Differential Embryo Development among Tibetan Chicken, DRW and Shouguang Chicken Exposed to Chronic Hypoxia*

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ABSTRACT: Avian embryos at high altitude are independent of maternal protection against hypoxia, which is contrary to mammals. It is well known that chronic hypoxic exposure at key points can significantly impact on avian development. Tibetan Chicken, a Chinese indigenous breed, living in Tibetan areas with an altitude of 2.2 to 4.1 thousand meters, has an adaptive mechanism to hypoxia. In the present study, fertilized eggs of Tibetan Chicken were incubated under 13% and 21% oxygen concentration. Two lowland chicken breeds, Shouguang Chicken, an indigenous chicken breed in Shandong Province of China, and Dwarf Recessive White Chicken, an imported breed in Beijing, were used as control groups. The embryo mass and some organs such as brain, heart, liver, stomach and eye weight in the three species were measured at Hamburger-Hamilton stage 39, 41, 43 and 45 under hypoxic and normal conditions. The results showed that in hypoxia Tibetan Chicken significantly differed from the two lowland chicken breeds in embryo mass at Hamburger-Hamilton stage 41, 43 and 45 (p<0.01). In particular, Dwarf Recessive White Chicken and Shouguang Chicken showed retarded growth in hypoxic incubation (p<0.01), whereas Tibetan Chicken showed no significant difference between hypoxic and normal conditions (p>0.05). In addition, heart and the other organs showed different susceptibility to hypoxia at the studied stages. In conclusion, chronic hypoxia induced a change in the embryo development of the three different species and Tibetan Chicken showed adaptation to hypoxia. Of note, the embryo developmental physiology of Tibetan Chicken in response to hypoxia will shed light on the process of physiological acclimation or evolutionary adaptation as well as the study of clinical disease. (Key Words: Adaptation, Chronic Hypoxia, Developmental Physiology, Tibetan Chicken)

INTRODUCTION

Oxygen concentration is essential for embryo development (Jaffee, 1974; Strick et al., 1991; Meuer et al., 1992; Miller et al., 2002; Rouwet et al., 2002; Villamor et al., 2004; Chan and Burggren, 2005). Hypoxia increased embryo mortality (Villamor et al., 2004), induced the retarded growth (Wangensteen et al., 1974; Stock and Metcalfe, 1987; Burton and Palmer, 1992; Rouwet et al., 2002; Villamor et al., 2004), depressed the metabolic rate (YC, 1993), blunted the cardiovascular response to the hypoxic challenge (Crossley and Altimiras, 2005), altered the physiological and morphological trajectories of developing chicken embryos (Dzialowski et al., 2002), also affected the rennin-angiotensin system, the hypothalamo-pituitary-adrenal axis, vascular endothelial function and sympathetic intervation (Jansson and Lambert, 1999; Ruijtenbeek et al., 2000; Ruijtenbeek et al., 2003). Chicken embryo is a good tool to study developmental physiology. In particular, fetal oxygenation, independent of maternal nutrition during development, plays a vital role in the control of embryonic growth. In the present study, three chicken breeds were involved, which included Tibetan chicken (T), a Chinese indigenous breed living in Tibetan areas with an altitude of 2.2 to 4.1 thousand meter for more than one thousand years; Shouguang Chicken (S), another Chinese indigenous breed from Shandong province with an altitude of 40 to 200 m, and Dwarf Recessive White Chicken (DRW), an imported breed, reared at the Experimental Farm of China Agricultural University in Beijing where altitude is less than 60 m. It was reported that T had a hatchability of 79.51% at high altitude of 2.9 thousand meter while the hatchability of DRW and S at the same altitude was 31.70% and 32.21%, respectively (Zhang, 2005). More and more embryos died at the late embryonic developmental stages during the incubation at high altitude,
which is due to that hypoxia produces greater effects later in gestation (Mulder et al., 1998; Dragon and Baumann, 2003) and compensatory mechanism such as activation of the autonomic nervous system and catecholamine release redistribute cardiac output to the heart and brain in response to hypoxia (Mulder et al., 1998; Mulder et al., 2002).

Chick embryonic red blood cell (RBC) played a role in this process of oxygen transportation from the environment to the tissue, which was regulated by hormonal effectors stimulating the cAMP pathway (Dragon and Baumann, 2003). RBC population expanded rapidly until Hamburger and Hamilton stage (HH) 43 (Dragon and Baumann, 2003). The erythroid carbonic anhydrase II (CAII) also improved the CO2 transport properties in hypoxia to respond to the adaptive regulation of embryonic blood gas transport properties as well as the control of cardiovascular development from HH 39 to 40 (Dragon, 1999). The peak of norepinephrine and epinephrine released at HH 45 in the chicken embryos. Collectively, the physiology regulated by hormone above in the avian development suggested that a critical window appeared to be present in response to hypoxia. Therefore, the development critical windows of the avian embryos were determined at HH 39, 41, 43 and 45 in the present study.

