e.g. Tukey-Kramer). All data are presented as means ± 1S.E.

Table 1
Organ masses and lengths during normoxic incubation

Measurement	Day 6	Day 12	Day 18
Egg and whole embryo			
Egg mass (g)	58.89 ± 1.40 (9)	58.03 ± 1.11 (12)	56.58 ± 0.99[a] (16)
Embryo mass (g)	0.39 ± 0.029 (9)	5.60 ± 0.38[b] (12)	15.49 ± 1.13[ac] (14)
Yolk mass (g)	34.84 ± 1.43 (9)	23.92 ± 0.74[b] (11)	17.14 ± 0.97[ac] (16)
CAM and internal organs			
CAM mass (mg)	20 ± 2 (9)	541 ± 31[b] (12)	559 ± 46[a] (14)
Heart mass (mg)	3 ± 0.4 (9)	59 ± 4[b] (12)	111 ± 7[ac] (14)
Lung mass (mg)	1 ± 0.2 (9)	60 ± 6[b] (12)	65 ± 11[a] (14)
Brain mass (mg)	22 ± 0. 3 (9)	363 ± 21[b] (12)	564 ± 42[ac] (16)
Kidney mass (mg)	1 ± 0.3 (8)	48 ± 7[b] (12)	89 ± 11[ac] (16)
Liver mass (mg)	3 ± 1 (9)	143 ± 20 (12)	350 ± 29[ac] (16)
Intestine mass (mg)	2 ± 1 (9)	80 ± 11 (12)	245 ± 26[ac] (16)
Stomach mass (mg)	2 ± 0.4 (9)	127 ± 12 (12)	692 ± 83[ac] (16)
External organs			
Eye mass (mg)	477 ± 5 (9)	540 ± 27[b] (12)	678 ± 28[ac] (16)
Beak length (mm)	2.4 ± 0.1 (9)	9.8 ± 0.4[b] (12)	13.4 ± 0.3[ac] (16)
Limbs			
Forelimb length (mm)	5.9 ± 0.2 (9)	22.2 ± 0.7[b] (12)	33.6 ± 1.4[ac] (16)
Humerus length (mm)	1.9 ± 0.1 (9)	7.3 ± 0.3[b] (12)	10.3 ± 0.5[ac] (16)
Ulna length (mm)	2.6 ± 0.1 (9)	6.3 ± 0.2[b] (12)	9.1 ± 0.4[ac] (16)
Radius length (mm)	2.6 ± 0.1 (9)	6.9 ± 0.2[b] (12)	9.6 ± 0.4[ac] (16)
Hindlimb length (mm)	6.6 ± 0.1 (9)	37.4 ± 1.1[b] (12)	63.5 ± 3.5[ac] (16)
Femur length (mm)	2.0 ± 0.2 (9)	9.0 ± 0.3[b] (12)	13.4 ± 0.8[ac] (16)
Tibia length (mm)	2.7 ± 0.2 (9)	11.5 ± 0.4[b] (12)	18.6 ± 1.2[ac] (16)
Tarsus length (mm)	1.3 ± 0.1 (9)	8.3 ± 0.3[b] (12)	15.0 ± 1.3[ac] (16)

Mean ± 1S.E. are presented.
[a] Day 18 values significantly different from Day 6.
[b] Day 12 values significantly different from Day 6.
[c] Day 18 values significantly different from Day 12.

3. Results

3.1. Normoxic development

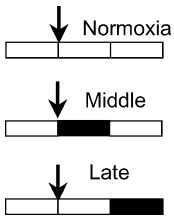
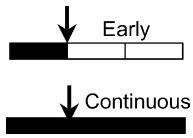
All organs increased significantly ($P < 0.001$) in mass or length during the incubation period. Most notably, body mass showed the largest proportional increase (about 14-fold) from Day 6 (0.38 ± 0.03 g) to Day 12 (5.59 ± 0.38 g), then showed a smaller proportional increase (about three-fold) as growth continued to Day 18 (16.13 ± 1.20 g) (Table 1). Organ masses (CAM, heart, lungs, brain, eyes, kidneys, liver, intestines and stomach) and organ lengths (beak, femur, forelimb, hindlimb, humerus, radius, tarsus, tibia, ulna) similarly showed their greatest proportional increase between Day 6 and Day 12 (Table 1). During normoxic development, all organ masses and lengths at Day 12 were significantly larger than at Day 6. Most, but not all variables at Day 18 were significantly higher than at Day 12. The only organs that did *not* grow significantly in mass or length from Day 12 to Day 18 were the lungs and CAM. As anticipated, yolk mass was significantly smaller on Day 18 as yolk was being consumed to fuel embryonic growth.

