MORPHOLOGICAL AND HEMATOLOGICAL RESPONSES TO HYPOXIA DURING

DEVELOPMENT IN THE JAPANESE QUAIL, *Coturnix coturnix*

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Hypoxic responses in quail development differ depending upon stage, duration and level of oxygen partial pressure of embryo. Incubation was switched to/from 110mmHg partial pressure (hypoxia), to/from 150mmHg (normoxia) during different stages in development, and control was incubated in normoxia throughout.  Hatchability and embryo survival resulted in no hatchlings in continuous hypoxia.  Responses to various hypoxic exposures throughout development resulted in recovery/repair of hypoxic damage by hatch. Heart and body mass, beak and toe length, hemoglobin, and hematocrit were measured to determine embryo responses to hypoxia during development at days 10, 15, and hatch. Hypoxia seemed to have the most deleterious effects on eggs in continuous hypoxia.  Collectively, data indicate critical developmental windows for hypoxia susceptibility, especially during mid-embryonic development.

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CHAPTER 1

INTRODUCTION

Although chicken eggs are commonly used in avian hypoxic studies

(Van Liere and Stickney, 1963; Olander et al., 1967; Meuer and Baumann,

1987; Hochachka, 1997), this study examines the influence of hypoxic

incubation on development of Japanese quail eggs (*Coturnix coturnix*). To date

there have been no studies of hypoxic effects on quail eggs or embryos. Quail

are precocious, hardy birds that have adapted to many different environments

(Microlivestock, 1991), but prefer temperate climates. Quail are not native to

the Americas and were introduced to the United States in 1870 (Woodard et al.,

1973). Domesticated quail were first used in laboratory, in the U.S. post World

War II importation, from Mount Fujiyama Japan (Woodard et al. 1973;

Microlivestock, 1991). Female quail incubate eggs and raise quail chicks,

while males leave and court other females when their partners begin nesting.

Nests are on the ground and well hidden in grass, dead brush, and under

bushes. Under favorable environments quail have the ability to produce 3 to 4

clutches per year, and produce for extended periods during their 1-2 year life

span; averaging 25 eggs per year. Quail exhibit north-south migratory behavior

(Woodard et al., 1973; Johnsgard, 1988). Their migration covers 400-1000 km,

which is notable for a bird not known for its flying capability (Hoffman, 1988).

Quail are similar to chickens and turkeys in their sexual development and egg production, while differing from these species in their distinct egg color patterns and pigmentation. The average quail egg is approximately 8% of the female quail body weight, while chicken and turkey eggs are 3% and 1% of their body weights, respectively. In contrast to first laid chicken and turkey eggs, first quail eggs are smaller than succeeding eggs. Quail lay 7-14 eggs per clutch (Hoffman, 1988). An egg averages 29.8 by 21.5 mm in size and weighs 7.6 g (Johnsgard, 1988). Incubation time is 15-18 days.

From the early assumption that bird embryos developed independent of the surrounding air (Erman, 1818), research in developmental biology of gas exchange has progressed steadily. Avian models have been particularly useful (Hoyt, 1987; Klika, 1997; Menna and Mortola, 2001; Jurgens and Gros, 2002; Maina, 2002). Hatchability of bird eggs depends primarily on temperature, ambient gas partial pressures of oxygen, carbon dioxide and water vapor (Lundy, 1969; Pettit and Whittow, 1982; Visschedijk, 1985). In most experimental studies, hatchability is the ultimate gauge for the successful gaseous exchange of the avian embryo (Visschedijk, 1980; Pettit and Whittow, 1982; Visschedijk, 1985). Not surprisingly, hatchability of eggs normally laid at or near sea level progressively decreases when eggs are incubated at higher altitudes (Smith et al., 1969; Carey et al., 1982; Christensen and Bagley, 1984; Menna, 2002). Oxygen is required by avian embryos for maintenance of existing tissues and for synthesis of new cells from raw materials contained within the egg (Olander et al., 1967; Carey et al., 1982; Richards et al., 1991; Meuer et al., 1992; Altimiras and Phu,

2000; Wieser, 2002).  Thus, the restriction of oxygen below critical levels, which could occur at high altitude or in fossorial species, could potentially retard growth and reduce survival.

Almost all studies on the effect of oxygen on avian development have exposed embryos to chronic hypoxia throughout the entire incubation.  However, it is still unknown at what developmental stage, if any, that egg survival is particularly vulnerable to low oxygen levels and why exposure at these stages leads to a decrease in hatching success.  The purpose of this study is to use hypoxic exposure at different stages during incubation to potentially provide insights on when hypoxia affects key morphological features of avian development.  Although there are many potential physiological changes contingent upon incubation in hypoxic conditions, my thesis will focus on developmental trajectories and systems involved in the delivery of oxygen in the developing embryo.

Debilitating effects of hypoxia on avian embryos are reported to lead to characteristic anatomical and physiological alterations (Olander et. al., 1967; Altimiras and Phu, 2000; Mulder et al., 2000; Hopkins and Powell, 2001; Miller et al., 2002), as well as pathophysiological conditions.  Hypoxia during incubation retards embryonic growth, prolongs incubation, and reduces hatching success (Carey et al., 1982; Altimiras and Phu, 2000).  Hypoxia also alters the timing of the switch from embryonic to adult hemoglobin (Baumann et. al., 1983).  As a result, these alterations lead to changes in concentration of red blood cell adenosine 5'-triphosphate (ATP), and

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the hypoxic embryo acquires an increased oxygen affinity that partially compensates for the effects of the reduced $PO_2$ (Baumann et. al., 1983; Weber et al., 1993; Jessen et al., 1991; Kavdia et al., 2002). Under normal incubation the switch from embryonic to adult hemoglobin occurs during embryonic development. Baumann (1983) found no significant changes on hemoglobin parameters except hemoglobin type, from blood samples of chicken taken daily from day 4 though day 9. Therefore, it is apparent from Baumann's (1983) blood sample results that hypoxic incubation altered only one aspect of the pattern of erythropoiesis in chicken embryos, namely the type of hemoglobin, but not hemoglobin concentration or hematocrit counts (Baumann, 1983; Jessen et al., 1991; Weber et al., 1993; Kavida et al., 2002).  These results were influenced by both the time course of the transition from embryo to adult hemoglobin as well as the oxygen transport properties of embryonic blood.

Given hypoxia reduces embryo growth retardation (Carey et al., 1982; Altimiras and Phu, 2000), questions arise as to whether these changes are an adaptation to hypoxia or a negative effect of retarded development.  However, it is of importance to note that Baumann (1983) reported no significant difference between normoxic and hypoxic embryos in hematological parameters, suggesting that different values obtained at the same age are only due to retarded growth rather that a specific effect of hypoxia (Baumann, 1983).

Avian embryos developing in chronic hypoxic environments exhibit cardiac decompensation, i.e. the heart has a minimal, limited ability to compensate for low

oxygen levels by pumping more blood.  Bird embryos also show congestive failure, dilation and hypertrophy of the heart.  Hypertrophy of the heart, which involves all chambers of the heart, is commonly characterized by a doubling of normal heart size (Olander et al., 1967; Hernandez, 1987; Julian, 1993).  Avian embryonic hearts that exhibit hypertrophy at high altitudes have a repressed heart growth rate over the first two weeks of incubation (Konarzewski et al., 2000).  Surprisingly, embryonic body growth rates are greater at high altitude in the third week, indicating the onset of systemic response to hypoxia (Smith, 1969), as well as vascular growth, which is stimulated by hypoxia (Hoper and Jahn, 1995; Yue and Tomanek, 1999, 2001).  In addition to these hypoxic responses, there are also morphological cardio pathologies, such as pericardial edema (Olander et al., 1967; Hernandez, 1987; Witzel et al., 1990; Julian, 1993; McGovern et al., 2001).  During hypoxic incubation in some birds, the lungs are over-distended and congested;  while edematous in others (Burton et al., 1969; Hernandez, 1987; Witzel et al., 1990).

Whether or not an animal's developmental trajectory is altered by environmental perturbation depends on whether that perturbation occurs during a "critical developmental window".  For major organ systems, a discrete period of susceptibility to experiencing unalterable developmental changes occurs (Burggren, 1998).  Finally, whether the embryo has the ability to recover from hypoxic damage after the various hypoxic treatments and throughout development depends on several factors which include, but may not be limited to: the stage in development when the embryo is

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exposed to hypoxia, duration of exposure to hypoxia, and the amount of time in which the embryo has to recover and/ or repair damage.

The alternate hypotheses tested were:

- Survival of the quail embryo is influenced by hypoxic exposure during incubation.

- Regardless of the timing and duration of hypoxic treatment, embryos will recover/repair any hypoxic damage and will follow a normal (control) developmental trajectory.

- Organogenesis and hematopoesis will vary depending on the stage in which the developing embryo was subjected to hypoxic exposure, and the length of that hypoxic exposure.

- Quail eggs will follow a similar developmental pattern to that found in previous studies on chicken eggs.

- Eggs exposed to continuous/chronic hypoxia will vary significantly developmentally from control eggs, in overall body size/mass, organ mass, beak length and toe length.

