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# Chronic hypoxia alters the physiological and morphological trajectories of developing chicken embryos<sup>☆</sup>

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## Abstract

Chicken embryos were chronically exposed to hypoxia ( $P_{O_2} \sim 110$  mmHg) during development, and assessed for detrimental metabolic and morphological effects. Eggs were incubated in one of four groups: control (i.e. 151 mmHg), or treated with continuous 110 mmHg (15%  $O_2$ ) during days 1–6 (H1–6), 6–12 (H6–12), or 12–18 (H12–18) with normoxia during the remaining incubation. Metabolism ( $V_{O_2}$ ), body mass, hemoglobin (Hb) and hematocrit (Hct) were measured in embryos on days 12 and 18 of incubation and in day-old hatchlings. Ability to maintain  $V_{O_2}$  was acutely measured during a step-wise decrease in  $P_{O_2}$  from normoxia to hypoxia (55 mmHg). On day 12,  $V_{O_2}$  of H1–6 eggs were significantly lower than in the control and H6–12 eggs.  $P_{crit}$  in H6–12 eggs was lower than in control and H1–6 eggs. Body mass of H1–6 and H6–12 embryos on day 12 was significantly lower than in control embryos, while in H6–12 embryos, Hct and Hb were higher. On day 18, H6–12 embryos had significantly lower  $V_{O_2}$  than control eggs. Body mass of H6–12 and H12–18 embryos was significantly lower than control embryos. Hct and Hb did not differ between treatments. In hatchlings, mass, Hb and Hct had returned to values statistically identical to controls. However, H6–12 embryos had significantly lower  $V_{O_2}$ . Long-term hypoxia altered  $V_{O_2}$  when hypoxic incubation occurred during the middle third of incubation, but not during earlier or later incubation. Thus, chronic hypoxic exposure during critical periods in development altered the developmental physiological trajectories and modified the phenotypes of the developing embryos. © 2002 Elsevier Science Inc. All rights reserved.

**Keywords:** Hypoxia; Metabolic rate; Development; Heart mass; Body mass; Hematocrit; Hemoglobin; Trajectories

## 1. Introduction

Response to hypoxia during development in chicken eggs has previously been studied with respect to changes in body mass, metabolic rate

and hematology (Wangensteen et al., 1974; McCutcheon et al., 1982; Baumann et al., 1983; Stock and Metcalfe, 1987). Several studies on the effects of chronic hypoxia or hyperoxia on the development of chicken embryos have shown that the availability of oxygen induces differential growth among individual organs (Smith et al., 1969; McCutcheon et al., 1982; Stock et al., 1983). Embryo mass decreases in response to hypoxia and increases with hyperoxia. The heart is the least affected by hypoxia, and in contrast to embryo mass, the hearts of hypoxic-incubated

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animals increase in mass in some species (McCutcheon et al., 1982; Snyder et al., 1982).

The metabolic rate of developing chicken embryos has been shown to have differential responses to hypoxia. Brief (72-h) exposure to hypoxia from day 16 through day 18 of incubation resulted in decreased metabolic rate on day 18 (Stock and Metcalfe, 1987). Similar patterns have been observed under more chronic hypoxia (Beattie and Smith, 1975). During chronic exposure to low levels of oxygen at high altitude, chickens exhibit a decreased metabolism and growth rate (Wangensteen et al., 1974). Along with the metabolic changes observed, hematocrit and hemoglobin concentrations tend to be higher in chicken embryos incubated in hypoxia or with lowered shell-gas conductance (Tazawa et al., 1971a; Dusseau and Hutchins, 1988). Developing chicken embryos tend to be more susceptible to exposure to chronic hypoxia than many wild birds (Wangensteen et al., 1974; Carey et al., 1982, 1993).

All previous studies have examined the effect of chronic hypoxia on development in chickens either during the entire incubation period, or during only one short segment of incubation. However, hypoxia may have different effects on development, depending on the timing within incubation of the hypoxic bout. Hypoxia occurring early in development may have quite different effects than hypoxia occurring in the later stages, since different organ systems have different 'critical windows' during which environmental perturbation will exert maximal effects (Burggren, 1999).

In the present study, we tested for critical windows of development in the metabolic physiology and morphology of developing chicken embryos, assessed by exposure to chronic hypoxia during different stages of development. By measuring during early, middle and late incubation, we were able to determine developmental trajectories leading to new physiological and morphological phenotypes, as measured by metabolic rate ( $V_{O_2}$ ),  $P_{crit}$  (the partial pressure of oxygen at which  $V_{O_2}$  begins to decrease), body and heart mass, morphology and hematological parameters.

