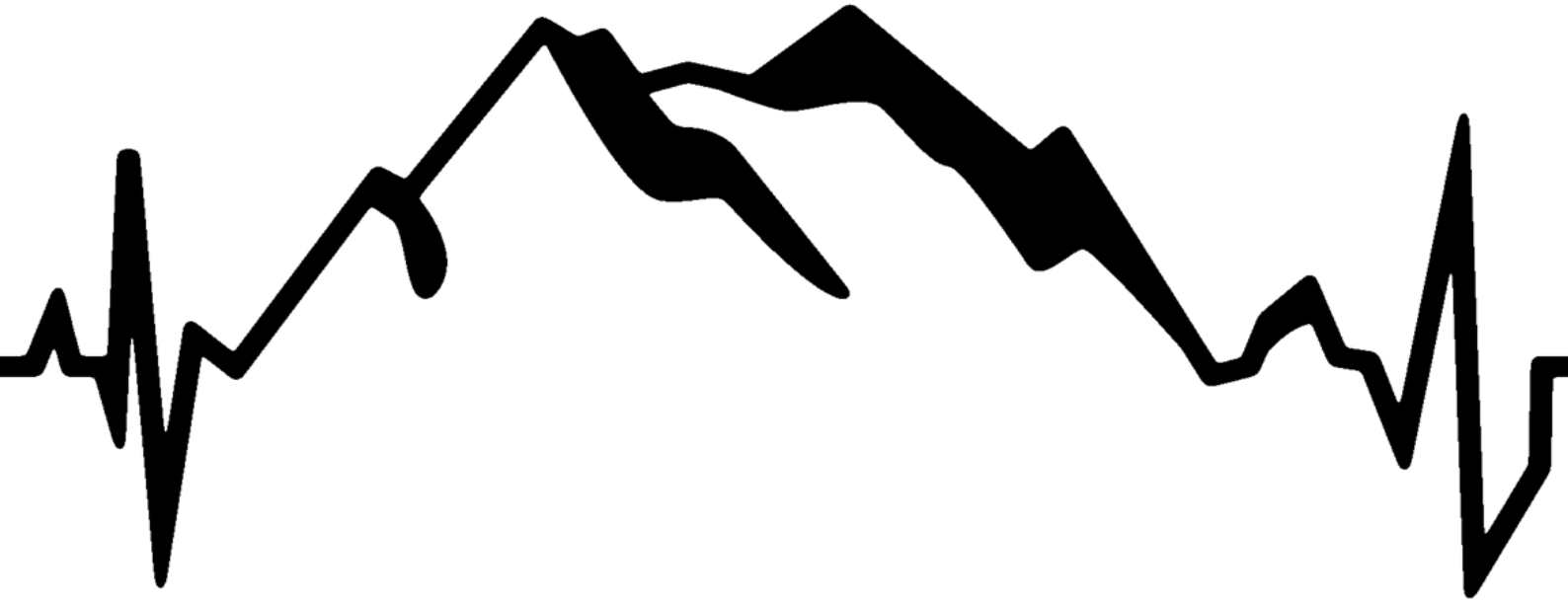
The highs and lows of programmed cardiovascular disease by developmental hypoxia

THE HIGHS AND LOWS OF PROGRAMMED CARDIOVASCULAR DISEASE BY DEVELOPMENTAL HYPOXIA: STUDIES IN THE CHICKEN EMBRYO



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STUDIES IN THE CHICKEN EMBRYO

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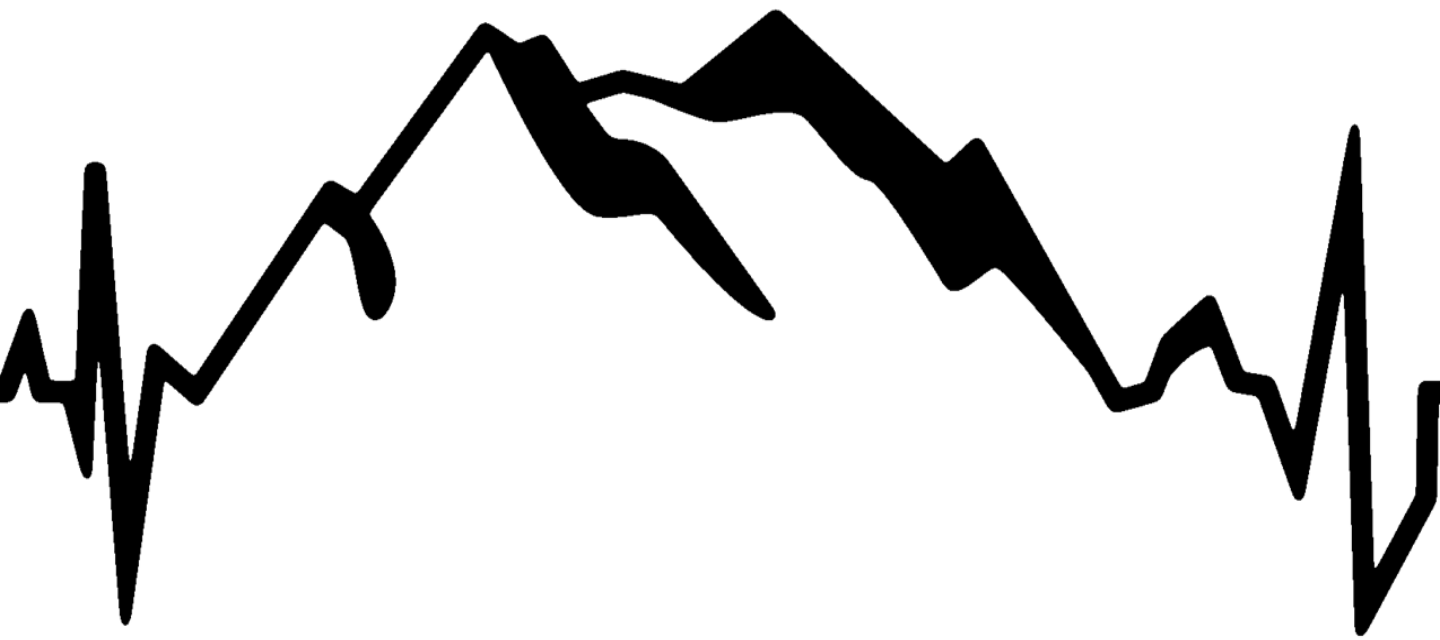
Chapter I

INTRODUCTION, AIMS AND OUTLINE OF THE THESIS



CHAPTER I: INTRODUCTION, AIMS AND OUTLINE OF THE THESIS

Developmental origins of cardiovascular health and disease

Cardiovascular disease is the leading cause of death in the world and is associated with high morbidity and cost (1, 2). It is widely accepted that the risk of developing the most common cardiovascular and metabolic diseases in our society - hypertension, diabetes, dyslipidaemia and coronary heart disease – is determined by the interaction between our genetic make-up and traditional lifestyle risk factors, such as smoking, obesity and/or a sedentary life. Nevertheless, we now understand that adverse environmental conditions during early life, such as pre-conceptually and during pregnancy, may be just as if not more important than postnatal lifestyle in interacting with genes to set a risk of cardiovascular disease (3, 4).

The Barker hypothesis states that low birth weight is associated with an increased risk of cardiovascular disease and its associated disorders (stroke, type 2 diabetes, hypertension and metabolic syndrome) in adulthood. In this scenario, low birth weight is a surrogate measure for adverse intrauterine conditions in complicated pregnancy. The first epidemiological studies, which support the Barker hypothesis came from Forsdahl (5). This study reported that the same regions in Norway shared an increased prevalence of complications during infancy, such as low birth weight and poor prognosis during infancy and an increased incidence of cardiovascular disease in adulthood (5). Later, Barker and Osmond reported that the prevalence of cardiovascular mortality coincided with greater neonatal mortality in the same areas of England and Wales during the first decades of the 20th century (6, 7). This finding triggered the idea that poor intrauterine growth, indexed by low birth weight, was associated with an increased risk of coronary heart disease in later life (8). Subsequently, fetal malnutrition was associated with an increased prevalence of insulin resistance (9), hypertension (10), hyperlipidaemia (11) and elevated plasma levels of fibrinogen in the offspring in later life (12). The idea that fetal malnutrition could have long term effects on the physiology of the offspring is not in fact new. The classical studies of Widdowson and McCance showed in animals that those with low weight at birth never reach the same size or maturity at adulthood compared with those born with normal birth weight (13, 14). It was postulated that a decrease in cellular endowment in key organs, such as in the heart and kidney imposed by adverse intrauterine conditions, might underlie the relationship (13, 14).

Normal and slow fetal growth

The Barker hypothesis has now been expanded into the concept of Developmental Origins of Health and Disease (DOHAD)(3, 4). Since intrauterine growth restriction (IUGR) is an established surrogate marker for adverse intrauterine conditions, there has been a keen scientific and clinical interest in identifying which and how sub-optimal environmental conditions may promote IUGR and programme an increased cardiovascular risk in the offspring. Fetal growth and development is believed to be determined by three main factors: the nutritional state of the pregnant mother, placental function and the fetal capacity to utilise substrates for energy production.

•**Maternal nutritional status during pregnancy.** It is known that low maternal food intake and/or impaired nutrient absorption can cause slow fetal growth and IUGR (15). Nevertheless, the individual variability in the response to protein and energy constraint is vast. It is now known that undernutrition during pregnancy can induce permanent effects in the offspring, such as a reduction in tissue cell number, organ structural remodelling, changes in key hormone levels as well as epigenetic changes. The specific long-term effects of maternal malnutrition will clearly depend on the duration and magnitude of the challenge. Every organ has its own critical and sensitive period of cellular replication during which it will be most affected by adverse developmental conditions (16). For instance, both hypo- and hyperglycaemia during early embryogenesis have been associated with low birth weight. While maternal undernutrition during early gestation triggers compensatory placental growth that protects fetal growth, maternal undernutrition during late gestation significantly impairs fetal growth and triggers cardiometabolic sequelae in the offspring. The latter is illustrated by the observations of the “Dutch Hunger Winter", which was a famine that took place in the German-occupied part of the Netherlands, especially in the densely populated western provinces north of the great rivers, during the winter of 1944–45, near the end of World War II. It was reported that children born to mothers with significantly reduced food intake (400- 800 kcal/day) during the last third of pregnancy were of low birth weight and developed glucose intolerance and insulin resistance at adulthood (17). This relationship between maternal malnutrition and glucose intolerance in the offspring in later life could be explained by a permanent effect of the challenge on fetal pancreatic beta cell endowment, with long term consequences for changes in insulin sensitivity in target organs later in life (17).

- **Placental function.** It is well established that placental insufficiency causes a slowing of fetal growth. The lack or inappropriate development of the placental vascular bed will

increase placental vascular resistance which will reduce delivery of oxygen and nutrients to the growing conceptus. In addition, infarcts and oxidative stress will further impact on functional placental tissue, aggravating the reduction in the appropriate supply of nutrients to the fetus (18, 19). It is also accepted that there may be placental adaptation to adverse or stressful conditions during pregnancy that may cushion the impact of adversity on fetal growth and development. For instance, anaemia during pregnancy (20), maternal exercise (21) and high altitude pregnancy (22) have all been reported to trigger an increase in placental mass with partial protection on fetal growth. Studies in animal models have also reported that pre-conceptual overnutrition rather than an increase in food intake during early pregnancy, leads to an increase in placental growth with benefits to fetal growth (23). Therefore, it is clear that impaired placental function will adversely affect fetal growth and that the placenta can adapt beneficially to ameliorate the effects of adverse pregnancy on fetal growth, depending on the timing, duration and magnitude of the suboptimal condition.

•**Fetal capacity to utilize energy substrate:** It must also be accepted that despite appropriate maternal nutrition and adequate placental development and function, there are circumstances in which fetal growth may still be compromised. This is the case in chromosomal abnormalities, uterine or fetal malformations or during intrauterine infection (24).

Chronic fetal hypoxia, fetal growth and cardiovascular risk in later life

In addition to alterations in maternal nutrition and placental function, significant data derived from human epidemiological studies and from experimental animal models of pregnancy resulting in IUGR now clarified that a component of the slowed fetal growth and the setting of an increased cardiovascular risk in later life may be due specifically to long-term reductions in fetal oxygenation or chronic fetal hypoxia. Nevertheless, the contribution of chronic fetal hypoxia in promoting IUGR and programmed cardiovascular risk has been difficult to isolate for a number of reasons. For instance, it is established that high-altitude pregnancy leads to IUGR (25-28). However, most high-altitude populations are impoverished with significant maternal malnutrition (26, 29, 30). Therefore, the contribution of chronic fetal hypoxia versus chronic fetal undernutrition in slowing fetal growth and in setting future cardiovascular risk under these conditions is uncertain. The same applies to sea level pregnancy complicated by preeclampsia, placental insufficiency, gestational diabetes and even maternal obesity. All these conditions are associated with an increase in placental vascular resistance and alterations in placental development and

function (31-35), which will decrease oxygen as well as nutrient delivery to the growing fetus. Similarly, several studies in mammalian experimental animal models have shown that maternal chronic hypoxia during pregnancy can lead to IUGR and programme increased cardiovascular risk in the offspring (36-40). However, because experimental induction of chronic hypoxia in rodents can reduce maternal food intake and/or alter the quality of the maternal milk (36, 41-43), the contribution of chronic fetal hypoxia versus chronic fetal and/or neonatal under-nutrition under these conditions, again, remains uncertain.

The chicken embryo model

By using the chicken embryo as an animal model, science has been able to circumvent a number of these problems highlighted above because, in contrast to mammals, with the exception of monotremes, in the chicken the effects of changes in oxygenation on the embryo can be isolated and determined directly, independent of changes in the maternal physiology, the secretion of placental hormones or imposed changes in postnatal maternal lactation (44-47).

The development of the chicken embryo during the 21 days of incubation is supported by three gas exchange organ systems: the yolk sac, the chorioallantoic membrane (CAM) and the lungs (48, 49). The vascular portion of the yolk sac is the principal gas exchange organ during early development (50). In addition to its role as an early gas exchange organ, the yolk sac provides the chicken embryo with essential nutrients for its growth. Protein, fat, carbohydrates and minerals are stored in the yolk of the egg for future utilization by the embryo (51, 52). At approximately embryonic day 8, the allantoic sac develops and fuses with the chorion to create the CAM (49). This highly vascular structure, in conjunction with the porosity of the eggshell, permits diffusion of oxygen and carbon dioxide between the environment and the blood, thereby replacing the yolk sac as the primary source of oxygen uptake (48, 49, 53). Finally, transition to *ex ovo* life in chickens is initiated around incubation day 19-20, when the embryo 'pips' internally through to the air cell of the egg with its beak and begins air breathing for the first time (54). From this point on, the embryo relies on both the CAM and the lungs for gas exchange. Therefore, during the second half of incubation, chicken embryos are endowed with a gas-exchange organ, the CAM, and a choriovitelline nutritional organ, the yolk sac (48, 49, 53, 54). Hypoxia is easily induced by incubating the egg in a low O_2 environment. Exposure to hypoxia during mid-incubation increased CAM vascularity, resulting in an increased functional surface area for gas exchange, which would be an appropriate acclimation to hypoxia (55-57).

AIMS AND OUTLINE OF THE THESIS

The present thesis is a collection of studies designed to isolate the effects of chronic fetal hypoxia on fetal growth, fetal cardiovascular and endocrine development and programming of future cardiovascular dysfunction in the adult offspring, using the chicken as an animal model. The combination of high altitude exposure with the use of the chicken embryo model is ideal as it permits investigation of the direct effects of high altitude hypoxia on growth and on cardiovascular development completely independent of alterations in placental function, independent of changes in the maternal physiology and independent of any effects of socioeconomic factors.

In **chapter II** (The role of oxygen in prenatal growth: studies in the chick embryo. *J Physiol*. 2007; 585:911-7), **chapter III** (Cardiac and vascular disease prior to hatching in chick embryos incubated at high altitude. *J Dev Orig Health Dis*. 2010; 1:60-6), and **chapter IV** (Adrenocortical suppression in highland chick embryos is restored during incubation at sea level. *High Alt Med Biol.* 2011; 12:79-87), we investigated the effects of high altitude hypoxia on chicken embryo growth and *in ovo* cardiovascular and endocrine development. For this purpose, we adopted an experimental design based on a three-prong approach using: (1) incubation at high altitude of fertilized eggs laid by sea-level hens; (2) incubation at sea level of fertilized eggs laid by high-altitude hens; and (3) incubation at high altitude of sea-level eggs with oxygen supplementation to equate sea level oxygen partial pressure.

In **chapter V** (High altitude hypoxia and blood pressure dysregulation in adult chickens. J Dev Orig Health Dis. 2013; 4:69-76) and **chapter VI** (High-altitude hypoxia and echocardiographic indices of pulmonary hypertension in male and female chickens at adulthood. *Circ J.* 2014;78:1459-64), we isolated the long-term consequences of chronic hypoxic incubation of chick embryos on the systemic and pulmonary circulations of the adult bird. This was achieved using noninvasive echocardiography as well as testing basal and stimulated cardiovascular function in the chronically instrumented adult bird. Additional specific points of interest were to determine whether there were any sex differences and whether any adverse effects of chronic hypoxia during the embryonic period could be ameliorated by generational exposure to hypobaric hypoxia in highland adapted chickens.

Finally, in **chapter VII** (The highs and lows of programmed cardiovascular disease by developmental hypoxia: Studies in the chicken embryo. *J Physiol 2017*), we discuss and

put into perspective the findings of this thesis. We summarise studies that have exploited the chicken embryo model to isolate the direct effects of chronic hypoxia on prenatal growth, cardiovascular and endocrine development and in triggering an increased risk of cardiovascular dysfunction and pathology at adulthood.

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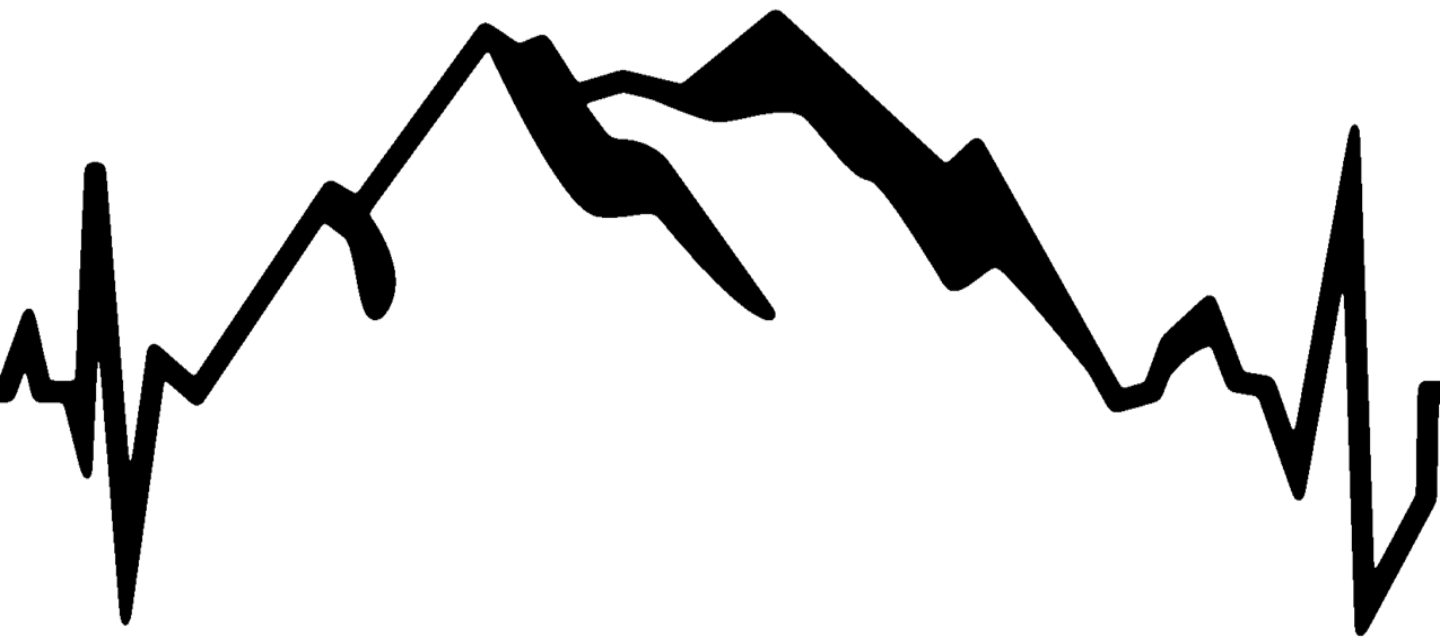
Chapter II

The role of oxygen in prenatal growth: studies in the chick embryo.



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The role of oxygen in prenatal growth: studies in the chick embryo

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The compelling evidence linking small size at birth with later cardiovascular disease has renewed and amplified scientific and clinical interests into the determinants of fetal growth. It is accepted that genes and nutrition control fetal growth; however, prior to this study, it had been impossible to isolate the effect of increases and decreases in fetal oxygenation on the regulation of prenatal growth. We investigated the role of oxygen in the control of fetal growth in the chicken because in contrast to mammals, the effects on the fetus of changes in oxygenation could be isolated, by assessing them directly without alteration to the maternal or placental physiology or maternal nutrition during development. The data show that incubation at high altitude of fertilized eggs laid by sea level hens markedly restricted fetal growth. Incubation at high altitude of fertilized eggs laid by high altitude hens also restricted fetal growth, but to a lesser extent compared to eggs laid by sea level hens. By contrast, incubation at sea level of fertilized eggs laid by high altitude hens not only restored, but enhanced, fetal growth relative to sea level controls. Incubation at high altitude of sea level eggs with oxygen supplementation completely prevented the high altitude-induced fetal growth restriction. Thus, fetal oxygenation, independent of maternal nutrition during development, has a predominant role in the control of fetal growth. Further, prolonged high altitude residence confers protection against the deleterious effects of hypoxia on fetal growth.

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It is generally accepted that hypoxia during pregnancy decreases birth weight; however, it has been difficult to demonstrate or isolate this effect because all human conditions or experimental animal models that induce fetal hypoxia are also accompanied by changes in nutrient delivery. For instance, elegant studies in mammalian animals have shown that maternal chronic hypoxia during pregnancy can lead to slow, disproportionate fetal growth (De Grauw *et al.* 1986; Jacobs *et al.* 1988). However, whether these effects are due to fetal under-oxygenation or fetal under-nutrition is uncertain as chronic experimental hypoxia also reduces maternal food intake (De Grauw *et al.* 1986). In humans, fetal hypoxia occurs most commonly under physiological conditions during the hypobaric hypoxia of pregnancy at high altitude (Moore, 1990), or under pathological conditions during pregnancy complicated by placental insufficiency (Baschat, 2004). Several investigators have reported reduced birth weight and asymmetric growth restriction in babies with increasing altitude (e.g. Lichty *et al.* 1957; McClung, 1969; Haas *et al.* 1980; Moore *et al.* 1998; Giussani *et al.* 2001; Zamudio *et al.* 2007). However, because most high altitude populations are also impoverished, and because placental insufficiency decreases nutrient as well as oxygen transfer to the baby, the extent to which the reduction in fetal growth under these conditions is governed by fetal under-nutrition or under-oxygenation, again, remains uncertain. By using the chick embryo as an animal model, this study could isolate the direct effects on the fetus of developmental hypoxia due to high altitude for the first time, completely independent of changes in maternal nutrition and of the physiology of the mother or the placenta. The study tested the hypothesis that fetal oxygenation, independent of maternal nutrition during development, has a predominant role in the control of fetal growth by investigating the effects of incubation at high altitude of fertilized eggs laid by sea level hens. In addition, the experiment could be done the

This study is dedicated to the memory of Professor Dr Lieselotte Bauer de Barragán, previous Director of the Hospital San Gabriel, who dedicated most of her life to the welfare of the mother and newborn at high altitude. The study received The Pfizer Award at the 52nd Annual meeting of the Society for Gynecologic Investigation.

other way around, by developing at sea level fertilized eggs laid by high altitude hens, to assess whether the hypoxia-induced effects on fetal growth could be reverted, something that is difficult if not impossible to assess in human populations. Finally, to discount the possibility that high altitude-induced fetal growth restriction is due to hypobaria rather than hypoxia, the effects of incubation at high altitude with oxygen supplementation of fertilized eggs laid by sea level hens was also investigated.

Methods

The study was done in Bolivia, in the high altitude city of La Paz (3600 m, 494 mmHg, approximate ambient dry P_{O_2} 100 mmHg) and the sea level city of Santa Cruz (420 m, 760 mmHg, approximate ambient dry P_{O_2} 160 mmHg). Fertilized eggs were obtained from Black Leghorn chickens that had been reared at the sea level city of Santa Cruz or at high altitude city of La Paz for at least six generations. Fertilized eggs from sea level hens, laid at sea level, were randomly divided and incubated either at sea level (SLSL, $n = 45$) or high altitude (SLHA, $n = 60$). Eggs from high altitude hens, laid at high altitude, were randomly divided and incubated either at high altitude (HAHA, $n = 70$) or sea level (HASL, $n = 50$). SLHA embryos were also incubated with oxygen supplementation (SLHA + O_2, $n = 42$) at rates to maintain sea level oxygen partial pressures according to Dalton's law (see West, 2004).

Egg storage is commonly practiced in the artificial incubation of domestic birds. If the storage temperature for freshly laid chicken eggs is kept below the physiological zero (25–27°C), dormancy of the embryo can be maintained and fertile eggs can be stored for 1–3 weeks (Butler, 1991). In this study, fertilized eggs from any one group were accumulated, maintained and transported over 2–3 days at 14°C to arrest and synchronize development, prior to incubation. Eggs were weighed prior to incubation. All incubations (Polyhatch, Brinsea Products Ltd, Sandford, UK) were carried under conditions to optimize development, with controlled temperature (38°C), humidity (60%) and appropriate egg rotation. On day 20, out of the 21 day incubation period, the egg was weighed, the air cell was exposed and chorio-allantoic venous blood was drawn into a 1 ml syringe for analysis of P_{O_2} (ABL 500; Radiometer, Copenhagen, Denmark) and haematocrit, whenever possible in duplicate (Hawksley centrifuge). Following killing by spinal transection, the fetus was removed from the eggshell and weighed. Head diameter and body length (crown rump length) were measured with a digital micrometer. The fetal brain was dissected and weighed.

Growth efficiency was calculated as the ratio of the 'measured fetal weight at day 20' and the product of 'the egg weight at day 20' minus 'the weight of the egg once the embryo had been removed at day 20'. An example, for a SLSL and a SLHA embryo is given in Scheme 1.

To calculate the partitioning of the resource, both the 'measured fetal weight at day 20' and 'egg weight at day 20 – the measured fetal weight at day 20' were expressed as a percentage of the egg weight at day 20 and plotted in histogram format. An example for the same SLSL and SLHA embryos as above is given in Scheme 2.

All procedures were approved by the local ethics committee (Consejo Tecnico) of the Bolivian Institute for High Altitude Biology (IBBA), Universidad Mayor de San Andrés, La Paz, Bolvia. All variables were analysed for normality of distribution and then expressed as means ± S.E.M. Comparisons between groups were assessed statistically using One-way ANOVA with an appropriate *post hoc* test (SigmaStat; Systat Software Inc., San Jose, CA, USA). Differences in mortality were compared using a contingency table and assessed by the chi-square (χ^2) test. For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

The data show that incubations at high altitude increased embryonic mortality relative to incubations at sea level, but this effect was reduced in HAHA embryos. The altitude-induced increase in mortality in the SLHA group was prevented by oxygen supplementation (mortality: SLSL = 28.8%, SLHA = 66.6%; HAHA = 52.8%; HASL = 28.1%; SLHA + O_2 = 30.2%; $P < 0.05$, χ^2 test). Surviving chick embryos incubated at high altitude were hypoxic relative to chick embryos incubated at sea level, with the exception of those with oxygen supplementation,

SLSL

$$\frac{\text{measured fetal body weight at day 20}}{\text{(weight of egg at day 20 – weight of egg without embryo at day 20)}} \quad \frac{29.6}{(60.1 - 24.9)} = 0.84 \text{ or } 84\%$$

SLHA

$$\frac{\text{measured fetal body weight at day 20}}{\text{(weight of egg at day 20 – weight of egg without embryo at day 20)}} \quad \frac{14.9}{(60.5 - 42.3)} = 0.82 \text{ or } 82\%$$

Scheme 1.

SLSL

$$\frac{\text{measured fetal body weight at day 20}}{\text{(egg weight at day 20)}} \times 100 \qquad \frac{29.6}{60.1} \times 100 = 49\%$$

$$\frac{\text{(egg weight at day 20 - measured fetal body weight at day 20)}}{\text{(egg weight at day 20)}} \times 100 \qquad \frac{(60.1 - 29.6)}{(60.1)} \times 100 = 51\%$$

SLHA

$$\frac{\text{measured fetal body weight at day 20}}{\text{(egg weight at day 20)}} \times 100 \qquad \frac{14.9}{60.5} \times 100 = 25\%$$

$$\frac{\text{(egg weight at day 20 - measured fetal body weight at day 20)}}{\text{(egg weight at day 20)}} \times 100 \qquad \frac{(60.5 - 14.9)}{(60.5)} \times 100 = 75\%$$

Scheme 2.

in which sea level oxygen partial pressure was maintained (Fig. 1*A*). Relative to SLSL and SLHA + O_2 chick embryos, haematocrit was elevated in the SLHA, HAHA and HASL groups ($P < 0.05$), despite the latter group being incubated at sea level (Fig. 1*B*).

Prior to incubation, fertilized eggs from high altitude hens were lighter than fertilized eggs from sea level hens (Fig. 2*A*). At the end of the incubation period, in terms of absolute weight, chick embryos incubated at high altitude showed pronounced growth restriction, relative to chick embryos incubated at sea level ($P < 0.05$), with the exception of the SLHA + O_2 group, in which the growth restriction was completely prevented (Fig. 2*B*). When embryonic weight was expressed as a percentage of the egg mass prior to incubation, to account for the initial differences in weights between high altitude and sea level eggs, fetal growth was restricted by 45.2% in the SLHA group and by 22.2% in the HAHA group relative to SLSL controls (Fig. 2*C*). Incubation at sea level of eggs laid by hens at high altitude not only restored growth, but these embryos grew heavier than the SLSL group. The embryonic growth restriction of incubations at high altitude was asymmetric as head growth was spared at the expense of body length (Fig. 3*A* and *B*). Hence, the relative brain weight, expressed as a percentage of the fetal body mass, was elevated in embryo groups, which had suffered body growth restriction during incubations at high altitude (Fig. 3*C*). The ratio of the head diameter to body weight was elevated in chick embryos incubated at high altitude. However, the increase in this ratio was markedly attenuated in the HAHA chicks relative to the SLHA group (Fig. 3*D*). Oxygen supplementation completely prevented the high altitude-induced asymmetry in fetal growth (Figs 2 and 3). Calculation of growth efficiency and the partitioning of the resource showed that reductions in oxygen availability during incubations at high altitude did not affect how well the resource is converted into fetal body mass (growth efficiency) (Fig. 4*A*). Rather, growth restriction at high

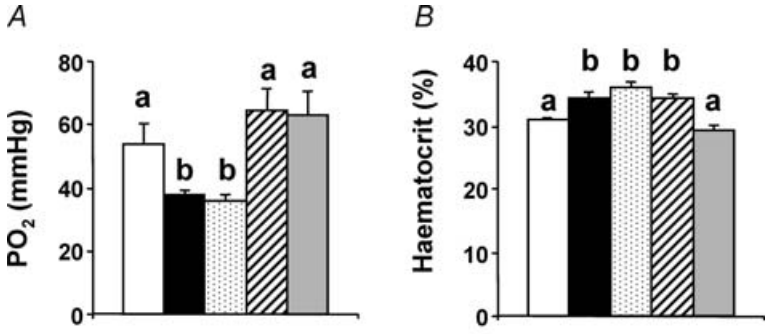

Figure 1. Partial pressure of oxygen and haematocrit
Values are means + S.E.M. for chorioallantoic venous blood taken from sea level chick embryos incubated either at sea level (SLSL, open bar, $n = 9$) or high altitude (SLHA, filled bar, $n = 12$), high altitude embryos incubated at high altitude (HAHA, stippled bar, $n = 10$) or sea level (HASL, hatched bar, $n = 7$), and from sea level chick embryos incubated at high altitude with oxygen supplementation (SLHA + O_2, grey bar, $n = 12$). Different letters are significantly different by one-way ANOVA with Student–Newman–Keuls test ($P < 0.05$).