- There will be a difference in hematocrit and hemoglobin of treatment groups from control.

This study examines whether incubation in hypoxic conditions alters embryonic anatomy (body and heart mass, toe and beak length, and hematological parameters) throughout the development of quail embryos.

CHAPTER 2

MATERIAL AND METHODS

ANIMALS AND INCUBATION

Quail eggs (*Coturnix coturnix*) were obtained from Texas A&M University and

Bear Bayou Quail Farm, Gainesville, Texas, as available.  Eggs were incubated in

compartments in 1.7-liter closed containers in Lyon incubators at 37°C and then

incubated.  Containers containing the eggs were automatically rotated 90° every four

hours.  Each container was ventilated with the appropriate 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% $O_2$), and humidified between 75 and

95% relative humidity.  The level of hypoxia and relative humidity was selected based

on previous protocols for hypoxic incubation of bird eggs (Baumann, 1983;

Ingermann, 1983; Altimiras and Phu, 2000; Dzialowski et al., 2002; Miller et al.,

2002).  In this study, the control group provides data for normal development during

normoxic incubation.  The various hypoxic treatments (acute and chronic) represent

the effects of hypoxic exposure on specific stages in development of the quail (Fig.1).

The target sample size was 20 eggs per group.

EXPERIMENTAL PROTOCOL

Eggs were divided into five different groups and exposed to normoxia or

different patterns of hypoxic exposure.  The five groups were: control ($P_{O2}$ of 151

mmHg throughout incubation), early hypoxia ($P_{O2}$ of 110 mmHg from day 1 to 5 with

normoxia for the remainder of incubation), middle hypoxia (110 mmHg exposure from day 5 to 10 with normoxia for the remainder of incubation), late hypoxia (110 mmHg exposure from day 10 to 15 with normoxia for the remainder of incubation), and continuous hypoxia (110 mmHg $O_2$ throughout incubation).

Anatomical and hematological variables described below were measured at days 10, 15, and at hatch for control and individuals of all treatment groups. Sample groups were defined as those taken from the initial population at days 10, 15, and hatch.

ANATOMICAL MEASUREMENTS

On days 10, 15, and at hatch, eggs were taken out of the control and each of the experimental groups for evaluation. Day 10 and 15 embryos were removed from the egg and freed of yolk and extraembryonic membranes. It should be noted that the use of embryo mass as a measure of growth neglects the contribution of the extraembryonic membranes to the total mass of the embryo. Growth and metabolic rate of the membrane have been reported to be an important aspect of the total mass, in early chicken incubation (Needham, 1932; Haas and Spratt, 1976). However, the importance of extraembryonic membranes declines rapidly as embryo size increases (Vleck and Hoyt, 1980). For the purpose of this study, extraembryonic membranes were removed from all embryonic stages because there was no compelling argument for including the membrane in wet body mass measurements, and which would be negligible in dry mass.

Wet mass of day 10 and 15 embryos, and hatchlings were determined to the nearest milligram with a top-pan balance Denver Instrument Company.  The heart was then removed from the embryo, blotted dry, and then weighed.  After drying for approximately 4 days at 60 ºC, dry masses of body and heart were also taken and water content as a percentage of body or heart mass was calculated by the following formula: [(Wet mass - dry mass)/wet mass] X 100.

Both wet mass and dry mass was measured to determine whether there was significant edema.  Toe and beak lengths are used as indices of development in the chick embryo in late incubation (Hamburger and Hamilton, 1951; Stock et al., 1983) and served as a comparative gauge throughout development, and for interpreting results.  Calipers were used to measure length (to the nearest millimeter) of beak, from the tip to the anterior end of the left nostril, and the length of the third toe, from the phalangeal-tarso-metatarsal joint to the tip of the claw.  Further anatomical observations made were: presence/absence of feathers, abnormal presence of fluids/edema, toe and beak deformities, wing malformations and deformities, and any gross developmental abnormalities.

## HEMATOLOGICAL MEASUREMENTS

To sample blood at day 10 and 15 embryos, eggs from each group were opened at the pointed end and approximately 1-2 milliliters of blood was drawn directly from the omphalomesentric artery of each embryo into a heparinized syringe. To sample blood from hatchlings, each animal was anesthetized by placing a cotton ball saturated

with halothane (Halocarbon Laboratories) into a sealed plastic container with the hatchling. After anesthetization, blood from hatchlings was taken out by direct cardiopuncture within 3-5 minutes of anesthetization. Blood was drawn by intracardiac puncture; about 50-100 microliters of blood was needed for analysis in the Radiometer OSM2 Hemoximeter to obtain hemoglobin (Hb) concentration. Hematocrit values (Hct) were measured by centrifugation of blood in capillary tubes to obtain packed red blood cell count. Two measurements were made for both hemoglobin and hematocrit, and an average was taken for each, per embryo/hatchling.

## STATISTICAL ANALYSIS

Data were assessed using SAS. Heart and body masses of quail, as well as hemoglobin and hematocrit were described with appropriate descriptive statistics, following assessment for normality of distributions (Shapiro-Wilk normality tests) and equality of variances (Hartley's F-max test). Within and between groups were tested for statistical significance with an ANOVA and significance between groups was determined with a Student-Newman-Keuls multiple range tests *post-hoc*. All statistical decisions were made with a 0.05 level of significance, and all values are presented as mean ± standard error.

CHAPTER 3

RESULTS

Despite some degree of popularity in developmental studies, there is a dearth of morphological and hematological information available for quail embryos, making the control (normoxic) population worthy of initial consideration in this study. In a subsequent section I compare the hypoxic populations to controls. The four treatment groups and the control (Fig.1) were compared at days 10, 15 and hatch. Samples sizes for each group were different depending on group and day (days 10, 15, or hatch).

NORMAL DEVELOPMENT

SURVIVAL

Cumulative survivorship was calculated based on the total number of eggs initially incubated for each treatment group, and was calculated by determining the percentage survival in each group at the time of sampling from the initial sample of incubating eggs. Survival of controls ranged from 75 to 82% (Fig. 2). At day 10, there was a 75% survival rate among the control quail embryos. At day 15, survivorship was 82% of the initial sample of incubating eggs taken out, and remained at 82% by hatch (Fig.2). Upon evaluation, feathers were not visually apparent until day 15 of embryo development. Similarly, eyes were covered by eyelid membrane and were not visually apparent until day 15.

BODY MASS

Growth as measured by increase in body mass (both wet and dry), heart mass (both wet and dry), and toe and beak length is compared among treatment groups and control at days 10, 15, and hatchling in figures 3, 4, 5, 6 and in tables 1, 2, 3, and 4. At day 10, wet body mass of embryos averaged $2.0 \pm 0.13$ grams, and $0.2 \pm 0.03$ grams for dry body mass (Table 1). At day 15, there was a significant increase in wet body mass ($F=25.59$, $p<0.0001$), and a significant increase in dry body mass ($F=86.85$, $p<0.0001$) (Fig.6). By hatch, there was a significant increase in wet body mass from day 10 ($p<0.0001$), and significant increase in dry body mass from day 10 ($p<0.0001$) (Table 1).

TOE AND BEAK LENGTH

At day 10, toe lengths averaged $6.5 \pm 0.24$ mm, and beak length averaged $2.2 \pm 0.05$ mm (Table 2). At day 15, toe length increased significantly to 14.1 mm ($F=20.68$, $p<0.0001$), and beak increased to 3.2 mm ($F=18.19$, $p<0.0001$), (Fig.4). By hatch, toe length increased significantly to 15.7 mm ($p=0.0027$). There was an insignificant increase in beak length (to 3.4 mm) ($p>0.61$).

HEART MASS

At day 10, the average wet heart mass was $33 \pm 5.4$ mg, while dry heart mass was $3.6 \pm 0.5$ mg (Table 3). At day 15, wet heart mass dropped to 30 mg, while dry heart mass increased to 5 mg (Fig.5). By hatch, there was an insignificant increase in wet heart

mass to 38 mg.  Dry heart mass increased insignificantly from day 10 to day 15, and by hatch dry heart mass had increased insignificantly from mass at day 10 (p=0.20).

HEART TO BODY MASS RATIO

Dry heart to body mass ratio dropped from 0.0147 at day 10 to 0.0055 at day 15, and went up to 0.0063 at hatch (Fig.6).

HEMATOLOGY

Hematocrit ranged from 23.4% to 26% across all measured stages (Table 4). Hemoglobin ranged from 6.6g% to 9.5g%.  Similarly, hemoglobin showed no significant change throughout development: 6.6g% (day 10), 9.3g% (day 15), 9.5g% (hatch) (Table 4).

EFFECTS OF HYPOXIC INCUBATION

Compared to the initial sample size of each group (day-0), survival at day 10 was 79% for early hypoxia, 61% for middle hypoxia, 74% for late hypoxia and 56% for continuous hypoxia.  At day 15, survival was 73% for early hypoxia, 57% for middle hypoxia, 48% for late hypoxia and 13% for continuous hypoxia. By the time of hatching, survival was 90% for early hypoxia, 33% for middle hypoxia and 86% for late hypoxia. No embryos survived continuous hypoxia (Fig.2).

