Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

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

Received October 29, 2013; revised manuscript received February 11, 2014; accepted February 27, 2014; released online April 16, 2014
Time for primary review: 21 days

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ISSN-1346-9843 doi:10.1253/circj.CJ-13-1329

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).

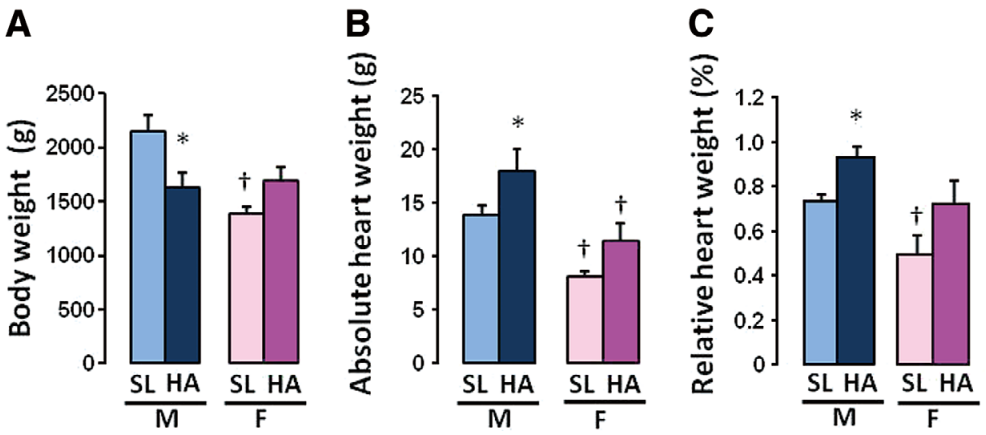

Figure 1. Bodyweight and heart weights in sea-level and high-altitude adult chickens. Values are the mean±SEM for bodyweight (**A**), absolute heart weight (**B**) and heart weight expressed as a percentage of bodyweight (**C**) in 7 males (M) and 7 female (F) chickens incubated, hatched and raised at 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

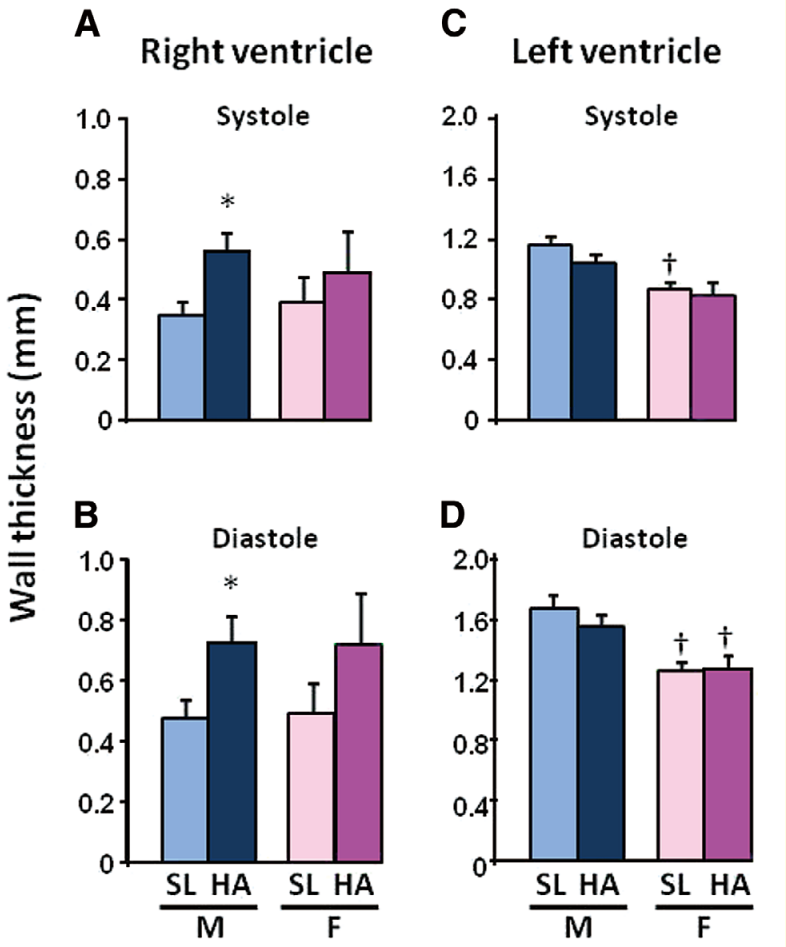

Figure 2. Ventricular wall thickness in sea-level and high-altitude adult chickens. Values are the mean±SEM for the wall thickness of the right (**A** and **B**) and left (**C** and **D**) ventricle during systole and diastole in 7 male (M) and 7 female (F) chickens incubated, hatched and raised at sea 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