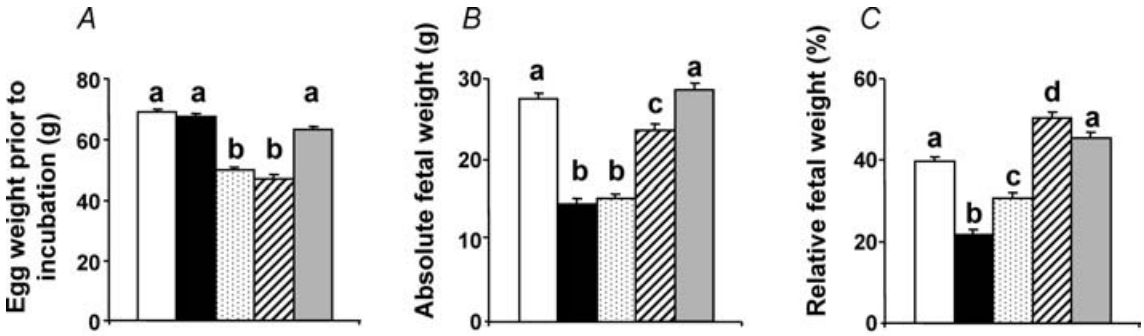

Figure 2. Fetal growth following high and lowland incubations
Values are means + S.E.M. for the egg weight prior to incubation (*A*), the absolute fetal weight at the end of the incubation period (*B*) and the fetal weight at the end of the incubation period expressed as a percentage of the initial egg mass (*C*). SLSL (open bar, $n = 31$), SLHA (filled bar, $n = 19$), HAHA (stippled bar, $n = 33$), HASL (hatched bar, $n = 30$), and SLHA + O_2 (grey bar, $n = 26$). Different letters are significantly different by one-way ANOVA with Student–Newman–Keuls or Dunn's tests, as appropriate ($P < 0.05$).

altitude was due to a reduction in fetal resource uptake (Fig. 4*B*).

Discussion

The data show that development of chick embryos at high altitude induced fetal hypoxaemia and led to an increase in fetal haematocrit. Whilst several studies have reported an increase in packed red cell mass in the umbilical blood of human infants following gestation at high altitude (Ballew & Haas, 1986; Buys de Jorge *et al.* 1988; Leibson *et al.* 1989; Niermeyer *et al.* 1995; Ramirez-Cardich *et al.* 2004), this is the first direct demonstration that the partial pressure of oxygen is actually reduced in the fetal blood during development at high altitude. The data also show that incubation at high altitude of fertilized eggs laid by sea level hens restricted fetal growth, in similar fashion to restriction of fetal growth in the chick embryo by isobaric hypoxia (Miller *et al.* 2002). Incubation at high altitude of fertilized eggs laid by high altitude hens also restricted fetal growth, but to a lesser extent compared to eggs laid by sea level hens. By contrast, incubation at sea level of fertilized eggs laid by high altitude hens not only restored, but enhanced, fetal growth relative to sea level

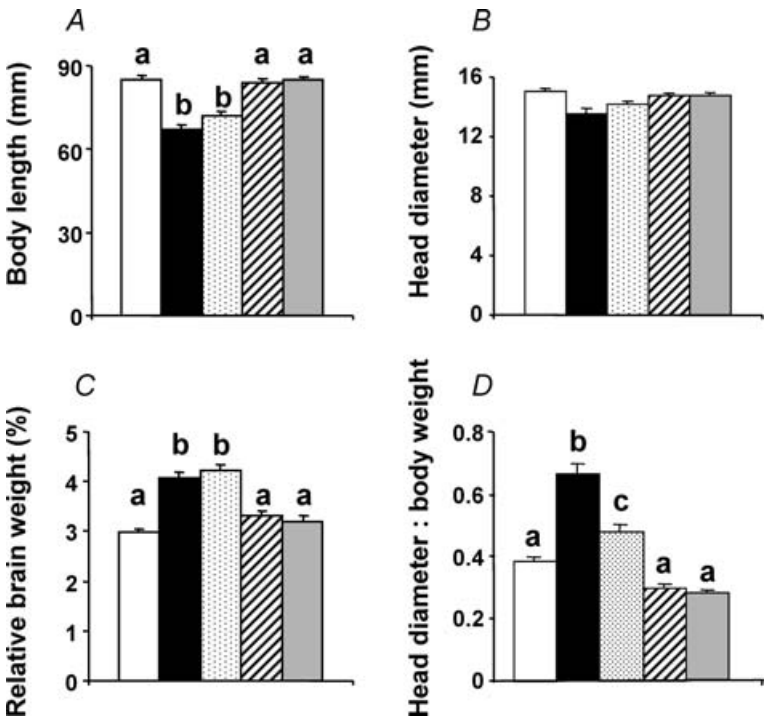

Figure 3. Symmetry of fetal growth following high and lowland incubations
Values are means + S.E.M. for the body length (*A*), the head diameter (*B*), the brain weight expressed as a percentage of the fetal body weight (*C*) and the ratio of the head diameter to body weight (*D*). SLSL (open bar, $n = 31$), SLHA (filled bar, $n = 19$), HAHA (stippled bar, $n = 33$), HASL (hatched bar, $n = 30$), and SLHA + O_2 (grey bar, $n = 26$). Different letters are significantly different by one-way ANOVA with Dunn's test ($P < 0.05$).

controls. Incubation at high altitude of sea level eggs with oxygen supplementation completely prevented the high altitude-induced fetal growth restriction.

The data obtained from the chick embryo in the current study resembles that presented in an epidemiological study of human populations in Bolivia (Giussani *et al.* 2001). In that study, birth weight records were obtained from term pregnancies in La Paz and Santa Cruz, especially from obstetric hospitals attended by wealthy or impoverished mothers. The data revealed pronounced asymmetric growth restriction in babies born at high altitude relative to sea level. When lowland babies born from mothers with high or low economic status were compared, birth weight was significantly reduced in low *versus* high income groups, but this difference was not as pronounced as the effect on birth weight of high altitude alone. Additional data also showed that highland babies from poor families did not have the greatest reduction in birth weight, as one would have expected. Rather, counter-intuitively, these babies were actually heavier than highland babies born from families with a high socio-economic status. The apparent conundrum was easily explained by assessing the ancestry of the families. In that study, the low socio-economic group of La Paz contained a high percentage (92%) of women from Amerindian origin with Aymara indian paternal and maternal surnames. In contrast, the high socio-economic group of La Paz contained a high European admixture. The findings of that study support the observations of Moore (1990) and Haas *et al.* (1980), who suggested that fetal growth restriction at altitude is correlated with the duration of high altitude residence: the longest resident population experiencing the least decline in birth weight with altitude, while the shortest historical residence groups, the greatest decline. Asymmetric growth restriction has been attributed, in part, to the sustained redistribution of the fetal cardiac output, to maintain oxygenation of essential circulations, such as those perfusing the brain, at the expense of the peripheral vascular beds (Akalin-Sel & Campbell, 1992; Giussani *et al.* 1993). Interestingly, a report by Martyn *et al.* (1996) suggested that, in human babies, an increase in the ratio of the head circumference to birth weight is associated with increased stroke in adulthood. Data in the present manuscript show that incubation of chick embryos at high altitude also produced an elevated ratio of the head diameter to body weight, and that this effect was also reduced in embryos from high altitude hens. Combined, our experiments in the chick embryo and observations in human babies in Bolivia therefore suggest that oxygen deprivation, independent of maternal nutrition during development, has a predominant role in the control of fetal growth. Further, prolonged high altitude residence confers protection against the effects of hypoxia on fetal mortality, fetal growth and the developing cardiovascular system. The overwhelming similarities between our findings in human babies and chick embryos make a serious case for the applicability of experiments with chicken eggs to model human conditions.

The physiology underlying the protection conferred by prolonged high altitude residence against the deleterious effects of hypoxia on fetal development remains unknown. In humans, a comparison of blood flow in Tibetan and Han Chinese pregnant women living at high altitude showed a greater redistribution of blood flow from the common iliac artery to the uterine artery in Tibetan than Han women. Thus, delivery of uteroplacental oxygen was increased and heavier babies were noted, despite lower arterial oxygen content in Tibetan women (Moore *et al.* 2001). A recent study by Zamudio *et al.* (2007) tested the hypothesis that greater maternal uteroplacental oxygen delivery would also explain increased human fetal growth at altitude in Andeans *versus* Europeans in Bolivia. They concluded that genetically mediated differences in maternal oxygen delivery did not explain the Andean advantage. Rather, the mechanism underlying this protection likely resides within the feto-placental unit. The present findings in the chick embryo confirm this expectation since protection conferred by prolonged high altitude residence could also express itself directly at the fetal, rather than the maternal or uteroplacental, levels. One possibility may be adaptations that enhance fetal arterial oxygen saturation.

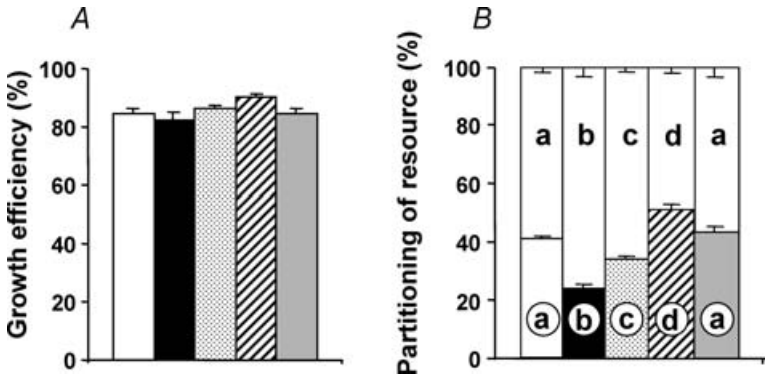

Figure 4. Use of resource for fetal growth during high and lowland incubations
Values are mean + S.E.M. for the growth efficiency (*A*) and the partitioning of the resource (*B*), both expressed as a percentage. SLSL (open bar, $n = 31$), SLHA (filled bar, $n = 19$), HAHA (stippled bar, $n = 33$), HASL (hatched bar, $n = 30$), and SLHA + O_2 (grey bar, $n = 26$). Values for partitioning of resource (mean + S.E.M.) are expressed as a histogram on top of the corresponding group. Different letters are significantly different by one-way ANOVA + Student–Newman–Keuls or Dunn's tests, as appropriate ($P < 0.05$). For calculations with examples, see Methods.

Whilst this has not been shown in the unborn child, an interesting study by (Beall, 2000) identified an autosomal major gene that confers higher resting oxygen saturation in sedentary Tibetan natives. In addition, Velarde *et al.* (1991) have also reported that Peruvian high altitude chickens have evolved a high haemoglobin-oxygen affinity, a finding suggested to be a genetic response to high altitude hypoxia. In our study, the highland chickens had lived at high altitude only for at least six generations. Whether this is sufficient time for physiological adaptations to high altitude to become genetically determined is questionable. However, the possibility exists that high altitude hypoxia may confer protection via epigenetic mechanisms, alterations in gene regulation that occur without a change in DNA sequence. Epigenetic marks such as CpG methylation and histone tail modifications have been shown to be affected by environmental conditions (Jirtle & Skinner, 2007), and because epigenetic marks are heritable (see Godfrey *et al.* 2007), gene expression and physiological responses can be altered in a comparatively shorter time frame, from one generation to the other, and for years to come.

To give further insight into the physiological mechanisms by which high altitude hypoxia depresses fetal growth, and the protection against this effect in highland chickens, we asked two further questions: do alterations in oxygen availability during development affect how well the resource is used by the fetus? Or does oxygen availability affect how well the resource is converted into fetal body mass? Calculation of growth efficiency and the partitioning of the resource in all five groups of chick embryos revealed that high altitude hypoxia did not affect growth efficiency, but it had a marked effect on fetal resource uptake or resource utilization for tissue accretion. For instance, incubation at high altitude of embryos from sea level hens had the greatest depressive effect on fetal growth because these embryos had the least uptake of resource. By contrast, despite development under similar conditions of fetal hypoxia, fetal resource uptake was greater in HAHA compared to SLHA groups. The mechanism by which development under hypoxic conditions reduces resource utilization by the fetus remains unresolved, but it is likely to be due to the depressive effects of oxygen deprivation on ATP synthesis and/or due to the effects of hypoxia on endocrine factors important in the regulation of fetal growth, such as insulin, thyroxine, cortisol and insulin-like growth factors (IGFs; see Fowden, 1995). Elucidation of changes in fetal nutrient utilization imposed by high altitude hypoxic pregnancy in humans and other mammals would require calculation of maternal glucose delivery and umbilical glucose uptake. In experimental mammalian models, such as sheep, this is highly technical and requires the chronic implantation of catheters into the umbilical vein, fetal dorsal aorta and caudal vena cava, and into the uterine ovarian vein and the maternal aorta with simultaneous blood sampling during experimentation (Comline & Silver, 1972). The simplicity with which answers to similar questions can be obtained in the chick embryo again highlights the excellence and applicability of this animal model.

A final component of the data presented in this study shows that when fertilized eggs laid by high altitude hens were incubated at sea level, the resulting embryos not only recovered their growth, but they grew heavier than sea level controls. The haematocrit data reveal that this group of embryos retained an increased oxygen carrying capacity despite incubation at sea level. This suggests that embryos from high altitude hens incubated at sea level had a greater oxygen content than sea level controls, further supporting a role for oxygen in the control of fetal growth. The mechanism by which elevated haematocrit levels are maintained in the absence of a hypoxic stimulus is unknown, but the data may reflect an adaptive response, transmitted by the mother to the oocyte prior to egg laying, predictive of fetal development in a hypoxic environment. Another example of a maternal predictive adaptive response (Gluckman & Hanson, 2004) is that of the meadow vole, in which the photoperiodic history of the dam prior to conception, rather than the perinatal thermal environment, can better determine the offspring's coat thickness at birth (Lee & Zucker, 1988). Alternatively, the maintained elevated haematocrit in HASL embryos may highlight that genetic or epigenetic control of factors determining oxygen carrying capacity is regulated very early on in the developmental process of the oocyte by the available oxygen concentration at that time.

In conclusion, we have isolated the effects of alterations in fetal oxygenation on fetal growth. Oxygen, independent of maternal nutritional factors, has a predominant role in the control of fetal growth and prolonged high altitude residence confers protection against the effects of hypoxia on fetal development. This discovery calls for the attention of this aspect of growth regulation not only in women undergoing pregnancy at high altitude, but also in sea level human pregnancies complicated with reduced oxygen delivery to the fetus, such as during placental insufficiency and pre-eclampsia (Many *et al.* 1996; Baschat, 2004).

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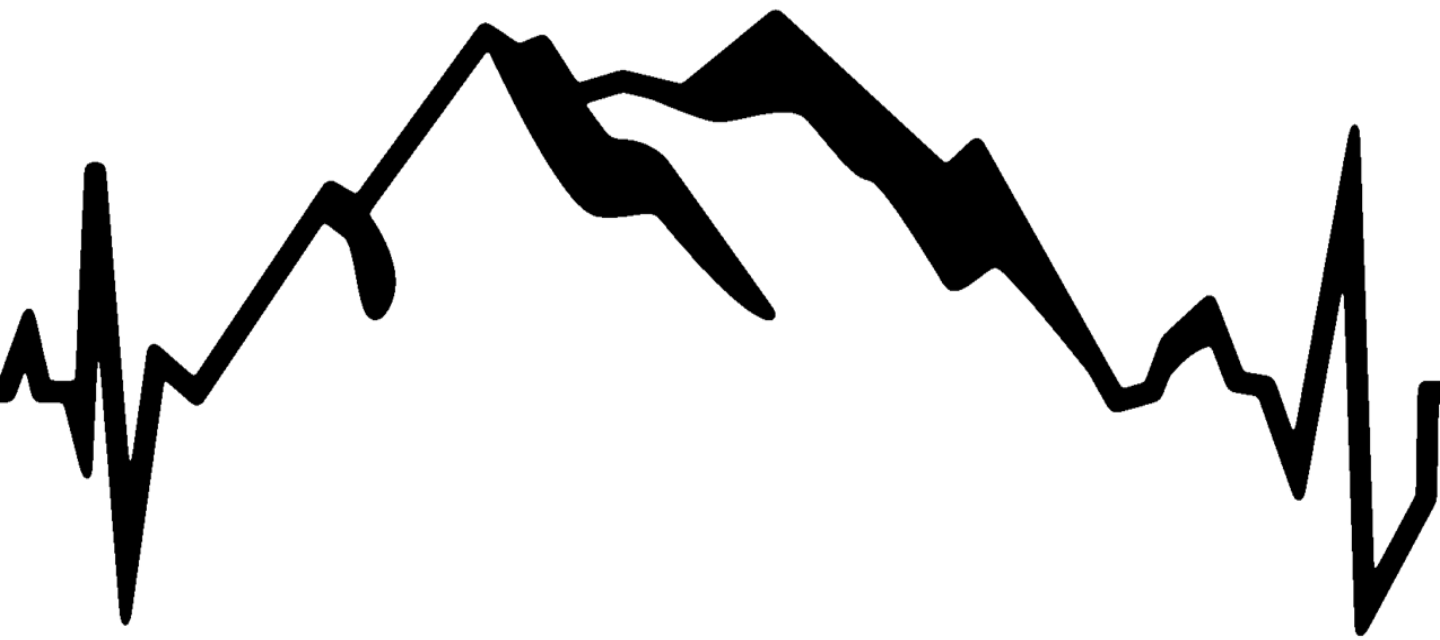
Chapter III

Cardiac and vascular disease prior to hatching in chick embryos incubated at high altitude.



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ORIGINAL ARTICLE

Cardiac and vascular disease prior to hatching in chick embryos incubated at high altitude

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The partial contributions of reductions in fetal nutrition and oxygenation to slow fetal growth and a developmental origin of cardiovascular disease remain unclear. By combining high altitude with the chick embryo model, we have previously isolated the direct effects of high-altitude hypoxia on growth. This study isolated the direct effects of high-altitude hypoxia on cardiovascular development. Fertilized eggs from sea-level or high-altitude hens were incubated at sea level or high altitude. Fertilized eggs from sea-level hens were also incubated at high altitude with oxygen supplementation. High altitude promoted embryonic growth restriction, cardiomegaly and aortic wall thickening, effects which could be prevented by incubating eggs from high-altitude hens at sea level or by incubating eggs from sea-level hens at high altitude with oxygen supplementation. Embryos from high-altitude hens showed reduced effects of altitude incubation on growth restriction but not on cardiovascular remodeling. The data show that: (1) high-altitude hypoxia promotes embryonic cardiac and vascular disease already evident prior to hatching and that this is associated with growth restriction; (2) the effects can be prevented by increased oxygenation; and (3) the effects are different in embryos from sea-level or high-altitude hens.

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Key words: cardiovascular disease, chick embryo, high altitude, hypoxia.

Introduction

Despite healthy skepticism,[1,2] evidence derived from human epidemiologic studies linking small size at birth with greater cardiovascular risk has gathered increasing support in recent years.[3,4] This risk of cardiovascular disease not only results from intrauterine growth retardation in complicated pregnancy, but the association also extends across the normal range of birth weight in healthy pregnancy.[1,3] A component of fetal growth is determined by the quality of the intrauterine environment. In turn, the quality of the intrauterine environment is largely determined by the available nutrient and oxygen supply to the growing young. Consequently, there have been many reports investigating the association between reduced fetal growth and increased risk of cardiovascular disease in animal models in which development has been complicated by reductions in fetal nutrition and/or in fetal oxygenation.[5–10]

Under physiologic conditions, in humans, fetal hypoxia occurs most commonly during the hypobaric hypoxia of pregnancy at high altitude.[11] Although several investigators have reported reduced birth weight in human babies with increasing altitude,[12–17] there have been no reports on the association between fetal growth restriction and alterations in cardiovascular development already evident prior to birth at high altitude in any species. Most high-altitude human populations are impoverished, therefore the extent to which any effects on fetal development during pregnancy at high altitude is governed by fetal under-nutrition or fetal under-oxygenation, remains uncertain. By using the chick embryo as an animal model, an earlier study in our laboratory isolated the direct effects of developmental hypoxia owing to high altitude on embryonic growth, independent of changes in maternal nutrition and of the physiology of the mother or the placenta.[18] The data in that study showed that high-altitude incubation of fertilized eggs laid by sea-level hens markedly restricted growth of the chick embryo. Incubation at high altitude of fertilized eggs laid by high-altitude hens also restricted embryonic growth, but to a lesser extent compared to eggs laid by sea-level hens. By contrast, incubation at sea level of fertilized eggs laid by high-altitude hens not only restored, but also enhanced growth relative to sea-level controls. Incubation at high altitude of sea-level eggs with oxygen supplementation completely prevented the high-altitude-induced growth restriction. Thus, the oxygenation of the chick embryo, independent of maternal nutrition, has a predominant role in the control of its growth during development at high altitude. Further, prolonged high-altitude residence confers protection against the deleterious effects of hypoxia on growth.

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The present study tested the hypothesis that development at high altitude is related to a prenatal origin of cardiovascular disease and that hypoxia is the mechanism underlying the relationship. The hypothesis was tested three-fold: (1) by investigating the effects on the cardiovascular development of fertilized eggs laid by sea-level hens when incubated at high altitude; (2) by investigating whether alterations in the embryonic cardiovascular system induced by development at high altitude could be prevented by incubation at sea level of fertilized eggs laid by high-altitude hens; and (3) by investigating whether alterations in the embryonic cardiovascular system induced by development at high altitude could be prevented by incubation at high altitude of sea-level eggs with oxygen supplementation. We were also interested in whether prolonged high-altitude residence conferred any protection against any deleterious effects of hypoxia on cardiovascular development.

Methods

The study was done in Bolivia, in the high-altitude city of La Paz (3600 m, 494 mmHg, approximate ambient dry PO_2 100 mmHg) and the sea-level city of Santa Cruz (420 m, 760 mmHg, approximate ambient dry PO_2 160 mmHg). The incubation procedures have been published earlier in detail.[18] In brief, fertilized eggs were obtained from Black Leghorn chickens that had been reared at the sea-level city of Santa Cruz or at the high-altitude city of La Paz for at least six generations. Fertilized eggs from sea-level hens, laid at sea level, were randomly divided and incubated either at sea level (SLSL, $n = 31$) or high altitude (SLHA, $n = 19$). Eggs from high-altitude hens, laid at high altitude, were randomly divided and incubated either at high altitude (HAHA, $n = 33$) or sea level (HASL, $n = 25$). SLHA embryos were also incubated with oxygen supplementation (SLHA + O_2, $n = 21$) at rates to maintain sea-level oxygen partial pressures according to Dalton's Law.[19]

All incubations (Polyhatch; Brinsea Products Ltd, UK) were carried under conditions to optimize development, with controlled temperature (38°C), humidity (60%) and appropriate egg rotation. On day 20, out of the 21-day incubation period, the egg was weighed, the air cell was exposed and chorioallantoic venous blood was drawn into a 1 ml syringe for analysis of PO_2 (ABL 500; Radiometer, Copenhagen, Denmark), whenever possible in duplicate. Following euthanasia by spinal transection, the embryo was removed from the eggshell and weighed. Head diameter and body length (crown–rump length) were measured with a digital micrometer.

The embryonic heart was dissected and weighed. In a subset of animals, following maximal dilatation using ethylenediaminetetraacetic acid (EDTA; 50 mg/kg), a 5 mm segment of the thoracic aorta was dissected at the level of the apex of the heart, and the heart and aortic segment were fixed in 4% phosphate buffered paraformaldehyde for 24 h and then stored in physiologic buffer. Hearts and vessels were then embedded in paraffin. To account for possible shrinkage because of paraffin processing, the diameter of erythrocytes in heart sections was measured and compared to that obtained by measuring fresh erythrocytes from chick embryos at the same stage of incubation.[20] All measurements were corrected using this factor. Mid-cardiac 4-μm coronal sections and 7-μm transverse aortic sections were stained with van Gieson's solution. Slices were digitally recorded and analyzed by computerized morphometric systems (Quantimet 570; Leica, The Netherlands and Hauppauge Computer Works, UK).

All procedures were approved by the local ethics committee of the Bolivian Institute for High Altitude Biology (Consejo Técnico, IBBA, Universidad Mayor de San Andrés, La Paz, Bolivia). Comparisons between groups were assessed statistically using one-way ANOVA with the Student–Newman–Keuls *post-hoc* test (Sigma-Stat; SPSS Inc., Chicago, IL, USA). The relationships between indices of cardiovascular remodeling and embryonic size or PO_2 were assessed using the Pearson Product-Moment correlation. A comparison between the slopes and intercepts of regression lines was conducted according to Armitage and Berry.[21] For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Oxygenation and biometry in the chick embryo

Analysis of this subset of animals confirms that incubation at high altitude induced embryonic systemic hypoxia and growth restriction (Table 1). The embryonic growth restriction is disproportionate as the ratio of the head diameter to body length was increased following incubation at high altitude (Table 1). When weight was expressed as a percentage of the initial egg mass, HAHA embryos showed partial protection against the effects of high-altitude incubation on growth. Further, the relative body weight in HASL embryos was greater than any other group (Table 1).

Cardiac measurements in the chick embryo

Relative to SLSL chick embryos, SLHA and HAHA groups showed significant increases of similar magnitude in the relative cardiac weight, and in the relative wall thickness of the left and right ventricles and septum (Fig. 1 and Table 2). In contrast, HASL and SLHA + O_2 embryos had cardiac measurements similar to SLSL embryos (Fig. 1). However, the relative thickness of the walls of the left and right ventricles was significantly reduced when compared to all other groups in SLHA + O_2 embryos (Fig. 1).

Aortic measurements in the chick embryo

SLHA embryos showed significant aortic medial thickening, as indexed by calculation of the aortic wall to lumen area ratio (Fig. 2). Aortae from HAHA embryos had the greatest wall

Table 1. *Oxygenation and biometry in the chick embryo*

	PO_2 (mmHg)	Absolute body weight (g)	Relative body weight (%)	Head diameter : body length
SLSL	56 ± 6 (9)[a]	28 ± 1 (31)[a]	40 ± 1 (31)[a]	0.177 ± 0.001 (31)[a]
SLHA	38 ± 2 (12)[b]	15 ± 1 (19)[b]	22 ± 1 (19)[b]	0.202 ± 0.005 (19)[b]
HAHA	36 ± 2 (10)[b]	15 ± 1 (33)[b]	31 ± 1 (33)[c]	0.201 ± 0.003 (33)[b]
HASL	64 ± 9 (7)[a]	24 ± 1 (25)[c]	55 ± 2 (25)[d]	0.176 ± 0.001 (25)[a]
SLHA + O_2	64 ± 4 (10)[a]	30 ± 1 (21)[a]	45 ± 1 (21)[a]	0.173 ± 0.003 (21)[a]

Values are mean ± SEM for the partial pressure of oxygen in chorio-allantoic venous blood, the embryonic weight expressed as a percentage of the initial egg mass and the ratio of the head diameter to crown–rump length in sea-level chick embryos incubated either at sea level (SLSL) or high altitude (SLHA), high-altitude embryos incubated at high altitude (HAHA) or sea level (HASL), and from sea-level chick embryos incubated at high altitude with oxygen supplementation (SLHA + O_2).

Number (*n*) of chicks for each variable in parentheses.

[a,b,c,d] Values within columns that have different letters as superscripts are significantly different from each other (one-way ANOVA with Student–Newman–Keuls test; $P < 0.05$).

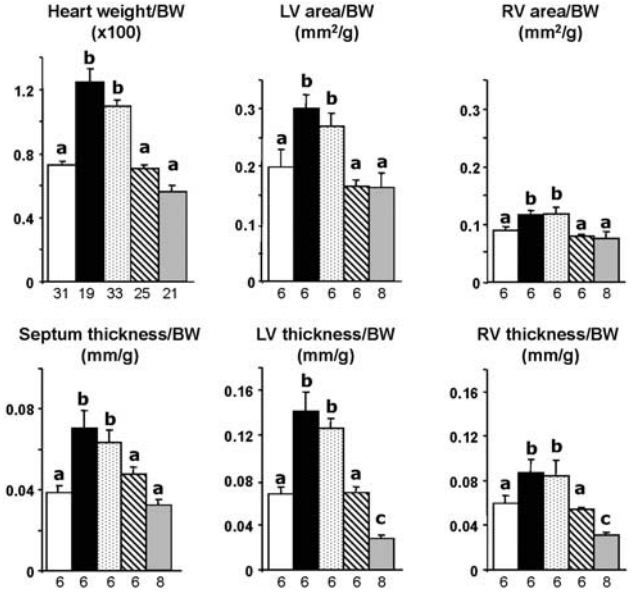

Fig. 1. Bars represent the mean ± SEM for the heart weight, area and thickness of the walls of the left and right ventricles and thickness of the cardiac septum expressed relative to body weight in sea-level chick embryos incubated either at sea level (SLSL, open bar) or high altitude (SLHA, filled bar), high-altitude embryos incubated at high altitude (HAHA, stippled bar) or sea level (HASL, hatched bar), and in sea-level chick embryos incubated at high altitude with oxygen supplementation (SLHA + O_2, gray bar). *n* of each group for each variable are shown at the bottom of the histograms. Values within columns that have different letters as superscripts are significantly different from each other (one-way ANOVA with Student–Newman–Keuls test; $P < 0.05$).

thickening relative to all other groups, as indexed by significant differences in all aortic measurements and derived calculations (Table 3 and Fig. 2). HASL or SLHA + O_2 prevented the aortic thickening induced by incubation at high altitude (Fig. 2).

Relation between cardiovascular remodeling and embryonic size or PO_2

Correlation analysis revealed that the embryonic body weight and PO_2 were negatively related to the aortic wall to lumen area ratio in all groups independent of treatment (Fig. 3). By contrast, the ratio of the embryonic head diameter to body length was positively related to the aortic wall to lumen area ratio in all groups independent of treatment (Fig. 3). When body weight was related to the cardiac weight across all groups, the association was best described by a reverse exponential ($y = 1.8951e^{-0.036x}$, $r = 0.85$) (Fig. 4*a*). Though SLSL, HASL and SLHA + O_2 embryos were distributed across the right-hand side, SLHA and HAHA groups were distributed across the left-hand side of the association (Fig. 4*a*). The relation between body weight and cardiac weight remained significant even across the normal range for body weight in SLSL embryos (Fig. 4*b*). Though the embryonic body and cardiac weights were obtained in every chick, only organs from smaller subgroups of embryos were prepared for histology. Similarly, chorio-allantoic PO_2 was obtained only from subgroups of embryos. Therefore, the relationship between embryonic body weight and any variable other than cardiac weight (for instance aortic wall to lumen area ratio or PO_2) within any one group could not be investigated.