## 2. Material and methods

### 2.1. Incubation

White leghorn eggs were obtained from Texas A&M University, transported to the University of

North Texas, and used within 1 week of arrival. Up to 24 eggs at a time were incubated in a number of 4-l closed containers, to allow for the control of ambient  $O_2$  concentration. Each container was ventilated with a gas mixture.  $O_2$  and  $N_2$  were mixed with a Cameron gas mixer (model GF-3) to produce a gas mixture of either 151 mmHg (21%  $O_2$ ) or 110 mmHg (15%  $O_2$ ), humidified to between 75 and 95% relative humidity. Six containers with eggs were placed in a Lyon egg incubator at 37.5 °C and turned every 4 h. Eggs from each treatment were randomly placed into containers. On day 19, all eggs were incubated in 151 mmHg.

### 2.2. Incubation protocol

Eggs were incubated in one of four treatment groups:  $P_{O_2}$  of 151 mmHg throughout incubation (control);  $P_{O_2}$  of 110 mmHg from day 1 to 6 with normoxia thereafter (H1–6); 110 mmHg exposure from day 6 to 12 with normoxia at all other times (H6–12); and 110 mmHg  $O_2$  exposure from day 12 to 18 with normoxia at all other times (H12–18). Measurements were carried out on days 12 and 18 of incubation, and on day-old hatchlings. Fig. 1 shows the experimental protocol.

### 2.3. Metabolic rate measurements

Metabolic rate was measured as oxygen consumption ( $V_{O_2}$ ) using flow-through respirometry. Eggs or hatchlings removed from the incubators were placed in individual PVC respirometers (approx. volume 225 ml). The  $O_2$  concentration flowing through each respirometer was controlled by mixing  $O_2$  and  $N_2$  with a Cameron gas mixer (model GF-3). Outflow  $O_2$  concentration from each respirometer was measured using a Beckman OM11  $O_2$  analyzer. Inflow  $O_2$  concentration to the respirometers was determined from the outflow of an empty respirometer.  $V_{O_2}$  was measured in eggs and hatchlings, while  $P_{O_2}$  was decreased step-wise from normoxia to hypoxia (approx. 151, 131, 111, 90, 78, 64 and 51 mmHg). Five eggs or day-old hatchlings in separate respirometers were measured at the same time. Initially, the eggs or hatchlings were allowed to equilibrate at 151 mmHg for 1 h in the chamber. For each egg or hatchling, measurement of outflow  $O_2$  level lasted 2 min, followed by 1 min of measurement of the inflow gas. Eggs or hatchlings were exposed to the next  $O_2$  level



differences. The level of significance for all tests was  $P < 0.05$ . All statistical tests were performed with SAS 8.0 using the Proc Mixed function.

Fisherex, an extension of Fisher's exact test (Sokal and Rohlf, 1995), which handles multiple-column contingency tables with small sample sizes, was used to test for differences in survival between incubation treatments after  $V_{O_2}$  measurements.

### 3. Results

#### 3.1. Body morphology

Changes in wet and dry body mass and heart mass, length of third toe and beak length as a function of incubation treatment through development are presented in Table 1. On day 12, eggs that had been exposed to hypoxia during either the first or second third of incubation had significantly smaller ( $P < 0.05$ ) wet and dry body masses than the control eggs. Eggs exposed to hypoxia from day 6 to 12 had significantly larger ( $P < 0.01$ ) hearts than control eggs after correction for embryo mass differences. The percent of water in either the embryo or the heart did not differ between treatments. There were no significant differences in toe or beak length between treatments on day 12.

On day 18, eggs exposed to hypoxia during either the second or last third of incubation had significantly smaller ( $P < 0.03$ ) wet and dry embryo masses. However, embryos from H1–6 eggs had actually regained body mass, and were not significantly different in mass from the control embryos. However, they had significantly less ( $P < 0.01$ ) water content in the embryo than the control embryos. Embryos from H6–12 eggs had significantly shorter ( $P < 0.01$ ) third toes than the embryos from control eggs, while H12–18 embryos had shorter beaks ( $P < 0.01$ ). On day 18, there were no differences in wet or dry heart mass between treatments (Table 1).

By the time of hatching, all morphological parameters were statistically identical in all treatments and the controls. The only exception was that the yolk-free dry mass of hatchlings from eggs exposed to hypoxia during days 12–18 was significantly smaller ( $P < 0.01$ ) than the control hatchlings (Table 1).

#### 3.2. Oxygen consumption

##### 3.2.1. $V_{O_2}$ on day 12

Mean  $V_{O_2}$  differed significantly between incubation treatments during acute exposure to decreasing levels of  $O_2$  (Table 2; Fig. 2a). H1–6 eggs had significantly lower mean  $V_{O_2}$  ( $0.077 \text{ ml } O_2 \text{ min}^{-1} \text{ egg}^{-1}$ ) than either the control ( $0.096 \text{ ml } O_2 \text{ min}^{-1} \text{ egg}^{-1}$ ) or the H6–12 eggs ( $0.095 \text{ ml } O_2 \text{ min}^{-1} \text{ egg}^{-1}$ ). There was a significant interaction between the incubation treatment effect and the acute hypoxic exposure levels (Table 2). The  $V_{O_2}$  of H1–6 eggs was significantly lower ( $P < 0.01$ ) than the control eggs at  $P_{O_2}$  of 130 and 110 mmHg. No differences in  $V_{O_2}$  were observed between the control eggs and H6–12 eggs, except at a  $P_{O_2}$  of 130 mmHg ( $P < 0.05$ ).