The previous study mainly focused on the subjects of White Leghorn chicken, turtle, fish as well as Alligator and the incubation stages divided into three discrete periods with equal length to assess physiological morphology (Dzialowski et al., 2002). Yet there remains some confusion about how the physiological and cellular designs of the avian respiratory system have evolved under the selective pressure of multiple requirements for gas exchange (Leon-Velarde and Monge, 2004). Moreover, there was little information of T during the mid-late incubation periods of the physiological development. The objective of the present study is to investigate differential effects induced by chronic hypoxia on tissue growth and development in DRW, S and T at HH 39, 41, 43 and 45 of avian incubation.

**MATERIALS AND METHODS**

**Eggs and incubation**

Fertilized eggs of DRW, S and T were obtained from the Experimental Chicken Farm of China Agricultural University. Prior to incubation, eggs in all of the three chicken breeds were weighed and they were incubated under hypoxic condition (13% O2). At the same time, control fertilized eggs were incubated in another incubator exposed to ambient oxygen (approximately 21% O2) for the same duration. And samples were collected from two groups at HH stage 39, 41, 43, 45, respectively (Hamburger and Hamilton, 1951; Corresponding to embryonic day: Day 13, 15, 17 and 19). The simulated hypoxic incubator was developed by Wu’s lab (Wu et al., 2005) with a gas mixture containing 13% oxygen and 87% nitrogen. Temperature (37.8°C) and humidity (60%) in the two incubators were adjusted to be the same. Oxygen concentration was maintained 12.5 to 13% during the exposure period.

To consider repeated sampling and mortality, the following numbers of eggs were placed in each incubator at the beginning of incubation. The eggs number of DRW was identical with that of S in hypoxia (n = 150) and normoxia (n = 120). And 120 fertilized eggs of T were put in each incubator under hypoxic or normoxic condition, respectively.

**Embryo and various organ mass**

At HH stage 39, 41, 43 and 45, the eggs were weighed, respectively. The survival individual was operated with surgical scissors to access to the internal organs of embryo. Brain, heart, liver, stomach, intestine and eyes were carefully isolated from the embryo. Then they were washed with 0.85% NaCl. The visible free liquid on the surface of the organ was removed. Furthermore, all the organs were weighed by electronic balance (Ohaus Corp. Pine Brook, NJ, USA). The scale is sensitive to 0.001 g. The number of the surviving individuals measured during the various portions of incubation was presented in Table 1.

**Statistical analysis**

Data were analyzed by least square analysis procedure of SAS (version 8.02, SAS Inst. InC., Cary, NC). The model was as follows: $Y_{ijk} = \mu + A_i + B_j + C_k + A_iB_j + A_iC_k + B_jC_k + e_{ijk}$, in which the meanings of the items are as follows: $Y_{ijk}$, the embryo or organ mass; $\mu$, the overall mean; $A_i$, environment stress; $B_j$, breed difference; $C_k$, embryonic stage; $A_iB_j$, interaction effect of stress and breed. In analogy, $A_iC_k$ and $B_jC_k$ are interaction effect between two factors; $e_{ijk}$, the residual error. The egg mass at four different developmental stages was used as a covariate for embryo mass. The organ mass was normalized by body mass. All data are presented as means±SE. The significance levels were set at p<0.05 and p<0.01.

**RESULTS**

**Hypoxic growth**

Prior to incubation, fertilized eggs from T are much lighter than those from DRW and S (p<0.01) (Figure 1).

In hypoxia, in the three chicken breeds, there was
significant difference in the embryo mass from stage 41 to 45 (p<0.01). For clarity of presentation, data for all the embryos and the key organs in all of the three chicken breeds incubated under hypoxic and normoxic conditions are presented in Table 1. It was obvious that T grew the fastest among all of the three chicken breeds at all studied embryonic stages in hypoxia. Interestingly, the embryo weight of DRW was similar to S at HH 39, and both of them were lower than T, though there was no significant difference in the embryo mass in all the three chicken breeds at HH 39. By HH 41, the embryo mass in T is much heavier than that in DRW (p<0.01) and S (p<0.05). However, there is no significant difference in the embryo mass of the two lowland chicken breeds (p>0.05). From HH 43 to 45, T gained more weight than DRW and S in hypoxia.

In hypoxia, significant effects of hypoxic exposure on brain mass of each species were shown at the four different developmental stages (p<0.01). However, the brain mass in all of the three chicken breeds at each developmental stage in hypoxic environment changed in a similar manner (p<0.05) (Figure 2). There are the same growth patterns for liver, stomach, intestine (data not shown) as well as eye (data not shown) in all of the three chicken breeds in hypoxia with the exception that T stomach mass was much lighter than those of DRW and S at HH 45 (p<0.05).