Discussion

Several experimental techniques, employed primarily in pregnant sheep, rats and guinea pigs, have been used to induce sustained fetal hypoxemia, including reductions in uterine and umbilical blood flow,[22–25] placental embolization,[26] pre-conceptual removal of endometrial caruncles[27,28] and maternal chronic hypoxia.[29–31] All these elegant studies have reported marked effects on the developing cardiovascular system. More recently, attention has focused on whether sustained prenatal hypoxia may have adverse consequences for

Table 2. *Cardiac measurements in the chick embryo*

	Heart weight (mg)	LV area (mm^2)	RV area (mm^2)	Septum thickness (mm)	LV wall thickness (mm)	RV wall thickness (mm)
SLSL	196 ± 4 (31)	5.5 ± 0.8 (6)	2.2 ± 0.2 (6)	1.11 ± 0.05 (6)	1.87 ± 0.16 (6)	1.59 ± 0.17 (6)
SLHA	185 ± 7 (19)	4.6 ± 0.2 (6)	1.8 ± 0.2 (6)	1.10 ± 0.05 (6)	2.13 ± 0.07 (6)	1.32 ± 0.07 (6)
HAHA	175 ± 5 (33)	4.2 ± 0.5 (6)	1.8 ± 0.3 (6)	1.02 ± 0.05 (6)	2.08 ± 0.09 (6)	1.31 ± 0.07 (6)
HASL	188 ± 6 (25)	4.0 ± 0.3 (6)	1.8 ± 0.1 (6)	1.00 ± 0.06 (6)	1.74 ± 0.07 (6)	1.47 ± 0.07 (6)
SLHA + O_2	194 ± 9 (21)	5.0 ± 0.6 (8)	2.3 ± 0.3 (8)	1.01 ± 0.09 (8)	0.93 ± 0.15 (8)	1.08 ± 0.07 (8)

Values are mean ± SEM for absolute cardiac measurements in sea-level chick embryos incubated either at sea level (SLSL) or high altitude (SLHA), high-altitude embryos incubated at high altitude (HAHA) or sea level (HASL), and from sea-level chick embryos incubated at high altitude with oxygen supplementation (SLHA + O_2).

Number (*n*) of chicks for each variable in parentheses.

*$P < 0.05$ *v.* SLSL, ANOVA + Student–Newman–Keuls test.

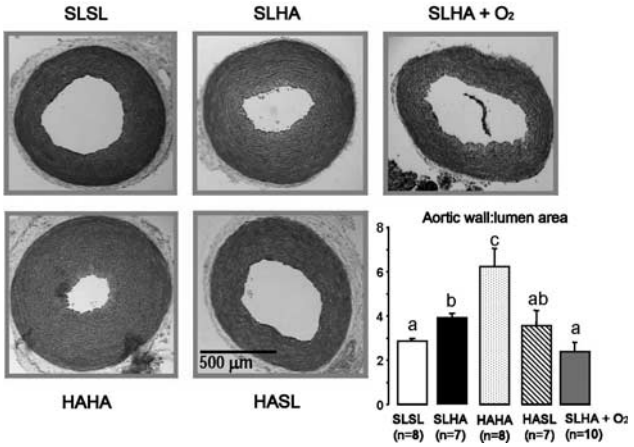

Fig. 2. Photomicrographs of representative examples of aortic sections and the mean ± SEM of the aortic wall to lumen area ratio for sea-level chick embryos incubated either at sea level (SLSL, open bar) or high altitude (SLHA, filled bar), high-altitude embryos incubated at high altitude (HAHA, stippled bar) or sea level (HASL, hatched bar), and in sea-level chick embryos incubated at high altitude with oxygen supplementation (SLHA + O_2, gray bar). Values within columns that have different letters as superscripts are significantly different from each other (one-way ANOVA with Student–Newman–Keuls test; $P < 0.05$).

the function of the cardiovascular system after birth and in later life. For instance, the groups of Zhang,[8] McMillen and Davidge[32–34] have reported that pregnant dams exposed to chronic hypoxia produce offspring with unequivocal cardiac and vascular dysfunction. However, because placental insufficiency decreases the delivery of nutrients as well as oxygen to the fetus and because chronic maternal hypoxia decreases maternal food intake,[32–35] the extent to which the effects on the developing cardiovascular system of all the above interventions are because of fetal under-nutrition or under-oxygenation remains uncertain. Employing the chick embryo as an animal model, a few studies have been able to isolate the effects on the developing cardiovascular system of chronic hypoxia, independent of changes in maternal nutrition and of the physiology of the mother and the placenta. Studies by Blanco and colleagues[36] and the group of le Noble[37] have confirmed that oxygen deprivation can act alone to remodel the developing cardiovascular system. Incubation of chick embryos with isobaric hypoxia induced embryonic aortic hypertrophic growth, left ventricular dysfunction and sympathetic hyperinnervation of peripheral arteries.[36–39] The present study combined the use of the chick embryo model with incubation at high altitude to determine for the first time: (1) whether chronic hypoxia during development at high altitude is the mechanism underlying the relationship between growth restriction and cardiovascular disease already evident prior to hatching; (2) whether such effects are different in embryos from sea-level or high-altitude hens; and (3) whether the effects could be prevented by incubation of fertilized eggs from sea-level hens at high altitude with oxygen supplementation, or by incubation of fertilized eggs from high-altitude hens at sea level.

Analysis of this subset of animals confirms that incubation at high altitude induced embryonic growth restriction and that this effect was diminished in embryos from high-altitude hens. These findings support the observations of other studies reporting that in human populations prolonged high-altitude residence ancestry can confer protection against the effects of high altitude on fetal growth.[11,14,16] The cardiovascular data in the present study show that incubation at high altitude leads to cardiac and aortic wall thickening in the chick embryo, independent of highland ancestry. Such cardiac and vascular remodeling could be prevented by incubation at sea level of fertilized eggs laid by high-altitude hens, or by incubation at high altitude of sea-level eggs with oxygen supplementation. Significant negative relationships were obtained between embryonic body weight or chorio-allantoic venous PO_2 (equivalent to umbilical venous PO_2 in mammalian pregnancy) with aortic wall thickening, and a significant positive relationship occurred between the ratio of

Table 3. *Aortic measurements in the chick embryo*

	Outer diameter (μm)	Lumen diameter (μm)	Wall thickness (μm)	Wall thickness/ lumen radius ratio	Wall area (mm^2)	Lumen area (mm^2)
SLSL	995 ± 63	508 ± 30	244 ± 17	0.96 ± 0.03	593 ± 78	207 ± 24
SLHA	1085 ± 39	488 ± 18	288 ± 14	1.14 ± 0.05	727 ± 57	184 ± 14
HAHA	1036 ± 39	396 ± 24*	320 ±16*	1.66 ± 0.14*	794 ± 58	126 ± 15*
HASL	996 ± 82	464 ± 36	266 ± 29	1.16 ± 0.12	637 ± 108	175 ± 27
SLHA + O_2	939 ± 68	515 ± 38	212 ± 28	0.85 ± 0.13	500 ± 85	216 ± 32

Values are mean ± SEM for aortic measurements in sea-level chick embryos incubated either at sea level (SLSL, $n = 8$) or high altitude (SLHA, $n = 7$), high-altitude embryos incubated at high altitude (HAHA, $n = 8$) or sea level (HASL, $n = 7$), and from sea-level chick embryos incubated at high altitude with oxygen supplementation (SLHA + O_2, $n = 10$).

*$P < 0.05$ *v.* SLSL, ANOVA + Student–Newman–Keuls test.

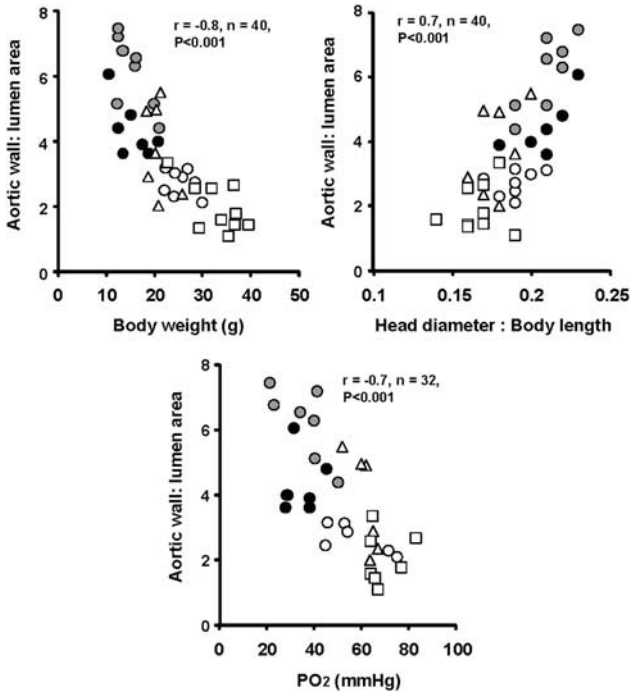

Fig. 3. Relationship between cardiovascular remodeling and embryonic size or PO_2. Body weight, the head diameter to body length ratio and chorio-allantoic venous PO_2 at the end of the incubation period were related to the aortic wall to lumen area ratio in all embryos independent of treatment. *r*, Pearson Product-Moment correlation coefficient; *n*, number of observations. SLSL (○); SLHA (●); HAHA (gray circles); HASL (△) and SLHA+O_2 (□).

the embryonic head diameter to body weight (an index of growth symmetry) and the vascular changes. Combined, therefore, the data presented strongly implicate that hypoxia owing to high altitude is an important mechanism, retarding embryonic growth as well as triggering a developmental origin of cardiovascular disease, already evident prior to hatching/birth. Interestingly, when body weight was related to cardiac weight, data in the present study also show that: (1) a significant negative relationship occurs across the normal range of weights; (2) that this relationship is shifted to the left and upwards by developmental high-altitude hypoxia; and (3) that the shift of the relationship could be restored by incubation at sea level of eggs from high-altitude hens, or by incubation at high altitude of sea-level eggs with oxygen supplementation. These observations have many commonalities with the original findings of Barker and colleagues,[1,3] who related birth weight with increased rates of cardiovascular disease in human populations. They also reported a phenotypic association between asymmetric fetal growth restriction and cardiovascular risk factors, and that this relationship extended across the normal range of birth weights.[1,3]

There is general agreement that cardiovascular remodeling of this type results from an increase in peripheral resistance.[26,29,37] The aortic thickening may be a response to restore wall stress, as is typical of an increase in load, and the ventricular wall thickening occurs in response to the increased cardiac afterload.[35,36] The hemodynamic overload may increase protein synthesis via a plethora of cellular and molecular pathways, including activation of stretch receptors, proto-oncogenes and vascular growth trophic factors.[40,41] Hypoxia may also affect hypoxia-sensitive growth factors, such as VEGF (vascular endothelial growth factor).[39,42] Consistent with the idea that this cardiovascular remodeling results from an increase in peripheral resistance, it has been reported that chronic hypoxia in the chick embryo promotes sympathetic hyper-innervation and enhanced norepinephrine release from perivascular sympathetic nerves;[36,37] that it decreases NO-dependent relaxation; and that it increases constrictor reactivity in the peripheral vasculature.[36]

The data presented using the chick embryo model are of important human relevance. Three separate clinical studies[43–45] have reported that babies born from pregnancies complicated by placental insufficiency show aortic thickening with increased vascular stiffness and reduced distensibility. A component of aortic thickening in the human fetus in pregnancies complicated by placental insufficiency may therefore be triggered by developmental hypoxia alone.

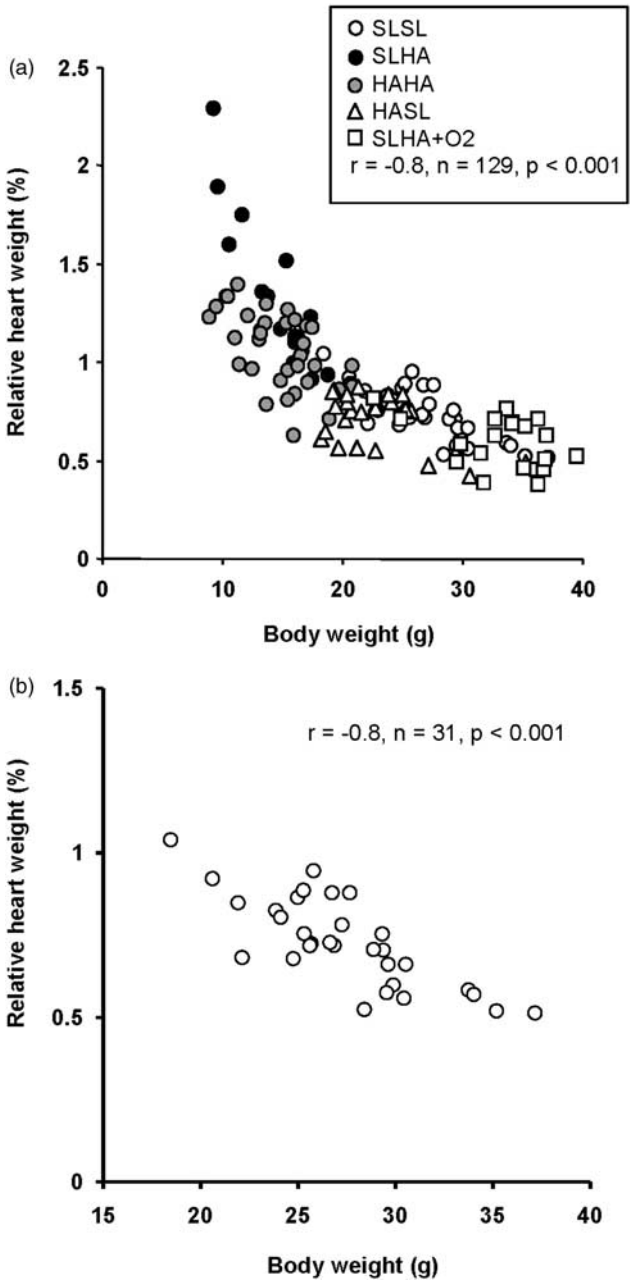

Fig. 4. Relationship between the cardiac weight and body weight in embryos following incubations at sea level and at high altitude (*r*, Pearson Product-Moment correlation coefficient; *n*, number of observations): (*a*) shows that a significant negative relationship occurs across all groups independent of treatment ($P < 0.001$); and (*b*) shows that a significant negative relationship occurs across the normal range of weights in SLSL embryos ($P < 0.001$).

In conclusion, the data show that hypoxia owing to high altitude induces pronounced cardiovascular changes associated with disease in the chick embryo, which are already evident by the end of the incubation period, and that these cardiovascular alterations are associated with disproportionate growth restriction. The effects can be prevented by incubation at sea level of fertilized eggs laid by high-altitude hens, or by incubation at high altitude of sea-level eggs with oxygen supplementation. Prolonged high-altitude residence ancestry confers partial protection against the effects of high-altitude incubation on growth but not on cardiovascular remodeling. It is of obvious interest whether these cardiovascular changes *in ovo* persist, resolve or amplify in later life.

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Statement of Interest

There are no conflicts of interest.

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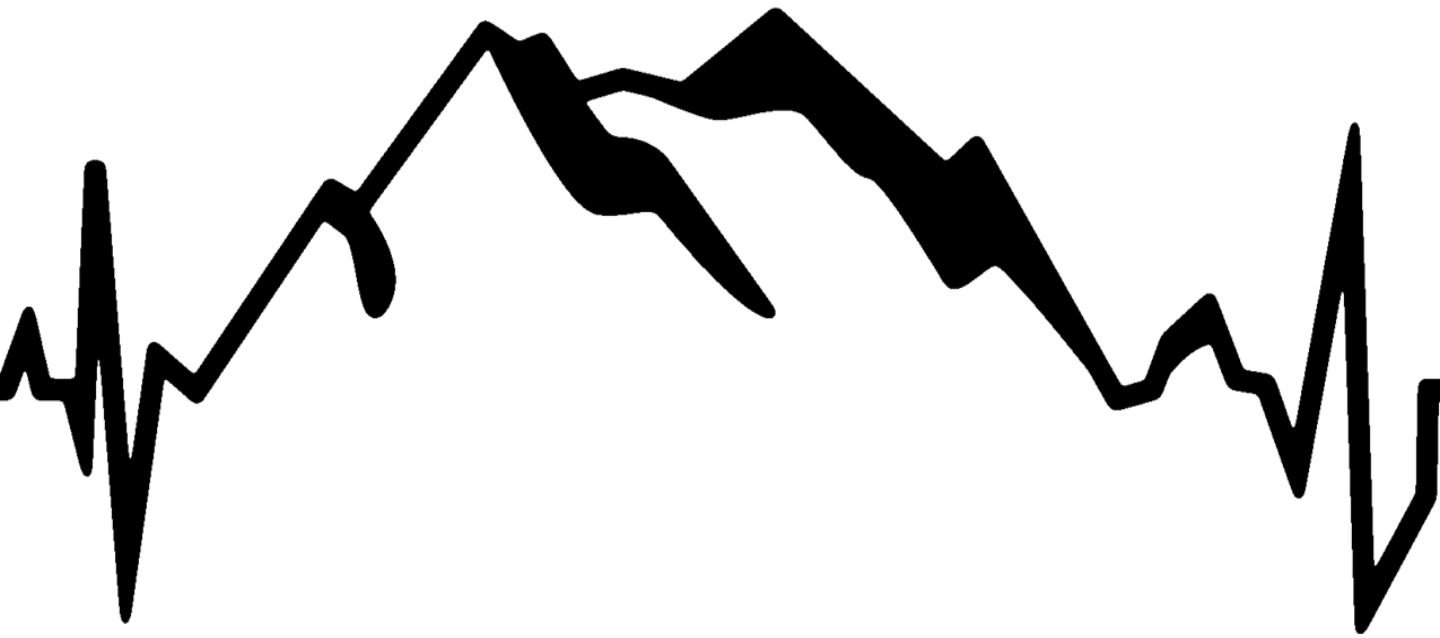
Chapter IV

Adrenocortical suppression in highland chick embryos is restored during incubation at sea level.



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Adrenocortical Suppression in Highland Chick Embryos Is Restored during Incubation at Sea Level

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Abstract

Salinas, Carlo E., Mercedes Villena, Carlos E. Blanco, and Dino A. Giusssani. Adrenocortical suppression in highland chick embryos is restored during incubation at sea level. *High Alt. Med. Biol.* 12:79–87, 2011.—By combining the chick embryo model with incubation at high altitude, this study tested the hypothesis that development at high altitude is related to a fetal origin of adrenocortical but not adrenomedullary suppression and that hypoxia is the mechanism underlying the relationship. Fertilized eggs from sea-level or high altitude hens were incubated at sea level or high altitude. Fertilized eggs from sea-level hens were also incubated at altitude with oxygen supplementation. At day 20 of incubation, embryonic blood was taken for measurement of plasma corticotropin, corticosterone, and Po_2. Following biometry, the adrenal glands were collected and frozen for measurement of catecholamine content. Development of chick embryos at high altitude led to pronounced adrenocortical blunting, but an increase in adrenal catecholamine content. These effects were similar whether the fertilized eggs were laid by sea-level or high altitude hens. The effects of high altitude on the stress axes were completely prevented by incubation at high altitude with oxygen supplementation. When chick embryos from high altitude hens were incubated at sea level, plasma hormones and adrenal catecholamine content were partially restored toward levels measured in sea-level chick embryos. There was a significant correlation between adrenocortical blunting and elevated adrenal catecholamine content with both asymmetric growth restriction and fetal hypoxia. The data support the hypothesis tested and provide evidence to isolate the direct contribution of developmental hypoxia to alterations in the stress system.

Key Words: corticotropin; cortisol; hypoxia; high altitude; chick embryo

Introduction

In the prenatal period, one of the most common forms of stress is fetal hypoxia (Giussani et al., 2001; Thakor and Giussani, 2009). In the late- gestation fetus, hypoxic episodes elicit an integrated defense response that facilitates fetal survival and the protection of hypoxia-sensitive tissues during the period of reduced oxygen availability (Giussani et al., 1994). Increases in fetal plasma catecholamines contribute to the fetal glucogenic (Fowden et al., 1998) and cardiovascular (Giussani et al., 1993) defenses against acute hypoxic stress. Increased fetal plasma concentrations of glucocorticoid amplify the actions of the sympathetic nervous system, contributing also to the fetal metabolic (Fletcher et al., 2000) and cardiovascular (Fletcher et al., 2003) responses to acute stress. However, prolonged elevations in fetal plasma glucocorticoid can reduce fetal growth (Ikegami et al., 1997), trigger preterm birth (Nathanielsz et al., 1988), and program cardiovascular and metabolic defects in later life (Seckl et al., 1999). Therefore, switching the pituitary–adrenocortical axis (HPA) off during prolonged stress is just as important as switching it on during short-term stress (Ducsay, 1998). For instance, in ovine pregnancy, if the fetal exposure to hypoxia lasts from hours to days, dissociation in the plasma corticotropin and cortisol responses occurs, such that glucocorticoid is still maintained high despite plasma corticotropin concentrations returning to basal levels (Murotsuki et al., 1996). However, if the fetal exposure to hypoxia is more prolonged, lasting months, such as during pregnancy at high altitude (>2500 m), plasma corticotropin remains high while plasma cortisol concentrations return to basal levels (Ducsay, 1998). Blunting of fetal basal adrenocortical output may therefore be an appropriate

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homeostatic response to prolonged periods of hypoxic stress to protect sensitive tissues from inappropriate and sustained elevations in plasma glucocorticoid levels during fetal development (Ducsay, 1998).

In human pregnancy, several studies have reported that newborn infants from pregnancies complicated by placental insufficiency are very low birth weight and show adrenocortical suppression (Kajantie et al., 2003; Watterberg et al., 2004). However, because placental insufficiency decreases the delivery of both nutrients and oxygen to the fetus, the extent to which the effects on the developing stress axes are owing to fetal hypoxia or underoxygenation is uncertain. Because most high altitude populations are also impoverished, with a high prevalence of maternal undernutrition (Julian et al., 2009), and because chronic maternal hypoxia in experimental animals also reduces maternal food intake (de Grauw et al., 1986), whether the associated adrenocortical blunting is owing to fetal underoxygenation or undernutrition again remains uncertain.

By combining the chick embryo model with incubation at high altitude, we have previously been able to isolate the direct effects of chronic hypoxia in the control of fetal growth and on fetal cardiovascular development, independent of changes in maternal nutrition or other confounders, such as the maternal and placental physiology (Giussani et al., 2007; Salinas et al., 2010). Since the stress axis is functional before hatching in chicks (Jenkins and Porter, 2004), the present study tested the hypothesis that high altitude is related to fetal adrenocortical but not adrenomedullay suppression. The hypothesis was tested in three ways:

1. By investigating the effects on the stress axes of incubation at high altitude of fertilized eggs laid by sea level-hens
2. By investigating whether alterations in the stress axes induced by development at high altitude could be prevented by incubation at sea level of fertilized eggs laid by high altitude hens
3. By investigating whether alterations in the stress axes induced by development at high altitude could be prevented by incubation at high altitude of sea-level eggs with oxygen supplementation

We were also interested in whether generational high altitude residence altered the effects of chronic hypoxia on the stress system and whether pituitary–adrenal hormone concentration correlated with fetal biometry and/or arterial Po_2.

Materials and Methods

The study was done in Bolivia, in the high altitude city of La Paz (3600 m, 494 mmHg, approximate ambient dry Po_2 100 mmHg) and in the sea-level city of Santa Cruz (420 m, 760 mmHg, approximate ambient dry Po_2 160 mmHg). The incubation procedures have been previously published in detail (Giussani et al., 2007; Salinas et al., 2010). In brief, fertilized eggs were obtained from Black Leghorn chickens that had been reared at the sea-level city of Santa Cruz or at the high altitude city of La Paz for at least six generations. Egg storage is commonly practiced in the artificial incubation of domestic birds. If the storage temperature for freshly laid chicken eggs is kept below the physiological zero (25° to 27°C), dormancy of the embryo can be maintained and fertile eggs can be stored for 1 to 3 weeks. In this study, fertilized eggs from Santa Cruz and La Paz were accumulated, maintained, and transported over 2 to 3 days at 14°C to arrest and synchronize development prior to incubation. This permitted incubation at different altitudes to start at day 1 of embryonic age (Giussani et al., 2007). Fertilized eggs from sea-level hens, laid at sea level, were randomly divided and incubated either at sea level (SLSL, $n = 35$) or high altitude (SLHA, $n = 24$). Eggs from high altitude hens, laid at high altitude, were randomly divided and incubated either at high altitude (HAHA, $n = 36$) or sea level (HASL, $n = 31$). SLHA embryos were also incubated with oxygen supplementation ($SLHA + O_2$, $n = 28$) at rates to maintain sea-level oxygen partial pressures according to Dalton's law (West, 1999).

All incubations (Polyhatch, Brinsea Products Ltd., Sanford, North Somerset, UK) were carried out under conditions to optimize development, with controlled temperature (38°C) and humidity (60%) and appropriate egg rotation. On day 20 of the 21-day incubation period, the egg was weighed, the air cell was exposed, and chorioallantoic venous blood was drawn into a 1-mL syringe for analysis of Po_2 (ABL 500, Radiometer, Copenhagen, Denmark), whenever possible in duplicate. At the same time, as much blood as possible (1 to 2 mL) was collected from chorioallantoic arterial vessels for subsequent measurement of plasma corticotropin and corticosterone. These samples were collected under sterile conditions into chilled EDTA tubes (K^+/EDTA, LIP Ltd., Shipley, West Yorkshire, UK); they were then centrifuged at 4000 rpm for 4 min at 4°C. The plasma obtained was then dispensed into prelabeled tubes, and the samples were stored at −80°C until analysis.

Following euthanasia by spinal transection, the embryo was removed from the eggshell, the adrenal glands were isolated, and embryonic body weight and the combined adrenal weight were recorded. The adrenal glands were snap frozen in liquid nitrogen for subsequent analysis of catecholamine content, because insufficient blood was available from any one embryo for analysis of circulating plasma concentrations of catecholamines.

Measurements for plasma corticotropin, corticosterone, and adrenal catecholamine content were performed in a subset of animals for each group within 2 months of sample collection, as previously described in detail, (Gardner et al., 2001; Fletcher et al., 2006). Plasma corticotropin and corticosterone concentrations were determined by radioimmunoassay (RIA) using commercially available kits (corticotropin: DiaSorin Inc., Stillwater, Minnesota, USA; corticosterone: ICN Biomedicals, Irvine, CA, USA). For corticotropin, the lower limit of detection for the assay was between 10 and 25 pg/mL^{-1}. The intraassay coefficients of variation for two plasma pools (37 and 150 pg/mL^{-1}) were 3.6% and 4.1%, respectively. The interassay coefficient of variation was 8.4%. For corticosterone, the assay sensitivity was 25 pg/mL^{-1}, and the intra- and interassay coefficients of variation were 5.8% and 7.5%, respectively. Noradrenaline and adrenaline concentrations were measured in both adrenal glands per chick embryo by high-pressure liquid chromatography (HPLC) using electrochemical detection. The samples were prepared by absorption of 250 μL onto acid-washed alumina, and 20 μL aliquots of the 100-μL perchloric acid elutes was injected onto the column. Dihydroxybenzylamine was added as the internal standard to each sample before absorption. Recovery ranged from 63% to 97%, and all catecholamine values were corrected for their respective recovery. The interassay coeffi-

cients of variation for adrenaline and noradrenaline were 7.3% and 6.2%, respectively. Measurements were made in duplicate on two dilutions of each purified sample, and the data were expressed as $\mu g/mg^{-1}$ for tissue content.

All procedures were approved by the local ethics committee of the Bolivian Institute for High Altitude Biology (Consejo Técnico, IBBA, Universidad Mayor de San Andrés, La Paz, Bolivia). Values for Po_2, embryonic and adrenal weights, and endocrine variables are expressed as mean ± SEM. Comparisons between groups were assessed statistically using one-way ANOVA with the Student Newman–Keuls post hoc test (Sigma-Stat, SPSS Inc., Chicago, IL, USA). The relationship between parallel measurements of plasma concentrations of corticotropin and corticosterone in all individual chick embryos was assessed using the Pearson product moment correlation. A comparison between the slopes and intercepts of regression lines was conducted according to Armitage and Berry (1994). For all comparisons, statistical significance was accepted when $p < 0.05$.

Results

Oxygenation and biometry

Embryonic systemic hypoxia and growth restriction occurred during incubation at high altitude (SLHA and HAHA) (Table 1). When weight was expressed as a percentage of the initial egg mass, embryos from high altitude hens (HAHA) relative to those from sea-level hens (SLHA) showed a diminished reduction in growth during high altitude incubation. Further, when this group was incubated at sea level (HASL), the relative body weight was greater than for any other group. The absolute combined adrenal weight was also reduced during incubation at high altitude (SLHA and HAHA); however, this effect no longer occurred when the combined adrenal weight was expressed relative to the embryonic body weight. Reductions in body and adrenal weights no longer occurred during incubation at high altitude with oxygen supplementation ($SLHA + O_2$).

Plasma corticotropin and corticosterone

Although plasma concentrations of corticotropin were significantly elevated, plasma concentrations of corticosterone were significantly depressed in embryos incubated at high altitude (SLHA and HAHA) relative to sea-level embryos (SLSL; Fig. 1). The magnitudes of the increment in plasma corticotropin and of the decrement in plasma corticosterone following incubation at high altitude were similar in embryos from hens native to sea level (SLHA) or to high altitude (HAHA). Plasma corticotropin in high altitude embryos incubated at sea level (HASL) was no longer different from sea-level embryos (SLSL). Plasma corticosterone concentrations in these embryos (HASL) were significantly greater than in SLHA and HAHA embryos, but still significantly depressed relative to SLSL embryos. Incubation of sea-level embryos at high altitude with oxygen supplementation ($SLHA + O_2$) prevented the high altitude-induced increase in plasma corticotropin and the high altitude-induced decrease in plasma corticosterone.

Correlation analysis, using the Pearson product moment test of paired plasma corticotropin and corticosterone values for all individual chick embryos (Fig. 2), revealed significant relationships for sea-level embryos incubated at sea level (SLSL, $r = 0.9$, $n = 10$, $p < 0.001$) and for sea-level embryos incubated at high altitude with oxygen supplementation ($SLHA + O_2$, $r = 0.9$, $n = 9$, $p < 0.003$). However, no significant relationship between plasma corticotropin and corticosterone was found in sea-level embryos incubated at high altitude (SLHA, $r = 0.7$, $n = 7$, $p = 0.07$), in high altitude embryos incubated at high altitude (HAHA, $r = 0.1$, $n = 7$, $p = 0.90$), or in high altitude embryos incubated at sea level (HASL, $r = 0.1$, $n = 9$, $p = 0.88$).

A comparison of slopes of the linear regressions (Fig. 2) also revealed that the slopes of sea-level chick embryos incubated at high altitude (SLHA, $y = 0.0101x + 2.4$), of high altitude embryos incubated at high altitude (HAHA, $y = 0.0007x + 5.3$), and of high altitude embryos incubated at sea level (HASL, $y = 0.0017x + 10.5$) were significantly depressed ($p < 0.05$) relative to sea-level embryos incubated at sea level (SLSL, $y = 0.1103x - 3.4$) or sea-level embryos incubated at high altitude with oxygen supplementation ($SLHA + O_2$, $y = 0.0838x + 11.0$).