Control eggs regulated  $V_{O_2}$  only down to a  $P_{crit}$  of 132 mmHg on day 12. However, H1–6 eggs were oxyconformers, with a  $P_{crit}$  at the highest  $P_{O_2}$  level. H6–12 eggs had the greatest ability to oxyregulate, with a  $P_{crit}$  of 111 mmHg.

There was significant differential survival on day 12 after acute exposure to increasing levels of hypoxia. Embryos from H6–12 eggs, which had been chronically incubated in hypoxia just prior to the measurement, had higher survival (12 of 14; 86%) than the control (7 of 15; 47%) or H1–6 (0 of 15; 0%) embryos ( $P < 0.01$ ).

Embryos exposed to hypoxia during the first third of incubation (H1–6) exhibited the largest effects of chronic hypoxia. Even though mass was smaller in H6–12 embryos, their mass-corrected  $V_{O_2}$  did not differ from the control embryos.

##### 3.2.2. $V_{O_2}$ on day 18

There was a significant treatment effect on  $V_{O_2}$  during acute decreases in  $O_2$  (Table 2, Fig. 2b). H6–12 eggs had a significantly lower (all  $P < 0.02$ ) overall  $V_{O_2}$  ( $0.24 \text{ ml } O_2 \text{ min}^{-1} \text{ egg}^{-1}$ ) than control ( $0.28 \text{ ml } O_2 \text{ min}^{-1} \text{ egg}^{-1}$ ), H1–6 ( $0.29 \text{ ml } O_2 \text{ min}^{-1} \text{ egg}^{-1}$ ), and H12–18 eggs ( $0.30 \text{ ml } O_2 \text{ min}^{-1} \text{ egg}^{-1}$ ). Acute decreases in  $P_{O_2}$  resulted in significant decreases in  $V_{O_2}$  in all levels of acute hypoxia.

There was no significant interaction between incubation condition and  $P_{O_2}$  level on  $V_{O_2}$  at day 18. However, independent contrasts revealed differences between treatments. At  $P_{O_2}$  levels of 155, 132, and 110 mmHg, H6–12 eggs had a significantly lower (all  $P < 0.02$ )  $V_{O_2}$  than the control eggs. H12–18 eggs had a significantly higher

Table 1

Wet mass, dry mass, and % water for embryos and hearts, and length of 3rd toes and beaks of day 12 and 18 embryos and hatchlings exposed to hypoxia during various portions of incubation

	Day 12			Day 18				Hatchling				
	Control	Hypoxia		Control	Hypoxia			Control	Hypoxia			
		d1–6	d6–12		d1–6	d6–12	d12–18		d1–6	d6–12	d12–18	
<i>n</i>	45	27	27	22	22	26	25	18	9	16	16	
<i>Embryo</i>												
Wet body mass (g)	6.0±0.1	5.5±0.2*	5.2±0.2**	23.4±0.5	22.4±0.5	20.7±0.5**	19.7±0.5**	33.6±0.6	35.4±0.9	33.5±0.6	32.1±0.6	
Dry body mass (g)	0.50±0.02	0.43±0.02*	0.41±0.02**	4.6±0.1	4.7±0.1	3.8±0.1*	3.5±0.1**	6.3±0.1	6.7±0.2	6.1±0.1	5.7±0.1*	
Body water (%)	91.7±0.1	92.1±0.2	92.1±0.2	81.8±0.6	79.0±0.6**	81.7±0.5	82.4±0.6	81.3±0.3	80.1±0.5	81.8±0.3	82.3±0.3	
<i>Heart</i>												
Wet heart mass (mg)	45.8±1.3	47.6±1.6	54.9±1.7**	121.8±5.6	130.1±5.4	121.9±5.0	114.9±5.4	196.4±8.4	235.0±14.2	223.0±8.8	220.8±8.9	
Dry heart mass (mg)	5.4±0.1	5.6±0.2	6.3±0.2	16.2±0.6	18.0±0.6	16.6±0.5	15.4±0.6	30.8±1.4	34.8±2.1	34.3±1.5	32.7±1.6	
Heart water (%)	88.3±0.2	88.3±0.2	88.5±0.2	86.5±0.2	85.9±0.2	86.3±0.2	86.6±0.2	84.6±0.3	84.0±0.4	84.7±0.3	85.0±0.3	
3rd toe length (mm)	9.0±0.1	8.7±0.2	8.7±0.2	20.9±0.4	20.6±0.4	19.4±0.3**	20.1±0.1	24.0±0.3	23.8±0.7	24.0±0.7	23.4±0.3	
Beak length (mm)	2.6±0.05	2.6±0.05	2.6±0.06	5.0±0.1	4.8±0.1	4.9±0.1	4.6±0.1**	5.4±0.1	5.8±0.1	5.7±0.1	5.3±0.1	

Significantly different from the control at the given time period  $P < 0.05^*$ ;  $P < 0.005^{**}$ .