Fig. 1 specifies the changes in mass of the CAM, lungs and heart, the three tissues that might be presumed a priori to be most sensitive to ambient oxygen during incubation. During normal development, heart growth continued during the last third of incubation, nearly doubling in size from 0.059 ± 0.004 to 0.111 ± 0.007 g. However, the lungs and CAM in normoxic embryos showed no significant increase in mass during the last third of incubation.

3.2. Hypoxic development

Significant effects of hypoxic exposure (relative to normoxic controls) were evident at different points of incubation in yolk, CAM, eyes and beak length (ANCOVA, $P < 0.05$). Only beak length showed an interaction between hypoxic exposure and development. No other organs were affected by any of the periods of hypoxic exposure during incubation (including continuous hypoxia). For clarity of presentation, data for all organs during normoxic incubation are presented in Table 1, but Tables 2–4 present data for only those organs affected by hypoxia at some point during development—i.e. yolk mass, CAM mass, eye mass and beak length. Total embryo mass is also presented in these tables for comparison purposes.

Table 2
Significant organ mass effects of hypoxic exposure measured on Day 6

	Population	
Measurement	Normoxia D1 to D6[a]	Hypoxia D1 to D6[b]
		
Embryo mass (g)	0.39 ± 0.03 (9)[c]	0.35 ± 0.03 (14)
Yolk (g)	34.84 ± 1.43 (9)	28.17 ± 1.08[d] (14)
CAM mass (g)	0.02 ± 0.002 (9)	0.03 ± 0.003 (14)
Eye mass (mg)	48 ± 5 (9)	33 ± 5 (14)
Beak length (mm)	2.4 ± 0.1 (9)	2.5 ± 0.3 (13)

Mean ± 1S.E. are presented. (Arrow indicates time of measurement. Filled block equals period of hypoxic exposure.)

[a] Combined value for normoxic, middle, and late populations on Day 6, prior to any hypoxic exposure.

[b] Combined value for early and continuous populations on Day 6, each of which is exposed to hypoxia during D1 to D6.

[c] *n* values in parentheses.

[d] Significantly different ($P < 0.05$) from normoxia.

3.2.1. Early incubation period (Days 1–6)

Both the early and continuous populations experienced early hypoxic exposure (Table 2). Concurrently, normoxia was experienced not only by the normoxia population, but also by the middle and late populations, which by protocol had not yet been exposed to low oxygen levels.

Hypoxic exposure during Days 1–6 (i.e. early and continuous groups) caused a significant reduction from normoxic values only in yolk mass. Interestingly, yolk mass was consumed at a greater rate by hypoxic populations than normoxic populations during early incubation (Table 2). All other measured variables were not significantly different from normoxic values.

3.2.2. Middle incubation period (Days 6–12)

By the end of the middle incubation period, neither the normoxic nor late populations had experienced hypoxia (Table 3). The middle and continuous populations had experienced hypoxia for 6 days (mid-