Adrenal catecholamine content

Adrenal concentrations of noradrenaline and adrenaline were significantly elevated in embryos incubated at high altitude (SLHA and HAHA) relative to sea-level embryos (SLSL; Fig. 3). The magnitudes of these increments in adrenal catecholamine content following incubation at high altitude were similar in embryos from hens native to both sea level (SLHA) and high altitude (HAHA). Adrenal catecholamine content in high altitude embryos incubated at sea level (HASL) were significantly depressed relative to SLHA and HAHA embryos, but still significantly elevated relative to SLSL embryos. Incubation of sea-level embryos at high altitude with oxygen

Table 1. Oxygenation and Biometry in the Chick Embryo

	PO_2 (mmHg)	Embryonic body weight (%)	Adrenal weight (mg)	Relative adrenal weight (%)
SLSL	57 ± 3 (12)[a]	41 ± 1 (35)[a]	5.5 ± 0.3 (35)[a]	0.020 ± 0.001 (35)
SLHA	34 ± 2 (14)[b]	21 ± 1 (24)[b]	3.6 ± 0.3 (24)[b]	0.025 ± 0.002 (24)
HAHA	35 ± 1 (13)[b]	29 ± 1 (36)[c]	3.7 ± 0.3 (36)[b]	0.024 ± 0.002 (36)
HASL	59 ± 5 (9)[a]	54 ± 2 (31)[d]	5.2 ± 0.3 (31)[a]	0.021 ± 0.002 (31)
$SLHA + O_2$	60 ± 4 (11)[a]	46 ± 1 (28)[a]	5.9 ± 0.4 (28)[a]	0.020 ± 0.002 (28)

Values are mean ± SEM for the partial pressure of oxygen in chorioallantoic venous blood, the embryonic weight expressed as a percentage of the initial egg mass, and the absolute combined adrenal weight and the combined adrenal weight expressed as a percentage of the embryonic body weight. Groups are sea-level chick embryos incubated either at sea level (SLSL) or at high altitude (SLHA), high altitude embryos incubated at high altitude (HAHA) or at sea level (HASL), and sea-level chick embryos incubated at high altitude with oxygen supplementation ($SLHA + O_2$). Numbers in brackets refer to n. Values within columns that have different letters as superscripts are significantly different from each other (one-way ANOVA with Student Newman–Keuls test; $p < 0.05$).

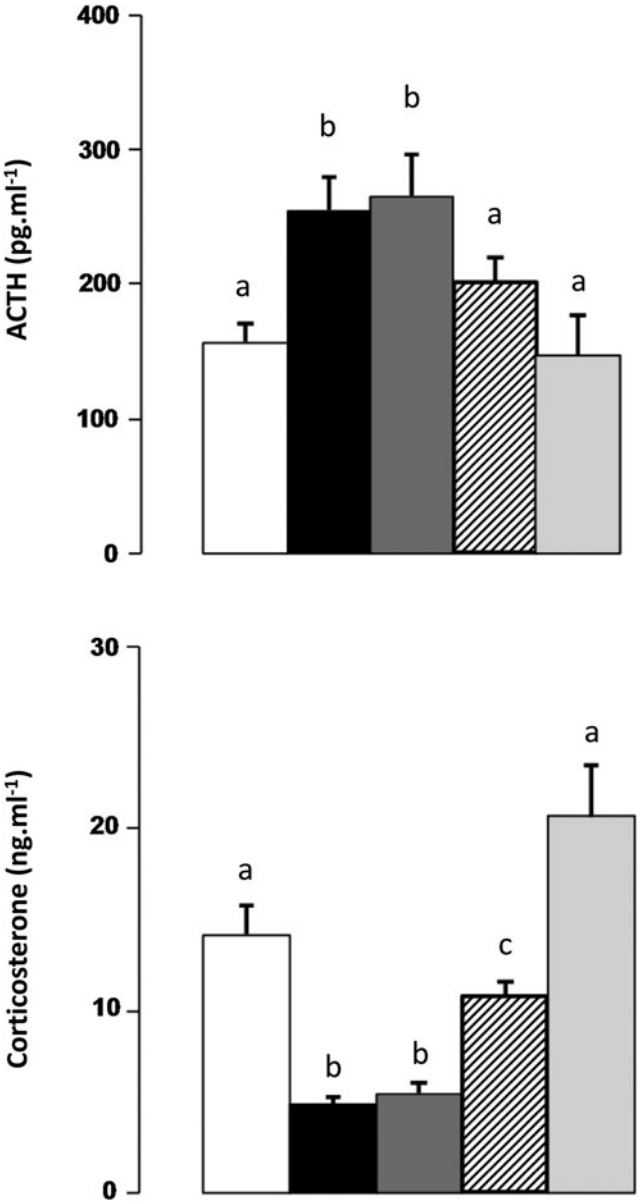

FIG. 1. Plasma corticotropin and corticosterone in the chick embryo. Values are mean ± SEM for the plasma concentrations of corticotropin and corticosterone in chorioallantoic arterial blood at day 20 of the incubation period. Groups are sea-level chick embryos incubated either at sea level (SLSL, $n = 9$, □) or at high altitude (SLHA, $n = 7$, ■), high altitude embryos incubated at high altitude (HAHA, $n = 9$, ■) or at sea level (HASL, $n = 7$, ▨), and sea-level chick embryos incubated at high altitude with oxygen supplementation (SLHA ± O_2, $n = 10$, ■). Values within columns that have different letters as superscripts are significantly different from each other (one-way ANOVA with Student Newman–Keuls test, $p < 0.05$).

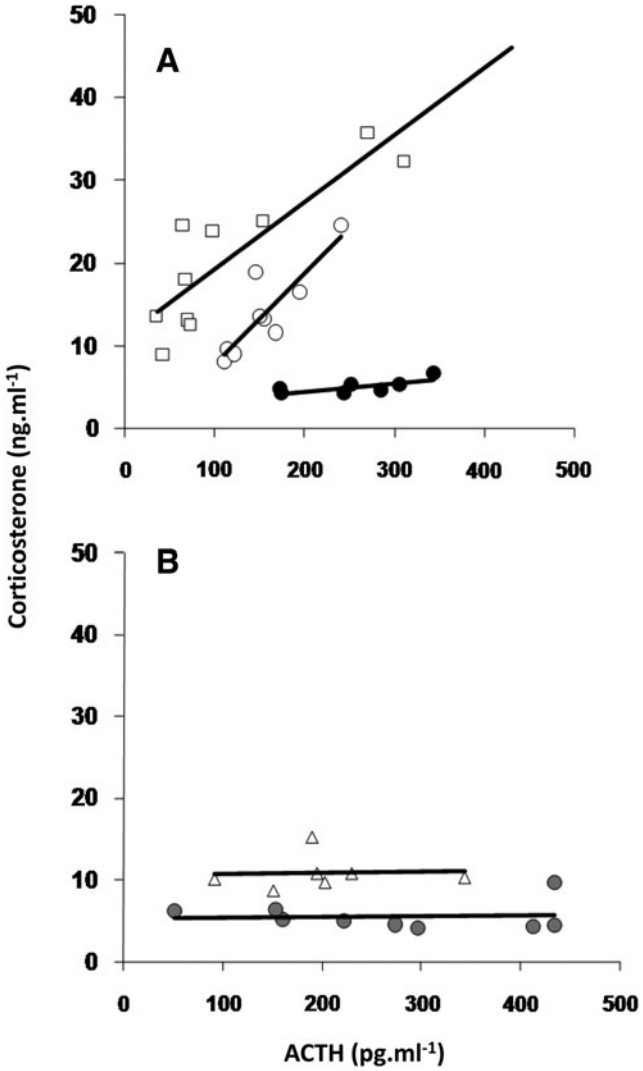

FIG. 2. Relation between plasma corticotropin and corticosterone in the chick embryo.Values are the paired plasma corticotropin and corticosterone concentrations in chorioallantoic blood at day 20 of the incubation period for all individual samples in chick embryos. (**A**) Sea-level chick embryos incubated either at sea level (SLSL, $n = 9$, ○), or at high altitude (SLHA, $n = 7$ ●), or at high altitude with oxygen supplementation (SLHA ± O_2, $n = 10$, □). (**B**) High altitude embryos incubated either at high altitude (HAHA, $n = 9$, ●) or at sea level (HASL, $n = 7$, △).

supplementation (SLHA + O_2) prevented the high altitude-induced increase in adrenal catecholamine content.

Relation between adrenal function and embryonic size or Po_2

Correlation analysis revealed that the plasma corticosterone–corticotropin ratio was positively related to the embryonic body weight and to Po_2, but negatively related to the head diameter–body weight ratio in all groups, independent of treatment (Fig. 4). Conversely, the adrenal catecholamine content was negatively related to embryonic body weight and to Po_2, but positively related to the head diameter–body weight ratio in all groups, independent of treatment (Fig. 4).

Discussion

The data show that the development of chick embryos at high altitude leads to pronounced adrenocortical blunting, but an increase in adrenal catecholamine content, by the end of the incubation period. These effects of high altitude incubation are similar whether the fertilized eggs were laid by sea-level or high altitude hens. The effects of high altitude on the stress axes are completely prevented by incubation at high

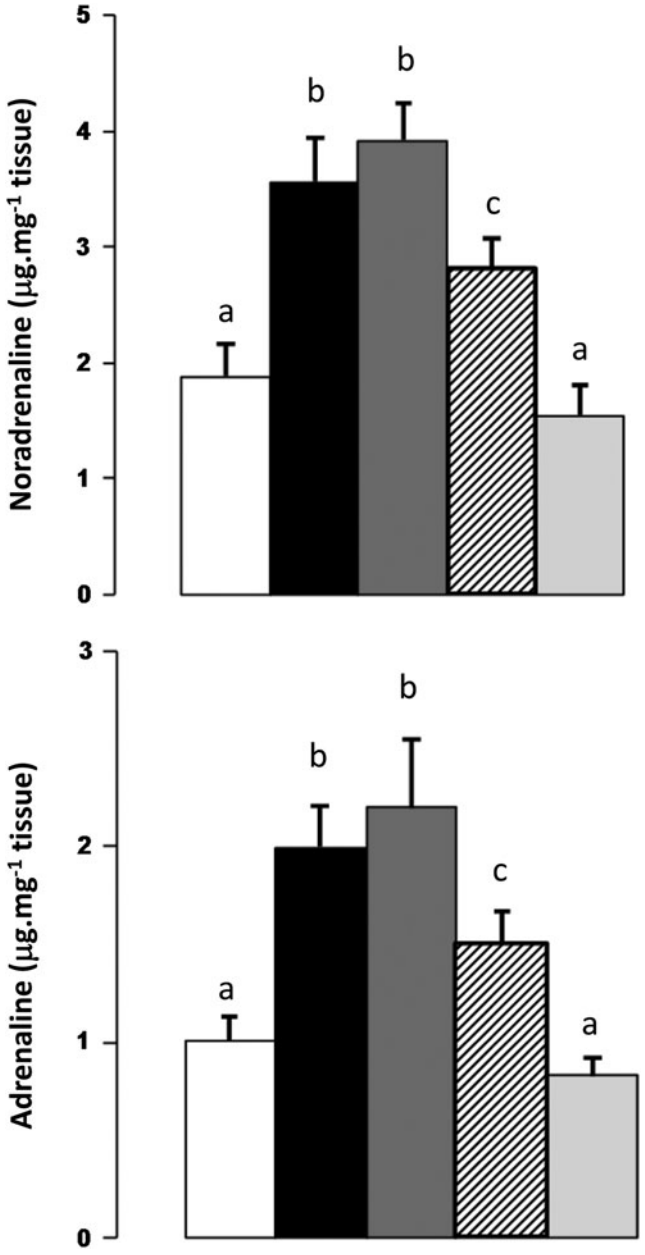

FIG. 3. Adrenal catecholamines in the chick embryo. Values are mean ± SEM for the content noradrenaline and adrenaline expressed per milligram of tissue at day 20 of the incubation period. Groups are sea-level chick embryos incubated either at sea level (SLSL, $n = 10$, □) or at high altitude (SLHA, $n = 10$, ■), high altitude embryos incubated at high altitude (HAHA, $n = 10$, ■) or at sea level (HASL, $n = 9$, ▨), and sea-level chick embryos incubated at high altitude with oxygen supplementation (SLHA ± O_2, $n = 10$, ■). Values within columns that have different letters as superscripts are significantly different from each other (one-way ANOVA with Student Newman–Keuls test, $p < 0.05$).

altitude with oxygen supplementation. When chick embryos from high altitude hens are incubated at sea level, plasma corticosterone and adrenal catecholamines by the end of incubation are partially restored toward levels measured in sea-level chick embryos. Adrenal cortical blunting and elevated catecholamine concentrations were significantly related to both growth restriction and fetal hypoxia.

Basal plasma concentrations of corticosterone in embryos from sea-level hens incubated at sea level were within the range of those reported by others for chick embryos at this stage of incubation or at hatching (Marie, 1981; Scott et al., 1981). Although several studies have reported that the adrenal cortex of the chick embryo is functionally responsive to corticotropin by the end of the incubation period (Woods et al., 1971; Wise and Frye, 1973), to our knowledge, this is the first report of circulating levels of corticotropin in the chick embryo during development at both at sea level and high altitude. One previous study has published the adrenaline content of the chick embryo adrenal gland during development (Wassermann and Bernard, 1970). In the present study, the levels of adrenaline content in the adrenal gland of embryos from sea-level hens incubated at sea level were similar to those reported by Wasserman and Bernard (1970) for chick embryos at this stage of incubation.

The physiology underlying the suppression of fetal adrenocortical function during development at high altitude has been studied extensively in a series of elegant contributions by Ducsay and Myers. Using the long-term hypoxemic (LTH) ovine model, whereby pregnant ewes are maintained at 3820 m above sea level from day 30 of gestation (term is ~150 days), they reported that the ability of the late-gestation sheep fetus to respond to a corticotropin challenge is markedly suppressed (Harvey et al., 1993). In addition, LTH sheep fetuses have significantly enhanced anterior pituitary processing of proopiomelanocortin (POMC) to corticotropin (Myers et al., 2005a) and an enhanced pituitary responsiveness to arginine vasopressin (Ducsay et al., 2009), both of which result in greater basal corticotropin concentrations, but in similar concentrations of cortisol (Ducsay, 1998). These findings are consistent with blunting of fetal basal adrenocortical output, which may be an appropriate adaptive fetal response to prolonged stress, to protect itself against the deleterious effects on fetal growth and the development of inappropriate and sustained elevations in plasma glucocorticoid levels (Ducsay, 1998). Reduced expression of adrenal corticotropin receptor and key steroidogenic enzymes (Myers et al., 2005b), as well as increased NO-mediated inhibition of steroidogenesis (Monau et al., 2009), may contribute to the physiology underlying adrenocortical blunting in the high altitude fetus. The data in the present article showed that adrenocortical blunting could be induced in chick embryos incubated at high altitude. Indeed, incubation at high altitude of fertilized eggs from sea-level hens produced severely growth restricted embryos with basal plasma corticosterone concentrations substantially lower than sea-level embryos. Adrenocortical blunting could be prevented by high altitude incubation with oxygen supplementation, and it could be reversed by incubating chick embryos from high altitude hens at sea level. Therefore, the present data extend the findings of Ducsay and Myers to confirm, by using a three-prong approach, that it is the direct effect of hypoxia during development at high altitude, rather than maternal or placental factors and/or hypobaria, that is responsible for adrenocortical blunting in the fetus. Of interest, our data also show that there is a strong positive relationship between adrenocortical blunting and intrauterine growth retardation (IUGR) and a strong negative relationship between adrenocortical blunting and arterial Po_2 in the chick embryo. These significant relationships further support the concepts that development under chronic hypoxia promotes IUGR (Giussani et al., 2007;

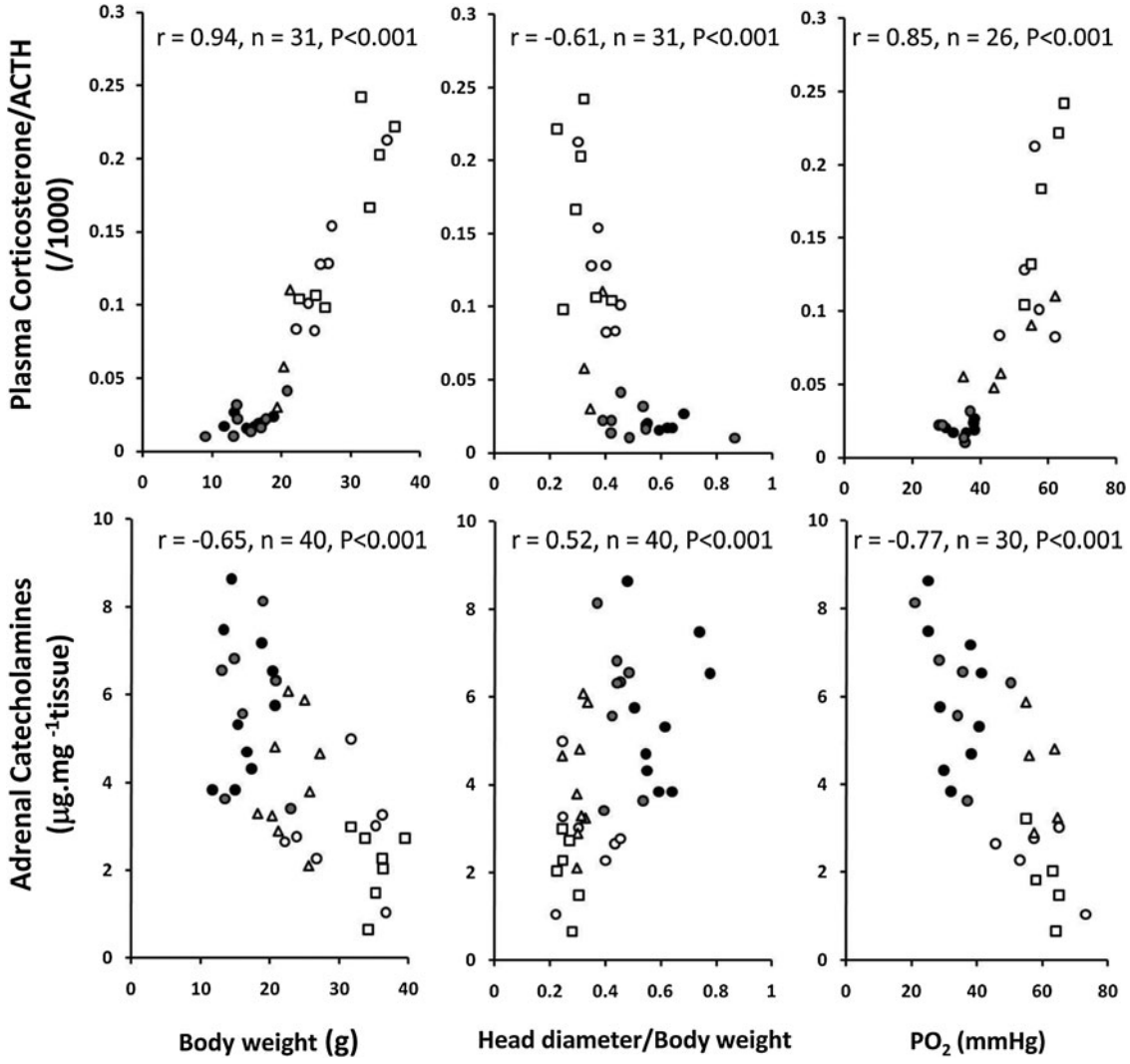

FIG. 4. Relationship between adrenal function and embryonic size or Po_2. Body weight, the head diameter to body weight ratio, and chorioallantoic venous Po_2 at the end of the incubation period were related to the ratio of corticosterone–corticotropin in plasma and the adrenal combined catecholamine content in all embryos independent of treatment. *r*, Pearson product moment correlation coefficient; *n*, number of observations. SLSL (○), SLHA (●), HAHA (◉), HASL (△), and SLHA ± O_2 (□).

Copeland and Dzialowski, 2009; Tintu et al., 2009) and that hypoxia-sensitive genes are involved in adrenocortical blunting.

Although blunting of basal adrenocortical output during long-term hypoxic development may protect the fetus against the deleterious effects of sustained elevations in glucocorticoid, it also renders the mammalian fetus susceptible to an inappropriate adrenocortical defense to a secondary acute stressor, such as the intrapartum hypoxia at the end of pregnancy. Our group (Riquelme et al., 1998; Riquelme et al., 2002) and Ducsay and Myers (Adachi et al., 2004; Imamura et al., 2004) have hypothesized that possible mechanisms exist to override suppression of fetal adrenocortical function during superimposed stress during development at high altitude. One strategy may be to increase acutely the gain of neural influences on adrenocortical function, which are triggered by a carotid chemoreflex (Riquelme et al., 1998; Riquelme et al, 2002) and mediated by splanchnic innervation to the adrenal gland (Myers et al., 1990). Compared to sheep, such neural influences on adrenocortical ouput during acute stress in the fetus are sensitized in the llama (Riquelme et al., 1998; Riquelme, 2002), a species adapted to the chronic hypoxia of life at high altitude for generational times (Giussani et al., 1999). Ducsay and Myers have recently reported (Vargas et al., 2010) that the enhanced plasma cortisol response to a second stressor in the LTH sheep fetus may involve enhanced adrenocortical intracellular signaling downstream from cAMP-dependent protein kinase A (PKA). Data in the present article also show that, when chick embryos from hens native to high altitude are incubated at sea level, plasma corticosterone by the end of incubation is partially restored toward the concentrations measured in sea-level chick embryos. However, the mechanism promoting this increase appears independent of corticotropin, because the relation between corticosterone and corticotropin in this group is still blunted relative to sea-level chick embryos. It is possible that the mechanisms employed by the highland chick embryo to restore adrenocortical output during incubation at sea level are similar to those

employed by the mammalian fetus to unleash an adrenocortical response of appropriate magnitude in response to a second stressor, despite basal adrenocortical blunting.

In contrast to the present study, Hassanzadeh and colleagues (2004) reported significantly higher plasma corticosterone concentrations in chick embryos following highland incubation compared to incubation at sea level. However, that study has several important differences. They incubated chick embryos at 2000-m altitude relative to ~4000- m altitude as in the present study. Also, their blood samples were obtained by cardiac puncture, rather than by chorioallantoic arterial blood sampling. It is likely that the difference in the effect of high altitude incubation on plasma corticosterone in the chick embryo between their and our studies is owing to the actual level of fetal hypoxia experienced. Blood sampling by cardiac puncture is stressful. Therefore, the significantly higher plasma corticosterone concentrations measured in highland chick embryos in the study of Hassanzadeh and colleagues (2004) may represent a greater capacity of the adrenal cortex to respond to acute stress, rather than basal concentrations during chronic exposure to high altitude. Therefore, the finding is in keeping with the hypothesis that possible mechanisms exist to override suppression of fetal adrenocortical function during superimposed stress following development at high altitude (Riquelme et al., 1998; Riquelme et al., 2002; Adachi et al., 2004; Imamura et al., 2004).

By late gestation in the developing ovine fetus, stress activates the sympathetic nervous system, eliciting the release of both noradrenaline and adrenaline. These catecholamines play an essential role in regulating fetal cardiovascular and metabolic responses to a wide variety of stresses and are fundamental in the adaptation of the neonate to the environment after birth (Giussani et al., 1993; Fowden et al., 1998; Olver et al., 2004). Several investigators have reported that fetal exposure to chronic hypoxia leads to upregulation of the sympathoadrenomedullary system. For instance, chronically hypoxemic fetal sheep (Gardner et al., 2002) and fetal llamas (Llanos et al., 2003) have higher resting plasma concentrations of noradrenaline than normoxic fetal sheep at equivalent stages of gestation. Studies by Ruijtenbeck and colleagues (2000) have reported that development of chick embryos under chronic isobaric hypoxia from 0.3 to 0.9 of the incubation period leads to elevations in the vascular noradrenaline content of peripheral circulations. Of interest, Camm and colleagues (2004) reported that the increase in catecholamine as a consequence of chronic hypoxia during development induced impaired memory in chicken. It has also been reported that both llama fetuses (Giussani et al., 1999) and chronically hypoxemic, growth-retarded fetal sheep (Creasy et al., 1973) have a greater dependence on α-adrenergic mechanisms to survive episodes of acute hypoxic stress than do control fetal sheep. Data in the present study show that in the chick embryo (1) adrenal catecholamine content is enhanced by development at high altitude, (2) this effect could be prevented by high altitude incubation with oxygen supplementation and could be reversed by incubating chick embryos from high altitude hens at sea level, and (3) there is a strong negative relationship between arterial Po_2 and adrenal catecholamine content and a strong positive relationship between adrenal catecholamine content and IUGR. Therefore, the present data also extend previous findings to confirm that it is the direct effect of reductions in oxygenation during chronic hypoxic development that leads to upregulation of the sympathoadrenomedullary system, of the type which is associated with IUGR.

In conclusion, the combined data in the present study provide strong evidence to support the hypothesis tested. High altitude is related to fetal adrenocortical but not to adrenomedullary suppression, and hypoxia is the mechanism underlying the relationship. Blunting of fetal basal adrenal cortical but not medullary function may be an appropriate homeostatic response to prolonged periods of hypoxia to protect sensitive tissues from sustained elevations of plasma cortisol levels while maintaining appropriate glucogenic capacity during fetal development. The biological trade-off may yield newborns with adrenocortical suppression.

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Disclosures

The authors have no conflicts of interest or financial ties to disclose.

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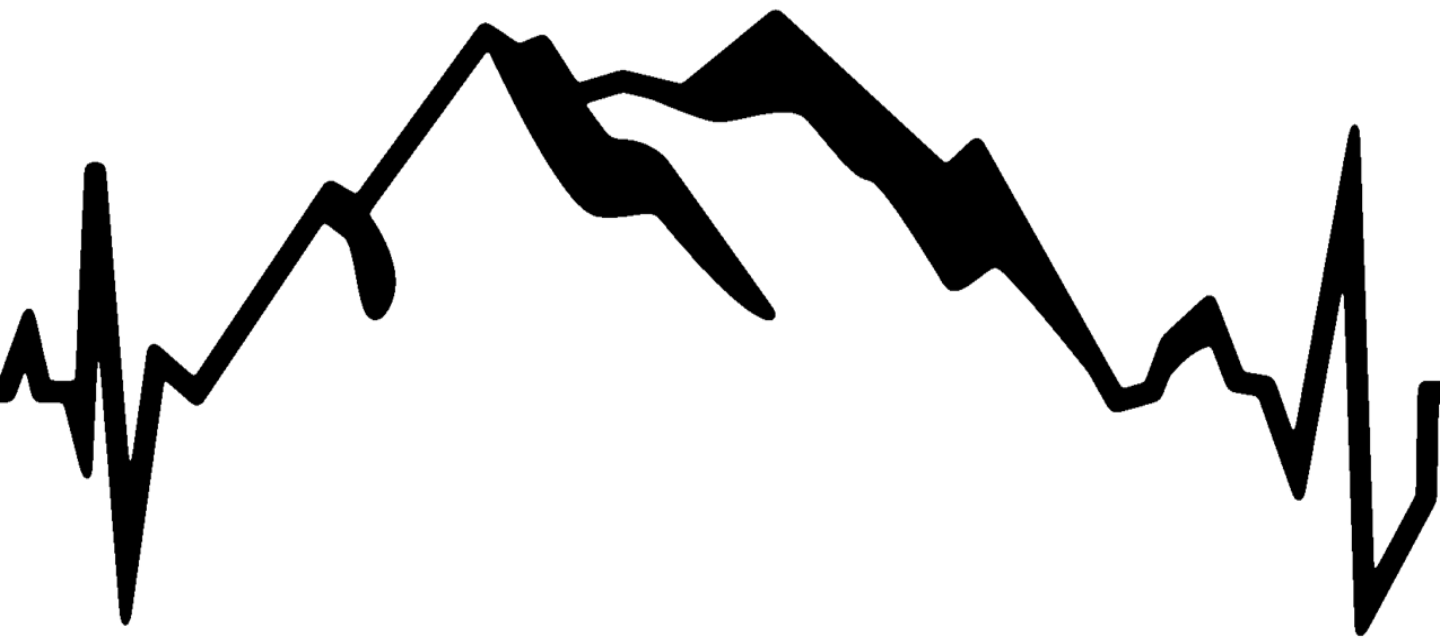
Chapter V

High altitude hypoxia and blood pressure dysregulation in adult chickens.



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ORIGINAL ARTICLE

High altitude hypoxia and blood pressure dysregulation in adult chickens

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Although it is accepted that impaired placental perfusion in complicated pregnancy can slow fetal growth and programme an increased risk of cardiovascular dysfunction at adulthood, the relative contribution of reductions in fetal nutrition and in fetal oxygenation as the triggering stimulus remains unclear. By combining high altitude (HA) with the chick embryo model, we have previously isolated the direct effects of HA hypoxia on embryonic growth and cardiovascular development before hatching. This study isolated the effects of developmental hypoxia on cardiovascular function measured *in vivo* in conscious adult male and female chickens. Chick embryos were incubated, hatched and raised at sea level (SL, nine males and nine females) or incubated, hatched and raised at HA (seven males and seven females). At 6 months of age, vascular catheters were inserted under general anaesthesia. Five days later, basal blood gas status, basal cardiovascular function and cardiac baroreflex responses were investigated. HA chickens had significantly lower basal arterial PO_2 and haemoglobin saturation, and significantly higher haematocrit than SL chickens, independent of the sex of the animal. HA chickens had significantly lower arterial blood pressure than SL chickens, independent of the sex of the animal. Although the gain of the arterial baroreflex was decreased in HA relative to SL male chickens, it was increased in HA relative to SL female chickens. We show that development at HA lowers basal arterial blood pressure and alters baroreflex sensitivity in a sex-dependent manner at adulthood.

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Key words: baroreflex, cardiovascular disease, chronic hypoxia, fetal programming

Introduction

Overwhelming evidence obtained from studies in human links development under sub-optimal intrauterine conditions with increased risk of cardiovascular disease.[1] Common sub-optimal intrauterine conditions include impaired fetal nutrition and oxygenation. Consequently, many studies have now accumulated to report that nutrient[2–4] and oxygen[2,5–9] restriction during development promotes intrauterine growth restriction (IUGR) and programmes cardiovascular dysfunction in the adult offspring.

Several studies, including our own, have used exposure of the pregnant rodent to a prolonged period of hypoxia during gestation and have studied adverse effects on fetal growth and on the cardiovascular system of the offspring at adulthood. These studies have reported that prenatal chronic hypoxia with or without IUGR programmes in the adult offspring impaired cardiac performance with increased susceptibility to cardiac ischaemia/reperfusion injury[6–10] and pronounced endothelial dysfunction in peripheral resistance circulations.[8,9,11] However, because maternal exposure to hypoxia can lead to a significant decrease in maternal food intake,[12,13] the extent to which any adverse effects on the offspring are due to fetal undernutrition and/or under-oxygenation remain unclear.

More than 140 million people live at altitudes higher than 3000 m, providing the largest single human group at risk for fetal exposure to hypoxia.[14] Studies of highland populations have reported fetal growth restriction and adverse cardiovascular alterations in offspring of high altitude (HA) relative to those of sea level (SL) pregnancies.[15–23] However, because most of HA populations are also impoverished, the relative contributions of fetal hypoxia and of malnutrition in slowing fetal growth and programming cardiovascular dysfunction, again, remain uncertain.

The chick embryo provides a useful model to investigate the direct effects on fetal growth and on programming of cardiovascular dysfunction of developmental hypoxia independent of additional effects on the nutrition of the mother and/or on the maternal or placental physiology and/or on lactation.[24–26] By combining HA exposure with the chick embryo model, we have previously been able to isolate the direct effects of HA hypoxia on fetal growth[27] and on fetal cardiovascular development.[28] Incubation of fertilized eggs

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from SL hens at HA promoted growth restriction, cardiomegaly and cardiac and aortic wall thickening in the chick embryo by the end of the incubation period.[27,28] These effects of HA incubation could be prevented by incubating eggs from HA hens at SL or by incubating eggs from SL hens at HA with oxygen supplementation.[27,28] In the present study, we have combined HA exposure with the chick embryo model, to isolate the effects of HA hypoxia during embryonic development and postnatal life on cardiovascular function measured *in vivo* at adulthood. Because in this model the hypoxic environmental condition persists before and after hatching, the study also permits the investigation of the effects of a 'double insult' in triggering cardiovascular dysfunction. As it is established that differences in sex affect developmental origins of cardiovascular disease,[29] this study investigated both male and female adult chickens.