(both  $P < 0.03$ )  $V_{O_2}$  at the low  $P_{O_2}$  levels of 80 and 50 mmHg. There were no differences at any of the  $P_{O_2}$  levels between control and H1–6 eggs. Both control and H1–6 eggs were oxyconformers at day 18, with a  $P_{crit}$  of 151 mmHg. H12–18 and H6–12 eggs were moderate oxyregulators, with a  $P_{crit}$  of 132 mmHg.

Exposure to hypoxia during the middle third of incubation had the largest effect on  $V_{O_2}$  when compared with control embryos. By day 18,  $V_{O_2}$  values of H1–6 eggs were no longer different from the control values.

### 3.2.3. $V_{O_2}$ in hatchlings

There was a significant effect of treatment and  $P_{O_2}$  level on the  $V_{O_2}$  of hatchlings (Table 2; Fig. 2c). H6–12 hatchlings had a significantly lower overall mean  $V_{O_2}$  ( $0.65 \text{ ml O}_2 \text{ min}^{-1} \text{ egg}^{-1}$ ) than the control ( $0.85 \text{ ml O}_2 \text{ min}^{-1} \text{ egg}^{-1}$ ), H1–6 ( $1.0 \text{ ml O}_2 \text{ min}^{-1} \text{ egg}^{-1}$ ) and H12–18 ( $0.84 \text{ ml O}_2 \text{ min}^{-1} \text{ egg}^{-1}$ ) hatchlings (all  $P < 0.01$ ). The H6–12 hatchlings had significantly lower (all  $P < 0.05$ )  $V_{O_2}$  than the control hatchlings at  $P_{O_2}$  levels of 90, 80, 70 and 50 mmHg. The  $V_{O_2}$  of H12–18 hatchlings did not differ from the control hatchlings. H1–6 hatchlings had a higher  $V_{O_2}$  than controls at 110 and 90 mmHg ( $P < 0.05$ ). All hatchlings except H6–12 had similar  $V_{O_2}$  levels and responses to decreasing  $P_{O_2}$ .

Exposure to hypoxia through incubation had little effect on hatchling phenotype when it occurred during the first or last third of incubation. Although no differences in  $V_{O_2}$  were observed on day 12 in H6–12 eggs, as hatchlings, they had significantly lower levels of  $V_{O_2}$ .

### 3.3. Hematocrit and hemoglobin

There was a significant increase ( $P < 0.01$ ) in hematocrit from day 12 to day 18, followed by a

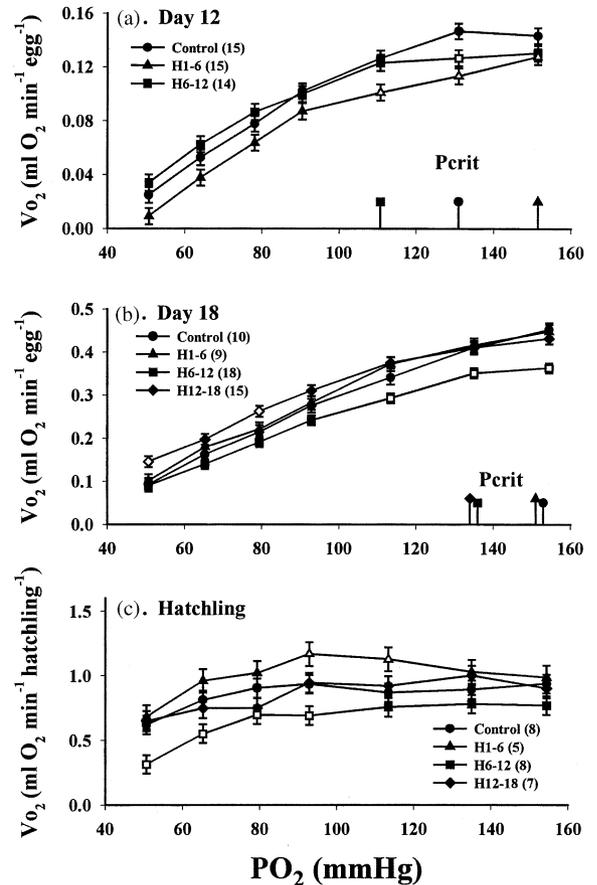


Fig. 2. Mean  $V_{O_2}$  of chicken embryos exposed to acute decreasing levels of oxygen on (a) day 12, (b) day 18 and (c) as day-old hatchlings. All  $V_{O_2}$  values are corrected for the animal's body mass and egg mass.  $P_{crit}$  is the  $P_{O_2}$  value at which the animal becomes an oxyconformer and is shown by the vertical lines along the x-axis.  $N$  for each group is given in the figure legends. All values are mean  $\pm$  S.E.M. Unfilled symbols represent values that are significantly different from the control values at that  $P_{O_2}$  level ( $P < 0.05$ ). We were unable to calculate a  $P_{crit}$  value for the hatchlings.