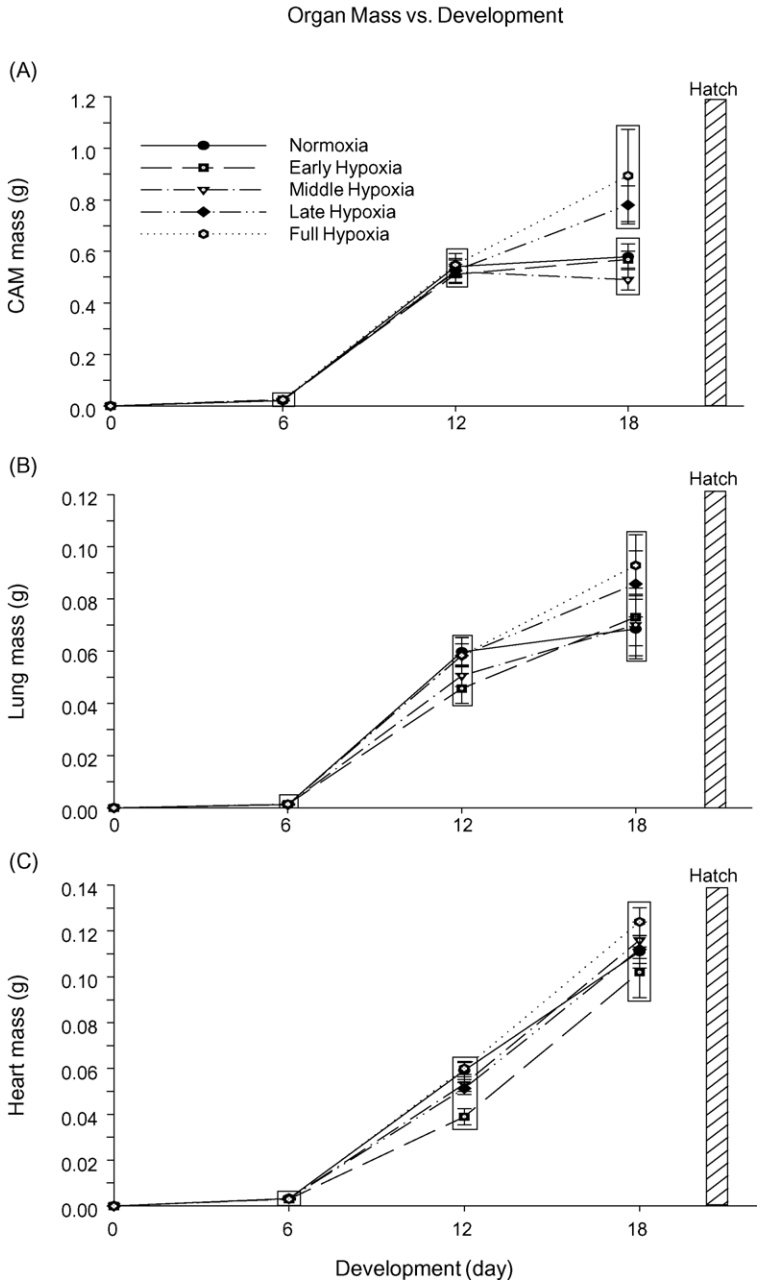
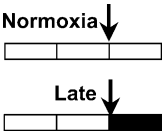
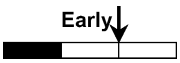
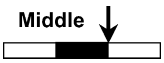
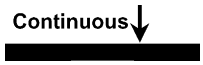


Fig. 1. Effects of different patterns of hypoxic exposure (15%) on the mass of the chorioallantoic membrane or CAM (A), lungs (B) and heart (C) of the developing chicken embryo. Mean values ± 1S.E. are plotted. All mean values within a box are not significantly different from each other. Data from five different protocols are shown. See text for additional details.

Table 3
Significant organ mass effects of hypoxic exposure measured on Day 12. Means ± 1S.E. are presented. (Arrow indicates time of measurement)

	Population			
Measurement	Normoxia D1 to D6[a]	Normoxia D6 to D12	Hypoxia D6 to D12	Hypoxia D1 to D12
				
Embryo mass (g)	5.59 ± 0.33 (25)[b]	4.78 ± 0.24 (7)	4.36 ± 0.29 (9)	4.63 ± 0.12 (7)
Yolk mass (g)	21.61 ± 0.61 (24)	18.96 ± 1.30 (7)	17.74 ± 0.76[c] (9)	23.43 ± 0.87 (8)
CAM mass (mg)	533 ± 28 (25)	511 ± 31 (7)	521 ± 45 (9)	548 ± 44 (7)
Eye mass (mg)	527 ± 31 (25)	409 ± 44 (7)	415 ± 26 (9)	351 ± 76[c] (8)
Beak length (mm)	9.3 ± 0.3 (25)	7.4 ± 0.4[c] (7)	8.5 ± 0.5 (9)	9.0 ± 0.2 (8)

[a] Combined value for normoxic and late populations on Day 12, prior to any hypoxic exposure.
[b] *n* values in parentheses
[c] Significantly different ($P < 0.05$) from normoxia.

dle) or 12 days (continuous). By the end of the middle period, the early hypoxic group had experienced 6 days of normoxic recovery from their early hypoxic exposure.