Methods

All procedures were approved by the local ethics committee of the Bolivian Institute for High Altitude Biology (Consejo Técnico, IBBA, Universidad Mayor de San Andrés, La Paz, Bolivia) and were performed under the UK Animals (Scientific Procedures) Act 1986.

Animals

The study was conducted in Bolivia, in the HA city of La Paz (HA, 3600 m, 494 mmHg, PO_2 100 mmHg) and the SL city of Santa Cruz (SL, 420 m, 760 mmHg, PO_2 160 mmHg). Eighteen (nine males and nine females) *Black Leghorn* chicken embryos were incubated, hatched and raised at SL and 14 (seven males and seven females) *Black Leghorn* chicken embryos were incubated, hatched and raised at HA. At 6 months, which in this species represents early adulthood, the animals were subjected to general anaesthesia (10 mg/kg Xylazine 2%, Millpledge Pharmaceuticals, United Kingdom and 15 mg/kg Ketamine, Ketaset, Fort Dodge Animal Health, Iowa, USA, i.m.) and the femoral artery and vein were isolated via a hind limb medial incision. Polyvinyl catheters (i.d. 0.58 mm; o.d. 0.96 mm; Critchly Electrical Products, NSW, Australia) were placed in the descending aorta and inferior vena cava. The catheters were filled with heparinized saline (100 i.u. heparin in 0.9% NaCl), plugged with a brass pin and tunnelled subcutaneously to exit between the origin of the wings at the back of the chicken. After surgery, the animals were returned to a recovery room and then to floor pens. At least 5 days of post-operative recovery were allowed before the beginning of any experiment. Catheters were maintained patent by daily flushing with heparinized saline. At this time, an arterial blood sample was taken to monitor well-being.

Experimental protocol

On the day of experiments, the chicken was placed in a sling inside a dark wooden box with appropriate ventilation. The catheters were extended. The arterial catheter was connected to a pressure transducer (COBE; Argon Division, Maxxim Medical, Athens, Texas, USA) at the level of the base of the heart. Mean (MAP), systolic (SAP) and diastolic arterial pressure (DAP) and heart rate (HR) were recorded continuously via a data acquisition system (MPAQ – Maastricht Programmable AcQuisition system, Maastricht Instruments, The Netherlands, 500 Hz sample rate).

During basal recording, before the start of any experiment, 0.5 ml of arterial blood was taken to determine pHa, PaO_2 and $PaCO_2$ (ABL 500, Radiometer, Copenhagen, Denmark, measurements corrected to 37°C), percentage saturation of haemoglobin (SaO_2) and haematocrit (Ht; OSM3, Radiometer, Copenhagen, Denmark).

The chicken was then subjected to a protocol consisting of a 10-min period of baseline (B), a 10-min intravenous infusion (I) and 10-min period of recovery (R). The infusion consisted of either phenylephrine (50 μg/kg per min) or sodium nitroprusside (20 μg/kg per min) in separate experiments. The experiments were performed on the same day with at least 2 h between them to allow the cardiovascular variables to return to basal values. The phenylephrine experiment always preceded the infusion of nitroprusside.

At the end of the experiments, the chicken was humanely killed with an overdose of anaesthetic (100 mg/kg, Thiopental injection BP, Link Pharmaceuticals Ltd, UK, i.v.). *Post mortem*, the chicken was weighed and several body measurements were taken.

Data and statistical analyses

Baseline values for MAP, HR, pulse interval (PI; HR/60,000) and the rate pressure product [RPP; (HR × SAP)/1000] were calculated. In brief, the calculation of the baroreflex gain required two steps. The first step was the calculation of the slope of the HR–blood pressure relationship and the maximum and minimum values for HR for each animal. This was achieved by plotting the minute-by-minute HR and blood pressure responses during the beginning of the infusion period from baseline to plateau. Only the period from baseline to plateau is used to avoid confounding by resetting of the baroreflex. Arterial blood pressure and HR responses to the drugs were fitted to sigmoidal curves as follows: $HR = HR_{min} + ((HR_{max} - HR_{min})/(1 + 10^{(Mid\text{-}point\text{-}MAP)} \times Gain\ \ coefficient)$. The value for gain represents the gain coefficient of the Hill slope that describes the steepness of the curve. The second step was to calculate the baroreflex sensitivity according to McDowall and Dampney[30] applying the values for gain, maximum and minimum HR to the following equation: $Baroreflex\ Gain = ((HR_{max} - HR_{min}) \times Gain\ coefficient)/4$. This was done for each individual animal and thereafter the mean ± S.E.M. of the baroreflex gain for each group was calculated.

All data are expressed as mean ± S.E.M. Comparisons between groups were assessed statistically using two-way ANOVA with the Student–Newman–Keuls *post-hoc* test, with altitude and sex

as factors (Prism 5, GraphPad Software, Inc.). For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Arterial blood gas status

There were no differences in basal arterial pH and pCO_2 between groups. However, HA chickens had lower arterial pO_2, SaO_2 and increased Ht compared with SL chickens (Fig. 1). There was no effect of sex on arterial blood gas status either at SL or at HA. Therefore, these data were grouped at each altitude (Fig. 1).

In vivo *basal cardiovascular function*

Values for SAP, DAP and MAP during basal conditions were significantly lower in HA compared with SL chickens, and these differences were independent of the sex of the animal (Fig. 2a–2c). In contrast, basal HR was significantly higher and basal PI significantly lower in female than in male chickens at SL. Although basal HR and PI were significantly altered in HA males compared with SL males, there was no effect of altitude on basal HR or PI in female chickens (Fig. 2d and 2e). The RPP, an index of myocardial workload and oxygen consumption,[31] was significantly elevated during basal conditions in female chickens compared with male chickens at SL. Although HA had no effect of RPP in males, values for RPP in HA females were significantly lower compared with SL females (Fig. 2f).

In vivo *baroreflex function*

Independent of sex and altitude, all animals responded with reciprocal changes in HR to increases and decreases in arterial

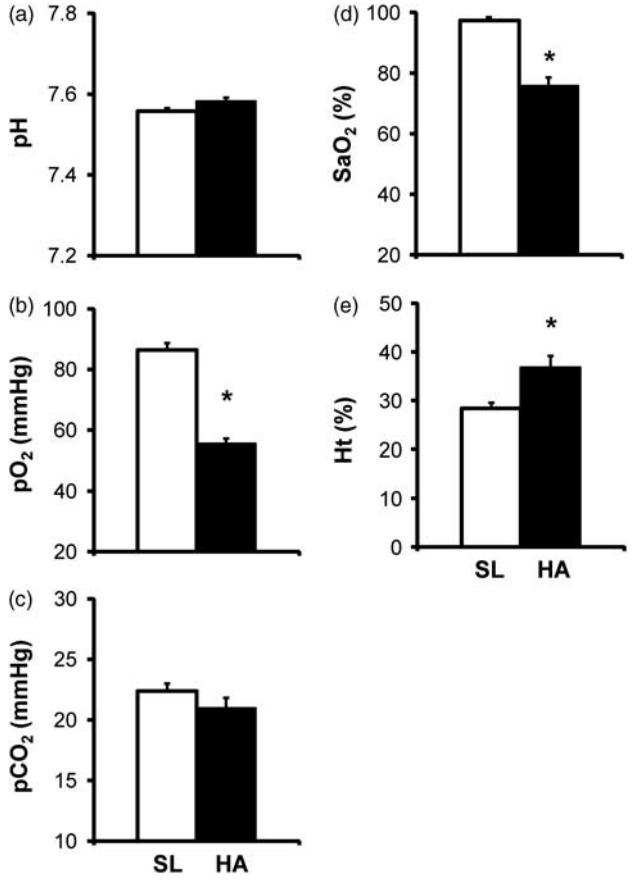

Fig. 1. Arterial blood gas status in sea level (SL) and high altitude (HA) adult chickens. Values are the mean ± S.E.M. for pH (*a*), pO_2 (*b*), pCO_2 (*c*), O_2 haemoglobin saturation (*d*, SaO_2) and haematocrit (*e*, Ht) in nine males and nine female chickens incubated, hatched and raised at SL (□) and in seven male and seven female chickens incubated, hatched and raised at HA (■). There was no effect of sex on arterial blood gas status either at SL or at HA. Therefore, these data were grouped at each altitude. Significant differences ($P < 0.05$) are: *SL *v.* HA (Two-way ANOVA + Student–Newman–Keuls *post-hoc* test).

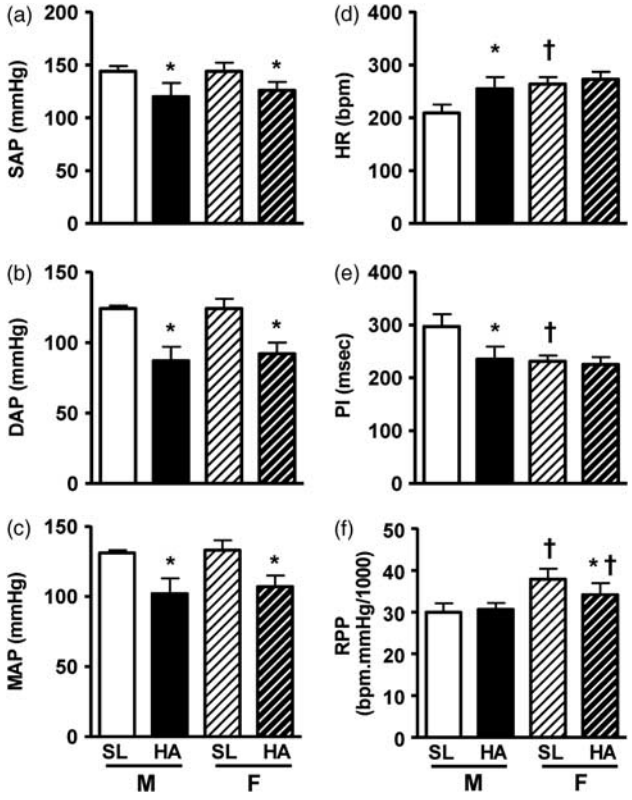

Fig. 2. Basal cardiovascular function in sea level (SL) and high altitude (HA) adult chickens. Values are the mean ± S.E.M. for systolic arterial pressure (*a*, SAP), diastolic arterial pressure (*b*, DAP), mean arterial pressure (*c*, MAP), heart rate (*d*, HR), pulse interval (*e*, PI) and the rate-pressure product (*f*, RPP) in nine males and nine female chickens incubated, hatched and raised at SL (white solid and white hatched bars) and in seven male and seven female chickens incubated, hatched and raised at HA (black solid and black hatched bars). Significant differences ($P < 0.05$) are: †male *v.* female, same altitude (hypoxia independent of sex) or *SL *v.* HA, for same sex (sex independent of hypoxia). There was no interaction between hypoxia and sex (Two-way ANOVA + Student–Newman–Keuls *post-hoc* test).

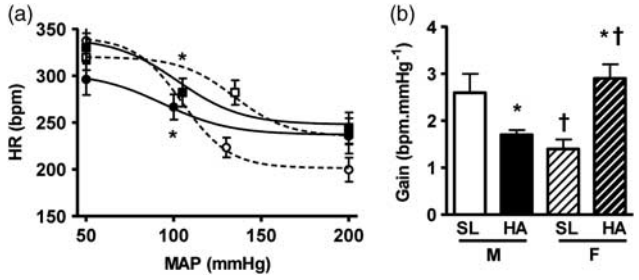

Fig. 3. Baroreflex function in sea level (SL) and high altitude (HA) adult chickens. Values are the mean ± S.E.M. for cardiac baroreflex function curves (*a*) and the gain of the cardiac baroreflex (*b*) in nine males and nine female chickens incubated, hatched and raised at SL and in seven male and seven female chickens incubated, hatched and raised at HA. Groups are sea level males (white circles, white bars), HA males (black circles, black bars), SL females (white squares, white hatched bars) and HA females (black squares, black hatched bars) chickens. Significant differences ($P < 0.05$) are: †male *v.* female, same altitude (hypoxia independent of sex) or *SL *v.* HA, for same sex (sex independent of hypoxia). There was no interaction between hypoxia and sex (Two-way ANOVA + Student–Newman–Keuls *post-hoc* test). The calculation of the baroreflex gain required two steps. The first step was the calculation of the slope of the heart rate (HR)–blood pressure relationship and the maximum and minimum values for HR for each animal. This was achieved by plotting the minute-by-minute HR and blood pressure responses during the beginning of the infusion period from baseline to plateau. Only the period from baseline to plateau is used to avoid confounding by resetting of the baroreflex. Arterial blood pressure and HR responses to the drugs were fitted to sigmoidal curves as follows: $HR = HR_{min} + ((HR_{max} - HR_{min})/(1 + 10^{(Mid\text{-}point\text{-}MAP)} \times Gain\ coefficient)$. The value for gain represents the gain coefficient of the Hill slope that describes the steepness of the curve. The second step was to calculate the baroreflex sensitivity according to McDowall and Dampney[30] applying the values for gain, maximum and minimum HR to the following equation: Baroreflex $Gain = ((HR_{max} - HR_{min}) \times Gain\ coefficient)/4$, The three points with S.E.M. on the curve represent the summary measures of the analysis: the gain and maximum and minimum HR values.

blood pressure induced by the administration of phenylephrine and sodium nitroprusside, respectively. Although values representing the cardiac baroreflex gain were significantly depressed in HA males compared with SL males, baroreflex gain was markedly increased in HA females compared with SL females (Fig. 3).

Biometry

Body weight was significantly lower in females than in males either at SL or HA. Body length [crown–rump length (CRL)] and other longitudinal measurements also tended to be shorter in females than in males at either SL or HA; however, only tibial and meta-tarsal length reached significant differences in SL animals (Table 1). Values for ponderal index were higher in male and female chickens at HA; however, the mean calculation was not significantly different from SL. The head length:CRL ratio was significantly elevated in HA compared with SL male but not female chickens (Table 1). The same trend was observed for the head diameter:body weight ratio, but the difference in males did not reach significance.

Discussion

By combining the chick embryo model with incubation at HA, we have previously isolated the effects of developmental hypoxia on fetal growth and on fetal cardiac and aortic wall remodelling.[27,28] Here, we isolate the effects of developmental hypoxia on cardiovascular function at adulthood measured in conscious chickens *in vivo*, and show that development at HA lowers basal arterial blood pressure and alters baroreflex sensitivity in a sex-dependent manner at adulthood.

Several experimental models of adverse intrauterine conditions, including maternal protein deprivation,[32–38] maternal overnutrition[39–43] and excess glucocorticoid exposure[44–47] have been reported to programme an increase in basal arterial blood pressure at adulthood. By contrast, there is little information on the effects of developmental hypoxia on the regulation of arterial blood pressure at adulthood, measured *in vivo*, particularly in the absence of anaesthesia. This is surprising as fetal hypoxia is the most common challenge in complicated pregnancy, such as during preeclampsia[48] and placental insufficiency.[49] The present study shows that developmental hypoxia is associated with a fall, rather than an increase, in mean, systolic and diastolic basal arterial blood pressure in adult conscious chickens and that this effect is independent of the sex of the animal. Given that hypotension occurred in the absence of bradycardia during basal conditions, an effect on lowering peripheral vascular resistance of development at HA is favoured. A lack of a programmed hypertensive effect in adult chickens of isobaric hypoxia *in ovo* has also been reported by Ruijtenbeek *et al.*[50] Similar findings have been reported in chick[51] and alligator[52] embryos incubated under chronic isobaric hypoxia, yielding basal hypotension without basal bradycardia, even before hatching. Coney and Marshall[53] reported that adult rats of chronically hypoxic pregnancies had significantly elevated resting femoral blood flow compared with adult rats of normoxic pregnancies, supporting a programmed fall in peripheral vascular resistance contributing to the programmed hypotensive effects of developmental hypoxia. Further, Herrera *et al.*[54] reported that HA chronic hypoxia during gestation yielded newborn lambs with significantly lower systemic vascular resistance than lowland newborn lambs, although basal systemic arterial blood pressure was not different. The mechanism via which developmental hypoxia may promote a fall in peripheral vascular resistance and a fall in resting blood pressure at adulthood is unclear. However, there is an established relationship between increased plasma Ht, blood viscosity and shear stress-induced increases in nitric oxide (NO) bioavailability.[55] Interestingly, moderate elevations in blood viscosity by increasing Ht by 10% from baseline could produce reductions

Table 1. *Body weight and dimensions in SL and HA adult chickens*

	Males		Females	
	SL	HA	SL	HA
Body (g)	2012 ± 159	1945 ± 341	1521 ± 76†	1614 ± 99
CRL (mm)	485 ± 15	436 ± 30	424 ± 14	393 ± 13
Femur (mm)	135 ± 4	138 ± 11	129 ± 5	123 ± 4
Tibia (mm)	166 ± 5	152 ± 6	141 ± 5†	131 ± 8
Meta-tarsal (mm)	124 ± 4	113 ± 7	98 ± 4†	100 ± 5
HD (mm)	33.1 ± 1.2	31.1 ± 0.9	30.1 ± 0.7	28.8 ± 1.1
HL (mm)	75 ± 1	78 ± 2	74 ± 1	75 ± 2
Beak (mm)	34.0 ± 2.8	37.5 ± 1.0	35.7 ± 1.1	35.4 ± 0.7
Ponderal index (kg/m^3)	19.01 ± 1.30	24.47 ± 4.76	22.30 ± 1.85	27.27 ± 2.43
HL:CRL	0.155 ± 0.005	0.183 ± 0.010*	0.176 ± 0.006	0.192 ± 0.009
HD:BW	0.016 ± 0.001	0.018 ± 0.002	0.018 ± 0.001	0.018 ± 0.001

SL, sea level; HA, high altitude; CRL, crown–rump length; HD, head diameter; HL, head length; BW, body weight. Values are mean ± S.E.M. for BW, CRL, femur, tibia and meta-tarsal lengths, HD, HL, beak length, ponderal index, the ratio of HL:CRL and of HD:BW in nine males and nine female chickens incubated, hatched and raised at SL and in seven male and seven female chickens incubated, hatched and raised at HA. Significant differences ($P < 0.05$) are: † male *v.* female, same altitude or *SL *v.* HA (Two-way ANOVA + Student–Newman–Keuls *post-hoc* test).

in basal blood pressure by 10 mmHg, an effect that could be abolished by treatment with the NO synthase blocker L-N omega-nitro-L-arginine methyl ester (L-NAME) or in NO synthase-deficient mice.[56] In the present study, chickens at HA had significantly lower arterial PO_2 and haemoglobin oxygen saturation and a persistent increase in Ht of ca. 8%.

Investigation of the effects of adverse intrauterine conditions on baroreflex function in the offspring is scarce relative to reported programmed changes in basal arterial blood pressure. Within the few studies available, it is known that maternal undernutrition can increase the set point and blunt baroreflex responses in the adult offspring[57–59] and that embryonic and fetal exposure to excess glucocorticoids can increase the set point and decrease the sensitivity of the baroreflex in fetal, newborn and adult life.[44,46,60–62] By contrast, as with effects on basal arterial blood pressure, there is less information on the effects of developmental hypoxia on baroreflex function. One study has reported that chronic hypoxic pregnancy decreases baroreflex sensitivity in the late-gestation ovine fetus[63] and Peyronnet *et al.*[64] predicted but did not measure alterations in baroreflex function in rat adult offspring of chronically hypoxic pregnancies.

Gilbert and Nijland,[65] in an elegant and useful review, brought to attention the growing body of evidence reporting sex differences in the developmental programming of alterations in arterial blood pressure and its regulation. Again, most of this evidence comes from studies involving alterations in maternal nutrition[2,62,66] or from models of maternal stress and glucocorticoid excess[62,67] than from models of prenatal hypoxia. The weight of the evidence has generally, but not exclusively, suggested that female offspring are less sensitive in manifestation of cardiovascular disease caused by prenatal stimuli.[65] Accordingly, Davidge and colleagues have reported that peripheral vascular function and cardiac hypertrophy is more profoundly affected in male than in female rat offspring of hypoxic pregnancies, particularly as they age.[68–70] Similarly, prenatal hypoxia caused an increase in heart susceptibility to ischaemia reperfusion injury in male but not in female adult rat offspring.[71] To our knowledge, only one study has reported sexually dimorphic effects in programming changes in baroreflex function induced by maternal malnutrition or fetal glucocorticoid exposure.[62] Certainly, there have been no reports on sex-dependent programming of baroreflex dysfunction by developmental hypoxia. In the present study, we show that while the effects of developmental hypoxia on resting arterial blood pressure at adulthood were sex independent, development at HA had reciprocal effects on the gain of the baroreflex in adult male and female chickens. Baroreflex gain was decreased in adult males, but it was increased in adult females at HA relative to SL controls. A decrease in baroreflex gain may render the male less able to respond to alterations in blood pressure homoeostasis. Further, the rate-pressure product, an index of myocardial workload and oxygen consumption,[31] although unchanged in males by altitude, was decreased in highland relative to lowland females. Finally, the head:CRL ratio, an index of blood flow redistribution, although unchanged in females by altitude, was increased in highland relative to lowland males. Collectively, therefore, the present data also suggest a greater resilience of the female offspring to development under conditions of HA hypoxia, again adding to the increasing body of evidence showing greater susceptibility to programmed cardiovascular dysfunction in male than female offspring. It is important to acknowledge that there are

important differences in sexual differentiation in avian and mammalian species. For instance, in the chicken, males rather than females are homogametic and sex determination is cell autonomous. Therefore, translation of the sexually dimorphic results between species should be considered cautiously.

It has been suggested that it is the mismatch between the pre- and postnatal environment that renders the offspring susceptible to developing disease at adulthood.[72] In the present study, the environmental condition of hypoxia occurs *in ovo* and persists after hatching. In this context, it is of interest that significant alterations in blood pressure homoeostasis were evident in male and female adult chickens despite matching of the incubation and post-hatching environments. Cardiovascular dysfunction may therefore have developed in response to a double insult; one occurring before and one after hatching. The partial contributions of incubation *v*. post-hatching HA hypoxia in triggering cardiovascular dysfunction at adulthood await investigation by study of adult offspring raised at SL following HA incubation, and by study of adult offspring raised at HA following SL incubation. However, evidence of similar alterations in blood pressure homoeostasis than those reported here at adulthood in the chronically hypoxic chick embryo even before hatching[51] supports a primary effect triggered during the incubation period rather than after hatching.

In conclusion, by combining the chick embryo model with incubation at HA, we have isolated the effects of developmental hypoxia on cardiovascular function at adulthood measured in conscious chickens *in vivo*, and show that development at HA promotes blood pressure dysregulation, altering baroreflex sensitivity in a sex-dependent manner at adulthood.

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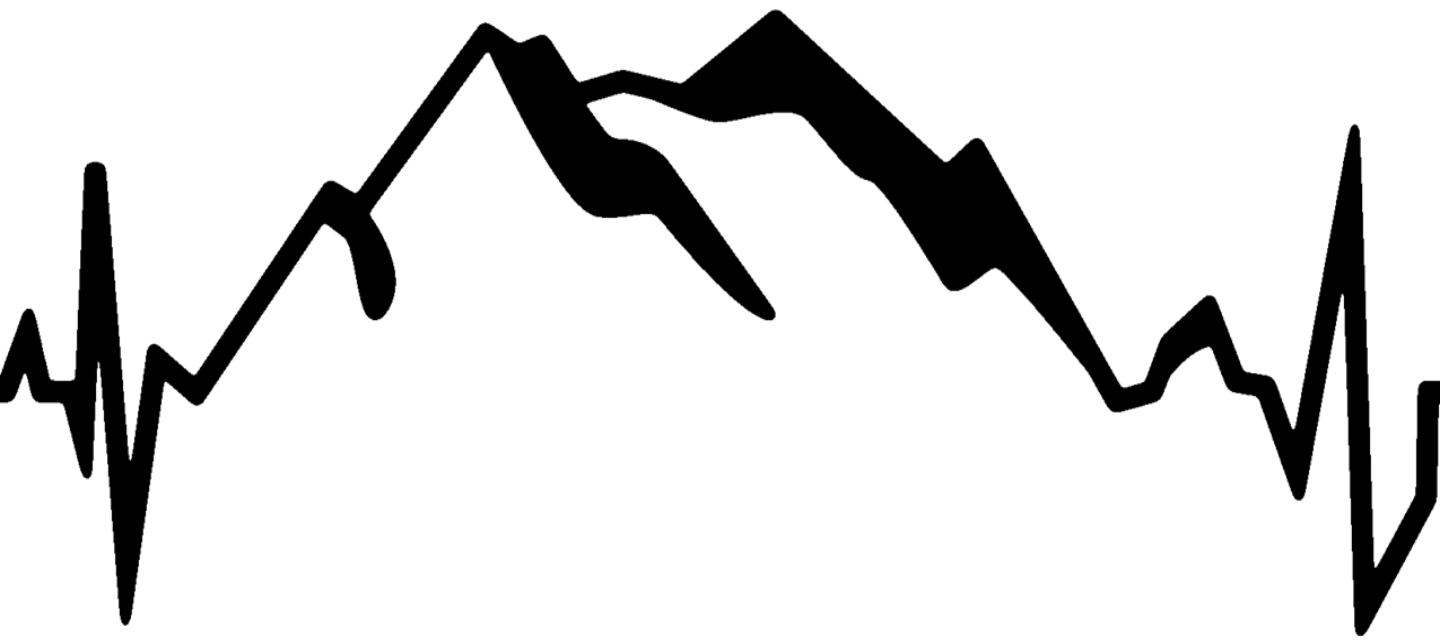
Chapter VI

High-altitude hypoxia and echocardiographic indices of pulmonary hypertension in male and female chickens at adulthood.



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ORIGINAL ARTICLE
Pulmonary Circulation

High-Altitude Hypoxia and Echocardiographic Indices of Pulmonary Hypertension in Male and Female Chickens at Adulthood

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Background: By combining the chick embryo model with incubation at high altitude (HA), the effects of chronic hypoxia on fetal growth, fetal cardiac and aortic wall remodeling and systemic arterial blood pressure at adulthood were reported. Using non-invasive functional echocardiography, here we investigated the in vivo effects of HA hypoxia on the pulmonary circulation at adulthood in male and female chickens.

Methods and Results: Chick embryos were incubated, hatched and raised at sea level (SL) or at HA. At 6 months of age, functional echocardiography was performed and the body and heart weights were taken. Heart weight was heavier in males but not in female HA chickens compared to their same sex SL counterparts. Similarly, male but not female HA chickens had greater in vivo right ventricular wall thickness compared to their same sex SL counterparts. The tricuspid pressure gradient was greatly enhanced in HA male and HA female chickens. However, the increment in the tricuspid pressure gradient was greater in HA males than in HA females. The pulmonary artery diameter was also enhanced in HA males than in SL males. In contrast, HA did not affect this variable in female chickens.

Conclusions: The data show that chronic hypoxia during development at HA is associated with echocardiocraphic indices of pulmonary hypertension at adulthood in a highly sex-dependent manner. (*Circ J* 2014; **78:** 1459–1464)

Key Words: Cardiovascular disease; Chronic hypoxia; Programming; Pulmonary hypertension

Pulmonary hypertension continues to be an important clinical problem.[1–8] Studies of populations at high altitude (HA) have unequivocally reported intrauterine growth restriction (IUGR) and a higher prevalence of pulmonary hypertension,[9–17] suggesting that a component of these conditions is associated with exposure to chronic hypoxia. However, because most highland populations are also impoverished, the relative contributions of chronic hypoxia or of chronic malnutrition during the fetal and postnatal periods in stunting growth and promoting pulmonary vascular disease during life at altitude remain uncertain.

Similarly, clinical studies at sea level (SL) have reported an association between the IUGR infant and the early development of right ventricular dysfunction and pulmonary hypertension.[18–20] However, because IUGR in human high-risk pregnancy normally occurs as a result of increased placental vascular impedance with consequent falls in oxygen and nutrient delivery to the baby, the relative contributions of chronic hypoxia or of chronic malnutrition during the fetal period in slowing growth and promoting pulmonary vascular anomalies under these conditions, again, remain uncertain.

Experimental studies in animal models, including our own, have used exposure of pregnant mammals to chronic hypobaric or isobaric hypoxia during gestation and have studied the effects on fetal growth and on the cardiovascular system of the offspring in the newborn and adult periods.[21–23] Studies such as these have reported that chronic fetal hypoxia can program persistent pulmonary hypertension in the newborn and pulmonary hypertension in the adult offspring.[24] However, because maternal exposure to hypoxia can lead to a significant decrease in maternal food intake,[25] the extent to which any adverse effects on the pulmonary circulation of the offspring are due to under-nutrition and/or under-oxygenation, once again, remain unclear.

The combination of HA exposure with the use of the chick embryo model permits investigation of the direct effects of HA hypoxia on growth and on cardiovascular development completely independent of alterations in placental function, independent of changes in the maternal physiology and independent of any effects of socioeconomic factors. Previously, we

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Table. Arterial Blood Gas Status in Sea-Level and High-Altitude Adult Chickens

	Males		Females	
	SL	HA	SL	HA
*pH*a	7.51±0.03	7.51±0.01	7.56±0.04	7.53±0.03
P_aCO_2 (mmHg)	27.8±2.2	25.9±2.1	28.9±2.4	26.2±2.1
P_aO_2 (mmHg)	87.1±3.6	45.4±3.2*	85.7±4.1	43.4±2.9*
SatHb (%)	97.4±0.4	58.5±7.2*	97.2±0.2	60.6±5.3*
Htc (%)	30.6±1.1	44.6±2.1*	27.7±1.8	46.1±2.3*

Values are the mean±SEM for arterial *pH* (*pH*a), arterial partial pressure of carbon dioxide (P_aCO_2), arterial partial pressure of oxygen (P_aO_2), hemoglobin saturation with oxygen (SatHb) and hematocrit (Htc) in 7 males and 7 female chickens incubated, hatched and raised at sea level (SL) and in 7 male and 7 female chickens incubated, hatched and raised at high altitude (HA). Significant differences (P<0.05) are: *SL vs. HA (Two-way ANOVA + Student-Newman-Keuls post-hoc test).