Surprisingly, hatchability appeared to be most successful at early hypoxia (Fig.2). Cumulative hypoxic exposure appeared to play a critical role in survival and hatchability

of the embryos exposed to continuous hypoxia, because none of these embryos survived past day 15 (Fig.2).

Developmental features of embryos in the early hypoxic treatment group appeared normal. Similar to embryos in the control group, feathers were not visually apparent until day 15 of embryo development and eyes were covered by eyelid membrane and were not visually apparent until day 15.

Middle hypoxic embryos exhibited normal developmental features at days 10, 15, and hatch. Feathers were visually apparent at day 15, and eyes appeared to be open at day 15. However, albumin in the egg at day 15 was visually thicker in than albumin in control, or early hypoxic eggs. Developmentally, late hypoxic embryos resembled those of control, early hypoxic, and middle hypoxic treatment groups, both at day 10 and day 15. Feathers were visually apparent at day 15, and eyes appeared to be open at day 15 as well.

The continuous hypoxic treatment group displayed many abnormal physiological and anatomical characteristics of embryos throughout development. At both day 10 and 15, the overall appearance of the embryo was edematous, with noticeable yellow/red fluids accompanying the clear fluid. In addition to the edematic embryos in this treatment group, there was a prominent edema of the head. Evaluation of the egg upon removal of the embryo from the yolk revealed water sacs surrounding the embryo, and trapped beneath the skin of the embryos, a condition referred to as ascites; excess amounts of a combination of lymph and blood plasma fluids leaking from the liver and accumulating in the body cavity. In addition to the morphological differences mentioned, there were

also notable beak and eye deformations in the continuous hypoxic group. Deformities included lack of lower or upper beak or eye, or incomplete formation of either or both. Eyes appeared to be open by day 10 in some cases, but appeared closed at day 15 in other cases. The full length of the wing was attached to the embryonic body in some embryos, and there were no apparent feathers present upon evaluation until day 15. The third toe was attached to other toes in some cases.

BODY MASS

Wet body mass of the early hypoxic embryo group ranged from $1.7 \pm 0.14$ up to $8.0 \pm 0.2$ grams over the measured period of development, while dry body mass ranged from $0.15 \pm 0.03$ up to $1.4 \pm 0.05$ grams (Table 1). Wet body mass significantly increased at day 15 ($p < 0.0001$), and was significantly different from control ($p = 0.003$) (Table 1 and Fig.3). Similarly, dry body mass was significantly different than control at day 15 ($p = 0.002$). At day 15, early hypoxic dry body mass was significantly different than that of continuous hypoxia ($p < 0.0001$). By hatch, dry body mass had significantly increased to 8.0 grams ($p < 0.0001$) from day 15. At hatch, dry body mass of the early hypoxic treatment group was also significantly different than the middle hypoxic treatment group ($p < 0.0001$) (Table 1 and Fig.3). Wet body mass at day 10 for middle hypoxic embryos was $1.9 \pm 0.2$ grams, increasing significantly to $5.5 \pm 0.15$ grams by day 15 ($p < 0.0001$), and further increasing significantly to $8.1 \pm 0.48$ grams by hatch ($p = 0.006$) (Table 1). Wet body mass was significantly lower than control values at day 15 ($p = 0.0003$). Dry body mass increased significantly, from $0.2 \pm 0.04$ grams (day 10), to $0.9 \pm 0.03$ grams

(day 15) (p<0.0001), and also increased significantly from day 15 to hatch (1.93 ± 0.31grams) (p=0.006). Similarly, at day 15 dry body mass was significantly lower than the control value (p<0.0001), and wet body mass was significantly different from control (p=0.0003). Dry body mass at hatch was also significantly lower from control (p<0.0001) (Table 1).

Late hypoxic wet body mass increased significantly from 1.6 ± 0.14 grams (day 10), to 5.4 ± 0.14 grams (day 15) (p<0.0001), to 8.7 ± 0.4 grams by hatching (p<0.0001). Dry body mass also increased significantly from 0.15 ± 0.03 grams (day 10), to 0.9 ± 0.03 grams (day 15) (p<0.0001), to 1.6 ± 0.05 grams by hatch (p<0.0001) (Table 1 and Fig.3). However, at day 15, both wet and dry body masses were significantly lower from control values (p<0.0001) (Fig.3).

Continuous hypoxic wet body mass increased significantly from 1.5 ± 0.13 grams (day 10) to 3.4 ± 0.27 grams (day 15) (p<0.0001), while dry body mass also increased significantly from 0.13 ± 0.03 grams (day 10) up to 0.60 ± 0.06 grams (day 15) (p<0.0001). Both wet and dry body masses were significantly lower from control values at day 15 (p<0.0001) (Table 1). There were no values for hatchlings since survival rate was 0%.

TOE AND BEAK LENGTH

Early hypoxic toe length increased significantly from 6.2 ± 0.21mm (day10) to 16.1 ± 0.34 mm (hatch) in length (p<0.0001), while beak length increased from 1.9 ± 0.04 mm (day 10) to 3.4 ± 0.07 mm (hatch) (p<0.0001) (Table 2). Toe length did not

differ significantly from control at any stage in development. Similarly, beak length in the early hypoxic group did not differ significantly from control at any stage in development (Fig.4).

Middle hypoxic toe length increased significantly from $6.4 \pm 0.31$mm (day 10) up to $16.3 \pm 0.5$ mm (hatch) (p<0.0001) (Table 2). Toe length also increased significantly from day 15 to hatch (p<0.0001). Beak length increased significantly from $2.2 \pm 0.06$ mm (day 10) up to $3.0 \pm 0.05$ mm (day 15) (p<0.0001), and also increased significantly from day 15 to $3.6 \pm 0.09$ mm at hatch (p<0.0001).

Late hypoxic toe length increased significantly from $6.1 \pm 0.22$ mm (day 10) to $12.9 \pm 0.22$mm (day 15) (p<0.0001), and increased significantly from day 15 to $15.4 \pm 0.4$ mm by hatch (p<0.0001). Beak length also increased from $2.0 \pm 0.04$ mm (day 10) to $2.9 \pm 0.05$ mm (day 15) (p<0.0001), but increased insignificantly from day 15 to $3.2 \pm 0.08$ mm by hatch (Table 2). Both toe length (p=0.031) and beak length (p=0.032) differed significantly at day 15 from control values (Fig.4).

Toe length in embryos exposed to continuous hypoxia increased significantly from $5.3 \pm 0.2$ mm at day 10 to $10.1 \pm 0.42$ mm at day 15 (p<0.0001) (Table 2). Toe length was significantly lower from control values at day 10 (p=0.0097), and day 15 (p<0.0001) (Fig.4). Beak length increased from $1.8 \pm 0.04$ mm at day 10 to $2.6 \pm 0.09$ mm at day 15 (p<0.0001). Beak length was also significantly lower from control values at day 10 (p<0.0001), and day 15 (p<0.0001) (Fig.4). Thus, the continuous hypoxia treatment group was directly affected by hypoxic incubation throughout development.

HEART MASS

Wet heart mass in early hypoxia ranged from $31 \pm 6$ mg (day 10) to $50 \pm 11$ mg at hatch (Table 3 and Fig.5). There was no significant increase from day 10 to day 15, or from day 15 to hatch. There were no significant differences in wet or dry heart mass from control values ($p>0.05$).

Middle hypoxic wet heart mass increased insignificantly from $28 \pm 7.3$ mg, at day 10 to $33 \pm 5.7$ mg, at day 15, and increased significantly from day 15 to $60 \pm 17$ mg, at hatch ($p<0.0001$) (Table 3 and Fig.5). Dry heart mass increased insignificantly from $3.6 \pm 0.7$ mg at day 10 to $5.3 \pm 0.5$ mg day 15, and increased significantly from day 15 to $11.4 \pm 0.4$mg at hatch ($p<0.0001$) (Table 3).

Late hypoxic wet heart mass increased insignificantly from $36.7 \pm 6$ mg (day 10), to $32 \pm 5$ mg (day 15), and also increased insignificantly from day 15 to $69 \pm 15$mg by hatch (Table 3). Dry heart mass increased insignificantly from $3.4 \pm 0.6$ mg (day 10), to $5.2 \pm 0.5$ mg (day 15), and increased significantly from day 15 to $10.3 \pm 1$mg by hatch ($p=0.0002$). Neither wet nor dry heart masses differed significantly from control values at day 10 or day 15(Fig.5).

Continuous hypoxic wet heart mass increased insignificantly from $26.4 \pm 6.2$ mg at day 10 to $42 \pm 8.7$ mg at day 15. Dry heart mass increased insignificantly from $3.2 \pm 0.6$ mg at day 10 to $4.9 \pm 0.8$ mg at day 15. There were no significantly different values from control at day 10 and 15 for the wet heart masses, or the dry heart masses at day 10 and 15 (Table 3 and Fig.5).