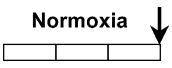
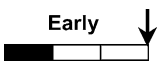
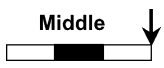
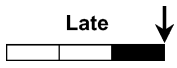
Hypoxic exposure caused a significant reduction in beak length in early embryos compared with those in normoxia. Continuous hypoxia also caused a significant reduction in yolk mass in the middle group. Although yolk mass had been lower in the continuous population after Day 6, by Day 12 it was not significantly different from normoxic populations. Eye mass was also reduced significantly by about 35% in the continuous population. All other values were not significantly different from normoxia on Day 12.

3.2.3. *Late incubation period (Days 12–18)*

By Day 18, all but the normoxic population had experienced some degree of hypoxic exposure (Table 4). The early and middle populations had 12 and 6 days, respectively, to recover from hypoxia. The late group had recently experienced hypoxia for 6 days, while the continuous group was finishing 18 days of hypoxic exposure.

Despite experiencing the most recent (late) and most prolonged (continuous) hypoxic exposure, neither population showed any significant differences in organ masses or lengths with the notable exception of the CAM, which was significantly heavier by ∼40% in the late population and ∼60% in the continuous population (Fig. 1). Associated with this heavier mass was an ap-

Table 4
Significant organ mass effects of hypoxic exposure measured on Day 18

	Population				
Measurement	Normoxia D 1 to D18	Normoxia D12 to D18	Normoxia D1 to D6 and D12 to D18	Hypoxia D12 to D18	Hypoxia D1 to D18
					
Embryo mass (g)	16.13 ± 1.20 (14)[a]	14.47 ± 1.71 (8)	15.81 ± 1.38 (10)	15.70 ± 1.30 (11)	14.74 ± 1.51 (8)
Yolk mass (g)	17.14 ± 0.97 (16)	14.97 ± 0.79 (8)	12.78 ± 0.88 (10)	16.79 ± 1.02 (11)	18.84 ± 1.73 (8)
CAM mass (mg)	559 ± 46 (14)	568 ± 33 (8)	490 ± 39 (10)	780 ± 74[b] (11)	894 ± 175[b] (7)
Eye mass (mg)	678 ± 276 (16)	590 ± 41 (8)	577 ± 65 (10)	705 ± 37 (11)	628 ± 31 (8)
Beak length (mm)	13.6 ± 0.3 (16)	13.5 ± 0.6 (8)	14.1 ± 0.4 (10)	13.2 ± 0.4 (11)	12.9 ± 0.4 (8)

Arrow indicates time of measurement. Mean ± 1S.E. are presented.
[a] *n* values in parentheses.
[b] Significantly different ($P < 0.05$) from normoxia.

parent increase in the vascular density of the CAM (not quantified). The early and middle populations showed no significant increase in CAM mass when compared to the normoxic population on Day 18, indicating no "carryover effect" on CAM mass from early hypoxic exposure.

4. Discussion

4.1. Critique of the methodology

The present study on chicken embryos, along with other recent studies that assess physiological and/or anatomical developmental landmarks and how they might be changed by experimental conditions, have divided the incubation period into thirds (e.g. Dzialowski et al., 2002; Elmonoufy, 2003). While convenient, the division of avian incubation into three discrete periods of equal length is arbitrary. Normal tables of avian anatomical development (e.g. Romanoff, 1960), as well as studies of physiological change during avian development (see reviews by Keller (1997), Hu et al. (2000), Tazawa and Hou (1997), Burggren and Crossley (2002)), suggest that structure and function do not progress in a linear function, nor do they progress towards increasing embryonic complexity at the same rate. That is, the amount of change from Day 1 to Day 6 may quite different from that evident from, say, Day 12 to Day 18. It is compelling to follow the emerging protocol of dividing incubation evenly into thirds for studies on environmentally induced alterations during avian development. Still, a study specifically investigating whether these arbitrary divisions represent the most appropriate division of an incubation period is certainly warranted.

The protocol used in the present study, with staggered periods of chronic hypoxic exposure, has been rarely used in avian studies (Dzialowski et al., 2002; Elmonoufy, 2003). However, this approach provides a powerful methodology for determining "critical windows" during development and how abnormal developmental trajectories may be ultimately still arrive at normal phenotype at hatching (Burggren, 1998). Unfortunately, this more complex protocol makes it somewhat difficult to compare our results with those previously published, which typically exposed embryos to a given level of hypoxia throughout the incubation period.