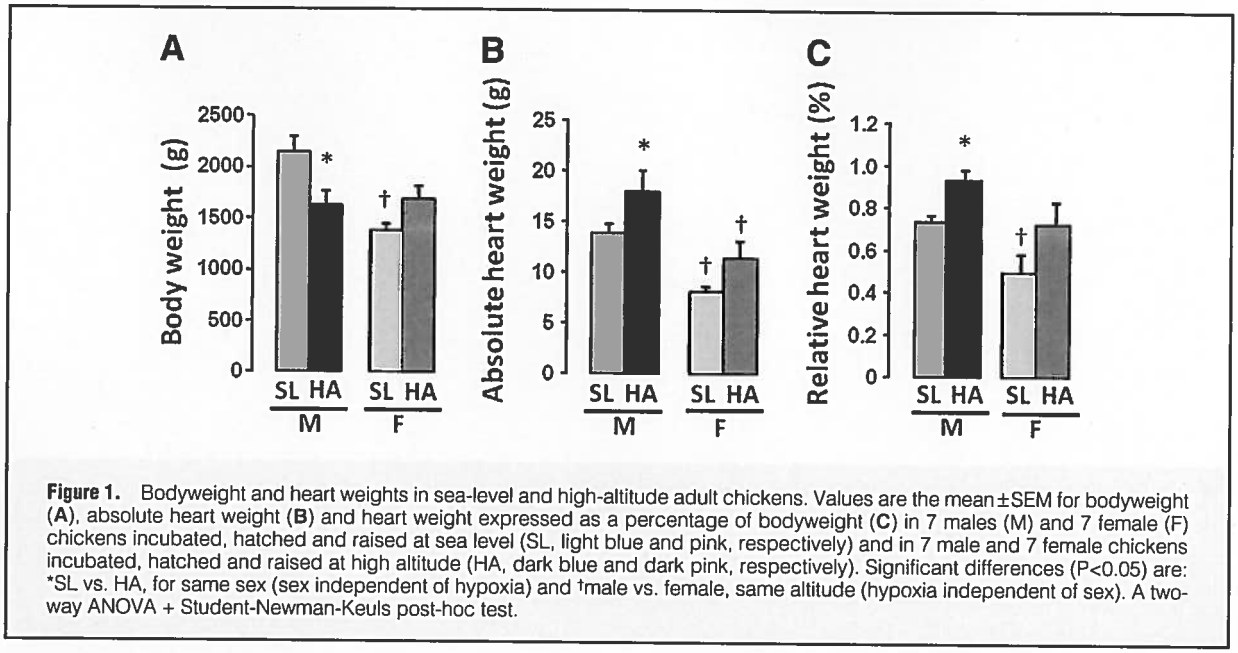

Figure 1. Bodyweight and heart weights in sea-level and high-altitude adult chickens. Values are the mean±SEM for bodyweight (**A**), absolute heart weight (**B**) and heart weight expressed as a percentage of bodyweight (**C**) in 7 males (M) and 7 female (F) chickens incubated, hatched and raised at sea level (SL, light blue and pink, respectively) and in 7 male and 7 female chickens incubated, hatched and raised at high altitude (HA, dark blue and dark pink, respectively). Significant differences (P<0.05) are: *SL vs. HA, for same sex (sex independent of hypoxia) and †male vs. female, same altitude (hypoxia independent of sex). A two-way ANOVA + Student-Newman-Keuls post-hoc test.

have reported that incubation of fertilised eggs from SL hens at HA promoted growth restriction, cardiomegaly, cardiac and aortic wall thickening in the chick embryo, and systemic blood pressure dysregulation in the adult chicken.[26–28] Using functional echocardiography, this study investigated in vivo in real time the effects of HA hypoxia on the pulmonary and systemic circulations in chickens at adulthood. As sexual dimorphic effects on cardiovascular disease are established,[29] we studied both male and female chickens.

Methods

All experiments were approved by the local ethics committee of the Bolivian Institute for HA Biology (Consejo Técnico, IBBA, Universidad Mayor de San Andrés, La Paz, Bolivia) and all procedures were performed under the UK Animals (Scientific Procedures) Act 1986.

The study took place in Bolivia, at the HA city of La Paz (HA, 3,600 m, 494 mmHg, PO_2 100 mmHg) and the SL city of Santa Cruz (SL, 420 m, 760 mmHg, PO_2 160 mmHg). Twenty-eight (14 male and 14 female) *Black Leghorn* chicken embryos were incubated, hatched and raised at SL and twenty-eight (14 males and 14 females) *Black Leghorn* chicken embryos were incubated, hatched and raised at HA. At 6 months of age (adulthood), in 7 males and 7 females in each group, the femoral artery was catheterised (polyvinyl catheters: i.d. 0.58 mm; o.d. 0.96 mm; Critchly Electrical Products, NSW, Australia) under anaesthesia (10 mg/kg Xylazine 2%, Millpledge Pharmaceuticals, UK and 30 mg/kg Ketamine, Ketaset, Fort Dodge Animal Health, Iowa, USA, i.m.) and arterial blood samples were taken after 5 days of post-operative recovery for determination of arterial blood gases, acid base status and hematocrit, in duplicate. Another 7 males and 7 females in each group were used for echocardiography studies. These chickens were mildly anaesthetised (10 mg/kg Xylazine 2%, Millpledge Pharmaceuticals, UK and 15 mg/kg Ketamine, Ketaset, Fort Dodge Animal Health, Iowa, USA, i.m.) and placed in a supine position on a heating pad, taking care to minimise body temperature loss. The feathers in the chest region were carefully plucked and echocardiography was performed (Acuson Siemens, Mountain View, CA) using a pediatric probe 7v3c (3.5–7 MHz), applying standard techniques similar to those described before.[20] Longitudinal and transverse images were obtained at different levels of the heart in the parasternal long- and short-axis using M-mode bi-dimensional (2D) echocardiography. The thickness of the ventricular walls in real time was measured using the

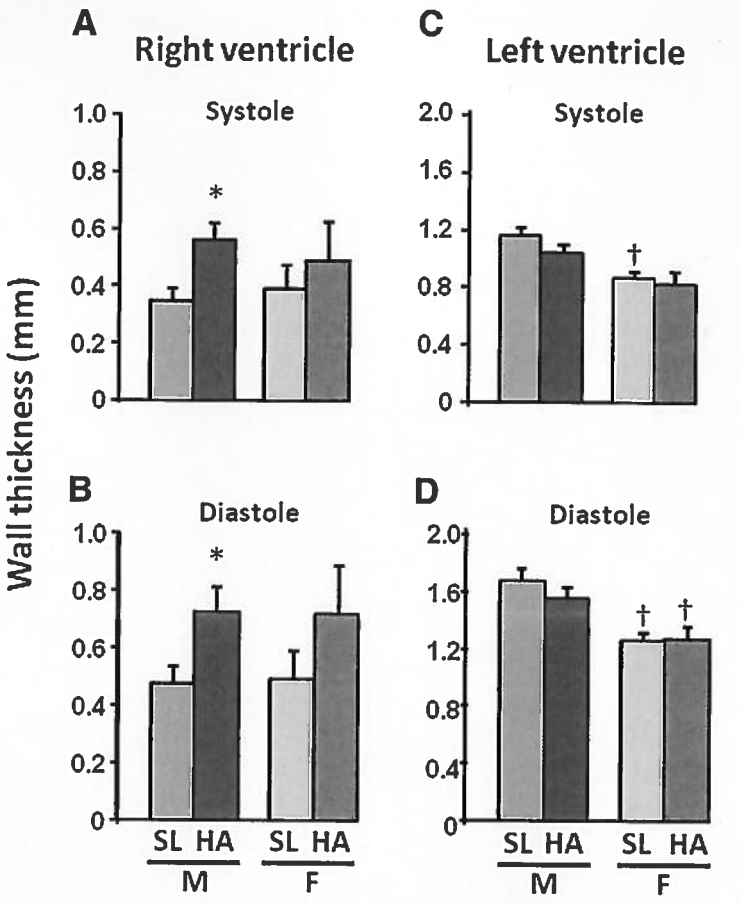

Figure 2. Ventricular wall thickness in sea-level and high-altitude adult chickens. Values are the mean±SEM for the wall thickness of the right (**A** and **B**) and left (**C** and **D**) ventricle during systole and diastole in 7 male (M) and 7 female (F) chickens incubated, hatched and raised at sea level (SL, light blue and pink, respectively) and in 7 male and 7 female chickens incubated, hatched and raised at high altitude (HA, dark blue and dark pink, respectively). Significant differences ($P<0.05$) are: *SL vs. HA, for same sex (sex independent of hypoxia) and †male vs. female, same altitude (hypoxia independent of sex). A two-way ANOVA + Student-Newman-Keuls post-hoc test.

parasternal long-axis view of the heart with the M-mode beam tip just beyond the atrioventricular valves, perpendicular to the long axis of either ventricle. The thickness of the walls of the major vessels was also determined using the parasternal long-axis view of the heart with M-mode. Doppler was used to determine the direction of blood flow and its velocity. In the parasternal long-axis orientation, with B-mode visualization of the pulmonary artery, the pulmonary artery Doppler was established. The peak flow velocity of the trans-tricuspid jet was measured and the pressure gradient between the right ventricle and the right atrium was calculated, as previously described and validated at HA.[20] The equivalent was determined for the left ventricle. At the end of the experiments, the chicken was humanely killed with an overdose of anaesthetic (100 mg/kg, Thiopental injection BP, Link Pharmaceuticals Ltd, UK, i.v.). Upon post mortem, the chicken was weighed. The heart was isolated, weighed and frozen in liquid nitrogen.

Statistical Analysis

All data are expressed as mean±SEM. Comparisons between groups were assessed statistically using a 2-way ANOVA with the Student-Newman-Keuls post-hoc test, with altitude and sex as factors (Prism 5, GraphPad Software, Inc). For all comparisons, statistical significance was accepted when $P<0.05$.

Results

Arterial Blood Gas Status and Hematocrit

At 6 months, there were no differences in arterial pH and pCO_2 between males and females or between SL and HA. However, HA male and female chickens had lower arterial pO_2, SaO_2 and increased hematocrit compared to SL chickens. Values for pO_2, SaO_2 and hematocrit were similarly altered in male and female chickens at HA relative to SL (**Table**).

Biometry

At 6 months, bodyweight, absolute heart weight and the heart weight expressed as a percentage of bodyweight were all significantly lower in SL female chickens than SL male chickens (**Figures 1A–C**). Male but not female chickens at HA were significantly lighter than their same sex SL counterparts (**Figure 1A**). Similarly, the absolute and relative heart weights were significantly greater only in male but not female HA chickens relative to their same sex SL counterparts (**Figures 1A–C**).

Echocardiography

At 6 months, the thickness of the right ventricular wall was similar during systole and diastole in SL male and SL female chickens (**Figures 2A,B**). However, the thickness of the left ventricular wall was significantly lower during systole and diastole in SL female than in SL male chickens (**Figures 2C,D**). Male but not female chickens at HA had significantly greater

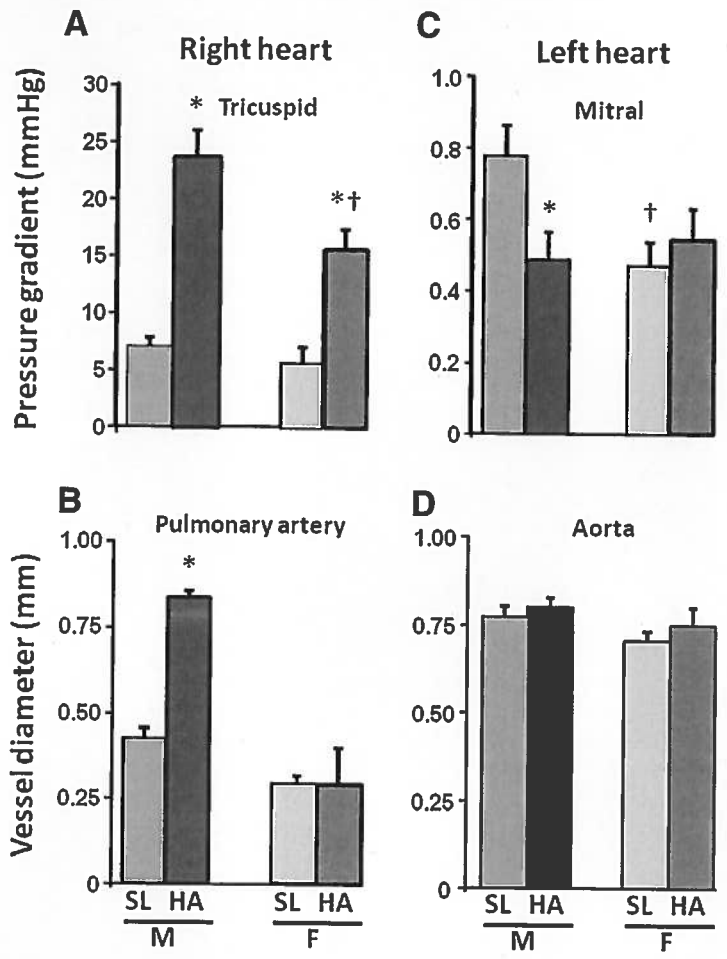

Figure 3. Ventricular pressure gradients and vessel diameter in sea-level and high-altitude adult chickens. Values are the mean±SEM for the ventricular pressure gradient and the main vessel diameter of the right (**A** and **B**) and left (**C** and **D**) heart in 7 male (M) and 7 female (F) chickens incubated, hatched and raised at sea level (SL, light blue and pink, respectively) and in 7 male and 7 female chickens incubated, hatched and raised at high altitude (HA, dark blue and dark pink, respectively). Significant differences ($P<0.05$) are: *SL vs. HA, for same sex (sex independent of hypoxia) and †male vs. female, same altitude (hypoxia independent of sex). A two-way ANOVA + Student-Newman-Keuls post-hoc test.

right ventricular wall thickness during systole and diastole than their same sex SL counterparts (**Figures 2A,B**). HA did not affect the wall thickness of the left ventricle in either males or females (**Figures 2C,D**).

At 6 months, the tricuspid pressure gradient was greatly enhanced in HA male and HA female chickens relative to their same sex SL counterparts. However, the increment in the tricuspid pressure gradient was significantly greater in HA males than in HA females (**Figure 3A**). The pulmonary artery diameter was also greatly enhanced in HA male than in SL males. In contrast, HA did not affect the pulmonary artery diameter in female chickens (**Figure 3B**). Overall, values for the mitral pressure gradient were much lower than values for the tricuspid pressure gradient (**Figures 3A,C**). The mitral pressure gradient was significantly lower in SL females relative to SL males and in HA males relative to SL males (**Figures 3C,D**). Neither sex nor HA affected the diameter of the aorta.

Discussion

Using non-invasive functional echocardiography, data in the present study show that chickens incubated, hatched and raised at HA develop significant indices of pulmonary hypertension at adulthood in a highly sex-dependent manner.

In contrast to the systemic circulation which dilates, the pulmonary vascular bed constricts during hypoxic conditions;[30] this is a physiological response, matching pulmonary perfusion to reduced oxygenation. However, excessive or prolonged increases in pulmonary vascular resistance can lead to pathology. Highland residents provide an excellent model to investigate the pathophysiology of the pulmonary vascular bed as they live in an environment of hypobaric hypoxia. Their hearts and pulmonary circulation show alterations that resemble those that occur in clinical conditions associated with alveolar hypoxia and polycythemia, exhibiting pulmonary hypertension and cardiomegaly due to right ventricular hypertrophy. As highlanders lose their capacity for adaptation with advancing age or due to additional risk factors, such as smoking, these findings become exaggerated leading to overt chronic mountain sickness. The expression of pulmonary hypertension and right heart remodelling in highland human and animal residents has been described for many years in a long and rich history of important studies.[10–15] Although sex differences in the prevalence of pulmonary hypertension at SL have been reported, there is disagreement about whether this is primarily a disease of male or female individuals.[31,32] In marked contrast, it is a widely held view that the highland female is relatively protected than the highland male against developing pulmonary hypertension during residence at HA. However, this has not been established in the literature. Data are beginning to surface to indicate

protection against pulmonary hypertension in native highland human residents, such as in the Aymaras, relative to newcomers, and relative protection in Aymara girls relative to Aymara boys.[13,33] Therefore, the present study advances the literature to report marked protection against echocardiographic indices associated with pulmonary hypertension in adult female chickens relative to male chickens when incubated, hatched and raised at HA. As our study involved chickens, the development of differential indices of pulmonary hypertension in males and females is clearly independent of possible alterations in socioeconomic factors, in alterations in the physiology of the mother animal, or in changes in placental function as may happen in humans, thereby isolating the effect to be due to HA hypoxia. The mechanism underlying protection against high altitude-induced pulmonary vascular dysfunction in highland natives or in females is not known. However, it might involve differences in the bioavailability of nitric oxide (NO).[13,34,35] There is considerable evidence highlighting the importance of both pulmonary vascular endothelial and alveolar epithelial NO synthesis in the appropriate regulation of the pulmonary circulation and its adequate response to hypoxia.[13] Therefore, it is of interest that exhaled NO is much greater in Andean and Tibetan natives than in SL residents,[34] and that estrogen dilates the pulmonary vascular bed and ameliorates pulmonary hypertension via NO-dependent mechanisms.[35]

In the fields of pulmonary hypertension and of programming of disease, there is increasing interest in establishing answers to 2 unknown questions: whether chronic fetal hypoxia might itself increase susceptibility to developing pulmonary hypertension at adulthood, and whether matching of the pre- and post-natal environments might ameliorate the development of pulmonary hypertension. It has been reported that it is the mismatch between the pre- and post-natal environments that might be more important than adverse intrauterine or post-natal conditions, per se, in rendering the offspring at increased risk of developing disease at adulthood.[36] In our study, because the environmental condition of hypoxia occurred in ovo as well as post-hatching, the partial contributions of HA hypoxia during the fetal or post-natal periods in triggering pulmonary vascular changes at adulthood cannot be distinguished. However, in the context of the mismatch hypothesis,[36] the present data are also novel because significant indices of pulmonary vascular dysfunction occurred in adult chickens despite matching of the environment pre- and post-hatching. In the present study, pulmonary vascular anomalies might therefore have developed in response to a double insult; one occurring prior and one after hatching, modeling precisely the continued pre- and post-natal hypoxic environment that HA human populations experience.

Evidence in the literature supporting a primary effect of chronic fetal hypoxia vs. chronic post-natal hypoxia in increasing susceptibility to the onset of pulmonary hypertension in later life is of mixed opinion. Independent evidence of cardiac biventricular hypertrophy and a significant increase in right ventricular wall area and thickness in chick embryos incubated at HA or during chronic isobaric hypoxia, even prior to hatching,[27,37] supports a primary effect triggered by chronic hypoxia already during the incubation period, which persists and/or becomes exacerbated by exposure to hypoxia after hatching. Similarly, there have been reports of children resident at HA diagnosed with pulmonary hypertension.[38–40] Experiments in ovine pregnancy at HA have also reported newborn offspring with basal pulmonary hypertension and an exaggerated increase in pulmonary arterial pressure to a superimposed episode of acute hypoxia.[41–46] Rueda-Clausen et al. have also reported that chronic hypoxic pregnancy in rodents, followed by post-natal normoxic conditions, leads to pulmonary hypertension in the adult offspring, becoming prominent with aging.[24] In contrast, there have also been reports in children resident at HA with no evidence of pulmonary hypertension, when socioeconomic factors were accounted for.[47] Similarly, experimental studies in newborn rats and guinea pigs exposed to chronic hypoxia in utero have reported no morphological evidence of pulmonary hypertension.[48–50] Finally, no evidence of early endothelial dysfunction was reported in small pulmonary arteries of fast-growing broilers raised in normoxia following incubation under hypoxic conditions.[51] Clearly, further insight into this debate could be obtained by exploiting the combination of the chick embryo model and HA exposure, but with a cross-over study design; by investigating adult offspring (pre- and post-puberty) incubated at HA but raised post-hatching at SL and vice versa. Although logistically rather more difficult, this is clearly an obvious extension of the present work and a path for future investigation.

In the present study, there are 2 additional findings that deserve some attention. First, the tricuspid pressure gradient was greatly enhanced in highland chickens. However, the increment in the tricuspid pressure gradient was significantly greater in highland males than in highland females. In contrast, the pulmonary artery diameter was also greatly enhanced in highland chickens, but only in males. HA did not affect the pulmonary artery diameter in female chickens. Second, the mitral pressure gradient was significantly decreased in highland males relative to SL males. The reasons for the dissociation between an effect of HA on the tricuspid pressure gradient but not on the pulmonary artery vessel diameter in female chickens are unclear. However, it might indicate the existence of a threshold tricuspid pressure gradient above which remodelling of the pulmonary vessel wall is triggered, as in highland male chickens. The lower mitral pressure gradient between highland vs. SL males might indicate relative systemic arterial hypotension in highland males. Of interest, a recent study by our group has reported that HA chickens had significantly lower arterial blood pressure than SL chickens, when measured in chronically instrumented animals in vivo. However, this effect was independent of the sex of the animal.[28]

In conclusion, by combining the chick embryo model with incubation at HA, we have investigated the in vivo effects of chronic hypoxia on the pulmonary system at adulthood, and show that pre- and post-hatching development at HA markedly enhances established echocardiographic indices of pulmonary hypertension at adulthood in a highly sex-specific manner.

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Conflict of Interest

The authors can confirm that they hold no conflict of interest.

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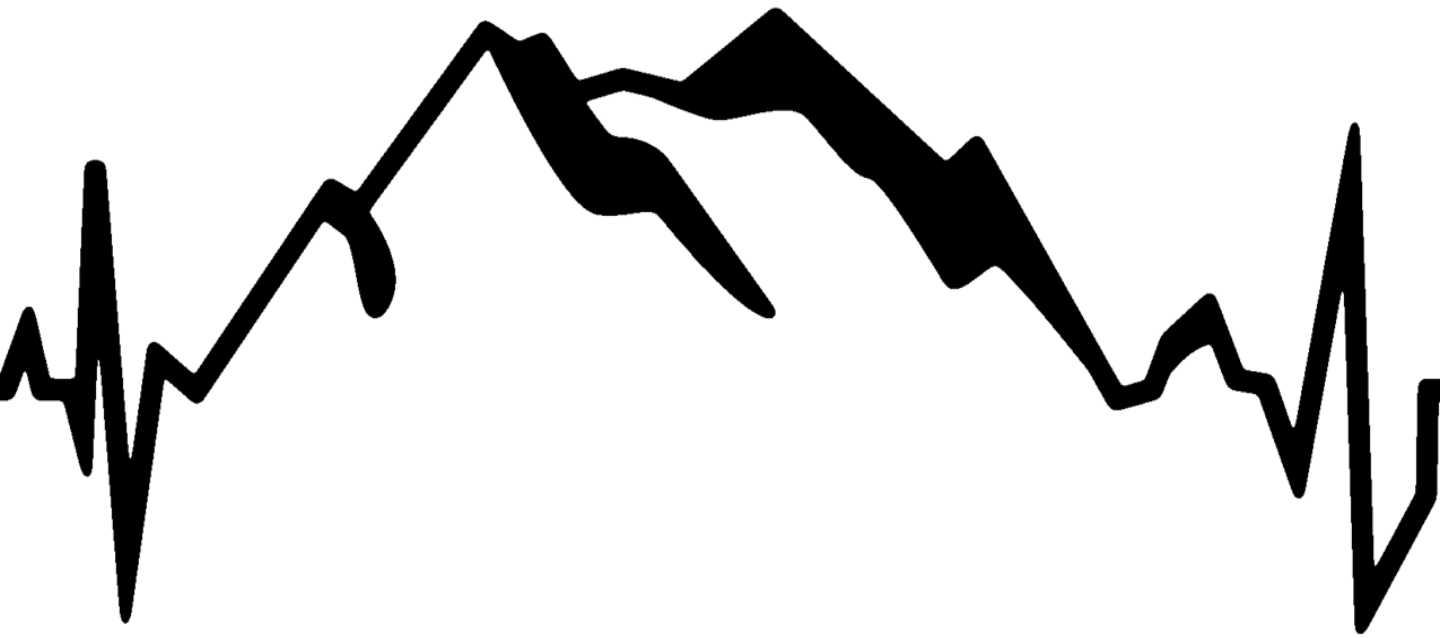
Chapter VII

General discussion



Adapted from
Itani N, Salinas CE, Villena M, Skeffington KL, Beck C, Villamor E, Blanco CE, Giussani DA. The highs and lows of programmed cardiovascular disease by developmental hypoxia: studies in the chicken embryo.
J Physiol. 2017 [Epub ahead of print]

It is now established that adverse conditions during pregnancy can trigger a fetal origin of cardiovascular dysfunction and/or increase the risk of heart disease in later life. Sub-optimal environmental conditions during early life that may promote the development of cardiovascular dysfunction in the offspring include alterations in fetal oxygenation and nutrition as well as fetal exposure to stress hormones, such as glucocorticoids. There has been growing interest in identifying the partial contributions of each of these stressors to programming of cardiovascular dysfunction. However, in humans and in many animal models this is difficult, as the challenges cannot be disentangled. By using the chicken embryo as an animal model, science has been able to circumvent a number of problems. In contrast to mammals, in the chicken embryo the effects on the developing cardiovascular system of changes in oxygenation, nutrition or stress hormones can be isolated and determined directly, independent of changes in the maternal or placental physiology. In this review, we summarise the findings of the present thesis and of other studies that have exploited the chicken embryo model to determine the effects on prenatal growth, cardiovascular development and pituitary-adrenal function of isolated chronic developmental hypoxia.

Chronic hypoxia and fetal growth: Studies in the chicken embryo

Incubation of fertilised eggs laid by sea level hens at high altitude (3600m) reduced fetal PO_2, increased haematocrit and reduced embryonic weight by 45% at the end of incubation period (Chapter II, Chapter III, Figure 1A). Importantly, the reduction in embryonic weight persisted when it was expressed relative to the egg mass at the start of the incubation period, to account for very real differences in egg size laid by sea level hens compared to high altitude hens (Figure 1B). Incubation of eggs laid by high altitude hens at high altitude also led to significant fetal growth restriction, although the change was smaller (by 22.2%) compared to the effect of high altitude on chicken embryos of sea level hens. This protection on prenatal growth by prolonged residence at high altitude has also been reported for human Andean populations (Giussani *et al.*, 2001; Moore *et al.*, 2004; Soria *et al.*, 2013), coining the effect as *the Andean curse on the conquistadors* (Giussani, 2007). Further data revealed that high-altitude induced growth restriction of fertilised eggs laid by sea level hens was prevented by supplementing the incubator with oxygen at pressures to equate sea level conditions (chapter II). Importantly, chicken embryos incubated at high altitude were asymmetrically growth restricted (Figure 1C), with a marked

increase in the brain weight and head diameter relative to the embryonic body mass (chapters II and III). It is well established that the asymmetric growth restriction is a consequence of fetal blood flow redistribution in response to chronic hypoxia; commonly referred to as the fetal brain sparing effect (Giussani & Davidge, 2013; Allison *et al.*, 2016; Giussani, 2016). This fetal phenotypic response to chronic fetal hypoxia is well conserved across species including the human, sheep and rat (Mulder *et al.*, 1998; Lang *et al.*, 2000; Ruijtenbeek *et al.*, 2000; Giussani *et al.*, 2001; Fowden *et al.*, 2006; Giussani *et al.*, 2007; Camm *et al.*, 2010), thereby supporting the chicken embryo as an appropriate and comparable animal model to study the consequences of chronic developmental hypoxia on asymmetric growth restriction prior to hatching.

The significant induction of embryonic growth restriction by developmental hypoxia has also been reported by us and others using incubation of chicken embryos under isobaric (sea level atmospheric pressure) hypoxic conditions (Figure 1 and Table 1, Ruijtenbeek *et al.*, 2000; Dzialowski *et al.*, 2002; Miller *et al.*, 2002; Rouwet *et al.*, 2002; Villamor *et al.*, 2004; Sharma *et al.*, 2006; Giussani *et al.*, 2007; Wei *et al.*, 2007; Lindgren & Altimiras, 2009; Van der Sterren *et al.*, 2009; Zoer *et al.*, 2009; Salinas *et al.*, 2010; Zoer *et al.*, 2010a; Lindgren & Altimiras, 2011; Lindgren *et al.*, 2011; Sahan *et al.*, 2011; Moonen *et al.*, 2012; Itani *et al.*, 2016b). Varying magnitudes of the reduction in embryo weight in these studies may reflect differences in the humidity, temperature, the length and timing of hypoxia as well as the breed of chicken used. However, combined, past studies describing the significant effect of incubation of fertilised eggs under hypobaric (lower than normal atmospheric pressure) or isobaric hypoxic conditions in slowing embryonic growth add robust evidence to the literature to strongly support a role for isolated fetal hypoxia in mediating fetal growth restriction.

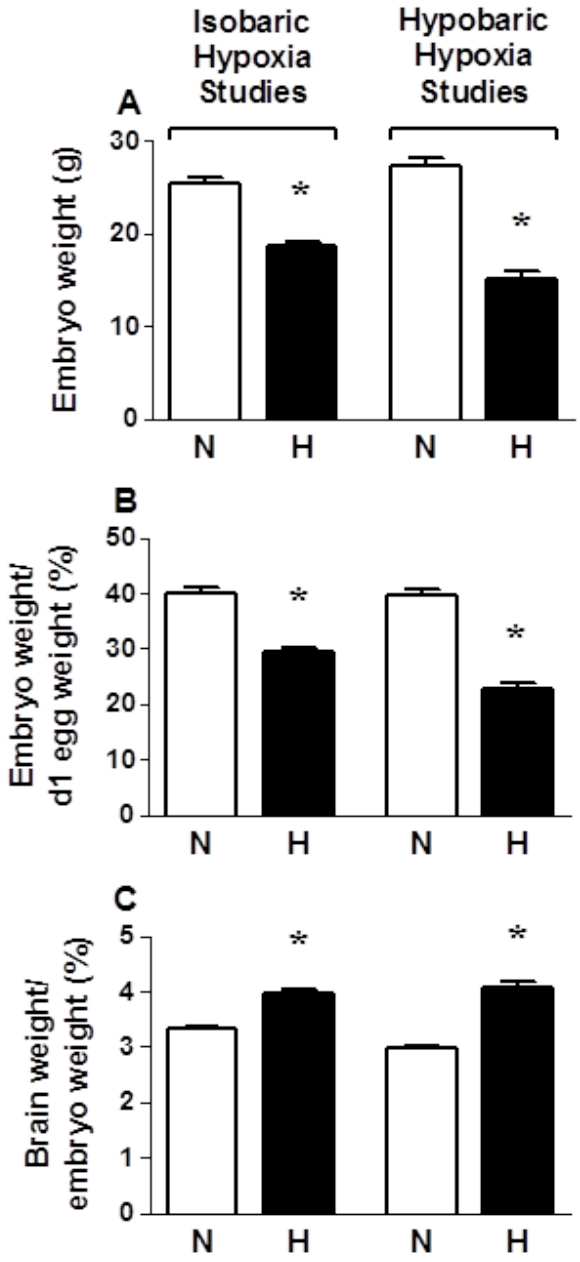


Figure 1. Fetal growth in the chicken embryo at day 19-20 of incubation. Values are mean ± S.E.M. at day 19-20 of absolute embryo weight (A), relative embryo weight (B), and brain weight relative to body weight (C) of chicken embryos incubated in either normoxia (N, n=21) or hypoxia (H, n=20) for isobaric hypoxia studies, and normoxia at sea level (N, n=31) or hypoxia at high altitude (H, n=16) for hypobaric hypoxia studies. *Significantly ($P<0.05$) different from corresponding control. Data adapted from chapter II; chapter III; Itani *et al.* (2016a) and Itani *et al.* (2016b).