HEART TO BODY MASS RATIO

At day 10, wet heart to body mass ratio of the early hypoxic treatment group was significantly different from control (p=0.0008), wet heart to body mass ratio of the late hypoxic group was significantly different from control (p<0.0001), and the wet heart to body mass ratio of the continuous hypoxic group was significantly different from control (p=0.035), while wet heart to body mass ratio of the middle hypoxic treatment group was not significantly different from control. Dry heart to body mass ratio of the early hypoxic group (p=0.499), and the late hypoxic group (p=0.743), did not differ significantly from control, while that of the middle hypoxic group, and the continuous hypoxic group (p=0.003) were significantly smaller than that of control.

At day 15, wet heart to body mass ratio of the early hypoxic treatment group (p<0.0001), and that of the continuous hypoxic group (p<0.0001) were significantly larger that of control. Dry heart to body mass ratio of the early hypoxic group (p=0.0001), and the continuous hypoxic group (p=0.026) were significantly larger than that of the control.

At hatch, none of the wet heart to body mass ratios of any of the treatment groups was significantly different than that of the control. However, the dry heart to body mass ratio of the late hypoxic group was significantly different from that of the control (p=0.002).

HEMATOLOGY

Hematocrit in early hypoxic embryos ranged from 21.4% to 27.8%, while hemoglobin ranged from 6.7g% to 8.8g% (Table 4). However, there were no significant differences in hematocrit and hemoglobin values from control values at any point in development (p>0.05). Middle hypoxic hematocrit ranged from 21.4% to 26.6% over development (Table 4). Hemoglobin ranged from 6.6g% to 9.1g%. There were no significant differences from control values at any point in development (p>0.05). Hematocrit in late hypoxic embryos ranged from 22.2% at day 10, to 29.9% at hatch (Table 4). Hemoglobin ranged from 6.5g% at day 10, to 8.1g% at day 15, and to 9.1g% by hatch. Neither hematocrit nor hemoglobin differed significantly from control values at any point in development (p>0.05) (Table 4). Hematocrit in embryos exposed to continuous hypoxia ranged from 22.2% at day 10 to 26.5% at day 15. Hemoglobin ranged from 6.6g% at day 10 to 7.4g% at day 15. There were no significant values in either measurement at both days (p>0.05) (Table 4).

CHAPTER 4

DISCUSSION

Birds are of particular interest in studies on the pathophysiological responses to hypoxia due to their sensitivity to hypoxia and high metabolic rate. Interestingly, there is great contrast between wild birds and the domestic chicken in the reduction of metabolism, hatchability, growth rate, and hatching mass upon exposure to moderate hypoxia (Carey et al., 1982). Consequently, the data derived from this study are not representative of all birds, especially wild ones.

During development the avian embryo goes through rapid growth rates supported by high basal metabolic rates (Vleck, 1980; Metcalfe et al., 1981; Hoyt, 1987; Nakane and Tsudzuki, 1999). This increased oxygen demand requires progressive increase in cardiac output, affected by an increased heart rate and stroke volume (Peacock, et al., 1989). When exposed to hypoxia, avian embryos develop an increase in pulmonary arterial pressure, then cardiac hypertrophy and finally liver congestion, edema and ultimately ascites.

HYPOXIC INCUBATION AND SURVIVAL, HATCHABILITY AND MORTALITY

The patterns of mortality in both normoxic and hypoxic incubation in chicken embryos appear to be reflected in this study on quail embryos, especially for the continuous hypoxic treatment group. Not only did the continuous hypoxic treatment group exhibit the highest mortality, but this group also exhibited further harmful hypoxic effects throughout development including: retardation of embryonic growth, edema, and

deformities in beak and eye formation.  These the harmful hypoxic effects culminated in

no success in hatchability and survival.  It is important to note that high altitudes and

hypoxic environments are major factors in hatchability, and decreased hatchability has

been recognized in chicken and turkey eggs at elevations above 1200 meters since 1895

(Smith, 1969, Christensen and Bagley, 1984, and Visschedijk, 1985).

Although the dramatic decrease in survival of the continuous hypoxic group was

expected, hypoxic incubation appeared exposure-dependent, because the survivorship of

the other hypoxic treatment groups lay between the control and the continuous hypoxic

group. The differences in survival between early, middle, and late hypoxia could be

indicative of critical windows of development in quail embryos.  Successful hatchability

of each treatment group, except for the continuous hypoxic group is indicative of the

quail embryo's capacity to overcome limited oxygen availability due to hypoxic

incubation.  So, although quail embryos were exposed to hypoxia for approximately 5

days at various stages in development their ability to recover/repair suggests that embryos

may take different developmental trajectories, leading to different embryo phenotypes,

but by hatch they have the same hatchling phenotype.

EMBRYONIC GROWTH AND DEVELOPMENT

Metabolic studies of quail hatchlings show that oxygen consumption is

allometrically related to total body mass (Lilja, 1997; Bishop, 1999).  It can be inferred

from this that the oxygen consumption was directly proportional to the combined masses

of the organs, i.e. to their growth pattern (Lilja, 1997). In chickens and turkeys, growth is divided into three phases: "early", up to 1-2 grams; "intermediate", 2-10 grams; "late", greater than 10 grams (Smith et al., 1969 and Hurwitz et al., 1991). Not only is there variation in embryo growth during development at particular growth phases in normoxic incubation (Altimiras and Phu, 2000), but variation in embryo growth also exists on a greater scale at different elevations (sea level 3100 and 3800 m) (Smith et al., 1969; Beattie and Smith, 1975; Altimiras and Phu, 2000). Although no significant differences exist in body mass or heart mass between the treatment groups and the control at day 10, or at day 15 in heart mass, these reported findings may provide additional information to assist in understanding the variation/and or significance in embryo body mass at day 15, and beak and toe lengths at days 10 and 15.

Smith (1969) reported that the repression by hypoxia of embryonic growth in quail is pronounced only earlier than 10 days and later than 17 days of incubation. Embryonic growth at the intermediate growth phase, in which the embryo increases in size from 2-10 grams, is essentially unaffected. This could account for the significant differences in beak and toe length in the continuous hypoxic treatment group at day 10. The significance of the toe and beak lengths, as mentioned previously, are not only used as indices of development in embryo in late incubation (Hamburger and Hamilton, 1951 and Stock et al., 1983) but serve as a comparative gauge throughout development, and for interpreting results. This would indicate that the continuous hypoxic treatment group was developmentally repressed due to hypoxic exposure from day 0- day 10.

However, the intermediate growth phase (which corresponds to the mid-incubation period) is characterized by an unusually high mortality at high altitudes (or low oxygen partial pressure) (Smith, 1969; Bjonnes et al., 1987; Richards et al., 1991-92; Altimiras and Phu, 2000; Mulder et al., 2000; Miller et al., 2002). In this study, high mortality at mid-incubation is shown at day 10 for the continuous hypoxic treatment group as well as the middle hypoxic treatment group (Fig.2). From this study it is unclear whether the mortality of the middle hypoxic treatment group was high due to hypoxic exposure earlier than day 10, or due to normal causes associated with the mid-incubation developmental phase. Nonetheless, the factors leading to this mortality did not appear to affect the hatchling phenotype for the middle hypoxic treatment group. In addition, the middle hypoxic treatment group did not exhibit any significant differences from the control in body mass, heart mass, or beak and toe length at day 10.

Although growth and differentiation, both of which are independent variables, are recognizable indicators of embryonic development, differentiation is not always estimated by embryonic mass criteria. After day 10 of incubation, the lengths of beak and third toe are the most consistent indicators of developmental stage (Smith et al., 1969). Although this (Smith et al., 1969) differs from what was reported by Stock et al. (1983), both reports were taken into consideration for the purpose of this study. In agreement with Smith et al., beak and toe lengths served as indicators of developmental delay, as shown in continuous hypoxic and late hypoxic treatment groups at day 15 (Fig.4). It appears that differentiation is repressed more than growth during development. Findings from this study are consistent with Smith et al. (1969).

Quail embryo growth follows similar patterns to that of chicken embryos during normoxic and hypoxic incubation. At day 15, both wet and dry body mass for all the treatment groups were significantly different than wet and dry body mass of control. These results are consistent with the findings of Stock and Metcalfe (1984 and 1987), in which growth was reported not only to be limited by the availability of oxygen during hypoxic incubation, but also during normal incubation in air. Since normoxic and hypoxic incubation were interchanged depending on treatment group, it cannot be inferred that growth was limited solely due to hypoxic incubation.

RESPONSES TO HYPOXIA

When confronted with hypoxia, the embryo has two alternatives for conserving oxygen that is not available to the adult chicken: it can temporarily stop growing (Metcalfe et al., 1981), or it can channel energy away from growth and into mere maintenance to cope with the stress of hypoxia (Barnas and Rautenberg, 1990). The first would imply a critical window, in which the embryo is developmentally hindered, by targeting an essential phase in development that cannot overcome the paucity of oxygen crucial for embryogenesis and organogenesis. This appears to be the detrimental developmental trajectory taken by the embryos of the continuous hypoxic treatment, which is exhibited in the high mortality level of this group.