Table 2

Results of repeated-measures ANCOVA showing  $P$  values for each age group for chronic hypoxic condition effects, acute hypoxia during measurement effects, chronic  $\times$  acute hypoxia interaction effects, and egg mass and residual of embryo mass covariate effects

	$P$ value for effects tested				
	Chronic hypoxic incubation	Acute hypoxia	Chronic $\times$ acute interaction	Egg mass	Residual of embryo mass
Day 12	<0.005	<0.0001	<0.05	NS	<0.0001
Day 18	<0.0001	<0.0001	NS	NS	<0.0001
Hatchling	<0.001	<0.0001	NS	<0.01	NS

significant decrease ( $P < 0.01$ ) between day 18 and hatching (Fig. 3). There was a significant interaction between the time of development and the incubation condition in both hematocrit and hemoglobin. Hematocrit in embryos from H6–12 eggs compared with embryos from control eggs was significantly higher on day 12 ( $P < 0.05$ ), while on day 18 and in hatchlings, there were no differences in hematocrit (Fig. 3a). Hemoglobin significantly increased ( $P < 0.01$ ) from day 12 to day 18 (Fig. 3b). There were no differences in hemoglobin between treatment groups on any of the measurement days. However, H6–12 embryos had a higher, although not significant hemoglobin concentration than the control embryos on day 12.

## 4. Discussion

### 4.1. Morphological responses to chronic hypoxia

A number of studies have shown that chronic hypoxia decreases the growth of developing chicken embryos (Smith et al., 1969; Wangenstein et al., 1974; McCutcheon et al., 1982). Similarly, in our study after a 6-day exposure to hypoxia during either the first, second, or third period of incubation, embryo masses in all of the treatments were significantly lower than the control embryos. This differential response in body mass to chronic hypoxia in developing chicken embryos was typically not observed in wild birds developing at high altitude (Carey et al., 1982, 1989). However, upon removal of the hypoxia, the chicken embryos in this study were able to recover body mass, such that all treatment groups had a statistically similar body mass as hatchlings.

Embryonic hypoxia has been achieved by covering portions of the chicken egg with a gas impermeable coating through the entire length of incubation (McCutcheon et al., 1982). Continuous hypoxia in this way resulted in decreased embryo, brain and liver mass, and a reduction in beak length. However, there were no significant changes in heart mass. Smith et al. (1969) found that exposure to high altitude affected chicken-embryo growth rates only prior to day 10 and after day 17 of incubation. Chicken eggs incubated at an altitude (and corresponding  $P_{O_2}$ ) in between that of Smith et al. (1969) and sea level resulted in no difference in embryo mass throughout development (Black and Snyder, 1980). Our study differs in that hypoxic exposure during all periods of

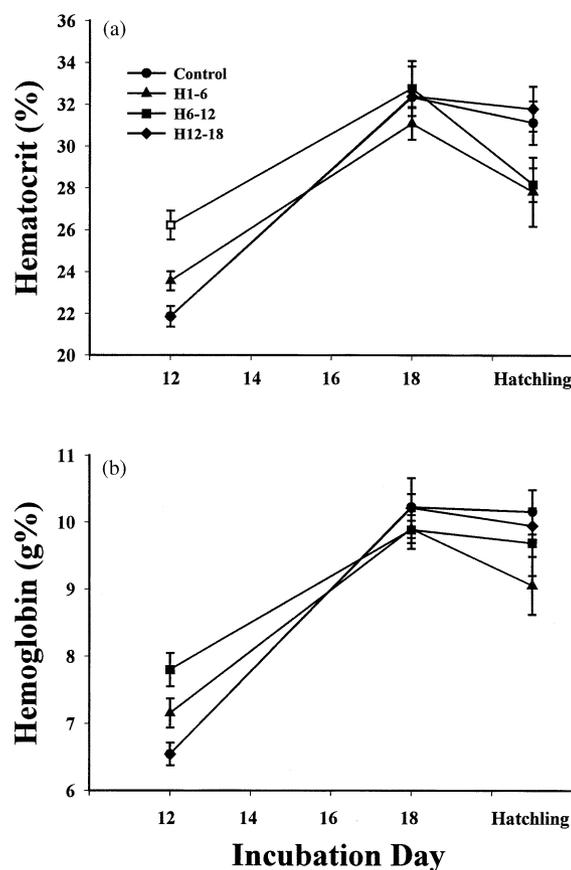


Fig. 3. (b) Hemoglobin and (a) hematocrit measured in embryos on days 12 and 18 of incubation and in day-old hatchlings. All values are mean  $\pm$  S.E.M. Unfilled symbols represent values that are significantly different from the untreated values on that incubation day ( $P < 0.05$ );  $N = 6-15$ .

development resulted in decreased embryo growth, but the embryos were all able to make up the deficit by the time they hatched.