Breed	Study design	Temp (°C)	Humidity (%)	Oxygen (%)		Body weight measured on	Body weight (g)		Difference (%)	Reference
				Control	Hypoxia		Control	Hypoxia		
Black Leghorn	Hypobaric hypoxia	38	60	20	13.7	d20	27.4 ±0.8	15.2 ±0.8	44.6	(Giussani *et al.*, 2007)
Black Leghorn	Hypobaric hypoxia	38	60	20	13.7	d20	28 ±1	15 ±1	46.4	(Salinas *et al.*, 2010)
Broiler Ross 308	Hypobaric hypoxia	37.5	55	20.7	18.5	d18	48.1 ±0.7	46.0 ±0.6	4	(Sahan *et al.*, 2011)
Bovans Brown	Isobaric hypoxia	37.9	45	21	14	d19	25.2 ±0.9	19.4 ±0.5	23	(Itani *et al.*, 2016b)
White Leghorn	Isobaric hypoxia from day 6	38	60	21	15	d19	25.4 ±0.6	21.9 ±0.4	13.8	(Ruijtenbeek *et al.*, 2000)
White Leghorn	Isobaric hypoxia from day 6	37.8	45	21	15	d19	29.9 ±5.6	25.3 ±5.5 SD	15.4	(Van der Sterren *et al.*, 2009)
White Leghorn	Isobaric hypoxia from day 6	38	60	21	15	d19	26.6 ±0.7	22.4 ±0.5	15.8	(Villamor *et al.*, 2004)
White Leghorn	Isobaric hypoxia	37.8	60	21	15	d19	28.0 ±2.9 SD	24.8 ±1.9 SD	11.4	(Moonen *et al.*, 2012)
White Leghorn	Isobaric hypoxia	37.8	45	21	15	d19	28.3 ±0.4 SD	23.7 ±0.6 SD	16.3	(Zoer *et al.*, 2010a)
White Leghorn	Isobaric hypoxia	37	60	21	15	d19	24.7 ±0.4	21.9 ±0.5	11.3	(Rouwet *et al.*, 2002)
White Leghorn	Isobaric hypoxia	38	60-70	21	15	d3.5	0.038 ±0.002	0.033 ±0.001	13.2	(Sharma *et al.*, 2006)
White Leghorn	Isobaric hypoxia	38.5	50-60	21	14	d15	12.2 ±1.7 SD	9.1 ±1.2 SD	25.5	(Miller *et al.*, 2002)
White Leghorn	Isobaric hypoxia from day 10	38.5	50-60	21	14	d15	12.2 ±1.7 SD	10.8 ±1.6 SD	10.8	(Miller *et al.*, 2002)
White Leghorn	Isobaric hypoxia between day 6-12	37.5	75-95	21	15	d18	23.4 ±0.5	20.7 ±0.5	11.5	(Dzialowski *et al.*, 2002)
White Leghorn	Isobaric hypoxia between day 12-18	37.5	75-95	21	15	d18	23.4 ±0.5	19.7 ±0.5	15.8	(Dzialowski *et al.*, 2002)
White Leghorn	Isobaric hypoxia from day 6	37.8	45	21	15	d19	29.2 ±0.3 SD	25.9 ±0.4 SD	11.3	(Zoer *et al.*, 2009)
Broiler	Isobaric hypoxia from day 6	37.8	45	21	15	d19	31.4 ±0.6 SD	25.6 ±0.5 SD	18.5	(Zoer *et al.*, 2009)
Broiler Ross 308	Isobaric hypoxia	37.8	45	20.95	14	d19	31.7 ±3.2 SD	21.5 ±2.8 SD	32.2	(Lindgren & Altimiras, 2009)
Broiler Ross 308	Isobaric hypoxia	37.8	45	21	14	d19	34.8 ±0.5	25.7 ±0.6	26.1	(Lindgren *et al.*, 2011)

Table 1. Studies showing a reduction in chicken embryo weight following exposure to chronic hypoxia. Body weight data are mean ± S.E.M unless otherwise stated. SD, standard deviation.

Chronic hypoxia and the fetal HPA axis: Studies in the chicken embryo

It is well established that the hypothalamo-pituitary-adrenal (HPA) axis is functional long before hatching in the chicken embryo (Woods *et al.*, 1971; Wise & Frye, 1973; Kalliecharan & Hall, 1974; Jenkins & Porter, 2004). Consequently, possible effects of hypoxia on the developing HPA axis can be isolated using this model. Within the few studies addressing HPA function in the chronically hypoxic chicken embryo, we reported (chapter IV) that incubation at high altitude of fertilised eggs laid by sea level hens significantly increased plasma ACTH concentrations by embryonic day 20, while circulating levels of corticosterone in these embryos were markedly reduced compared to chicken embryos of sea level hens incubated at sea level. In addition, incubation at high altitude of eggs laid by high altitude hens produced similar changes in the levels of fetal plasma ACTH and corticosterone. Moreover, supplementing sea level eggs with oxygen during incubation at high altitude completely prevented the observed changes in ACTH and corticosterone production, thereby supporting hypoxia rather than hypobaria in inducing the adrenocortical blunting. Correlation analysis revealed that the levels of plasma ACTH and corticosterone were positively related in sea level embryos incubated at sea level or at high altitude with oxygen supplementation. However, this correlation was no longer apparent in all other groups. Therefore, data in this study support previous human and mammalian studies reporting dissociated HPA activities in development complicated by chronic fetal hypoxia (Challis *et al.*, 1989; Hooper *et al.*, 1990; Harvey *et al.*, 1993; Murotsuki *et al.*, 1996; Stratford & Hooper, 1997). Importantly, the chicken embryo data showed that the plasma corticosterone-ACTH ratio was positively correlated to embryonic body weight and to chorioallantoic blood PO_2, providing further evidence for a direct relationship between changes in HPA activity and changes in oxygenation during embryonic development (chapter IV).

In line with our findings in the chicken embryo (chapter IV), a study using the sheep fetus demonstrated that development at high altitude promoted the enhanced processing of the precursor proopiomelanocortin into ACTH (Myers *et al.*, 2005). In addition, ACTH receptor expression and enzymatic capacity to synthesise cortisol were both reduced in the fetal sheep adrenal glands in the same experimental model (Myers *et al.*, 2005). In subsequent studies, Myers and Ducsay proposed that nitric oxide (NO) and leptin could be potential mediators of the inhibition of cortisol

synthesis in the adrenal glands of fetal sheep exposed to long-term high altitude hypoxia (Monau *et al.*, 2009; Myers & Ducsay, 2012). The authors suggested that leptin and NO limit the capacity of elevated fetal plasma ACTH levels to stimulate cortisol production in the chronically hypoxic fetus. In sheep, this prevents the premature induction of labour while allowing for the maintenance of the normal prepartum surge in fetal plasma cortisol that occurs close to term, which is essential for appropriate fetal maturation.

In addition to adrenocortical blunting, high altitude incubation of chicken embryos from hens native to sea level or to high altitude induced a significant increase in adrenal concentrations of both adrenaline and noradrenaline, and oxygen supplementation during high altitude incubation prevented this (chapter IV). Chronic hypoxia-induced sensitisation of the sympatho-adrenal medullary system has been reported in a range of animal models including the sheep, llama, rat and chicken (Roigas *et al.*, 1996; Simonetta *et al.*, 1997; Ruijtenbeek *et al.*, 2000; Gardner *et al.*, 2002; Llanos *et al.*, 2003; Lindgren & Altimiras, 2013). For instance, in the chicken embryo, chronic hypoxia sensitises cardiac β-adrenergic receptors (Lindgren & Altimiras, 2009, 2013). In addition, chronically hypoxic chicken embryos have increased noradrenaline levels and enhanced sympathetic innervation in the peripheral vasculature (Ruijtenbeek *et al.*, 2000). Importantly, there was a strong negative correlation between arterial PO_2 and adrenal catecholamine content in chicken embryos incubated at sea level and high altitude (chapter IV). These data therefore not only support a similar relationship between plasma noradrenaline levels and PO_2 reported in fetal sheep (Simonetta *et al.*, 1997), but provide further evidence for a direct effect of isolated developmental hypoxia on the reactivity of the sympathoadrenomedullary system.

Chronic fetal hypoxia and programmed cardiovascular risk: Studies in the chicken embryo and in the adult bird

It is widely established that adverse conditions during development not only promote IUGR and alterations in the HPA axis, but they also induce early origins of cardiovascular remodelling and dysfunction (see Gluckman *et al.*, 2008; Giussani & Davidge, 2013). In humans, IUGR pregnancy is associated with ventricular hypertrophy, and increased aortic stiffness and thickness in the offspring before and after birth (Veille *et al.*, 1993; Skilton *et al.*, 2005; Akira & Yoshiyuki, 2006; Cosmi *et al.*, 2009). Studies in rodent animal models have provided evidence to

support that the human IUGR-induced programming of cardiovascular remodelling is at least partly due to suspected chronic fetal hypoxia. For instance, hypoxic pregnancy induces similar thickening of the aortic wall and aortic stiffening in the rodent as well as ovine fetus (Williams *et al.*, 2005a; Camm *et al.*, 2010; Thompson *et al.*, 2011; Giussani *et al.*, 2012). Studies by Zhang and colleagues have also reported that hypoxic pregnancy in rodents leads to cardiac remodelling in the offspring (Bae *et al.*, 2003; Tong & Zhang, 2012). Aortic hypertrophy often precedes the clinical manifestation of hypertension, atherosclerosis and coronary heart disease (Arnett *et al.*, 1994). Consequently, the remodelling of the heart and major vessels early in life due to suspected chronic fetal hypoxia may increase the risk of developing cardiovascular disease in adulthood (Williams *et al.*, 2005b; Crispi *et al.*, 2010; Giussani *et al.*, 2012; Giussani & Davidge, 2013).

Again, studies using the chicken embryo have shown that hypoxic incubation can recapitulate the adverse cardiovascular phenotype described in studies either using mammalian animal models of chronic hypoxia or in human clinical studies of IUGR pregnancies, supporting that chronic fetal hypoxia is an important mechanism. The relatively large size of the chicken embryo at term (> 25 g on day 19, term is 21 days, Table 1) compared to the rodent fetus (< 4 g on day 20, term is 21 days, see Camm *et al.* (2010)) means that the chicken embryo model has the added advantage of facilitating study of cardiovascular function *in ovo* or in isolated organs, for instance using the Langendorff preparation (Itani *et al.*, 2016b) or the myograph (Ruijtenbeek *et al.*, 2003b; Villamor *et al.*, 2004; Van der Sterren *et al.*, 2009; Moonen *et al.*, 2012; Itani *et al.*, 2016a; Itani *et al.*, 2016b). Interestingly, and perhaps surprisingly, the chronology of cardiac development in the chicken is much more comparable to the human than is the rodent, in which cardiac development continues into the postnatal period (Sissman, 1970; Monie, 1976; Marcela *et al.*, 2012. See Figure 2). Combined, these advantages have convinced many investigators to use the chicken embryo as a useful animal model to isolate the effects of chronic hypoxia on cardiovascular development (Table 2). In chapter III, we reported that chicken embryos of both sea level and high altitude hens following incubation at high altitude have increased relative heart weight as well as relative left (LV) and right (RV) ventricular area and wall thickness). These embryos also had a higher aortic wall:lumen area ratio, indicating that developmental exposure to hypobaric hypoxia induced aortic hypertrophy prior to hatching. This morphometric analysis of the cardiovascular system of the high altitude chicken embryo has been

expanded recently to include analysis on cardiovascular morphology of chicken embryos exposed to isobaric hypoxia throughout the incubation period (Itani *et al.*, 2016b). In contrast to high altitude incubation, chronic isobaric hypoxia induced cardiac dilatation with reduced LV wall while enhancing LV lumen volume, yielding a marked reduction, rather than an increase, in the LV wall:lumen area ratio (Itani *et al.*, 2016b). Similarly, both dilatation as well as hypertrophy of the aorta in chronically hypoxic chicken embryos have been reported by independent groups (Figure 2 and 3. Rouwet *et al.*, 2002; Salinas *et al.*, 2010; Itani *et al.*, 2016b). The reasons underlying the differential effects of chronic hypobaric versus isobaric hypoxia of similar magnitude and duration on cardiovascular remodelling in the developing embryo are not clear at present.

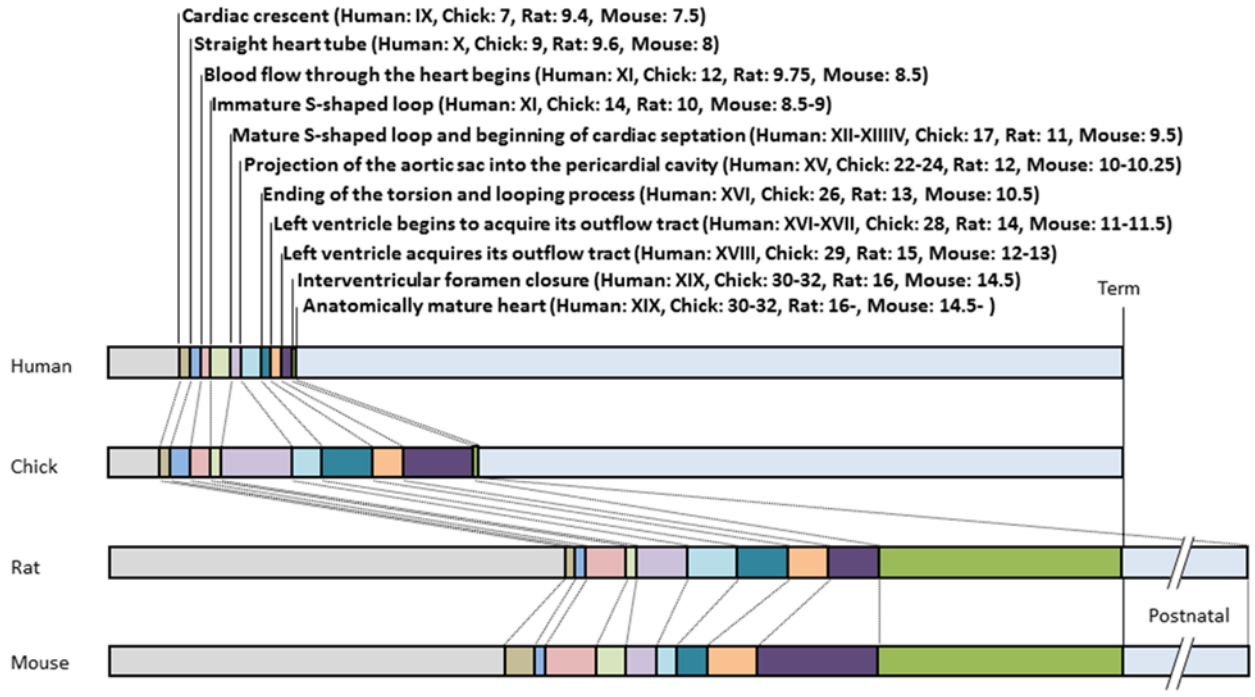


Figure 2. Comparison of the key stages of cardiac development in different animal models. Bars represent the proportion (%) of development. Roman and Arabic numerals refer to: Human Carnegie Stage (Streeter Horizons), Chick Hamburger and Hamilton stage, Rat and Mouse embryonic days. Term: Human, 38 weeks; Chick, 21 days; Rat, 21 days; Mouse, 19 days. While the main anatomical development of the heart is complete in the rat and mouse by day 16 and 14.5, respectively, the acquisition of mature septum, valves and tendinea cords is not yet complete until after birth (Marcela et al., 2012). Drawn from data provided in Sissman (1970) and Marcela *et al.* (2012).

Breed	Study design	Temp (C), Humidity (%)	Oxygen (%) N, H	Effects on cardiovascular function	Morphological and/or histochemical effects on the heart	Morphological and/or histochemical effects on vessels	Reference
Black Leghorn	Hypobaric hypoxia	38, 60	20, 13.7		Increased HW, LV and RV area and wall thicknesses relative to body	Increased aortic wall:lumen area ratio on embryonic day 20	(Salinas et al., 2010)
Black Leghorn	Hypobaric hypoxia		20, 13.7	Significantly lower systolic, diastolic and mean arterial pressure at 6 months of age. Increased HR and lower pulse interval in the males but not females			(Herrera et al., 2013)
Black Leghorn	Hypobaric hypoxia	38, 60	20, 13.7	Increased tricuspid pressure gradient. Reduced mitral valve pressure gradient in males	Thicker right ventricular wall	Increased pulmonary artery diameter in males	(Salinas et al., 2014)
Bovans Brown	Isobaric hypoxia	37.9, 45	21, 14	Reduced systolic pressure and higher diastolic pressure, impaired peripheral endothelial vasodilation	LV dilatation	Aortic dilatation	(Itani et al., 2016a; Itani et al., 2016b)
White Leghorn	Isobaric hypoxia from day 6	38, 60	21, 15	Reduced sensitivity of femoral artery to noradrenaline	Increased noradrenaline content	Higher perivascular sympathetic innervation in femoral artery	(Ruijtenbeek et al., 2000)
White Leghorn	Isobaric hypoxia from day 6	38, 60-70	21, 15	Impaired NO-dependent endothelial relaxation to Ach. Enhanced contractile response of the femoral artery to electrical stimulation 14-15 weeks post hatching		Perivascular hyperinnervation no longer present 14-15 weeks after hatching	(Ruijtenbeek et al., 2003a)
White Leghorn	Isobaric hypoxia from day 6	38, 60	21, 15	Impaired NO-dependent vasodilatation of the femoral artery on d19			(Ruijtenbeek et al., 2003b)
White Leghorn	Isobaric hypoxia from day 6	38, 60	21, 15	Impaired contractile function of the pulmonary artery. Impaired femoral arterial relaxation to ACh,	Increased heart mass and LV and RV wall thickness and areas relative to body		(Villamor et al., 2004)

Breed	Study design	Temp (C), Humidity (%)	Oxygen (%) N, H	Effects on cardiovascular function	Morphological and/or histochemical effects on the heart	Morphological and/or histochemical effects on vessels	Reference
White Leghorn	Isobaric hypoxia from day 6	37.8, 45	21, 15	Shortened ductus arteriosus. Enhanced DA contractile response to α-adrenergic agonists and impaired endothelial-dependent, -independent and β-adrenoreceptor agonist-induced relaxation			(Van der Sterren et al., 2009)
White Leghorn	Isobaric hypoxia from day 6	37.8, 45	21, 15	Endothelium-dependent relaxation of intrapulmonary arteries are not affected			(Zoer et al., 2009)
Broiler	Isobaric hypoxia from day 6	37.8, 45	21, 15	Endothelium-dependent relaxation of intrapulmonary arteries are not affected			(Zoer et al., 2009)
White Leghorn	Isobaric hypoxia	37.8, 45	21, 15	Hypoxia did not affect the Rho-kinase inhibitor hydroxyfasudil-induced relaxation			(Zoer et al., 2010)
White Leghorn	Isobaric hypoxia	37.8, 60	21, 15	Enhanced vascular response to noradrenaline on d19. Enhanced response to endothelin-1 on d15 and postnatal d1. Diminished response to ACh on d15.			(Moonen et al., 2012)
White Leghorn	Isobaric hypoxia	37, 60	21, 15	Reduced systolic pressure, enhanced peripheral arterial tone	Heart weight increased relative to body, reduced septum thickness	Hypertrophic growth of the aorta	(Rouwet et al., 2002)
White Leghorn	Isobaric hypoxia	37, 60	21, 15	LV systolic and diastolic dysfunction at d20 and 8 months post hatching	Dilated left ventricle with enhanced fibrosis, cardiomyocyte degeneration and disorganisation		(Tintu et al., 2009)
White Leghorn	Isobaric hypoxia	38, 60-70	21, 15	Reduced peak systolic pressure, systolic volume and cardiac output			(Sharma et al., 2006)

Breed	Study design	Temp (C), Humidity (%)	Oxygen (%) N, H	Effects on cardiovascular function	Morphological and/or histochemical effects on the heart	Morphological and/or histochemical effects on vessels	Reference
Broiler strain Ross 308	Isobaric hypoxia	37.8, 45	20.95, 14	Decreased density and enhanced sensitivity of β-AR at d19, followed by decreased sensitivity to β-AR stimulation by d35 post hatching	Increased relative heart mass in the embryo and in 14d old hatchlings, but the effect disappears by d35 post hatching		(Lindgren & Altimiras, 2009)
Broiler strain Ross 308	Isobaric hypoxia	37.8, 45	21, 14	Hypotension and enhanced βAR sensitivity			(Lindgren et al., 2011)
Broiler strain Ross 308	Isobaric hypoxia	37.8, 45	21, 14	Increased β1AR activity and in vivo systolic dysfunction	Increased relative heart mass and increased systolic lumen diameter		(Lindgren & Altimiras, 2013)
White Leghorn	Isobaric hypoxia	38, 60	21, 15	Blunted in vivo cardiovascular response to superimposed acute hypoxia and NO stimulation			(Iversen et al., 2014)
White Leghorn	Isobaric hypoxia	38, 60	21, 15	Reduced stroke volume and cardiac output, and impaired left ventricular contractility and relaxability	Increased relative heart and LV mass. Reduced expression of the genes involved in excitation-contraction coupling		(Jonker et al., 2015)
Broiler strain Ross 308	Isobaric hypoxia	37.8, 45	21, 14		Increased relative heart mass. No effect on cardiomyocyte density or size, indicating a reduction in the cell number by d19		(Osterman et al., 2015)

Table 2. Studies showing effects of chronic developmental hypoxia on the cardiovascular system in chicken embryos and /or in adult birds. BW, body weight; HW, heart weight; LV, left ventricle; RV, right ventricle.

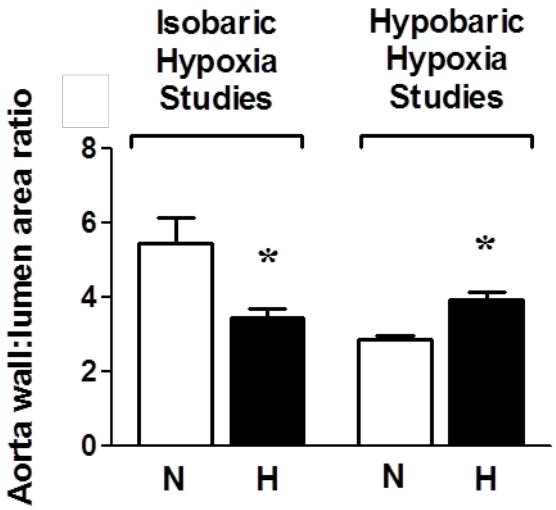


Figure 3. Aortic morphology in the chicken embryo at day 19-20 of incubation. Values are mean ± S.E.M at day 19-20 of aorta wall:lumen area ratio of chicken embryos incubated in either normoxia (N, n=10) or hypoxia (H, n=10) for isobaric hypoxia studies, and normoxia at sea level (N, n=8) or hypoxia at high altitude (H, n=7) for hypobaric hypoxia studies. *Significantly ($P<0.05$) different from corresponding control. Data adapted from chapter III and Itani *et al.* (2016b).

In addition to morphological remodelling of the developing heart, a number of studies in humans and mammalian animal models have reported that chronic fetal hypoxia has pronounced adverse effects on cardiac and vascular function in the offspring. In humans, IUGR pregnancy is associated with impaired cardiac contractility and ventricular filling, together with reduced ventricular ejection force in the offspring before and after birth (Rizzo *et al.*, 1995; Gardiner *et al.*, 2001). Human epidemiological studies have also reported endothelial dysfunction in children and adults with low birth weight (Goodfellow *et al.*, 1998; Martin *et al.*, 2000; Leeson *et al.*, 2001). In line with observations in human IUGR offspring, there are now elegant non-human primate data derived from cardiac magnetic resonance imaging that reports IUGR also being associated with cardiac remodelling in young adult baboons (Kuo *et al.,* 2017a; Kuo *et al.,* 2017b). Studies in rodent mammalian animal models have also reported that chronic fetal hypoxia can programme cardiac and vascular dysfunction in later life (Thompson & Weiner, 1999; Kim *et al.*, 2005; Williams *et al.*, 2005a; Giussani *et al.*, 2012; Giussani & Davidge, 2013; Kane *et al.*, 2013).

Collectively, studies in the chicken embryo suggest that cardiovascular dysfunction in children and in mammalian animal models of IUGR pregnancy may again be attributable to chronic fetal hypoxia. Thus, incubation of chicken embryos under hypoxic conditions was also associated with reduced left ventricular ejection fraction and contractility, and diminished left ventricular developed pressure, all indicative of significant systolic dysfunction (Rouwet *et al.*, 2002; Sharma *et al.*, 2006; Tintu *et al.*, 2009; Jonker *et al.*, 2015; Itani *et al.*, 2016b). Several candidate pathways may contribute to the hypoxia-induced cardiovascular dysfunction, including those involving VEGF (Tintu *et al.*, 2009; Moonen *et al.*, 2012; Itani *et al.*, 2016b) and Rho-kinase (Zoer *et al.*, 2010a). Exposure to chronic hypoxia increased both gene and protein expressions of VEGF in the embryonic heart, and systemic administration of recombinant VEGF mimicked the hypoxia-induced cardiac dilatation (Tintu *et al.*, 2009; Itani *et al.*, 2016b). Itani and colleagues have also reported that hypoxic incubation increases indices of oxidative stress, reduces the expression and activity of endogenous antioxidant enzymes and impairs levels of nitric oxide species in the chick embryo heart by the end of the incubation period (Itani *et al.*, 2016 a; Itani *et al.*, 2016b).

Incubation of chicken embryos under hypoxic conditions can also recapitulate impaired vasodilatation and/or enhanced contractile responses in pulmonary and systemic arteries in response to pharmacological or electrical stimulation (Table 2, Ruijtenbeek *et al.*, 2003b; Villamor *et al.*, 2004; Van der Sterren *et al.*, 2009; Moonen *et al.*, 2012; Itani *et al.*, 2016a; Itani *et al.*, 2016b). Endothelial function has been mainly studied by the use of acetylcholine (ACh). In the chicken embryo, ACh induced an endothelium-dependent and, at least partially, NO-mediated relaxation of pulmonary (Villamor *et al.*, 2002), femoral (le Noble *et al.*, 2000; Villamor *et al.*, 2002), mesenteric (Moonen & Villamor, 2011), and carotid arteries (le Noble *et al.*, 2000), as well as in the ductus arteriosus (Agren *et al.*, 2008). Interestingly, chronic isobaric hypoxia led to impairment of ACh-induced relaxation in the systemic (Ruijtenbeek *et al.*, 2003a; Ruijtenbeek *et al.*, 2003b; Villamor *et al.*, 2004; Van der Sterren *et al.*, 2009; Moonen *et al.*, 2012) but not in the pulmonary arteries (Villamor *et al.*, 2004). In contrast, chronic hypobaric hypoxia impaired endothelium-independent relaxation in the ductus arteriosus (Van der Sterren *et al.*, 2009), did not affect it in femoral and pulmonary arteries (Ruijtenbeek *et al.*, 2003a; Ruijtenbeek *et al.*, 2003b; Villamor *et al.*, 2004) and increased it in mesenteric arteries (Moonen *et al.*, 2012). Altogether, this suggests that the effects

of hypoxia in endothelium-dependent and –independent relaxation in chicken embryo vessels are strongly vascular bed-dependent.

Fewer studies have investigated the consequence of hypoxic incubation on the cardiovascular system of the adult bird (Table 2). Chronically instrumented adult chickens raised from eggs incubated at high altitude were significantly hypotensive with lower systolic and diastolic arterial pressures (chapter V). Adult chickens raised from eggs incubated at high altitude also showed echocardiographic indices of pulmonary hypertension and right heart dysfunction, relative to birds raised from eggs incubated at sea level (chapter VI). One important consideration for studies of cardiovascular function at high altitude in humans and experimental animals at adulthood is that the effects on cardiovascular dysfunction triggered by post-natal *versus* pre-natal hypoxia cannot be disentangled. However, studies from independent laboratories have reported that adult birds raised in a normoxic environment but incubated under hypoxic conditions do indeed show altered contractile and relaxant responses in the peripheral vasculature (Ruijtenbeek *et al.*, 2003a; Ruijtenbeek *et al.*, 2003b), systolic and diastolic dysfunction and marked increases in indices of myocardial fibrosis (Tintu *et al.*, 2009; Lindgren & Altimiras, 2013). These studies therefore support an effect of hypoxia during the incubation period rather than after hatching as the relevant stimulus in programing future cardiovascular risk at adulthood.

There is growing evidence for the importance of addressing sex differences in the programming of cardiovascular disease by adverse developmental conditions (Gilbert & Nijland, 2008; Aiken & Ozanne, 2013). The chicken embryo model offers a cost-effective experimental means to address some of these questions. Indeed, incubation of eggs at high altitude has been reported to have differential effects on cardiovascular function in male and female adult chickens (chapter V). Relative to sea level controls, hens that were incubated and raised at high altitude displayed an increased cardiac baroreflex gain, while this was significantly impaired in cockerels which were incubated and raised at high altitude (chapter V). Pre- and post-hatching development of chickens at high altitude is also associated with echocardiographic indices of pulmonary hypertension at adulthood in a highly sex-dependent manner (Chapter VI). Male but not female chickens incubated and reared at high altitude had significantly greater right ventricular wall thickness during systole and diastole than their same sex sea level counterparts. Further, the tricuspid pressure gradient was greatly enhanced in highland male and female

chickens relative to sea level controls. However, the increment in the tricuspid pressure gradient and the pulmonary artery diameter was significantly greater in highland cockerels than in highland hens. In fact, pre- and post-hatching development at high altitude did not affect the pulmonary artery diameter significantly in female chickens (chapter VI)

Summary and perspectives

In contrast to observations in humans and experiments in mammalian animal models, the direct effects of prenatal hypoxia on the individual can be isolated using the chicken embryo. Importantly, these effects on the embryonic and adult bird are independent of effects of hypoxic exposure on the maternal physiology, on the release of placental hormones and on the quality of the milk for lactation, as in mammalian species. The chicken compared to the rat or the mouse is a precocial rather than altricial species. Therefore, the temporal developmental trajectory of cardiovascular structure and function is also much more similar between chickens and humans. Data generated from chicken embryo incubations under hypoxic conditions are now beginning to reveal cellular and molecular mechanisms through which chronic hypoxia directly affects growth and the HPA axis during prenatal development and the setting of a future risk of cardiovascular, metabolic or endocrine disease. An additional advantage of the chicken embryo model is that it is high throughput and cost-effective. Therefore, interventional strategies to protect against growth restriction and the developmental programming of cardiovascular disease by chronic hypoxia can be tested in parallel, in different doses and at varying times of administration within one experimental design. Recent studies have reported that treatment of hypoxic incubations with antioxidants or agents that increase NO bioavailability, such as with melatonin or sildenafil (Itani *et al.*, 2016a; Itani *et al.*, 2016b), can not only protect but rescue the cardiovascular phenotype of the offspring even when therapy is started long after the induction of chronic developmental hypoxia. The latter is an important advance for human translational therapy, as in the clinical setting, IUGR resulting from chronic fetal hypoxia in adverse pregnancy needs to be diagnosed before it can be treated.

Blunting of fetal basal adrenal cortical but not medullary function may be an appropriate homeostatic response to prolonged periods of fetal hypoxia to protect sensitive tissues from sustained elevations in plasma glucocorticoid levels (Ducsay,

1998; Myers *et al.*, 2005). Conversely, sensitisation of the sympathoadrenal system may maintain appropriate glucogenic capacity during fetal development under conditions of chronic fetal hypoxia. The biological trade-offs of the divergent adaptations in adrenal responses may yield newborns with adrenocortical suppression and adrenergic hyper-reactivity. Both will have clear consequences for the programming of cardio-metabolic and endocrine dysfunction in later life (Reynolds *et al.*, 2001; Peyronnet *et al.*, 2002; Kajantie *et al.*, 2003; Watterberg *et al.*, 2004) and this warrants further investigation.

