© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb197970. doi:10.1242/jeb.197970

RESEARCH ARTICLE

Parabronchial remodeling in chicks in response to embryonic hypoxia

Lara do Amaral-Silva[1,2], Markus Lambertz[3,4], Fernando José Zara[5], Wilfried Klein[2,6], Luciane Helena Gargaglioni[1,2] and Kênia Cardoso Bícego[1,2,*]

ABSTRACT

The embryonic development of parabronchi occurs mainly during the second half of incubation in precocious birds, which makes this phase sensitive to possible morphological modifications induced by O_2 supply limitation. Thus, we hypothesized that hypoxia during the embryonic phase of parabronchial development induces morphological changes that remain after hatching. To test this hypothesis, chicken embryos were incubated entirely (21 days) under normoxia or partially under hypoxia (15% O_2 during days 12 to 18). Lung structures, including air capillaries, blood capillaries, infundibula, atria, parabronchial lumen, bronchi, blood vessels larger than capillaries and interparabronchial tissue, in 1- and 10-day-old chicks were analyzed using light microscopy-assisted stereology. Tissue barrier and surface area of air capillaries were measured using electron microscopy-assisted stereology, allowing for calculation of the anatomical diffusion factor. Hypoxia increased the relative volumes of air and blood capillaries, structures directly involved in gas exchange, but decreased the relative volumes of atria in both groups of chicks, and the parabronchial lumen in older chicks. Accordingly, the surface area of the air capillaries and the anatomical diffusion factor were increased under hypoxic incubation. Treatment did not alter total lung volume, relative volumes of infundibula, bronchi, blood vessels larger than capillaries, interparabronchial tissue or the tissue barrier of any group. We conclude that hypoxia during the embryonic phase of parabronchial development leads to a morphological remodeling, characterized by increased volume density and respiratory surface area of structures involved in gas exchange at the expense of structures responsible for air conduction in chicks up to 10 days old.

KEY WORDS: Incubation, Blood capillary, Air capillary, Lung, Blood–gas tissue barrier, Chicken

INTRODUCTION

The adequate supply of oxygen is an absolute requirement for the survival of aerobic tissues. Birds may face low oxygen levels (hypoxia) in natural environments (such as at high altitude and in burrows), during diving, and under pathological conditions (Ramirez et al., 2007). Even within the egg at the end of incubation, a deficit in oxygen delivery is normally observed when the chicken embryo increases its metabolic rate by up to 60% but the gas exchange through the egg shell is not sufficiently increased to supply enough oxygen (Visschedijk, 1968; Freeman and Misson, 1970; Szdzuy et al., 2008).

Chronic hypoxia is related to morphological changes affecting the pulmonary system, which may lead to diseases such as ascites (pulmonary hypertension) (Hislop and Reid, 1976; Rabinovitch et al., 1979; Stenmark and McMurtry, 2005; Rabinovitch, 2012). In contrast, transcriptional activators involved in oxygen homeostasis can induce gene responses that might promote cellular modifications in order to increase oxygen delivery or facilitate metabolic adjustments to hypoxia (Semenza, 2000; Semenza, 2012).

During embryonic development, contrasting morpho-physiological changes induced by chronic hypoxia have been demonstrated in chickens. On the one hand, there are data on decreased organ mass proportional to reduced embryonic body mass (Azzam and Mortola, 2007; Chan and Burggren, 2005; Zhang and Burggren, 2012), reduced metabolic rate (Bjønnes et al., 1987; Mortola and Labbè, 2005; Azzam et al., 2007; Lourens et al., 2007; Mortola and Cooney, 2008) and delayed ductus arteriosus maturation (Copeland and Dzialowski, 2008). On the other hand, hypoxia can lead to increased O_2 transport in the blood (Baumann et al., 1983), intensified chorioallantoic membrane vascularization (Xu and Mortola, 1989; Tazawa et al., 1971; Ruijtenbeek et al., 2000; Strick et al., 1991) and greater lung mass (Xu and Mortola, 1989).