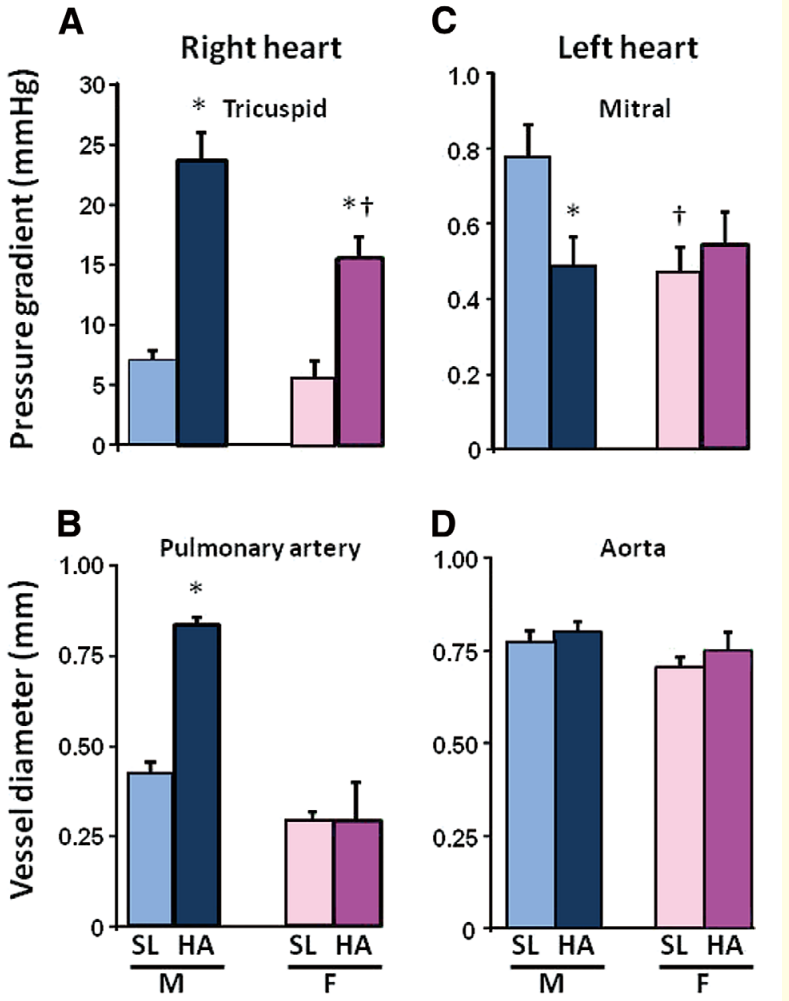

Figure 3. Ventricular pressure gradients and vessel diameter in sea-level and high-altitude adult chickens. Values are the mean±SEM for the ventricular pressure gradient and the main vessel diameter of the right (**A** and **B**) and left (**C** and **D**) heart in 7 male (M) and 7 female (F) chickens 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.

Acknowledgments

This work was supported by The British Heart Foundation and The Royal Society. Dino A. Giussani is the Professor of Cardiovascular Physiology & Medicine at the Department of Physiology Development & Neuroscience at the University of Cambridge, Professorial Fellow and Director of Studies in Medicine at Gonville & Caius College, a Lister Institute Fellow and a Royal Society Wolfson Research Merit Award Holder.

We thank Dr Diva Bellido, Dr Wilma Tellez, Mr Armando Rodriguez, Mrs Martha Aguilar, Mrs Loyola Riveros, Mr Wilmar Velasquez and Mr Didi Maquera at IBBA, La Paz, and Dr Regien Biesma, Dr Ginella, Dr Nioshi and Dr Roca for their help with these studies at CENETROP, Santa Cruz.

Conflict of Interest

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

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