Channeling energy away from growth implies that the embryo overcomes hypoxic incubation, and follows a normal developmental trajectory once normoxic incubation is

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resumed. Although no data was collected on ATP levels in this study, it is possible that quail embryos that follow an abnormal developmental trajectory succumb to the reduction of ATP, ultimately leading to physiological retardation of growth. This reduction may lead to less energy that is apportioned for development, and the embryo tries to survive by acclimating to hypoxia.

Since this alternate pathway is the only means (besides stopping growth altogether) in the face of limited availability of oxygen, quail embryos could possibly be acclimating metabolically to their hypoxic environment, by responding at the cellular level to hypoxia. Therefore, low oxygen levels, which have replaced plentiful oxygen supplies present during normal incubation, lead to the sparing usage of the limited oxygen available for the developing embryo. The developing embryo has less ATP to expend on development, and possibly saves energy for the energetically costly stage of pipping (Wieser, 2002). However, since no data were collected from this study to support this, it is unknown whether this pathway occurs in quail embryo or not.

It is also possible that the earlier in development that embryos are exposed to hypoxic incubation, the longer time they have to repair any damage that may have been caused by hypoxia, which would be consistent with what was reported by Miller et al. (2002). At later developmental stages, exposure to hypoxia may not have too much of an effect because the embryo has already gone through its vital developmental process, and it is able to fend off the detrimental effects of hypoxia - i.e. the embryo is past its major critical windows. Embryos possibly consume such minimal amounts of energy in these

early stages that they could survive hypoxia.  By acclimating to hypoxic incubation, less energy is expended in these oxygen and energy costly stages, and embryos may exhibit smaller body masses and shorter beak and third toe lengths as a result.

In conclusion, despite following different developmental trajectories during incubation, which lead to different embryological phenotypes, by hatch embryos of the different hypoxic treatment groups have hatchling phenotypes.

HYPOXIC INCUBATION AND HEMATOLOGY

Despite a protocol that included intervals of hypoxic incubation (approximately 5 days in length) and continuous hypoxic incubation, I was surprised to find no significant changes in hematology induced in quail embryos. Hypoxic exposure is known to lead to erythrocytic polycythemia in vertebrates, which is thought to be an adaptation to hypoxia (Burton et al., 1969). However, the lack of significance in hematological measurements in my study could be offset by production of hemoglobin with a greater affinity for oxygen, consistent with Hall (1934) and Velarde et al. (1991).  There are at least two distinct forms of hemoglobin in the blood of the chick embryos around day 6 (Baumann, 1983).  It is likely that hemoglobin having the greater affinity for oxygen is produced earlier in development and is replaced gradually by hemoglobin having lesser affinity (Baumann, 1983; 1984; Kavdia et al., 2002), however from my study it cannot be inferred because elaborate hematological studies were not conducted.  Additionally, it is possible that hypoxic incubation in this study did not occur during a critical window

lethal for hematopoeisis, or exposure was not long enough and/ or oxygen levels were not low enough to cause permanent changes in hematology.

The failure to increase the red cell count during hypoxia could indicate that red cell production is at a maximum rate under normal incubation conditions, as has been suggested for chicken embryos (Baumann, 1984; Kavida et al., 2002). If this is the case, then properties of each type of hemoglobin is suited to the specific oxygen needs of the embryo at the time of its production and depending on the hypoxic exposure and duration of exposure. Again, from this study it cannot be inferred that this occurs in developing quail embryos.

Although most of the findings in this study deal with the physical retardation in growth (body mass, heart mass, third toe length, and beak length), the lack of distinct change in Hb concentration and Hct could be indicative of the general retardation of the hypoxic embryo. During early development the chick embryo is unable to counter hypoxia by an increased production of red blood cells and or the expansion of total blood volume (Baumann and Meuer, 1992), indicating that the expansion of blood volume and changes in hemoglobin (embryonic to adult) occurs at maximum speed during normal development. This could be occurring in the quail embryos as well, however, it is unknown. The adaptation to hypoxia by display of increased erythropoetic polycythemic concentration is thought to increase the blood viscosity, which would thus increase blood flow resistance (Burton, 1969; Shams and Scheid, 1989; Hoper and Jahn, 1995). These adaptations would surely force additional work

requirements on the heart of the hypoxic animal, and could possibly lead to a pathological increase in cardiac output and stroke volume.

The sigmoid growth curve of the embryo is paralleled by both total oxygen consumption (Vleck et al., 1980 and Hopkins and Powell, 2001) and eggshell gas diffusing capacity (Temple and Metcalfe, 1970 and Tazawa et al., 1988). All three curves flatten perceptibly at the time when the oxygen content of the allantoic venous blood is declining (Metcalfe et al., 1981). The mechanisms by which variations in blood oxygen partial pressure influence embryonic growth are entirely speculative. However, it is believed that embryonic growth in the chick is regulated by the oxygen partial pressure in embryonic blood (Metcalfe et al., 1981, and Kavida et al., 2002). In this study there were no significant differences in hemoglobin and hematocrit between the treatment groups and the control, at days 10, 15, or hatch, but that is not to say that there is not an underlying mechanism by which there are variations in blood content. In hypoxic studies by Bjonnes et al. (1987) blood lactate measurements taken from chickens indicate significant changes. Although I did not find hypoxic stimulation of red blood cell production, this may have occurred with lower hypoxic incubation or longer hypoxic incubation treatments.

In summary, significant differences occurred in the anatomical measurements of quail exposed to hypoxia at different times during incubation as well as non-measurable differences in the embryos. These differences occurred depending on the stage in which they were exposed to hypoxia during embryological development in each of the treatment

groups.  However, hatchlings appeared phenotypically similar by hatch, thus following developmental trajectories leading to a common phenotype. The debilitating effect hypoxic exposure has on quail embryo was examined anatomically. In this study the critical window was the point in development in which hypoxic exposure to the developing quail embryo was the main contributor to the "developmental change", or where significant differences in physiological/hematological parameters existed after acute hypoxic incubation. Regardless of the experimental/hypoxic treatment and the stage in development when the embryo was exposed to hypoxia, all hypoxic treatment groups, except the continuous hypoxic group ended up phenotypically the same.

CHAPTER 5

FUTURE EXPERIMENTS

Although hemoglobin and hematocrit were measured at the same stages in which

embryos were weighed and measured, a more extensive and detailed hematological study

with daily measurements would be more reflective of the effects of hypoxia on blood.

Further future experiments include metabolic studies particularly lactate levels that would

be indicative of whether or not anaerobic metabolism replaces aerobic metabolism when

oxygen levels decrease.

A more detailed analysis of organs within the embryo body could explain whether

hypoxia causes any cellular and or molecular damage (apoptosis).  The traditional view

of cell level responses to oxygen limitation is encompassed in the concept of the Pasteur

Effect (Hochachka, 1997; Wieser, 2002).  This hypoxic cell level response could be

studied further in quail to determine whether or not hypoxia ultimately has a cellular and

molecular effect.

Quail growth rates would be interesting to study because what determines the

overall growth rate of the chicken embryo is based on the long-established concept of the

limiting factor to growth i.e. the speed of the complex process of growth is determined by

the speed of its slowest component (Robertson, 1923; Byerly, 1932).  Furthermore,

changes in oxygen availability influence embryo growth, and normally limits the speed of

the slowest component and thereby the chicken embryo's growth (Byerly, 1932).  Quail

growth rate studies could determine whether the concept of limiting factor to growth

follows the same pattern as that for chickens. Additionally, supportive evidence for the hypothesis that oxygen availability limits the growth rate can be drawn from the growth curve of the chicken embryo, which further supports that early growth restriction induced by hypoxia is reversed by the restoration of normoxia in incubation (Metcalfe et al., 1981 and Miller et al., 2002). So, similarly, quail growth rates would be beneficial to determine growth restriction induced by hypoxia. Organ growth rates would also provide further insight on growth restriction induced by hypoxia.

The use of the following equation for the calculation of growth rates, and further analysis may be the basis for the aforementioned future direction in quail experimentation. Embryo growth is typically exponential, fitting the equation: $W=ae^{kt}$, where (W) is embryo weight in grams, at time (t), days of incubation, (a) is the integration constant, and (k) is the growth rate constant (Smith et al., 1969 and Hurwitz et al., 1991).

Sustaining high metabolic rates requires oxygen, particularly during development. Bird embryos have a genetic potential for growth than potential to provide oxygen to sustain that growth (Witzel et al., 1990). The demand for oxygen may exceed cardiopulmonary capacity to supply sufficient oxygen, which ultimately leads to an oxygen deficit. Thus the heart responds by increasing its output of blood for oxygenation. Prolonged exposure to hypoxia causes the blood vascular system to adapt its structure to allow greater amounts of blood to flow to the tissues at any given pressure gradient, thus yielding embryos with red colored skin at any given time in development. Due to these

physiological characteristics of birds, it would be worthwhile to study the cardiovascular system as well as the vasculature of embryos in each of the hypoxic treatment groups. Furthermore, analyzing the possible increase in the rate of growth of blood vessels in the chorioallantoic membrane of the embryo could further help explain the developmental aspects of the vascular system. This increase in growth of exchange vessels would help increase oxygen delivery to the tissue cells by 1) increasing capillary surface area and 2) decreasing diffusion distance (Adair, 1987, Hoper and Jahn, 1995, and Mulder et al., 2000). This could be compensation for life in a hypoxic environment.