The consequences of hypoxia on the morphological and physiological development is, however, dependent on the specific embryonic stage of exposure. This is because there are critical windows when environmental stressors can have lasting effects and alter the trajectory of ontogenetic development (Chan and Burggren, 2005). The first half of embryonic development is considered a critical window for the embryo's survival to hypoxia, whereas the second half of incubation is related to compensatory responses of key organs to low O_2 (Zhang and Burggren, 2012). In this context, the main development and differentiation of the parabronchi, the organ for gas exchange in post hatching birds, occurs during the second half of incubation in chickens (Maina, 2003). The lumen of the parabronchi starts to develop and proliferate, connecting the secondary bronchi, on the eighth day of incubation. The atria are visible not earlier than day 15 and the infundibula on day 16; the air capillaries form and connect with blood capillaries on day 18 (Maina, 2003; Mortola, 2009). On the day of hatching (day 21), the lungs are fully formed and functional, and the blood–gas tissue barrier becomes extremely thin (Maina, 2003). Based on the ontogeny of the parabronchial structures, a reduced O_2 supply specifically during the main phase of their

[1]Department of Animal Morphology and Physiology, College of Agricultural and Veterinary Sciences, São Paulo State University, Unesp. Jaboticabal, São Paulo 14884-900, Brazil. [2]National Institute of Science and Technology – Comparative Physiology (INCT- Fisiologia Comparada), UNESP-Jaboticabal, São Paulo 14884-900, Brazil. [3]Institut für Zoologie, Rheinische Friedrich-Wilhelms-Universität Bonn, 53115 Bonn, Germany. [4]Sektion Herpetologie, Zoologisches Forschungsmuseum Alexander Koenig, 53113 Bonn, Germany. [5]Invertebrate Morphology Lab, Department of Applied Biology, IEAMar and CAUNESP College of Agricultural and Veterinary Sciences, São Paulo State University, Unesp. Jaboticabal, São Paulo 14884-900, Brazil. [6]Department of Biology, School of Philosophy, Sciences and Literature of Ribeirão Preto, University of São Paulo, São Paulo 14040-901, Brazil.

*Author for correspondence (k.bicego@unesp.br)

K.C.B., 0000-0002-1180-1132

Received 8 December 2018; Accepted 18 April 2019

Journal of Experimental Biology (2019) 222, jeb197970. doi:10.1242/jeb.197970

List of symbols and abbreviations

ADF	anatomical diffusion factor
Hx	hypoxia
M_b	body mass
Nx	normoxia
OLS	ordinary least squares
V_L	lung volume
V%	volume density

development and maturation may affect their morphology. In addition, this effect would last longer than the incubation phase, even under normoxic conditions after hatching. This latter idea is based on evidence of long-lasting physiological and morphological effects of hypoxia in hatchlings that returned to normoxia (Dzialowski et al., 2002; Hassanzadeh et al., 2004; Szdzuy and Mortola, 2007; Mortola, 2009; Ferner and Mortola, 2009; Amaral-Silva et al., 2017). Thus, we hypothesized that hypoxia exposure during the embryonic development of parabronchial structures affects their morphology, and that this effect endures after hatching. To test this hypothesis, chicken embryos were exposed to normoxia during the entire incubation or to 15% O_2 during days 12–18 of incubation. The parabronchial structures of normoxic 1- and 10-day-old chicks were analyzed via light and transmission electron microscopy using stereological methods.

MATERIALS AND METHODS

Animals

Fertile eggs of the domestic fowl [*Gallus gallus domesticus* (Linnaeus 1758); Cobb500®] were incubated (incubator Premium Ecologica®, Belo Horizonte, Minas Gerais, Brazil) at 37.5°C and a relative humidity of 60%, and turned every 2 h. The eggs were subjected to two treatments: normoxia (Nx; 21% O_2 throughout incubation) and late hypoxia (Hx; 21% O_2 until day 11 and 15% O_2 on days 12–18). For the exposure to hypoxia on days 12–18, the Hx eggs were transferred to a hypoxic incubator kept at 14.5–15.5% O_2 by leaking a small stream of N_2 (0.2–0.4 ml min^{-1}) from a pressurized tank controlled by a flowmeter (White Martins, Osasco, São Paulo, Brazil, pure N_2). The O_2 concentration and temperature inside the hypoxic incubator were monitored continuously by an O_2 analyzer (Sensepoint XCD, Honeywell, Morris Plains, NJ, USA) and three thermometers in different places inside the incubator, respectively. At day 19, all eggs (Nx and Hx) were taken to the same hatcher and hatched together under the same environmental conditions in normoxia. After hatching, animals remained in normoxic ambient conditions until day 1 (at ∼32°C) or day 10 (at ∼29°C) for lung sampling. The protocol was performed in agreement with the guidelines of the National Council of Control in Animal Experimentation (CONCEA-MCTIC-Brazil), and was approved by the local Animal Care and Use Committee (CEUA, FCAV-UNESP, protocol number 024166/13).