Only a few studies have examined chronic hypoxia over shorter time scales of development in the chicken. Exposure to 15%  $O_2$  during the late stages of development (day 16–18) resulted in decreased embryo and heart mass (Stock and Metcalfe, 1987; Asson-Batres et al., 1989). Similar differences in embryo mass were observed in our H12–18 embryos on day 18, but we did not observe any differences in heart mass. This may be due to our more sophisticated statistical analysis using body and egg mass as a covariate. Experiments with Canada geese that accounted for body mass revealed that embryos incubated in hypoxia had larger hearts than their normoxic counterparts (Snyder et al., 1982). In our study, these differ-

ences in embryo mass in response to late hypoxia disappeared in the hatchlings of this treatment, indicating that between day 18 of incubation and hatching, growth rates of the H12–18 embryos may actually be accelerated compared with the controls.

#### 4.2. Metabolic responses to chronic hypoxia

This is one of the first studies to examine how chronic hypoxic exposure in early, middle and late incubation *differentially* affects  $V_{O_2}$  and its regulation. Exposure during the middle third of incubation had long-term effects on the  $V_{O_2}$  of the chicken eggs. Initially, it resulted in a greater ability to cope with hypoxic exposure, but ultimately led to a decreased  $V_{O_2}$  at hatching when compared with the control group. The reverse was true of H1–6 embryos, which initially had a low  $V_{O_2}$  on day 12, with no ability to regulate in response to acute hypoxic exposure. By hatching, they had recovered to exhibit  $V_{O_2}$  values similar to the control hatchlings.

A number of studies have examined the effect of either acute or chronic exposure to hypoxia on  $V_{O_2}$  in chicken embryos. Tazawa et al. (1992) examined the response to acute hypoxic exposure and found that  $V_{O_2}$  of eggs on days 12 and 18 and of hatchlings significantly decreased in response to 1-h exposure to either 15 or 10%  $O_2$ . Exposure of up to 4 h resulted in continuous decreases in  $V_{O_2}$  for the day-12 and -18 embryos, but not the hatchlings. None of the  $V_{O_2}$  measurements made in this study during acute hypoxic exposure at 110 (15%  $O_2$ ) and 78 mmHg (10%  $O_2$ ) resulted in  $V_{O_2}$  changes as large as those observed by Tazawa et al. (1992). However, our exposure times at each  $P_{O_2}$  level were shorter than the 1 h used by Tazawa et al. (1992), which may account for the slightly higher  $V_{O_2}$  in our study. On day 12, exposure to chronic hypoxia during incubation resulted in a decreased response to changes in  $V_{O_2}$  to acute hypoxic exposure, as indicated by the lower  $P_{crit}$  in the H6–12 embryos (Fig. 2). Previous hypoxic exposure tended to decrease the  $P_{crit}$  of the eggs in relation to the control eggs. Similarly, Canada geese raised in mild hypoxia throughout incubation had  $V_{O_2}$  prior to internal pipping that was less sensitive to acute hypoxia than the controls (Snyder et al., 1982).

Stock and Metcalfe (1987) exposed eggs to 15%  $O_2$  hypoxic conditions on days 16, 17 and

18. They found a decrease in  $V_{O_2}$  from 0.41 to 0.34 ml  $O_2$  min<sup>-1</sup> egg<sup>-1</sup> between the control eggs and those exposed to chronic hypoxia from day 16 to 18, suggesting that short periods (72 h) of hypoxia elicit a decrease in  $V_{O_2}$  late in incubation. However, they only measured  $V_{O_2}$  at the  $P_{O_2}$  at which the eggs were incubated. In our study,  $V_{O_2}$  in normoxia (151 mmHg) did not differ between the control and the H12–18 embryos. However, if  $V_{O_2}$  is examined at a  $P_{O_2}$  of 151 mmHg in control eggs (0.45 ml  $O_2$  min<sup>-1</sup> egg<sup>-1</sup>) and in H12–18 eggs at a  $P_{O_2}$  of 110 mmHg (0.38 ml  $O_2$  min<sup>-1</sup> egg<sup>-1</sup>), a similar difference appears. However, when the control eggs were measured at a  $P_{O_2}$  of 110 mmHg, they had a lower  $V_{O_2}$  (0.34 ml  $O_2$  min<sup>-1</sup> egg<sup>-1</sup>). If Stock and Metcalfe (1987) had measured  $V_{O_2}$  at the same  $O_2$  level for both treatments, the differences observed may have disappeared.

Exposure to hypoxia during the development of a number of wild species had little effect on metabolic rate throughout development. There were no differences in  $V_{O_2}$  from hypoxic- and normoxic-incubated alligator eggs throughout all of incubation (Warburton et al., 1995). Wild bird species developing along an altitudinal gradient showed no difference in metabolic rates or hatchling mass (Carey et al., 1982). At higher elevations, 4150 m, coot embryos exhibited a decreased metabolic rate, even though they had similar mass (Carey et al., 1989). Thus, developing chicken embryos have a larger negative response to hypoxic conditions than wild birds that typically develop along a range of altitudes with various hypoxic conditions. These differences may be due to the long-term adaptation of wild species that have evolved at high altitude under hypoxic conditions.