The suite of characters in this study, including 20 different morphological measures quantified by weight or length, is among the most comprehensive that has been examined in response to hypoxic exposure in the chicken embryo. Major systems involved include skeletal (numerous bones), cardiovascular (heart), respiratory (lungs), renal (kidney), neural (brain) and sensory (eyes). While length and/or weight are useful indicators of gross morphological disturbance, this approach would not reveal malformations per se, nor changes in the mode of growth. For example, we measured heart mass, but not ventricular wall thickness, muscle hypertrophy or other such variables that might have actually resulted in changes in cardiovascular performance. Future studies could profitably concentrate on more detailed studies of individual organs or organ systems to determine graded anatomical effects short of those revealed by changes in mass or dimension.

The level of hypoxic exposure used in the present study (15%) was equivalent to that experienced at 2900 m. Various investigators attempting to perturb avian development through oxygen deprivation have used a variety of levels of hypoxic exposure, for example, 10% by Hoper and Jahn (1995), 12% by Adair et al. (1987), 14% by Burton and Palmer (1992), and 15% by Dzialowski et al. (2002) and the present study (Table 5). There are numerous species of birds that live above up to 6500 m (Dragon et al., 1999; Leon-Velarde et al., 1997), and certainly domesticated chickens are routinely reared between 400 and 6500 m (Leon-Velarde and Monge, 2004). Yet, there is no doubt that 15% O_2 or lower represents a significant hypoxic challenge to the embryo (see Table 5). Once the effects of hypoxia on avian development are better understood, it would be interesting to conduct "dose-response" experiments to determine the thresholds for various types of anatomical and physiological effects induced by oxygen deprivation.

4.2. A study in frustration: comparisons and contrasts with previous studies

Previous studies of the effects of hypoxic incubation on chicken embryo morphology have yielded highly inconsistent findings (Table 5). For example, Stock and Metcalfe (1987), Burton and Palmer (1992) and Rouwet et al. (2002) report significant declines in embryo weights during exposure to 14–15% O_2, whereas

Table 5
Survey of morphological and physiological effects of hypoxic exposure during incubation on the chicken embryo

Study	Level of hypoxia (% O_2)	Duration of hypoxia	Observations
Adair et al. (1987)	12, 16%	3 days, starting on D14	Increased arterial blood flow capacity
Akiyama et al. (1999)	10%	2 or 4 h; embryos range from D3 to D9	Bradycardia
Altimiras and Phu (2000)	10%	2, 4, and 6 h, starting on D2, D3, or D4	Head, eye, limb malformations; dwarfism; heart defects (heart to body mass ratio unchanged); bradycardia
Asson-Batres et al. (1989)	15%	3 days, starting on 16th day	Ventricular mass decrease (wet mass of brain unchanged); tissue protein/DNA ratios decreased
Baumann et al. (1983)	13.5%	Entire incubation, measurements on D4–D9	Accelerated transition from embryonic to adult Hb; blood volume and O_2 capacity unchanged; O_2 affinity increased
Burton and Palmer (1992)	14%	Entire incubation	Diminished embryo and CAM growth; accelerated growth of capillary plexus
Crossley et al. (2003b)	15, 10, 5%	Exposed to step changes for 5 min each	Hypotension; bradycardia
Dzialowski et al. (2002)	15%	6 days, starting at D0, D6 or D12	Decreased body mass (heart mass unchanged); decreased VO_2; elevated hematocrit (no differences in [Hb] between groups)
Hoper and Jahn (1995)	10%	D0–D4	Increased vascular density; enlargement of area vasculosa
Richards et al. (1991–1992)	15%	3 days, starting on D15	Reduced embryo, heart, brain, and liver wet mass; increased CAM wet mass; Cyto- and mieloarchitecture of the tectum severely affected
Stock and Metcalfe (1987)	15%	3 days, beginning on D16	Exaggeration of normal growth deceleration; decreased embryo mass (no change in relative decrease in brain mass); decreased VO_2
Strick et al. (1991)	12, 16, 21, 45, 70%	7 days, beginning D7	Cam vascularity inversely related to oxygen level
Xu and Mortola (1989)	10%	D14–D18	Increased lung:body mass ratios; elevated hematocrit

we found that embryo weight was protected during hypoxic exposure. Heart mass has variously been reported to increase (Stock and Metcalfe, 1987; Rouwet et al., 2002), remain unchanged (present study, Elmonoufy, 2003), or decrease during hypoxic exposure (Richards et al., 1991–1992). Similarly, brain weight in chicken embryos in response to hypoxia has variously been reported to decrease (Stock and Metcalfe, 1987) or remain unchanged (Asson-Batres et al., 1989; present study).