Tissue sampling and histological procedure

Five 1- and five 10-day-old chicks (both sexes) were used for each treatment. After induction of death by overdose of anesthetic (90 mg kg^{-1} ketamine+4.5 mg kg^{-1} xylazine), a solution of 2.5% glutaraldehyde (Sigma-Aldrich, St Louis, MO, USA) in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.5; Synth, Diadema, São Paulo, Brazil) was driven by gravity into the trachea for fixation of the lungs. This method (adapted from Maina et al., 1989) consisted of inserting, in supine animals, an intratracheal cannula (1 and 2 mm diameter for 1- and 10-day-old chicks, respectively) connected to another cannula (4.5 mm diameter) attached to a funnel positioned 20 cm above the insertion point. The fixative solution was gravity-fed to air sacs and lungs through the funnel and cannulas. After this, the trachea was ligated and the respiratory system remained immersed in the same fixative solution for 24 h inside the coelomic cavity at 4°C. Following fixation, the coelomic cavity was opened, both whole lungs were removed from the rib cage, and the lungs were 2-mm parallel sectioned in random orientation (independent uniform random) to measure volumes by the Cavalieri principle (Michel and Cruz-Orive, 1988; Nielsen et al., 2001; Howard and Reed, 2005). A total of 12 small lung pieces were collected from both whole lungs using systematic random sampling by covering all slices with a translucent sheet marked with Cavalieri points and used as a biopsy plate.

The samples remained immersed in the same fixative solution for 15–20 h, rinsed three times in the same buffer, and post-fixed in phosphate buffered 1% osmium tetroxide (EMS, Hatfield, PA, USA) for 2 h. Dehydration was carried out in an ascending series battery through increasing concentrations of acetone (30–100%; Synth, Diadema), and the samples were embedded and included in Epon/Araldite resin (EMS).

Light microscopy

For volume density and absolute volume estimation of lung components, 12 semi-thin sections (500 nm), one from each lung sample, were taken per animal using an ultramicrotome (Leica UC7, Wetzlar, Hessen, Germany), stained with 4% Toluidine Blue and photographed at a magnitude of 20× (Zeiss, Axio Image Z2 and camera AxioCam MRC5, Oberkochen, Baden-Württemberg, Germany). Micrographs were assembled (Mosai X function of the microscope's software) and were overlapped with frames, and point counting was used to estimate the volume densities (V%; relative volume in %) of the lung structures (Howard and Reed, 2005; Weibel et al., 2007). This method is based on point counting of each structure and its relation to point counting of the total lung volume. We also estimated the absolute volume (cm^3) of each structure, which was calculated from the relationship between the structures' volume densities (V%) and the total lung volume (V_L; in cm^3), i.e. the absolute volume of a certain structure was V%×V_L (Maina, 1984). The absolute volumes of each lung structure have also been standardized by body mass (M_b; cm^3 g^{-1}).

The lung structures quantified were air capillaries, blood capillaries, atria, infundibula, parabronchial lumen, interparabronchial tissue, bronchi (primary and secondary) and blood vessels larger than capillaries (lung arteries, arterioles, veins and venules; Makanya and Djonov, 2009) (Fig. 1).