It was well known that the heart was the first organ to develop during chicken embryonic stages. Under hypoxic condition, heart mass altered significantly in the three chicken breeds (p<0.01). Obviously, the heart mass of DRW was much more than that of S and T at any developmental stage (Table 1).

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**Table 1. Relative mass of embryo, brain, liver, heart and stomach in the three chicken breeds at HH 39, 41, 43 and 45 in hypoxia and normoxia**

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The means of the abbreviations are as follows: DRW = Dwarf recessive white chicken; S = Shouguang chicken; T = Tibetan chicken.

Values of the relative mass are listed in a way of means±SE. The numbers of the embryos tested are presented in parentheses.
In contrast to hypoxia, there was not significant difference in embryo mass of the three groups of DRW, S and T in normoxia (p>0.05). In the three chicken breeds incubated under normoxic condition, embryo mass as well as organ mass developed rapidly at the four different embryonic stages (p<0.01). Especially, DRW and S grew significantly faster than T at the last stage in normoxia.

The means of the relative embryo mass of the three chicken breeds under hypoxic and normoxic conditions were as follows (Figure 3): 22.86±0.62 (hypoxia) and 29.27±0.64 (normoxia) in DRW (p<0.01), 23.72±0.59 (hypoxia) and 27.90±0.64 (normoxia) in S (p<0.01), 27.54±0.52 (hypoxia) and 28.71±0.60 (normoxia) in T (p>0.05), respectively. From these data, it was easy to draw a conclusion that the embryonic growth of DRW and S was significantly retarded in hypoxia (p<0.01), whereas there was no significant difference between the embryo mass of T in hypoxia and that in normoxic incubation conditions (p>0.05).

In the three chicken breeds, breed difference in brain mass in normoxia was showed in Figure 2, which was not seen in hypoxia. T had heavier brain weight than DRW and S in both hypoxic and normoxic incubation conditions (Figure 2). Furthermore, in normoxia, T significantly differed from DRW in brain mass (p<0.01), also differed from S (p<0.05), while there was no difference between DRW and S (p>0.05).

DRW had heavier heart mass in hypoxia than that in normoxia (Figure 4). However, the heart mass of S was showed in an opposite manner, and the increasing patterns of heart mass of T in normoxia were almost identical to that in hypoxic condition (Figure 4).

Besides the change in the organs mentioned above, in the three chicken breeds, the mass increase of liver, intestine mass (data not shown) and eye (data not shown) during the studied stages in normoxia was similar to that in hypoxia.

**DISCUSSION**

**Chronic hypoxia**

A number of studies have shown that hypoxia exerted the impact on the developmental growth. Chicken embryo exposed to chronic hypoxia at high altitude decreased the metabolic rate (Wangensteen et al., 1974; Stock and Metcalfe, 1987), cell repair and tissue growth (Stock and Metcalfe, 1987; Xu et al., 1989), induced cardiovascular disorder (Crossley and Altimiras, 2005), triggered cardiac hypertrophy in chickens (Villamor et al., 2004), altered peripheral artery function and structure at the level of sympathetic innervation (Ruijtenbeek et al., 2000). Oxygen gradient was ranged from 10% to 15% for a few days or hours to the developmental stages in the previous studies, of course, including the studies above, which was summarized by Chan and Burggren (2005). In addition, Chan and Burggren pointed out that 15% O2 in their study was equivalent to the high altitude of 2.9 thousand meter. In general, how a species deals with hypoxia depends on the time available for orchestrating the response. Usually the timeline for response is divided into three categories: acute, acclimatory and genetic or phylogenetic (Hochachka et al., 1999). Yet there is no doubt that 13% O2 used in the present study can stimulate the hypoxic challenge at high altitude to study physiological acclimation or evolutionary adaptation of Tibetan Chicken.

**Comparative physiology**

T, a kind of highland indigenous animal, having successfully adapted itself to chronic hypoxia, can develop normally at high altitude (2.2 to 4.1 thousand meter). However, DRW and S are two lowland chicken breeds
where altitude is less than 400 m. Unfortunately, T has been rarely used in chronic hypoxia studies, though hypoxic incubation of avian development has been studied for decades.

In the present study, three subjects (DRW, S and T), two stress factors (hypoxia and normoxia) and four stages (HH 39, 41, 43 and 45) were considered together to design the experiment, which was more comprehensive than the previous studies (single subject and the equal length of incubation) (Dzialowski et al., 2002). The selection of the studied developmental stages was based on the knowledge of hormonal regulated physiology. The elaborate experimental design and the results from the present study may be helpful to study animal physiology of the adaptation in hypoxic environment.

Morphological responses to chronic hypoxia

The results of the present study showed that chronic incubation induced significant changes in embryo and organ mass including brain, heart, liver in the three chicken breeds.