Prolonged exposure to high altitudes or hypoxia increases capillary density in skeletal muscle, but decreases the rate of growth of individual muscle fibers (Adair, 1987). However, studies done by Ingermann (1983) suggest that late in development, erythrocytes of control chicken embryos respond to oxygen limitation "in a manner that facilitates" chorioallantoic oxygen uptake. Examining muscle from hypoxic incubated embryos by basic histochemical staining as well as molecular studies could reveal at what level muscle is responding to hypoxic exposure if at all and how hypoxia is responded to at the cellular level. Not only are there many essential questions that still remain unanswered, but there is also a multitude of experiments that can be performed to facilitate answering them. In addition, there is a hypoxic exposure effect that must be taken into consideration.  So, not only can further experiments be carried out on the same hypoxic levels, but on different hypoxic levels, as well as for longer treatment periods.

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**Figure 1**

| | D0-5 | D5-10 | D10-15 | Pip | Hatch |
|---|---|---|---|---|---|
| Control | 150 mmHg | | | | → |
| Early Hypoxia | 110 mmHg | 150 mmHg | | | → |
| Middle Hypoxia | 150 mmHg | 110 mmHg | 150 mmHg | | → |
| Late Hypoxia | 150 mmHg → | | 110 mmHg | 150 mmHg → | |
| Continuous Hypoxia | 110 mmHg → | | | | |

Fig.1. Experimental protocol illustrating the various hypoxic treatments (acute and chronic) on development of quail. Hypoxic incubation is presented as oxygen tension of 110 mmHg, while normoxic incubation is oxygen tension of 150 mmHg. D=Day. "Pip" is defined as stage in development when the hatching bird breaks through the shell. "Hatch" is defined as stage in development has emerged from shell and absorption of yolk is complete.
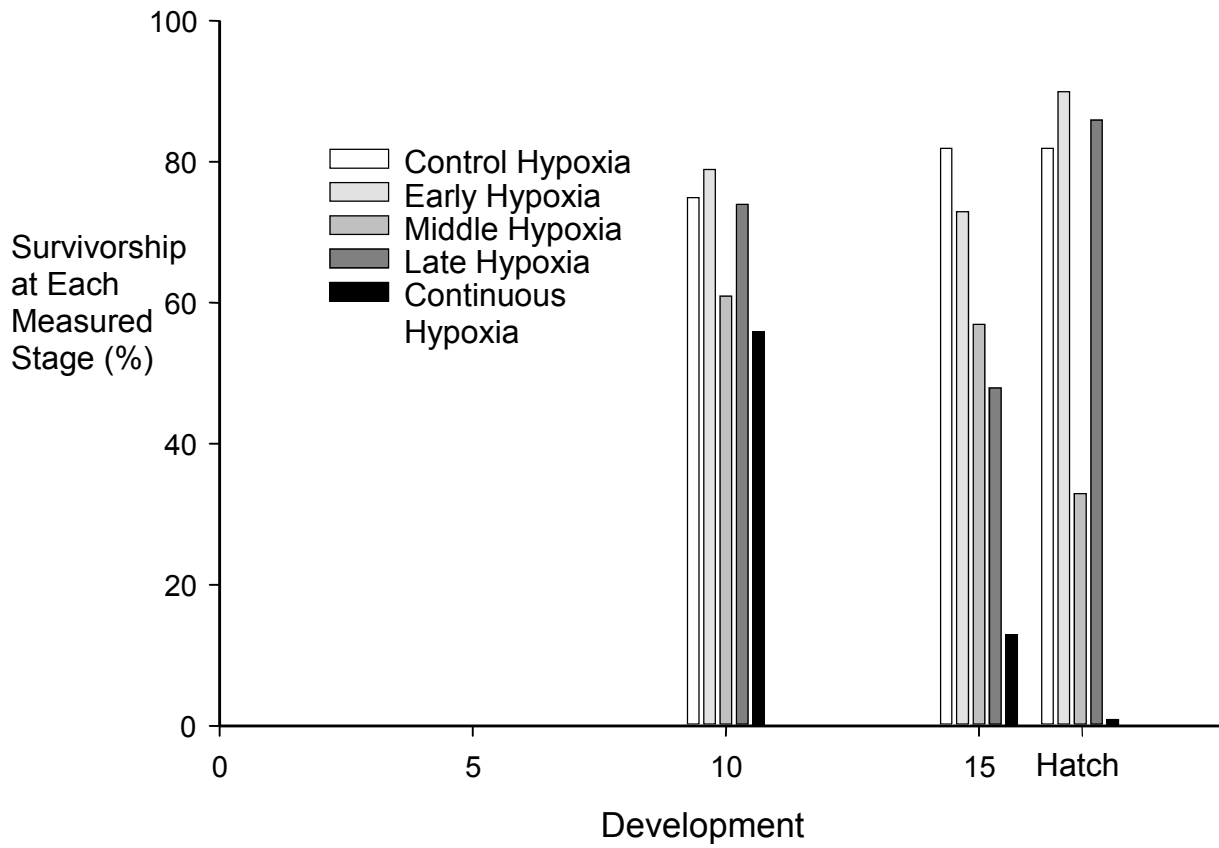
**Figure 2**



Fig.2. Survivorship of control and treatment groups measured at days 10, 15, and hatch.

Figure 3


Mean body mass during development.   A) Mean wet body mass
(mg) measured on days 10, 15, and hatch for early, middle, late and
continuous hypoxia. (B) Mean dry body mass (mg) measured on
days 10, 15, and hatch for early, middle, late and continuous
hypoxia.   Boxed points are not significantly different.

**Figure 3**



A

Wet Body Mass

Mass (g)

Development (Days)

B

Dry Body Mass

Mass (g)

- ● Control
- ○ Early Hypoxia
- ▼ Middle Hypoxia
- ▽ Late Hypoxia
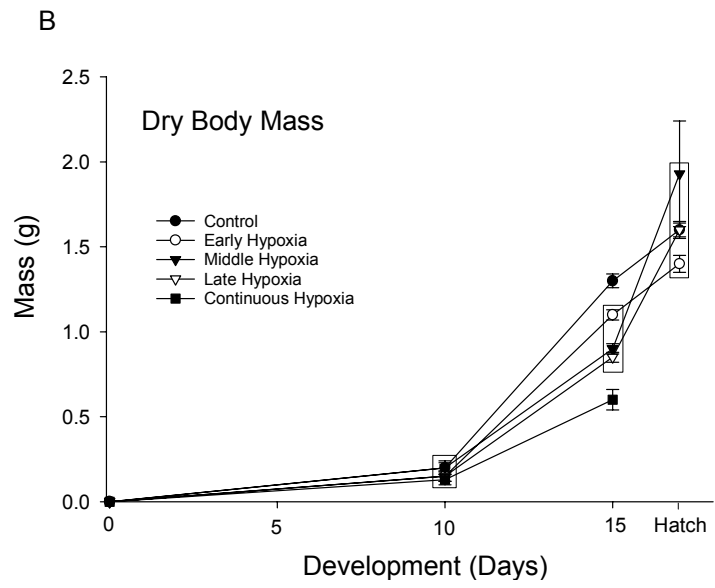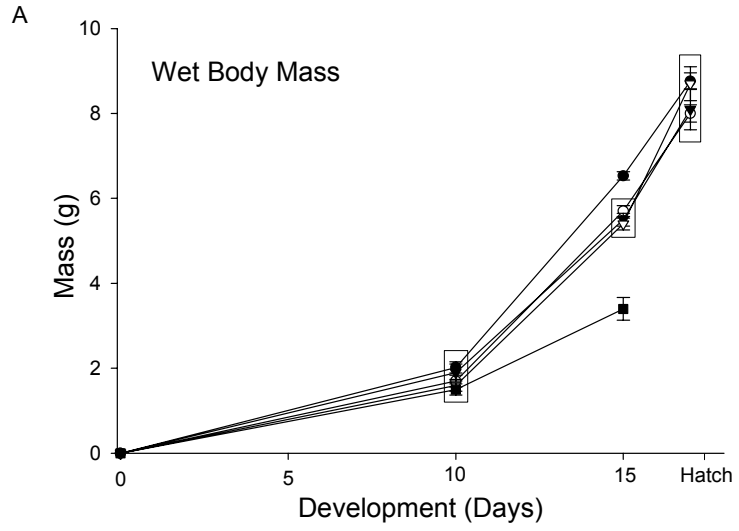- ■ Continuous Hypoxia

Development (Days)

Figure 4

Morphology during development.  A) Mean beak length (mm) at days 10, 15, and hatch for early, middle, late and continuous hypoxia.  (B) Mean third toe length (mm) at days 10, 15, and hatch for early, middle, late and continuous hypoxia.  Boxed points are not significantly different.