#### 4.3. Hematological responses

Our measurements of both hematocrit and hemoglobin through development of the chicken embryo agree with values reported by a number of studies (Yosphe-Purer et al., 1953; Johnston, 1955; Tazawa, 1971, 1984; Tazawa et al., 1971b; Baumann et al., 1983). A handful of studies have examined the effect of chronic hypoxia during short periods of development on hematocrit and hemoglobin levels. Dusseau and Hutchins (1988) found that chronic 15%  $O_2$  from day 7 to 10 did not produce differences in hematocrit on day 10, but hypoxia continued to day 14 produced a

significant increase in hematocrit, similar to the increase observed on day 12 in our H6–12 embryos.

Both hematocrit and hemoglobin have been found to increase in response to hypoxia during late stages of development in chicken embryos. A reduction in gas exchange across the shell by covering one-quarter of the shell resulted in gradual increases from day 14 to 18 in hemoglobin and hematocrit when compared with the controls (Tazawa et al., 1971a). Similarly, decreased egg conductance for the entire incubation resulted in a larger variance in both hematocrit and hemoglobin in embryos, with little change in the mean values on day 16 (Nakazawa and Tazawa, 1988). Short-term decreases in conductance occurring on day 17–18 or day 18–19 caused either no changes in hemoglobin with increases in hematocrit, or increases in both values (Tazawa et al., 1988). Throughout development, montane coot embryos incubated at 4150 m had significantly higher hematocrit levels compared with lowland coots incubated at 150 m (Carey et al., 1993). In contrast, we did not observe a differential hypoxic response in either hemoglobin or hematocrit over the last third of incubation in the H12–18 embryos. The changes that were observed in embryos from H6–12 eggs on day 12 disappeared by day 18. Thus, these changes were not long-lasting.

Changes in hematocrit and hemoglobin can occur earlier in development in response to hypoxia. Baumann et al. (1983) measured hematocrit and hemoglobin on days 6–9 on embryos that had been chronically exposed to 13.5% O<sub>2</sub> from the beginning of incubation. They found that hematocrit and hemoglobin did not differ on day 6 of incubation. Hemoglobin decreased in comparison to the controls as development in hypoxia continued. Hypoxic-incubated eggs tended to switch from the embryonic form of hemoglobin to the adult form at an earlier stage (Baumann et al., 1983).

Other changes have been observed in the cardiovascular system of avian eggs in response to hypoxia. Under hypoxic incubation conditions, the timing of the change in ATP and 2,3-bisphosphoglycerate levels occurs earlier in development, resulting in changes in the affinity of hemoglobin for oxygen (Baumann et al., 1986; Dragon et al., 1996, 1999). The vascular density of the chorio-allantoic membrane (CAM) has been shown to increase under hypoxia in a number of studies

(Dusseau and Hutchins, 1988; Corona and Warburton, 2000). Capillary density, but not myoglobin concentration, increases in Canada geese in response to hypoxic incubation (Snyder et al., 1984). All of these physiological changes in response to hypoxia may have critical windows of development, when they are most sensitive to hypoxia and may influence the V<sub>O<sub>2</sub></sub> and growth of embryos in different ways.

#### 4.4. Critical windows and developmental trajectories

Burggren and Fritsche (1997) and Burggren (1999) have proposed a model by which environmental perturbations (abiotic or biotic) may affect the genetically programmed developmental trajectory of a fertilized animal egg. Generally, a fertilized egg follows a genetically dictated developmental trajectory in undisturbed environmental conditions, resulting in a given hatchling phenotype. Environmental perturbations may result in the embryo developing along a new developmental trajectory.

Fig. 4 schematically shows how the physiological and morphological developmental trajectories of chicken embryos differed between the three treatments of hypoxic incubation in this study. All of the treatments resulted in a change in the developmental trajectory from the control trajectory. Two of the treatments, H1–6 and H12–18, resulted in different phenotypes at times before hatching that deviated from the control phenotypes, but ended up with similar phenotypes as hatchlings (Fig. 4a,c). Embryos exposed to hypoxia during the first third of incubation (H1–6) followed a trajectory that had a lower V<sub>O<sub>2</sub></sub> and smaller mass on day 12 of incubation, but no statistical differences in these parameters by the time they hatched (Fig. 4a). Exposure to hypoxia during the last third of incubation (H12–18) initially resulted in lower wet and dry mass on day 18, but by hatching they had only a smaller dry mass (Fig. 4c).