Prior to incubation, the egg weight of DRW is the heaviest while the ones of S is the second, and T egg weight is the lightest among the three chicken breeds (p<0.01) (Figure 1). However, in hypoxia, embryo mass in T is always much heavier than those in DRW and S at the four different developmental stages. The results suggested that the embryonic growth of DRW and S was significantly retarded in hypoxia (p<0.01), whereas the embryo mass of T had no significant change between hypoxic and normoxic conditions (p>0.05). At the same time, group of feed withdrawal of broiler chickens weighed less (p<0.05) compared to control group of heat acclimation on male broiler and layer chickens responses to acute heat stress at four weeks of age (Mahmoud and Yaseen, 2005).

Similarly, our present results in part were in good agreement with previous reports and further confirmed that hypoxic exposure during development resulted in decreased embryo growth in chicken (Ruijtenbeek et al., 2000; Dzialowski et al., 2002; Ruijtenbeek et al., 2003; Crossley and Altimiras, 2005). There was also the demonstration that the embryo of fertilized eggs that laid by sea level hens incubated at high altitude showed the restricted fetal growth (Miller et al., 2002). In particular, the retarded growth is correlated with the duration of high altitude residence: the longest resident population exposed to hypoxia, the least decline in birth weight with altitude, whereas the shortest historical residence groups, the greatest decline (Hass et al., 1980; Moore, 1990). For instance, the Black Leghorn chickens had lived at high altitude city of La Paz (3.6 thousand meter) only for at least six generations and sea level hens lived in the city of Santa Cruz (420 m). The fertilized eggs of both high altitude and lowland chickens were carried out the experiment in Bolivia (Giussani et al., 2007). As a result, incubation at high altitude of fertilized eggs laid by sea level hens markedly restricted fetal growth. Similarly, the embryo mass of fertilized eggs laid by high altitude hens that were incubated at high altitude also showed retarded fetal growth, but to a lesser extent compared to that of the lowland chicken embryos. Giussani et al. (2007) doubted in the discussion whether six generations for the chicken inhabited at high altitude is sufficient time for physiological adaptations to high altitude. Giussani (2007) also described that epigenetic mechanisms maybe highlight the hypoxia protection, which can lead to the alteration of gene expression and physiological response in a comparatively shorter period. On the other hand, the body mass of high altitude coot embryos (Fulica americana peruviana) incubated in the Peruvian Andes (3.5 thousand meter) did not differ from those at the sea level embryos at hatching (Cynthia Carey, 1989), which was consistent with the embryo mass change of T between the two different treatments in the present study. Of note, according to the previous reports (Dragon and Baumann, 2003; Leon-Velarde and Monge, 2004), the abilities of the elevated embryo mass in response to the gas exchange in hypoxia could increase the oxygen carrying capacity, decline the erythrocyte ATP level, early stimulate 2,3-bisphosphoglycerate (2,3-BPG) synthesis during embryonic development and promote oxygen utilization. Therefore, the results in the current study indicate that T has a genetically based adaptation to hypoxia, while DRW and S showed the blunted growth in hypoxic incubation, supporting that chronic hypoxia has a significant influence on fetal growth efficiency in different chicken breeds. Furthermore, fetal resource uptake for tissue accretion was induced by hypoxia (Giussani et al., 2007). For instance, fetal resource uptake was greater in fertilized eggs laid by high altitude hens compared to the ones laid by lowland chicken. However, the mechanism of resource utilization trigged by hypoxia remains unknown.

In the present study, chronic hypoxia had no significant impact on the brain mass in DRW, S and T in the present study (p>0.05) (Figure 2). This response to hypoxia was consistent with the report (Asson-Batres et al., 1989). In addition, to our knowledge, growth rate of the whole body is faster than that of all the organs from HH 39 to 45. And the brain mass was normalized by embryo mass in the present study. In this way, the decreased relative brain weights were shown in Table 1 during the chicken development under both hypoxic and normoxic incubation conditions.

Stress-induced responses vary among different tissues in broiler chickens (Yu and Bao, 2008). Acute hypoxia induced a redistribution of cardiac output from the periphery to key organs such as brain and heart in fetal mammals to maintain oxygenation of essential circulations.
The present study showed different susceptibility to hypoxia in chickens (DRW and S). In addition, the studied organs in embryonic development compared with the lowland demonstrated a substantial tolerance to hypoxia during nutrition during development, plays a predominant role in Dzialowski et al., 2002; Rouwet et al., 2002). However, S in heart mass were reported (Stock and Metcalfe, 1987; Asson-Batres, M. A., M. K. Stock, J. F. Hare and J. Metcalfe. 1989. O2 effect on composition of chick embryonic heart and brain. Respir. Physiol. 77(1):101-109.