**Figure 4**



Beak

Length (mm)

Development (Days)

Third Toe

Length (mm)

Control
Early Hypoxia
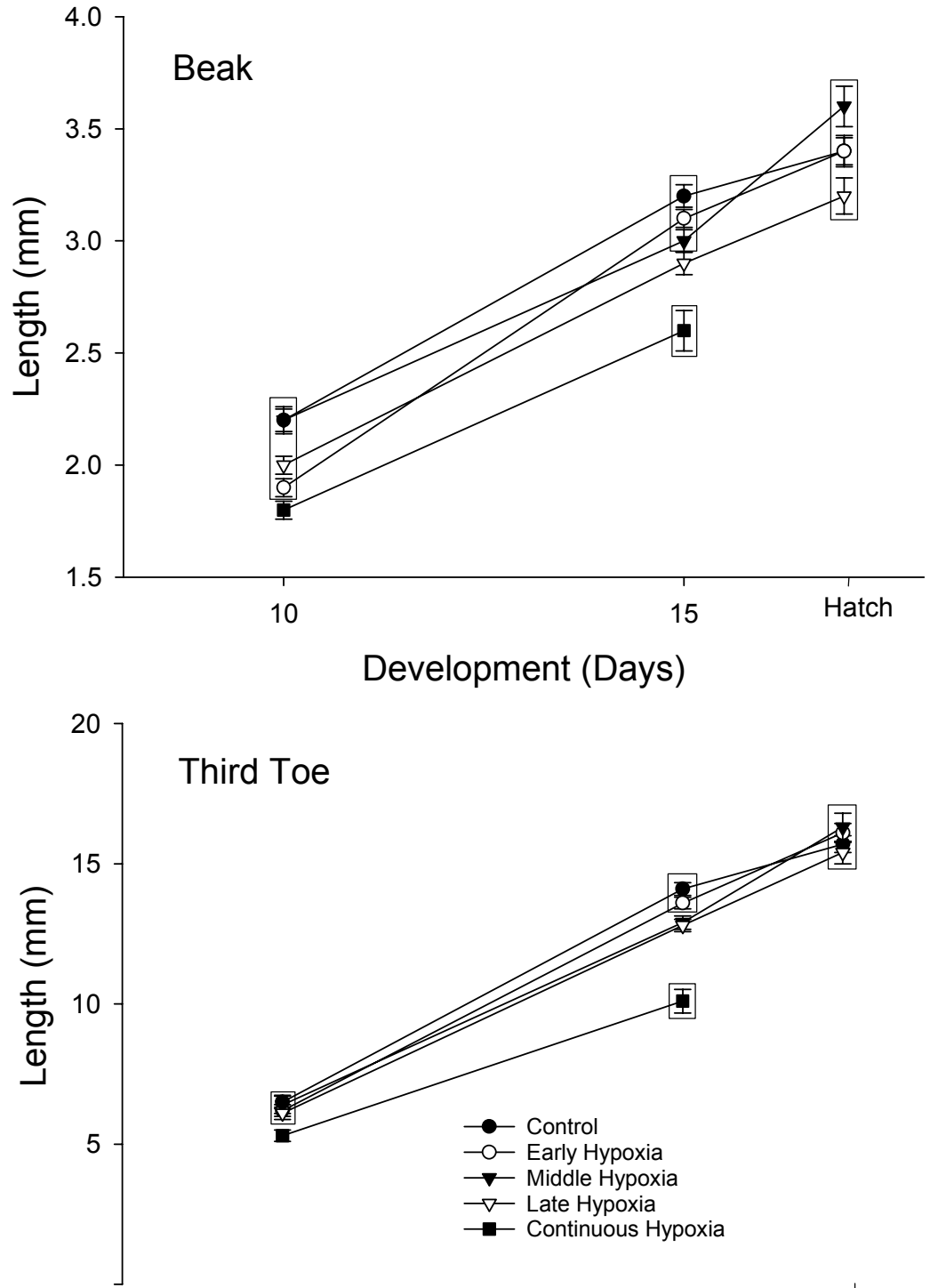Middle Hypoxia
Late Hypoxia
Continuous Hypoxia

Figure 5

Mean heart mass during development.   A) Mean wet heart mass
(mg) measured on days 10, day 15, and hatch for early, middle, late
and continuous hypoxia. (B) Mean dry heart mass (mg) measured
on days 10, 15, and hatch for early, middle, late and continuous
hypoxia.   Boxed points are not significantly different.

**Figure 5**

A



B

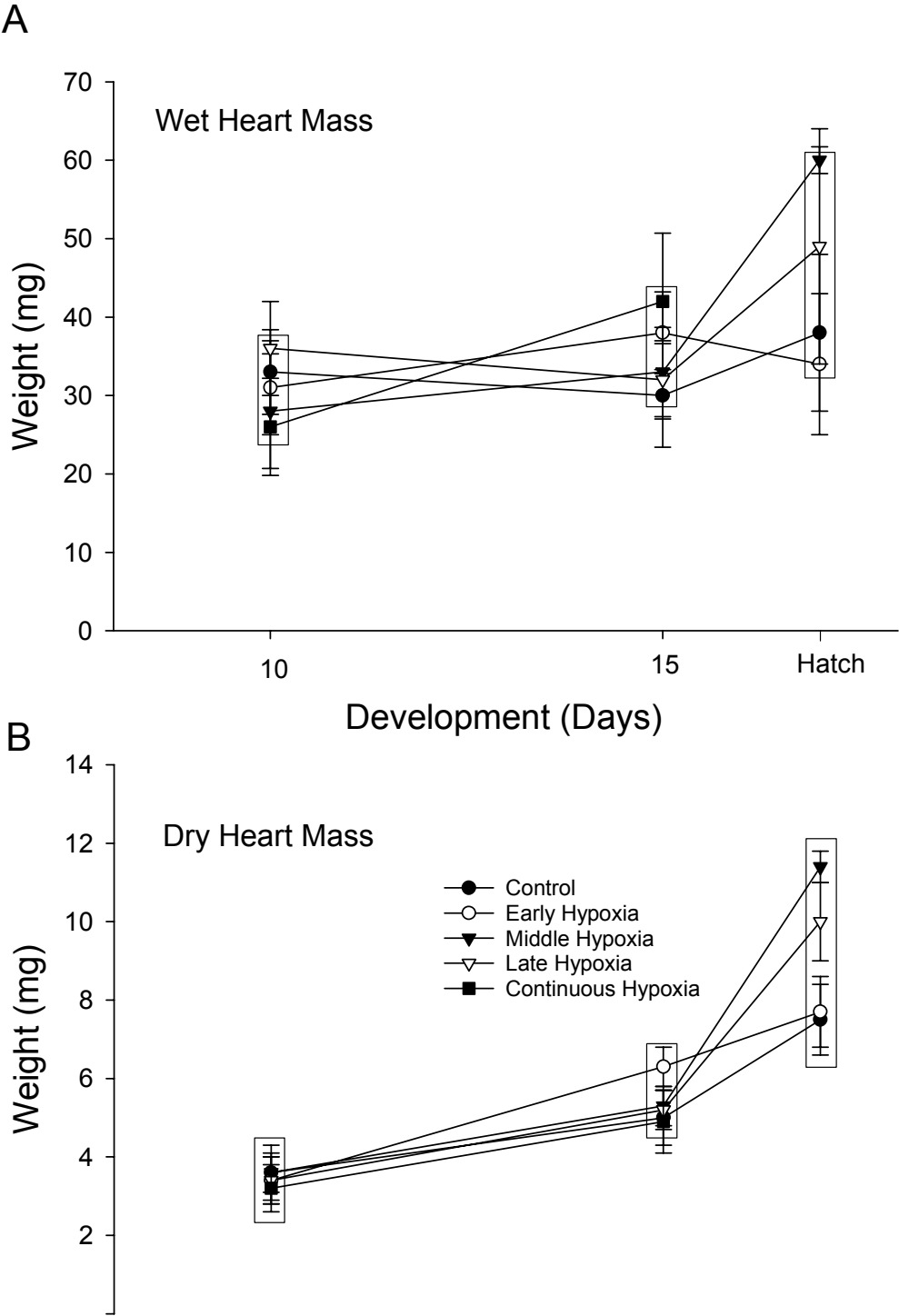Figure 6

Mean heart to body mass ratio during development.   A) Mean wet
heart to body mass ratios measured on days 10, day 15, and hatch
for early, middle, late and continuous hypoxia. (B) Mean dry heart
to body mass ratios measured on days 10, 15, and hatch for early,
middle, late and continuous hypoxia.   Boxed points are not
significantly different.