Transmission electron microscopy

Five resin blocks containing a randomly selected lung sample from each animal were used for this analysis. Each block was trimmed and cut into ultrathin sections (50–70 nm) using an ultramicrotome (Leica UC7) with a diamond knife. The copper grids with sections were stained with aqueous 2% uranyl acetate and 0.4% lead citrate (Reynolds, 1963; Venable and Coggeshall, 1965) and examined using a JEOL JEM 1010 (Akishima, Tokyo, Japan) transmission electron microscope at 80 kV electron beam. For each block, 10 micrographs were taken at a magnification of 7500×, totaling 50 photomicrographs per specimen, 250 photomicrographs per treatment.

The anatomically measurable component of the diffusing capacity is the 'anatomical diffusion factor' (ADF), which is

Journal of Experimental Biology (2019) 222, jeb197970. doi:10.1242/jeb.197970

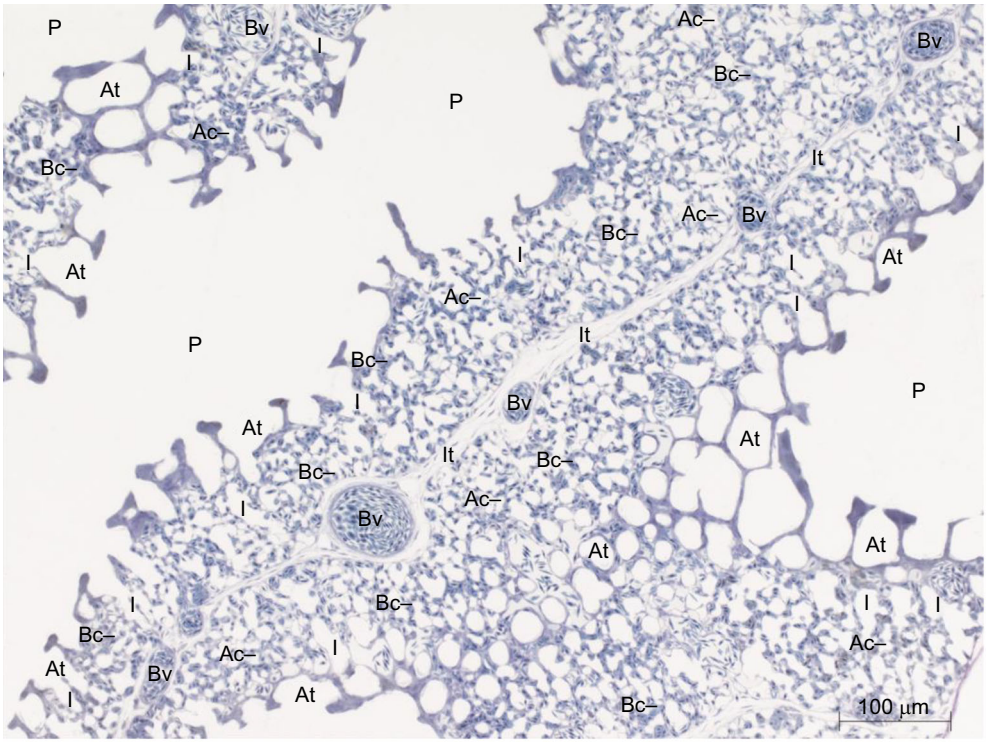

Fig. 1. Photomicrograph showing parabronchial structures in a 1-day-old chick. The parabronchial lumen (P), atria (At), infundibula (I), air capillaries (Ac), blood capillaries (Bc), interparabronquial tissue (It) and blood vessels larger than capillaries (Bv) are identified in the image. This is a single 2D image created as a result of using the *z*-stack tool (Zeiss, Axio Image Z2 and camera AxioCam MRC5, Oberkochen, Baden-Württemberg, Germany), in that it is possible to have images from bottom to top (*z*-sections), which are stacked one on top of the other (*z*-stacking). Magnification=20×.