The chicken embryo is a valuable model for the study of the pathophysiological effects not only of chronic but also of acute hypoxia. The fetus can be exposed to acute hypoxic challenges, which if severe can lead to injury or death (Maltepe & Saugstad, 2009; Vento *et al.*, 2012; Giussani, 2016). Reoxygenation following exposure to hypoxia may exacerbate the hypoxic damage (Maltepe & Saugstad, 2009; Vento *et al.*, 2012). Either in the mammalian fetus or the chicken embryo the cardiovascular responses to acute hypoxia includes a redistribution of the cardiac output away from the periphery towards high priority organs such as the heart, brain, and adrenal glands (Giussani *et al.*, 1993; Mulder *et al.*, 2000; Mulder *et al.*, 2002; Giussani, 2016). An important component of hypoxia-induced flow redistribution is mediated by increased release of catecholamines (Mulder *et al.*, 2000; Mulder *et al.*, 2001; Mulder *et al.*, 2002; Fletcher *et al.*, 2006), whose effects are modulated by the different responsiveness of the vascular beds. However, hypoxia-mediated redistribution of blood flow is also accomplished by a local vascular effect, which results in contraction or relaxation depending on the blood vessel. Hypoxia-induced contraction of isolated pulmonary artery (Zoer *et al.*, 2009; van der Sterren *et al.*, 2011; Moreno *et al.*, 2014) and mesenteric artery (Brinks *et al.*, 2016), and hypoxic relaxation of isolated femoral artery (Zoer *et al.*, 2010b; van der Sterren *et al.*, 2011) and ductus arteriosus (Agren *et al.*, 2007; Greyner & Dzialowski, 2008; Cogolludo *et al.*, 2009; van der Sterren *et al.*, 2011; Van der Sterren *et al.*, 2014) have been described in chicken embryos**.** In addition, the effects of chronic *in ovo* hypoxia on the *ex vivo* responsiveness of these vessels to acute hypoxia as well as to vasoactive mediators have been extensively studied (Van der Sterren *et al.*, 2009; Zoer *et al.*, 2009; Zoer *et al.*, 2010b; Lindgren *et al.*, 2011; Moonen *et al.*, 2012). However, the effects of acute *in ovo* hypoxia-reoxygenation on vascular reactivity have not been yet characterized and warrant further investigation.

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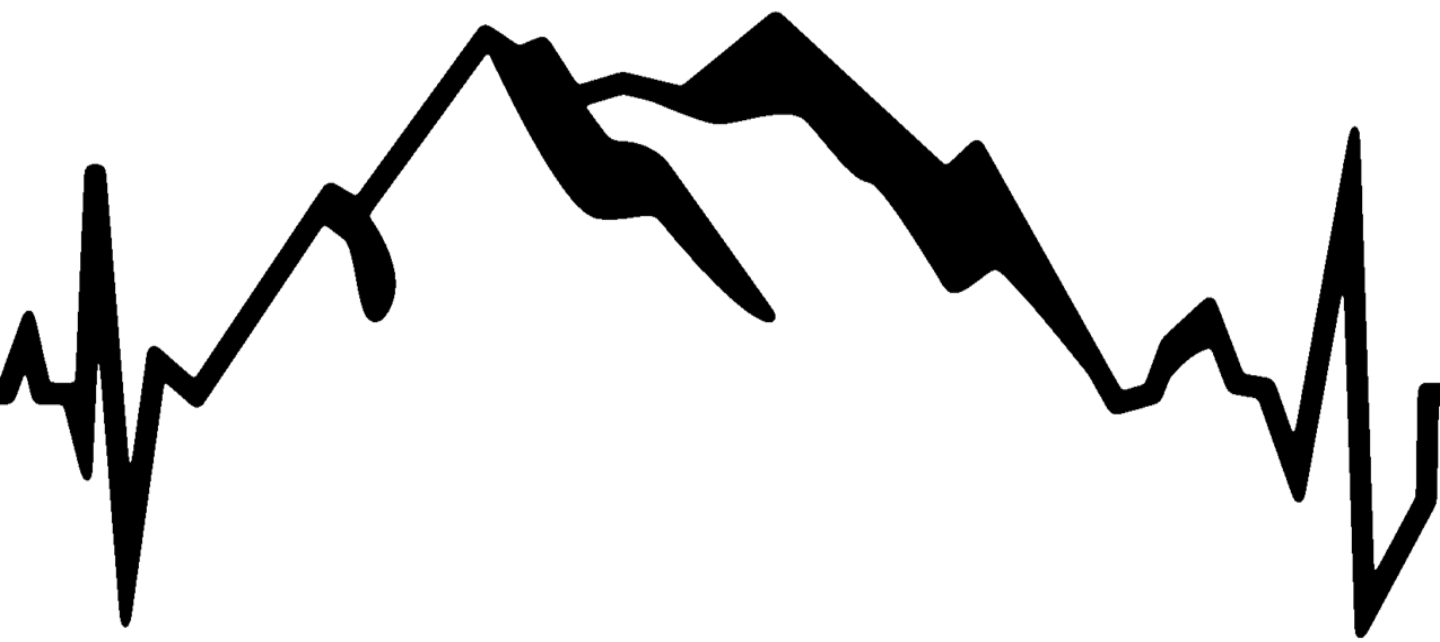
Summary



Summary

The present thesis is a collection of studies designed to isolate the effects of chronic fetal hypoxia on fetal growth, fetal cardiovascular and endocrine development and programming of future cardiovascular dysfunction in the adult offspring, using the chicken as an animal model. The combination of high altitude exposure with the use of the chicken embryo model is ideal as it permits investigation of the direct effects of high altitude hypoxia on growth and on cardiovascular development completely independent of alterations in placental function, independent of changes in the maternal physiology and independent of any effects of socioeconomic factors.

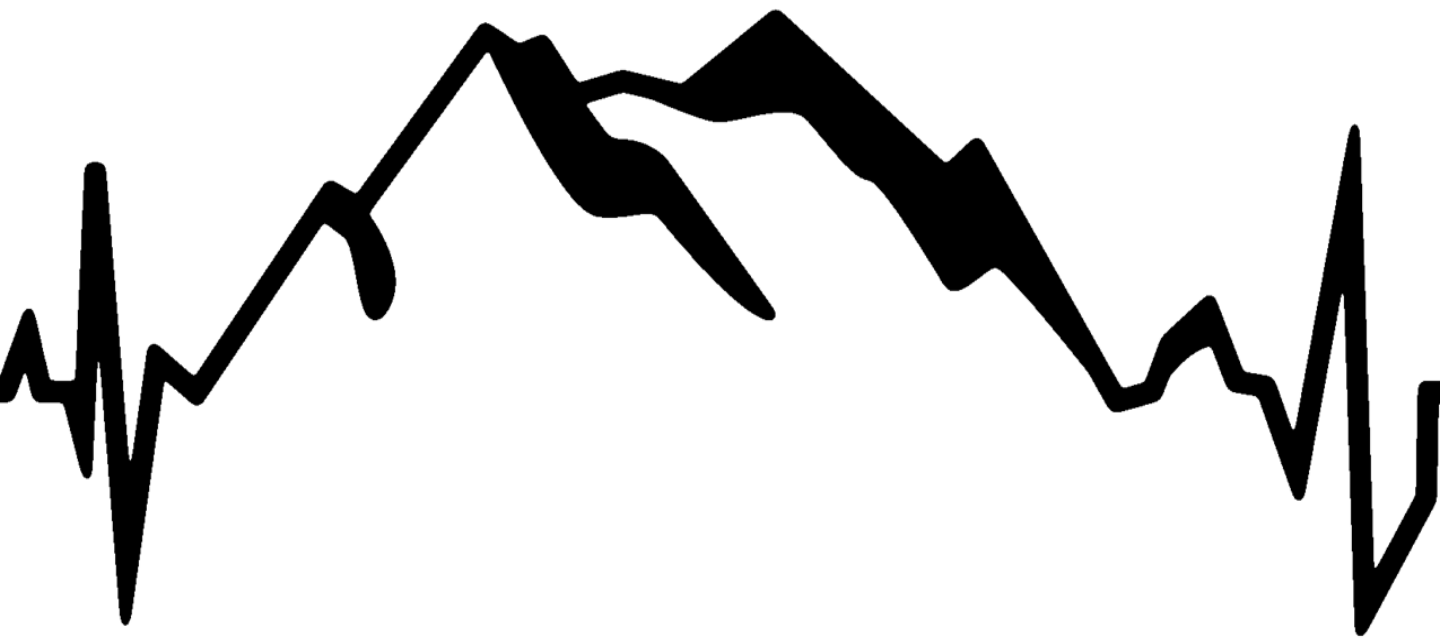
In **chapter II** (The role of oxygen in prenatal growth: studies in the chick embryo. *J Physiol*. 2007; 585:911-7), **chapter III** (Cardiac and vascular disease prior to hatching in chick embryos incubated at high altitude. *J Dev Orig Health Dis*. 2010; 1:60-6), and **chapter IV** (Adrenocortical suppression in highland chick embryos is restored during incubation at sea level. *High Alt Med Biol.* 2011; 12:79-87), we investigated the effects of high altitude hypoxia on chicken embryo growth and *in ovo* cardiovascular and endocrine development. For this purpose, we adopted an experimental design based on a three-prong approach using: (1) incubation at high altitude of fertilized eggs laid by sea-level hens; (2) incubation at sea level of fertilized eggs laid by high-altitude hens; and (3) incubation at high altitude of sea-level eggs with oxygen supplementation to equate sea level oxygen partial pressure. The data show that: (1) high-altitude hypoxia promotes embryonic cardiac and vascular disease already evident prior to hatching and that this is associated with growth restriction; (2) the effects can be prevented by increased oxygenation; and (3) the effects are different in embryos from sea-level or high-altitude hens. We conclude that fetal oxygenation, independent of maternal nutrition during development, has a predominant role in the control of fetal growth and cardiovascular development. Further, prolonged high altitude residence confers protection against the deleterious effects of hypoxia.

In **chapter V** (High altitude hypoxia and blood pressure dysregulation in adult chickens. J Dev Orig Health Dis. 2013; 4:69-76) and **chapter VI** (High-altitude hypoxia and echocardiographic indices of pulmonary hypertension in male and female chickens at adulthood. *Circ J.* 2014;78:1459-64), we isolated the long-term consequences of chronic hypoxic incubation of chick embryos on the systemic and pulmonary circulations of the adult bird. This was achieved using noninvasive

echocardiography as well as testing basal and stimulated cardiovascular function in the chronically instrumented adult bird. Additional specific points of interest were to determine whether there were any sex differences and whether any adverse effects of chronic hypoxia during the embryonic period could be ameliorated by generational exposure to hypobaric hypoxia in highland adapted chickens. We show that development at high altitude hypoxia lowers basal arterial blood pressure, alters baroreflex sensitivity, and induces pulmonary hypertension in a sex-dependent manner at adulthood.

Finally, in **chapter VII** (The highs and lows of programmed cardiovascular disease by developmental hypoxia: Studies in the chicken embryo. *J Physiol 2017*), we discuss and put into perspective the findings of this thesis. We summarise studies that have exploited the chicken embryo model to isolate the direct effects of chronic hypoxia on prenatal growth, cardiovascular and endocrine development and in triggering an increased risk of cardiovascular dysfunction and pathology at adulthood.

Samenvatting



Samenvatting

Het huidige proefschrift is een verzameling van studies die zijn ontworpen om de effecten te isoleren van chronische foetale hypoxie op de foetale groei, foetale cardiovasculaire en endocriene ontwikkeling en programmering van toekomstige cardiovasculaire dysfunctie bij volwassen nakomelingen, waarbij de kip als een diermodel wordt gebruikt. De combinatie van blootstelling op grote hoogte met het gebruik van het kippenembryo-model is ideaal omdat het onderzoek van de directe effecten van hypoxie op grote hoogte op groei en op cardiovasculaire ontwikkeling volledig onafhankelijk van veranderingen in placenta-functie mogelijk maakt, onafhankelijk van veranderingen in de maternale fysiologie en onafhankelijk van de effecten van sociaaleconomische factoren.

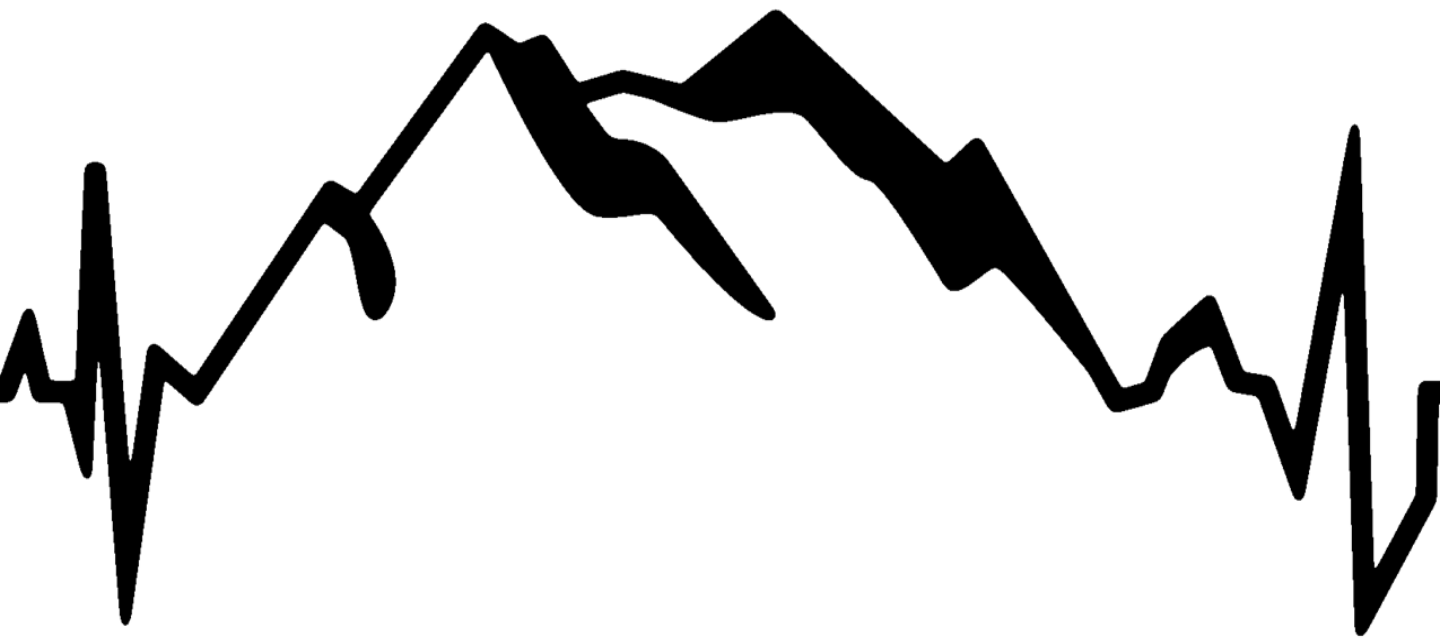
In hoofdstuk II (*J Physiol.* 2007; 585:911-7), hoofdstuk III (*J Dev Orig Health Dis.* 2010; 1:60-6) en hoofdstuk IV (*High Alt Med Biol.* 2011; 12:79-87), hebben we de effecten onderzocht van hypoxie op grote hoogte op de groei van kippenembryo en *in ovo* cardiovasculaire en endocriene ontwikkeling. Voor dit doel hebben we een experimenteel ontwerp aangenomen op basis van een drieledige aanpak met behulp van: (1) incubatie op grote hoogte van bevruchte eieren gelegd door kippen op zeeniveau; (2) incubatie op zeeniveau van bevruchte eieren gelegd door kippen op grote hoogte; en (3) incubatie op grote hoogte van eieren gelegd op zeeniveau met zuurstofsuppletie om de partiële zuurstofdruk op zeeniveau gelijk te stellen. De gegevens tonen aan dat: (1) hypoxie op grote hoogte embryonale hart- en vaatziekte reeds voorafgaand aan het uitkomen van de eieren bevordert en dat dit geassocieerd is met groeirestrictie; (2) de effecten kunnen worden voorkomen door verhoogde oxygenatie; en (3) de effecten verschillen in embryo's van zeeniveau- of hooggelegen kippen. We concluderen dat foetale oxygenatie, onafhankelijk van maternale voeding tijdens de ontwikkeling, een overheersende rol speelt in de beperking van foetale groei en cardiovasculaire ontwikkeling. Verder verleent langdurig verblijf op grote hoogte bescherming tegen de schadelijke effecten van hypoxie.

In hoofdstuk V (*J Dev Orig Health Dis.* 2013; 4:69-76) en hoofdstuk VI (*Circ J.* 2014;78:1459-64), isoleerden we de langetermijngevolgen van chronische hypoxische incubatie van kippenembryo's op de systemische en pulmonale circulaties van de volwassen vogel. Dit werd bereikt met behulp van niet-invasieve

echocardiografie en het testen van de basale en gestimuleerde cardiovasculaire functie bij de chronisch geïnstrumenteerde volwassen vogel. Aanvullende specifieke aandachtspunten waren om te bepalen of er sekse-verschillen waren en of eventuele nadelige effecten van chronische hypoxie gedurende de embryonale periode konden worden verlicht door generationele blootstelling aan hypobare hypoxie bij op hoogte aangepaste kippen. We laten zien dat ontwikkeling bij hypoxie op grote hoogte de basale arteriële bloeddruk verlaagt, de baroreflexgevoeligheid wijzigt en pulmonale hypertensie op een geslacht-afhankelijke manier induceert op volwassen leeftijd.

Tot slot bespreken we in hoofdstuk VII (*J Physiol 2017*) de bevindingen van dit proefschrift. We vatten studies samen die het kippenembryo-model hebben geëxploiteerd om de directe effecten van chronische hypoxie op de prenatale groei, cardiovasculaire en endocriene ontwikkeling te isoleren en om op volwassen leeftijd een verhoogd risico op cardiovasculaire disfunctie en pathologie te veroorzaken.

Valorization



Relevance

Cardiovascular disease kills 1 in 3 people. Every 3 minutes someone in the UK has a heart attack and 30% of these are fatal . Globally, around 17 million people die from cardiovascular disease each year. In 2010, the total costs of cardiovascular disease in the USA was $444 billion and this number is predicted to increase to 800 billion dollars by 2030. In the UK, according to the British Heart Foundation, the annual costs to the nation of premature death, lost productivity, hospital treatment and prescriptions relating to cardiovascular disease is of the order of £19 billion. Therefore, there is no question that cardiovascular dysfunction is a vast problem imposing a significant burden on every country's health and wealth (1).

It is widely accepted that our genes interact with traditional lifestyle factors, such as smoking, obesity and/or a sedentary lifestyle to promote an increased risk of heart disease (2). It has also become established that the gene-environment interaction early in life may be just as, if not more, important in 'programming' heart health and heart disease (3). This is unsurprising because our physiology is much more plastic and malleable during early life and the younger we are, the greater the impact the environment has upon us (3). These concepts have brought attention to adverse pregnancy and whether it can increase the risk of cardiovascular disease in the offspring. Accordingly, we now know that fetal development during suboptimal conditions can indeed trigger a fetal origin of cardiovascular dysfunction and increase the risk of chronic heart disease in the offspring in later life (3-5).

One of the most common adverse intrauterine conditions in complicated pregnancy is chronic fetal hypoxia (4-6). However, the contribution of chronic fetal hypoxia in promoting intrauterine growth restriction (IUGR) and programmed cardiovascular risk has been difficult to isolate for a number of reasons. For instance, it is established that high altitude pregnancy leads to IUGR (5). However, most high altitude populations are impoverished with significant maternal malnutrition (5). Therefore, the contribution of chronic fetal hypoxia versus chronic fetal undernutrition in slowing fetal growth and in setting future cardiovascular risk under these conditions is uncertain. The same applies to sea level pregnancy complicated by preeclampsia, placental insufficiency, gestational diabetes and even maternal obesity. All these conditions are associated with an increase in placental

vascular resistance (see 6), which will decrease oxygen as well as nutrient delivery to the growing fetus. This makes it impossible to disentangle the effects of chronic hypoxia versus chronic fetal undernutrition in promoting IUGR and programming of cardiovascular disease in human complicated pregnancy at sea level. Similarly, several experimantal studies including our own in mammalian animal models have shown that maternal chronic hypoxia during pregnancy can lead to IUGR and programme increased cardiovascular risk in the offspring (5,7). However, because experimental induction of chronic hypoxia in rodents can reduce maternal food intake and/or alter the quality of the maternal milk (5,7), the contribution of chronic fetal hypoxia versus chronic fetal and/or neonatal under-nutrition under these conditions, again, remains uncertain.

Innovation

By combining the chick embryo model with incubation at high altitude, this PhD thesis has been able to isolate the direct effects of chronic hypoxia due to high altitude on fetal growth, cardiovascular development, effects on the fetal stress axes and on the developmental programming of systemic and pulmonary vascular disease in the offspring. This is because in contrast to mammals, with the exception of monotremes, in the chicken the effects of changes in oxygenation on the embryo can be isolated and determined directly, independent of effects of chronic hypoxia on maternal food intake or the qulity of the maternal milk for lactation. In addition, chickens have a short incubation period (21 days), meaning embryonic studies can be conducted quickly. Maternal lives do not have to be taken in order to study the fetus and so for a fetal study the use of chickens could be described as more ethical, reducing the number of animal use, thereby complying with the 3R principle enshrined by the Home Office. The relatively large size of the chicken embryo at term (> 25g on day 19, term is 21 days) compared to the rodent fetus (< 4 g on day 20, term is 21 days) means that the chicken embryo model has the added advantage of facilitating study of cardiovascular function *in ovo* or in isolated organs, for instance by using the Langendorff preparation or the myograph (8). Interestingly, and perhaps surprisingly, the chronology of cardiac development in the chicken is much more comparable to the human than is the rodent, in which cardiac development continues into the postnatal period (8). Rodents are also polytocous, and the physiology of multiple pregnancies can be quite different to singletons, with different adaptations in place to support more

than one fetus (8). Combined, these advantages make the chicken embryo an animal model to isolate the effects of chronic hypoxia on cardiovascular development and programming of cardiovascular dysfunction.

The data provided by the chicken studies performed in this thesis adds considerably to the existing body of literature to demonstrate that chronic fetal hypoxia can programme a developmental origin of cardiovascular disease independent of any effects of undernutrition. This is important because of the similarities between the patterns of cardiovascular disease in hypoxic animals and the cardiovascular disease found in human offspring of complicated pregnancy. For example, IUGR human fetuses and neonates show decreased cardiac ejection force, diastolic dysfunction, decreased cardiomyocyte numbers, hypertrophic hearts and increased aortic stiffness (8-10). Some factors, including aortic stiffness and endothelial dysfunction have also been reported to be more prevalent in adults who were growth restricted as fetuses (11). Whilst IUGR is not necessarily caused by chronic fetal hypoxia alone, the fact that animal models which isolate the effects of chronic hypoxia produce similar phenotypes confirms that chronic fetal hypoxia is likely to be an important factor in the correlations between complicated human pregnancy, IUGR and an increased risk of cardiovascular disease later in life.

Target groups

This sound knowledge base generated in this PhD study has the potential to significantly facilitate the national and international development of the field, providing a significant academic advance within the field and between related disciplines. The PhD candidate and supervisors attend national and international scientific meetings routinely and are deeply involved in undergraduate and graduate teaching. Therefore, we will refer to this PhD project at least at three levels: 1) at international and national scientific meetings; 2) in laboratory meetings to postgraduate and graduate students and technicians, and 3) in lectures to undergraduate students, especially those in their final year who may be contemplating potential PhD research projects. It is important that investment is made to train new researchers, particularly in the vulnerable skills of fetal surgery and studying fetal cardiovascular function. To deliver maximum benefit in development of skills, capacity and capability, this PhD work will also serve as the basis to: 1) Provide cross-disciplinary training for emerging scientists in all

components of the thesis, spanning in vivo cardiovascular experimentation and stereological and histological analyses of the cardiovascular system; 2) Ensure that other scientists worldwide are exposed to the expertise available through international communications at scientific meetings; 3) Ensure that other scientists at the University of Cambridge, University of Maatricht and The Bolivian Institute for High Altitude Biology (IBBA) are exposed to the expertise available through maintained international collaboration. Thereore, this PhD also serves in outreach programmes.

The sound knowledge base generated in this PhD has also been published in high impact journals as original research articles. Combinations of original articles have been further disseminated in Topical Reviews and letters to the Editor of high impact journals. To further achieve excellence with impact, the University Communications Office at Cambridge has been alerted of the potential influence for human health of the scientific findings. Therefore, the work has had an immediate impact on clinicians, basic scientists, healthcare professionals, expectant mothers and their families in terms of providing them with information on the effects on the health of the offspring of high altitude pregnancy or reductions in fetal oxygenation in sea level complicated pregnancy. In addition, the data will benefit the design of therapeutic strategies to protect pregnancy complicated by chronic fetal hypoxia and/or improve fetal growth and development in adverse pregnancy with drugs that limit the adverse effects of fetal hypoxia, such as with spcific antioxidants. The proposed research is therefore likely to be of significant interest and benefit not only to researchers carrying out similar or related research in the field, but also to national and international researchers in other disciplines, such as experts in the metabolic syndrome, diabetes and gestational diabetes, scientists in biochemistry, pharmacology and nanotechnology, as well as cross-disciplinary teams in the pharmaceutical industry. Therefore, the data generated directly and indirectly has the potential to reduce the burden of cardiovascular disease throughout the life course, thereby having a major clinical, economic and societal impact on health.

Conclusion

It is now established that adverse conditions during pregnancy can trigger a fetal origin of cardiovascular dysfunction and/or increase the risk of heart disease in later life. Sub-optimal environmental conditions during early life that may promote

the development of cardiovascular dysfunction in the offspring include alterations in fetal oxygenation and nutrition as well as fetal exposure to stress hormones, such as glucocorticoids. There has been growing interest in identifying the partial contributions of each of these stressors to programming of cardiovascular dysfunction. However, in humans and in many animal models this is difficult, as the challenges cannot be disentangled. By using the chicken embryo as an animal model and intertwining this with high altitude incubation, this PhD has been able to circumvent a number of problems. The work has isolated an important direct contribution of chronic fetal hypoxia in regulating fetal growth, cardiovascular and endocrine development as well as in the programming of systemic and pulmonray vascular disease in the adult offspring.

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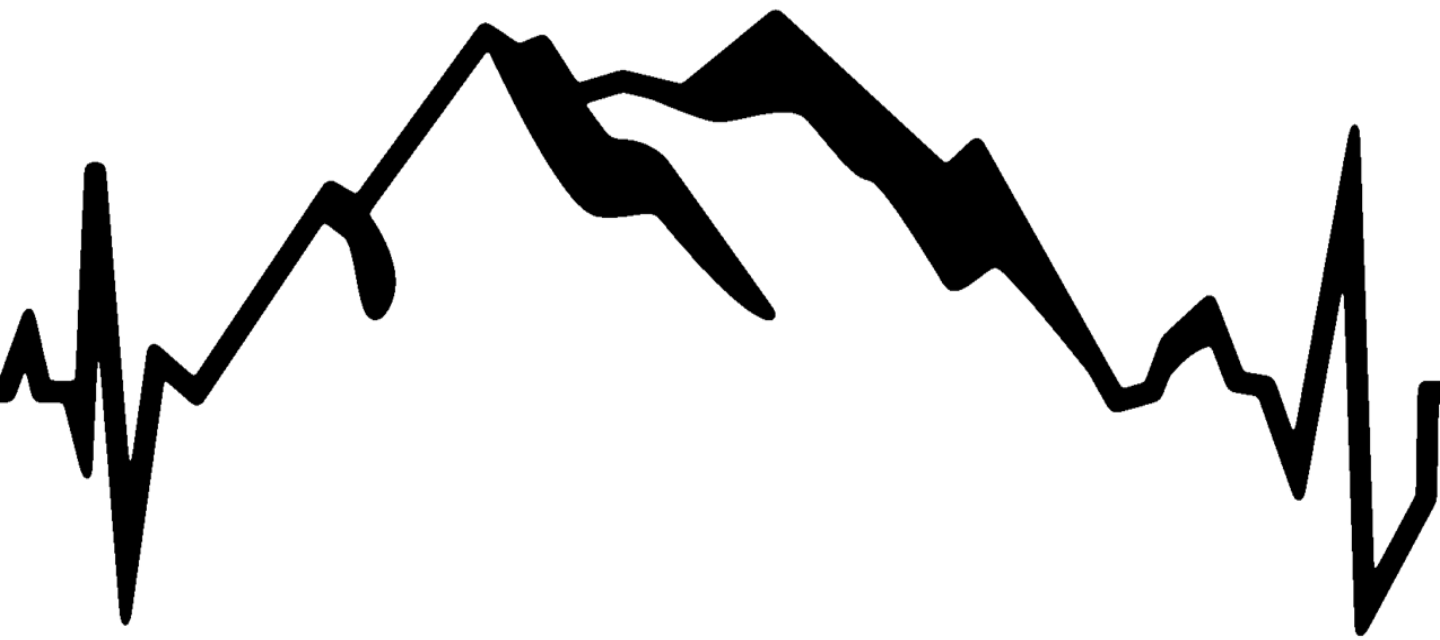
Acknowledgements



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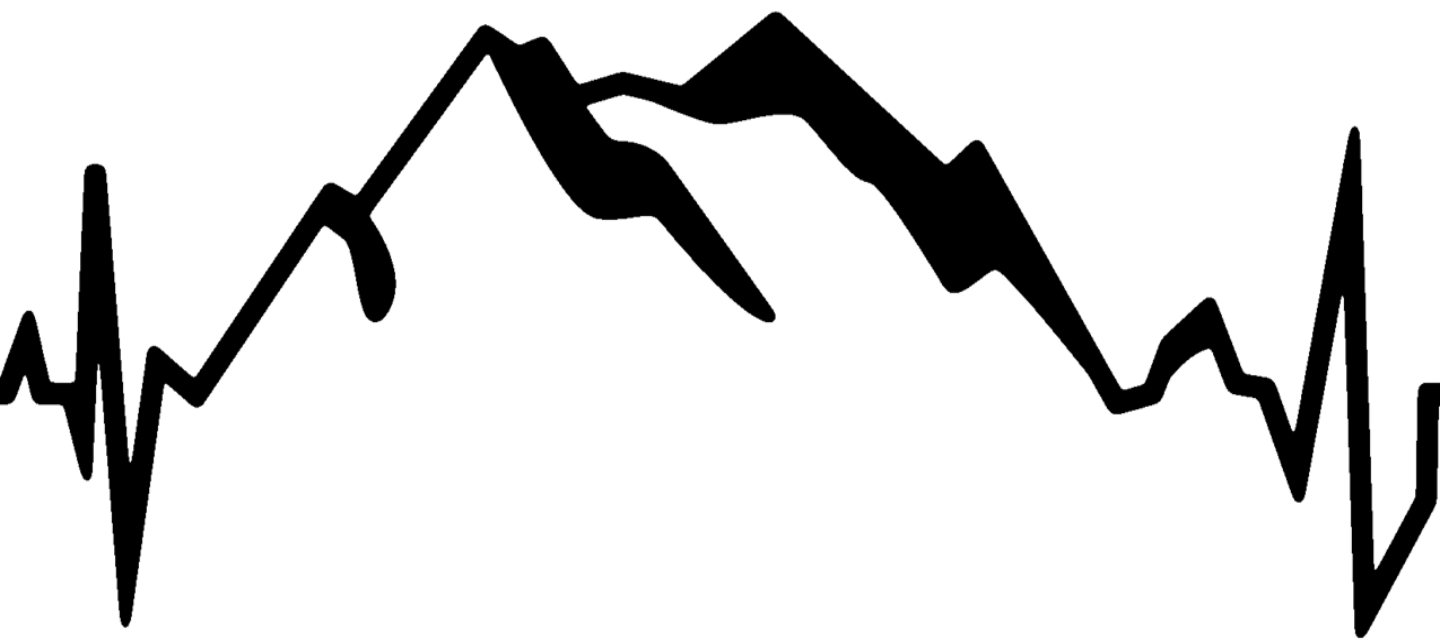
Curriculum Vitae



Carlos Eduardo Salinas Salmón achieved his Medical Doctor degree at the Universidad Mayor de San Andrés, Bolivia in 1992. He finished his specialization in Cardiology in 1997 at the same University. Since that time, he has been a researcher at the Bolivian Institute for High-Altitude Biology in La Paz, Bolivia. Since 1997 Carlos has been Assistant Professor at the department of Cardiology and in 2010 he completed a master in Health Education at the Universidad Mayor de San Andrés. Presently, he is the director of the Bolivian Institute for High-Altitude Biology in La Paz, Bolivia.

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