While additional comparisons and contrasts could be generated, the key point is that, is evident from Table 5, all of these studies have used slightly to fundamentally different *levels* of hypoxic exposure (e.g. 10%, Hoper and Jahn versus 15% for the present study and many others) as well as different *periods* of exposure (e.g. acutely for hours, Altimiras and Phu, 2000; chronically for the whole incubation period, Rouwet et al., 2002) or combinations of multi-day hypoxic periods during incubation (Adair et al., 1987; Richards et al., 1991–1992; present study). It is hardly surprising that such inconsistent patterns emerge. It is not the intent of the present study to attempt to disprove or support previous studies, but rather to learn how the developing chick responds to hypoxic challenge. In this respect, each of the previous studies make important contributions. Having said that, we urge future studies to at the very least use what appears to be an emerging standard for level of hypoxic exposure of 15% (Stock and Metcalfe, 1987; Asson-Batres et al.,

1989; Richards et al., 1991–1992; Rouwet et al., 2002; Dzialowski et al., 2002; Elmonoufy, 2003; Crossley et al., 2003b; present study). In those cases where milder or more severe hypoxia is desirable in the experimental protocol, we strongly suggest also running parallel experiments at 15% hypoxia to allow "calibration" of that study with the preponderance of those already carried out at 15% O_2. Similarly, standardized periods of exposure would be desirable (see our critique, above, of the arbitrary division of incubation into "thirds" or "quarters" when not based on real morphological or physiological landmarks).

Finally, emerging evidence indicates that different lines of *G. gallus* (e.g. Rhode Island Red, White Leghorn, or even genetic lines within these groups) may actually show differences in timing of onset of physiological regulatory processes during embryonic development (E. Dzialowski, unpublished) as well as differences in metabolic rate, gas exchange, etc. (Tona et al., 2004). Such differences could account in part for reported differences in the literature with respect to hypoxic responses during incubation. Our study also reports some organ masses that differ from those reported by Romanoff (1967). For example, Romanoff (1967) reports embryo and CAM masses on Day 18 of 22.09 and 1.37 g, respectively, while in the present study we report 15.49 g (embryos) and 0.60 g (CAM). Reported organ mass depends on a myriad of factors including not only the potential genetic mechanisms described above, but also nutritional state of the hen, incubation temperature and humidity, as well as the techniques used for dissecting out and separating organs and tissues. This renders comparisons across development *within* a study more consistent than comparisons at the same time in development *between* studies, arguing for experimental protocols that encompass as much of an animal's developmental range as feasible.

Having discussed the considerable variations in data and the corresponding differences in interpretation of the previously published studies, let us now consider the implications of our own findings.

4.3. Normal development

These data provide a comprehensive measure of the size and rate of growth of key organs in *G. gallus* during development. An organ-by-organ analysis of growth rate is beyond the scope of the present study, but it is interesting to note that most organs examined showed the greatest proportional growth in organ mass or dimension between Day 6 and Day 12, with relatively little proportional organ growth occurring between Day 12 and Day 18. This suggests that the earlier period of incubation focuses on rapid creation and subsequent growth of organs, while the later period of incubation reflects a period of slower growth but presumably considerable organ maturation.

It would be very interesting to compare changes in organ mass with corresponding linear dimension as development progresses. That is, for example, as a femur gets longer at what rate does its mass increase? While dimensional changes with total body mass increase are very well understood in an interspecific context (e.g. "mouse to elephant"), the corresponding *intraspecific* changes that accompany development (e.g. "embryo to adult") have been not been well studied and their implications are poorly understood.