defined as the ratio of the respiratory surface area and the harmonic mean thickness of the blood–gas barrier (Perry, 1978, 1981). We separately estimated the ADF for the lungs of each of the four groups of samples (1 and 10 days after hatching, both incubated under normoxia and hypoxia). We randomly selected 100 electron micrographs per group (20 samples per individual) and determined the respiratory surface area of the air capillaries by counting all intersections with the surface, as well as the points that fell over the reference volume, to obtain the surface-to-volume ratio. In order to do so, we superimposed a squared grid over the electron micrographs in Gimp (version 2.8.16). The grid was randomly positioned for each of the electron micrographs. Surface area was calculated based on standard procedures (Howard and Reed, 2005), using the estimate of air capillary volume (see above) as the reference volume. Blood–gas tissue barrier thickness was calculated as two-thirds of the harmonic mean of the measured distances at each of the intersections (Weibel and Knight, 1964). In addition to a specimen-based approach leading to mean values for each group that could be subjected to statistical analyses, we also conducted a population-based approach using the pooled data for each group, yielding only a single value per age and treatment that could not be evaluated statistically (Table 4).

Statistical analyses

Data are presented as means±s.d. The results were subjected to two-way ANOVA (factors: age and incubation treatment) and, in the case of statistically significant differences ($P \leq 0.05$), means were compared by Tukey's test (5% probability). Data were tested for unequal variance and normality and appropriate transformations were performed when necessary. To compare lung volume data obtained in the present study with values given in the literature, we extracted total lung volume and body mass results from other studies, $\log_{10}$-transformed the values and performed linear ordinary least squares (OLS) regression analyses.

RESULTS

General characteristics of chicks and lungs, such as number of chicks, incubation time, M_b, V_L and V_L/M_b, are presented in Table 1. No significant differences between treatments were observed regarding the above structures. M_b and V_L were greater, whereas V_L/M_b were lower, in the older animals independent of treatment.

All the structures analyzed in the present study are identified in a representative photomicrograph of a 1-day-old chick lung in Fig. 1 and a more detailed view of air and blood capillaries of 1- and 10-day-old chick lungs is shown in Fig. 2.

Table 1. Characteristics of 1- and 10-day-old chicks and their lungs after exposure to normoxic (Nx) or hypoxic (Hx; 15% O_2, during days 12–18) incubation

	1-day old chicks		10-day-old chicks	
	Nx	Hx	Nx	Hx
Number of chicks	5	5	5	5
Incubation duration (h)	488.4±8.1	488.4±8.1	492.0±7.2	489.6±5.4
Body mass, M_b (g)	43.8±1.2	43.0±1.2	167.9±17.2$^{\#}$	151.6±22.1$^{\#}$
Lung volume, V_L (cm³)	0.98±0.07	1.01±0.10	2.58±0.38$^{\#}$	2.09±0.49$^{\#}$
V_L/M_b (cm³ g^{-1})	0.022±0.002	0.024±0.002	0.015±0.002$^{\#}$	0.014±0.003$^{\#}$

Data are means±s.d. No significant differences were observed in the different oxygen conditions during incubation. $^{\#}$Significant difference between ages within the same treatment (Tukey's test, $P<0.01$).