Chronic hypoxic exposure of eggs during the middle third of incubation (H6–12) had the longest-lasting effect (Fig. 4b). On day 12, these eggs had a greater ability to regulate V<sub>O<sub>2</sub></sub> and a higher survival in the face of decreasing acute P<sub>O<sub>2</sub></sub> levels. The body size difference observed on day 12 disappeared in the yolk-free hatchlings. Although embryos from H6–12 eggs were able to achieve a

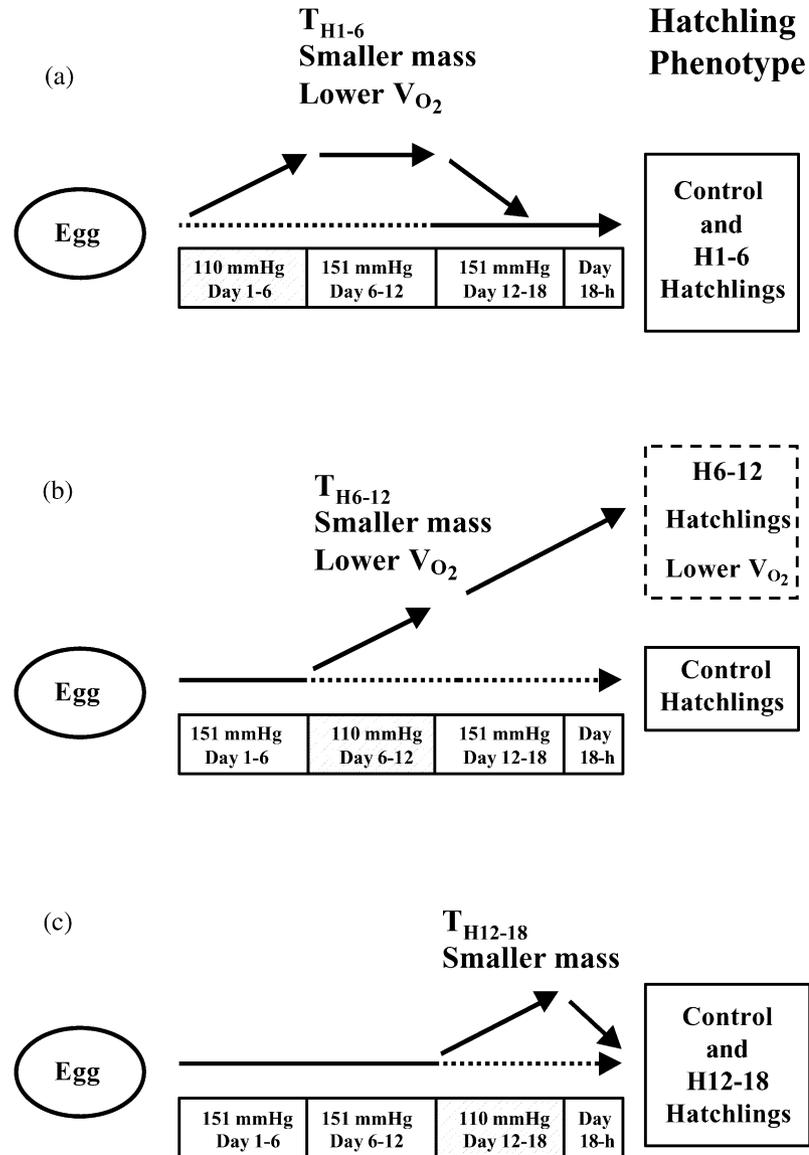


Fig. 4. Developmental trajectories of chicken embryo phenotypes developing under various regimes of chronic hypoxia. (a) The developmental trajectory of embryos exposed to hypoxia from day 1 to 6. The horizontal solid and dashed line represents the developmental trajectory of the control animals. Hatchlings from treatments in the same box share a similar phenotype. (b) Developmental trajectory of embryos exposed to hypoxia from day 6 to 12. (c) Developmental trajectory of embryos exposed to hypoxia from day 12 to 18.

mass not significantly different from embryos of control eggs by the time of hatching, the  $V_{O_2}$  differed in H6–12 hatchlings.

Collectively, these data indicate that a critical window appears to be present in the development of  $V_{O_2}$  in response to chronic hypoxia. During the middle third of incubation (H6–12), the developing embryo was most affected by the hypoxic exposure, with long-term changes in  $V_{O_2}$  occurring.

What mechanism(s) could account for this difference? From day 6 to day 12, the chorioallantoic membrane grows from 35% to 100% coverage of the egg surface area (Ackerman and Rahn, 1980). Although the density of the capillaries increases (Dusseau and Hutchins, 1988) in response to hypoxia, the overall CAM development may be inhibited. This would translate into metabolic changes for these embryos. Further experiments

are required to identify the mechanisms involved in the  $V_{O_2}$  changes.

Chicken embryos are very sensitive to hypoxia during development (Wangenstein et al., 1974; McCutcheon et al., 1982; Stock and Metcalfe, 1987). We found that embryos were most sensitive to mild chronic hypoxia during the middle third of incubation, with metabolic rates remaining significantly different from the control animals through hatching. Embryos were able to correct their developmental trajectories after exposure to hypoxia during the first or last third of incubation, ultimately resulting in the normal phenotype at hatching. The timing of hypoxic exposure plays a role in the developmental trajectories of these animals. Further investigation into the timing of developmental changes in the cardiovascular system is needed to determine the cause for the differences observed in the H6–12 chicken embryos.

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