4.4. Hypoxic influence on development

4.4.1. Early incubation

The growth of embryos during their early incubation period (Day 1 to Day 6) was completely unaffected by chronic hypoxic exposure. Certainly in the first hours to few days of development, vertebrate embryos exhibit relatively high rates of anaerobic metabolism (see Burggren and Just, 1992 for earlier literature), which would of course not be directly affected by hypoxic exposure. However, by Day 3 there is a vigorous consumption of oxygen (see Romanoff, 1967; Burggren et al., 2000), and hypoxic effects were anticipated. Interestingly, yolk mass was consumed at a significantly greater rate in hypoxic compared with control (normoxic) embryos. Development of any embryo is characterized by dual requirements of ongoing organogenesis as well as the maintenance of existing structures. That our hypoxic embryos showed growth equivalent to controls, but at the "cost" of increased yolk utilization, suggests that the costs of aerobic tissue maintenance were appreciably higher during continuous hypoxic exposure. If this holds, we would anticipate that hypoxic embryos from Day 1 to Day 6 might have an elevated level of oxygen consumption at the cost of accelerated yolk consumption, with both of these increases reflecting the increased cost of maintenance.

It is interesting to note that the early population showed only smaller beaks at Day 12, and were statistically indistinguishable in all respects by Day 18. Thus, the greater rate of yolk consumption during Day 1 to Day 6 had no lasting deleterious effects. This suggests that the chicken embryo has a considerable "safety factor" with respect to the amount of yolk available at the beginning of incubation. It would be very illuminating to repeat these experiments while systematically altering the initial amount of yolk to determine the magnitude of this safety factor (e.g. the "alloengineering" advocated by Sinervo and Huey (1990)).

4.4.2. Middle incubation

Assessment of hypoxic effects on Day 12 revealed that populations exposed to hypoxia in either early or middle incubation were nonetheless able to maintain overall embryo and CAM mass at levels not significantly different from the control population. We did not assess CAM vascular density, but a protocol for hypoxic exposure essentially equivalent to that used for our middle group produced large increases in CAM vascular density (Dusseau and Hutchins, 1988, 1989; Strick et al., 1991). The eyes, which had not been affected by early hypoxic exposure, proved susceptible to the cumulative effects of hypoxic exposure over 12 days (Table 3). Why beak length was significantly lower following early hypoxic exposure in the early population but not in the continuous population is not clear.

Yolk consumption was stimulated by hypoxic exposure between Day 6 and Day 12 in the middle population, as was found for earlier hypoxic exposure. Curiously, however, the continuous population, which to this point in the experiments had received continuous hypoxic exposure, no longer had a reduced yolk size, indeed showing rates of yolk consumption not different from the controls (Table 3). In fact, reduced yolk size (i.e. greater yolk consumption) during the first 2/3 of incubation appears to be a function of the *first* 6 days of hypoxic exposure, rather than *which* 6 days. Thus, the early population showed reduced yolk mass between Day 1 and Day 6 (their first exposure), while the middle population showed reduced yolk mass between Day 6 and Day 12 (their first exposure). It is tempting to speculate that by Day 12 the early and continuous population had physiologically and biochemically compensated for hypoxic exposure—in the case of the early population having recovered from the effects of hypoxia, in the case of the continuous population having acclimated to them.

4.4.3. Late incubation

By late incubation (Days 12–18) the deleterious effects of hypoxic exposure experienced previously had been corrected, and no new deleterious effects were induced. Irrespective of whether chicken embryos were experiencing hypoxia for the first time in late development (late population), or had been incubated constantly under hypoxic conditions (continuous population), CAM masses were significantly higher in chicken embryos exposed to hypoxia during the last 6-day period (Table 4). The approximate 40–60% increase in CAM mass that we measured in these populations, coupled with past observations of CAM vascular density increases stimulated by hypoxia (e.g. Dusseau and Hutchins, 1988, 1989; Strick et al., 1991), suggests an increased functional ("physiological") surface area for gas exchange, which would be an appropriate acclimation to hypoxia. The oxygen consumption of the embryo is greatest during the last 1/3 of incubation (Romanoff, 1967; Metcalfe et al., 1984; Howe et al., 1995), and the CAM has already grown to occupy most of the available space lining the eggshell (Ackerman and Rahn, 1981). Thus, when combined with hypoxic exposure, the functional surface area could become limiting to embryonic oxygen consumption during the later stages of incubation. This may account for why hypoxia did not stimulate CAM mass until the later stages of incubation, when an increased functional surface area of the CAM would certainly help ensure sufficient tissue oxygenation for the measured normal growth of the other tissues.