Journal of Experimental Biology (2019) 222, jeb197970. doi:10.1242/jeb.197970

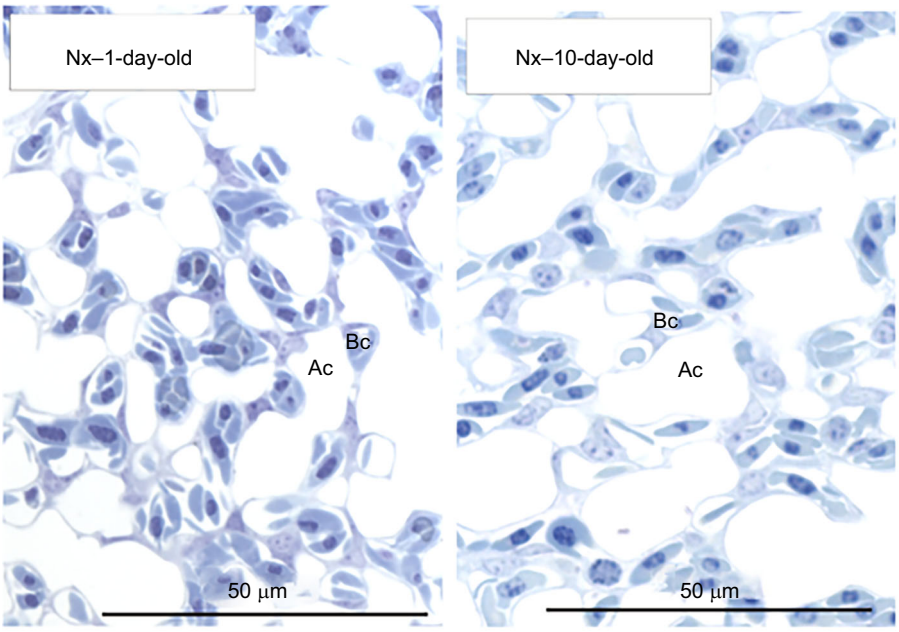

Fig. 2. Detailed view of air capillaries and blood capillaries of lungs in 1- and 10-day-old chicks incubated in normoxia (Nx). Each image is a single 2D image created as a result of using the *z*-stack tool (Zeiss, Axio Image Z2 and camera AxioCam MRC5, Oberkochen), in that it is possible to have images from bottom to top (*z*-sections), which are stacked one on top of the other (*z*-stacking). Magnification=62× immersion. Ac, air capillary; Bc, blood capillary.

Air capillaries of normoxic animals occupied 13.6% of the lung in 1-day-old and 15.3% in 10-day-old animals. Hypoxia caused an increase of 27.4% and 42.7% in air capillary volume densities in 1- and 10-day-old chicks, respectively (Tukey's test, $P<0.001$; Table 2). In addition, air capillary volume was significantly greater in 10-day-old than in 1-day-old chicks incubated in hypoxia, but no change was observed between those incubated in normoxia (Tukey's test, $P<0.001$; Table 2).

Normoxic incubated animals had 21.6% of their lungs occupied by blood capillaries at 1 day of age; this value was 21.3% in 10-day-old chicks. Hypoxia during incubation increased blood capillary volume density in both 1-day-old (23.5% increase) and 10-day-old (13.8% increase) chicks (Tukey's test, $P<0.001$; Table 2).

The atrial volume densities of 1- and 10-day-old animals incubated in hypoxia were significantly lower than those of animals incubated in normoxia (Tukey's test: 1-day-old, $P<0.001$; 10-day-old, $P=0.041$; Table 2); the atrial volumes decreased with age in chicks incubated in normoxia, but not in hypoxia (Tukey's test, $P<0.001$). In chicks incubated under normoxia, the parabronchial lumen volume density was significantly higher in older animals when compared with younger animals, an effect that was inhibited by hypoxic incubation (Tukey's test, $P<0.025$; Table 2). Regarding the other structures assessed, such as the infundibula, bronchi, blood vessels larger than capillaries and interparabronchial tissue, no significant effect was observed to be caused by the conditions during incubation.

The absolute volumes and volumes/M_b of lung structures are shown in Table 3. The absolute volumes of air capillaries, blood capillaries, atria, infundibula, parabronchial lumen and blood vessels larger than capillaries were greater in the older chicks (Tukey's test, $P<0.001$ to 0.004) whereas the volume/M_b of air capillaries, blood capillaries, atria, parabronchial lumen and normoxic incubated chicks' infundibula and bronchi were lower (Tukey's test, $P<0.001$). Hypoxia during incubation increased absolute volumes of air and blood capillaries (Tukey's test, both $P<0.001$) in 1-day-old chicks, but decreased atria volume at both ages (Tukey's test, $P<0.001$ for both), and reduced parabronchial lumen in 10-day-old chicks (Tukey's test, $P<0.001$; Table 3). Similar results were observed for volume/M_b, except that air capillaries were significantly increased after hypoxia incubation in 10-day-old chicks and infundibula were decreased in 1-day-old chicks.