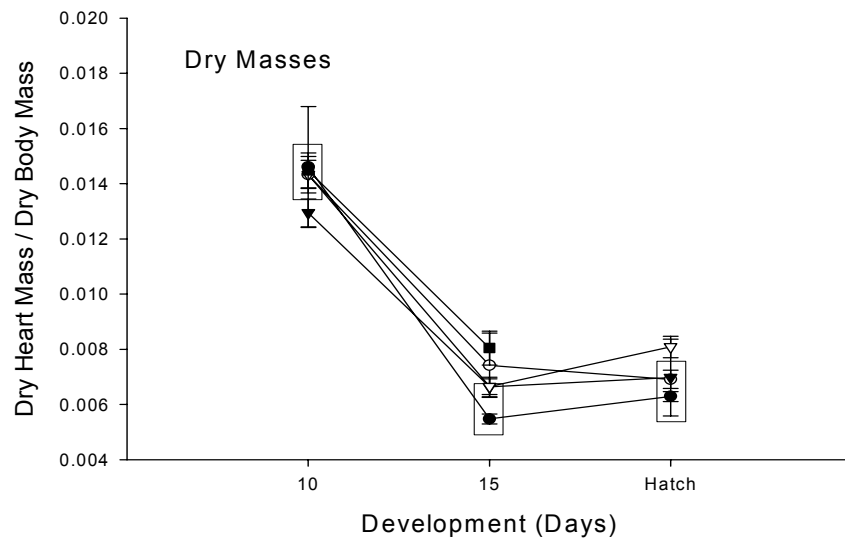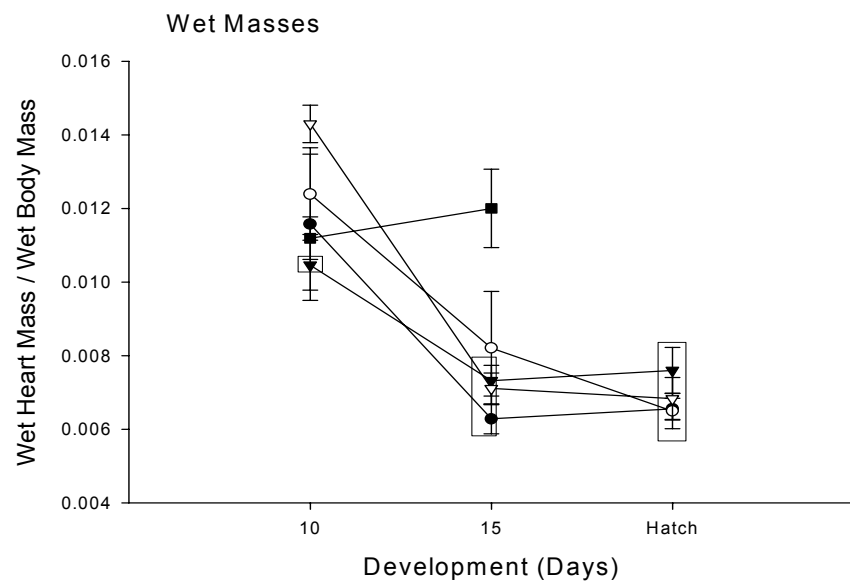**Figure 6**



Wet Masses



Dry Masses

Table 1. Mean body mass (g) and calculated mean water content during development, for control and each treatment group at days 10, 15, and hatch.  Asterisks and bold values indicate significant values from control (ANOVA followed by SNK multiple range tests).

| | Day 10 | | | | Day 15 | | | | Hatch | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Wet | Dry | Water Content (%) | n | Wet | Dry | Water Content (%) | n | Wet | Dry | Water Content (%) |
| Control | 33 | 2.0 ± 0.13 | 0.2 ± 0.03 | 91 | 37 | 6.5 ± 0.1 | 1.3 ± 0.04 | 81 | 18 | 8.8 ± 0.2 | 1.6 ± 0.04 | 82 |
| Early Hypoxia | 27 | 1.7 ± 0.14 | 0.15 ± 0.03 | 91 | 38 | **\*5.7 ± 0.13** | **\*1.1 ± 0.03** | 81 | 18 | 8.0 ± 0.2 | **\*1.4 ± 0.05** | 82 |
| Middle Hypoxia | 19 | 1.9 ± 0.2 | 0.2 ± 0.04 | 90 | 28 | **\*5.5 ± 0.15** | **\*0.9 ± 0.03** | 82 | 7 | 8.1 ± 0.48 | 1.93 ± 0.31 | 86 |
| Late Hypoxia | 28 | 1.6 ± 0.14 | 0.15 ± 0.03 | 91 | 33 | **\*5.4 ± 0.14** | **\*0.9 ± 0.03** | 83 | 12 | 8.7 ± 0.4 | 1.6 ± 0.05 | 82 |
| Continuous Hypoxia | 47 | 1.5 ± 0.13 | 0.13 ± 0.03 | 91 | 10 | **\*3.4 ± 0.27** | **\*0.6 ± 0.06** | 81 | 0 | N/A | N/A | N/A |

Table 2.  Mean toe and beak length (mm) during development, for control and each treatment group at days 10, 15, and hatch.

Asterisks and bold values indicate significant values from control (ANOVA followed by SNK multiple range tests).

| | | Day 10 | | | Day 15 | | | Hatch | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | n | **Toe (mm)** | **Beak (mm)** | n | **Toe (mm)** | **Beak (mm)** | n | **Toe (mm)** | **Beak (mm)** |
| Control | 33 | 6.5 ± 0.24 | 2.2 ± 0.05 | 37 | 14.1 ± 0.23 | 3.2 ± 0.05 | 18 | 15.7 ± 0.30 | 3.4 ± 0.06 |
| Early Hypoxia | 27 | 6.2 ± 0.21 | 1.9 ± 0.04 | 38 | 13.6 ± 0.21 | 3.1 ± 0.04 | 18 | 16.1 ± 0.34 | 3.4 ± 0.07 |
| Middle Hypoxia | 19 | 6.4 ± 0.31 | 2.2 ± 0.06 | 28 | 12.9 ± 0.24 | 3.0 ± 0.05 | 7 | 16.3 ± 0.5 | 3.6 ± 0.09 |
| Late Hypoxia | 28 | 6.1 ± 0.22 | 2.0 ± 0.04 | 33 | **\*12.9 ± 0.22** | **\*2.9 ± 0.05** | 12 | 15.4 ± 0.4 | 3.2 ± 0.08 |
| Continuous Hypoxia | 47 | **\*5.3 ± 0.2** | **\*1.8 ± 0.04** | 10 | **\*10.1 ± 0.42** | **\*2.6 ± 0.09** | 0 | N/A | N/A |

Table 3.   Mean heart mass (mg) and calculated mean water content during development, for control and each treatment group at days 10, 15, and hatch.  No significant differences were measured.

| | Day 10 | | | | Day 15 | | | | Hatch | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Wet | Dry | Water Content (%) | n | Wet | Dry | Water Content (%) | n | Wet | Dry | Water Content (%) |
| Control | 33 | 33 ± 5.4 | 3.6 ± 0.5 | 89 | 37 | 30 ± 6.6 | 5.0 ±0.7 | 83 | 18 | 96 ±17 | 7.5 ±0.9 | 80 |
| Early Hypoxia | 27 | 31 ±6 | 3.4 ±0.6 | 89 | 38 | 38 ±5.2 | 6.3 ±0.5 | 83 | 18 | 50 ±11 | 7.7 ±0.9 | 77 |
| Middle Hypoxia | 19 | 28 ±7.3 | 3.6 ±0.7 | 87 | 28 | 33 ±5.7 | 5.3 ±0.5 | 84 | 7 | 60 ± 17 | 11.4 ±0.4 | 90 |
| Late Hypoxia | 28 | 36.7 ±6 | 3.4 ±0.6 | 91 | 33 | 32 ±5 | 5.2 ±0.5 | 84 | 12 | 69 ±15 | 10.3 ±1 | 79 |
| Continuous Hypoxia | 47 | 26.4 ±6.2 | 3.2 ±0.6 | 88 | 10 | 42 ±8.7 | 4.9 ±0.8 | 88 | 0 | N/A | N/A | N/A |

Table 4. Mean hematocrit (%) and hemoglobin (g%) during development for control and each treatment group at days 10, 15, and hatch. No significant differences were measured.

| | Day 10 | | | | Day 15 | | | | Hatch | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | Hematocrit (%) | n | Hemoglobin (g%) | n | Hematocrit (%) | n | Hemoglobin (g%) | n | Hematocrit (%) | n | Hemoglobin (g%) |
| Control | 16 | 23.4 ± 3.2 | 18 | 6.6 ± 0.8 | 8 | 29.6 ± 6.1 | 9 | 9.3 ± 1.3 | 18 | 26 ± 3.9 | 17 | 9.5 ± 1.3 |
| Early Hypoxia | 21 | 21.4 ± 3.5 | 17 | 6.7 ± 1.1 | 20 | 26.9 ± 7.6 | 10 | 8.9 ± 1.2 | 12 | 27.8 ± 4.9 | 14 | 8.8 ± 1.7 |
| Middle Hypoxia | 8 | 21.4 ± 4.3 | 10 | 6.6 ± 1.2 | 13 | 25.2 ± 4.2 | 6 | 9.4 ± 1.0 | 5 | 26.6 ± 4.3 | 5 | 9.1 ± 1.3 |
| Late Hypoxia | 19 | 22.2 ± 3.4 | 19 | 6.5 ± 1.3 | 9 | 27.8 ± 7.6 | 7 | 8.1 ± 1.8 | 8 | 29.9 ± 3.7 | 8 | 9.1 ± 1.4 |
| Continuous Hypoxia | 13 | 22.2 ± 5.3 | 16 | 6.6 ± 1.6 | 5 | 26.5 ± 1.0 | 5 | 7.4 ± 0.0 | 0 | N/A | 0 | N/A |