4.4.4. General observations

The embryonic heart showed no evidence of hypoxia-induced hypertrophy during any combination of hypoxic exposure in these experiments. One option for a vertebrate facing hypoxic challenge is to increase heart rate and/or stroke volume, leading to an increase in blood oxygen transport. In the dynamic embryonic heart, stroke volume increases maintained over time are likely to manifest themselves in a ventricular hypertrophy, and thus higher heart mass (see Burggren and Keller, 1997; Harvey and Rosenthal, 1999). That we recorded no hypoxia-induced changes in heart

mass suggests that other mechanisms are likely to have been at play, and could include adjustments in heart rate, or changes in blood oxygen transport characteristics. The in vivo characteristics of the blood of chicken embryos are known to respond to chronic hypothermia (Black and Burggren, 2004) and hypoxia (Dragon and Baumann, 2003).

The lungs of the embryonic chicken were unaffected by hypoxic incubation in any combination during hypoxia, suggesting no "preadaptive" pulmonary hypertrophy that would assist gas exchange after hatching. However, the strong CAM hypertrophy recorded in this study, and for the CAM and area vasculosa previously by others (Dusseau and Hutchins, 1988, 1989; Strick et al., 1991; Richards et al., 1991; Hoper and Jahn, 1995) indicates that gas-exchanging tissues of the chicken embryo can respond with increased mass and vascular density (and presumably functional surface area for gas exchange) in response to hypoxic exposure. This differential response of the CAM and lungs also suggests that these two structures, though each having the same ultimate function of gas exchange, are developmentally quite distinctive. In addition, the effects of altered oxygen availability on the growth of individual organs vary considerably (McCutcheon et al., 1982; Stock et al., 1983; Bartels et al., 1985).

A basic tenet of developmental biology is that organs/organ systems exhibit a "critical window" during development, in which they are more highly susceptible to teratogens. The present study has shown that certain organs show different periods of susceptibility to hypoxia. For example, normal growth of the beak was disrupted by hypoxic exposure between Day 1 and Day 6, with negative effects not showing up until Day 12. The eyes also proved susceptible during Day 6–Day 12, but this was a cumulative effect, since hypoxic exposure in either the early or middle period had no effect, whereas exposure during both periods resulted in small eyes on Day 12. Similarly, the CAM has a critical window in the final third of incubation, during which CAM mass was stimulated irrespective of whether there had been any previous hypoxic exposure (that is, stimulation of CAM growth in the last third did not depend upon cumulative hypoxic exposure). Interestingly, embryos showed the capacity for "self-repair" of hypoxia-induced stunting, since both eyes and beak recovered—at least in terms of their dimensions—if given at least 6 days of normoxia following hypoxic exposure. A similar ability to follow an abnormal developmental phenotype but still arrive at the normal phenotype upon hatching following the induction of morphological anomalies by hypoxia during middle incubation has been demonstrated for the quail *Coturnix coturnix* (Elmonoufy, N). Future studies more narrowly delineating the critical windows for these and other aspects of both anatomical and physiological development would be very informative.

Perhaps the most remarkable finding of this study is the ability of the chicken embryo to exhibit many aspects of normal growth (e.g. normally sized structures), despite chronic hypoxic stress. Adjustments in cardiovascular performance (e.g. increased cardiac output) could counteract the potential decrease in blood oxygen transport that might result from environmental hypoxia. Alternatively, or in addition, stimulation of erythropoiesis leading to increased blood [Hb], or modifications of existing Hb-O_2 binding characteristics (e.g. increased Hb-O_2 affinity), could also help to protect the developing chicken embryo against chronic hypoxic exposure. Certainly, the blood of chicken embryos is responsive to changes in oxygen and temperature (Bjonnes et al., 1987; Ingermann et al., 1983; Dragon and Baumann, 2003; Black and Burggren, 2004). Future experiments should be directed towards understanding the physiological underpinnings of the hypoxic resistance during development revealed in this study.

Acknowledgements

This study was supported by a summer research grant from the American Physiological Society (TC) and an operating grant from the National Science Foundation (WB).

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