Table 4 summarizes the functional anatomical parameters of the lung analyzed by electron microscopy for the specimen-based approach and also the population-based approach (pooled data of all the individuals in a group). The population-based estimates correspond well with the means of the individual-based approach. Surface-to-volume ratio increased under hypoxia incubation in 1- and 10-day-old chicks (treatment: $F_{1,16}=5.84$,

Table 2. Volume densities (V%; relative to total lung volume) of lung structures in 1- and 10-day-old chicks after exposure to normoxic (Nx) or hypoxic (Hx; 15% O_2, during days 12–18) incubation

	1-day old chicks		10-day old chicks	
Lung structures	Nx	Hx	Nx	Hx
Air capillaries	13.62±0.04	17.35±0.04*	15.31±0.04	21.87±0.05*,#
Blood capillaries	21.58±0.04	26.63±0.06*	21.29±0.05	24.24±0.05*
Atria	11.88±0.05	6.25±0.03*	8.14±0.04#	6.0±0.04*
Infundibula	4.81±0.03	5.36±0.02	5.19±0.02	4.99±0.02
Parabronchial lumen	32.90±0.11	31.23±0.07	38.35±0.11#	29.15±0.11*
Bronchi	5.66±0.10	3.47±0.07	2.65±0.06	1.80±0.05
Blood vessels larger than capillaries	7.82±0.09	7.41±0.06	6.99±0.09	8.71±0.08
Interparabronchial tissue	1.74±0.02	2.30±0.02	2.07±0.03	3.27±0.05

Data are means±s.d. Bronchi=primary+secondary. *Significant difference between treatments within the same age; #significant difference between ages in the same treatment (Tukey's test, $P\leq0.05$). $N=5$.

Table 3. Absolute volume (cm^3) and volume/body mass ($cm^3\ g^{-1}$) of lung structures in 1- and 10-day-old chicks after exposure to normoxic (Nx) or hypoxic (Hx; 15% O_2, during days 12–18) incubation

	1-day old chicks		10-day old chicks	
Lung structures	Nx	Hx	Nx	Hx
Air capillaries	0.13±0.04	0.18±0.03*	0.41±0.13#	0.45±0.13#
	0.0033±0.0010	0.0042±0.0007*	0.0025±0.0008#	0.0031±0.0009*,#
Blood capillaries	0.21±0.05	0.27±0.07*	0.57±0.19#	0.51±0.18#
	0.0048±0.0012	0.0063±0.0014*	0.0034±0.0011#	0.0033±0.0010#
Atria	0.10±0.05	0.07±0.03*	0.21±0.10#	0.13±0.12*,#
	0.0024±0.0012	0.0015±0.0007*	0.0012±0.0006#	0.0008±0.0006*,#
Infundibula	0.11±0.15	0.06±0.03	0.13±0.06#	0.10±0.03#
	0.0027±0.0036	0.0013±0.0006*	0.0008±0.0004#	0.0007±0.0002
Parabronchial lumen	0.32±0.11	0.32±0.07	1.00±0.30#	0.59±0.24*,#
	0.0073±0.0025	0.0074±0.0016	0.0059±0.0017#	0.0040±0.0017*,#
Bronchi	0.06±0.10	0.04±0.07	0.06±0.14	0.05±0.13
	0.0013±0.0024	0.0008±0.0016	0.0004±0.0008#	0.0003±0.0007
Blood vessels larger than capillaries	0.08±0.09	0.08±0.06	0.18±0.23#	0.17±0.16#
	0.0017±0.0020	0.0018±0.0014	0.0011±0.0015	0.0011±0.0004
Interparabronchial tissue	0.02±0.02	0.02±0.03	0.05±0.08	0.08±0.13#
	0.0004±0.0005	0.0006±0.0006	0.0003±0.0005	0.0005±0.0007

Data are given as means±s.d. Top and bottom values of each lung structure give absolute volume and volume/body mass, respectively. Bronchi=primary+secondary. *Significant difference between treatments within the same age; #significant difference between ages within the same treatment (Tukey's test, $P \leq 0.05$). $N=5$.

$P=0.028$; age: $F_{1,16}=0.20$, $P=0.661$; no interaction: $F_{1,16}=0.00006$, $P=0.99$). Surface area of the air capillaries also increased under hypoxia incubation in both 1- and 10-day-old chicks (treatment: $F_{1,16}=6.01$, $P=0.026$; age: $F_{1,16}=4.74$, $P=0.045$; no interaction: $F_{1,16}=0.174$, $P=0.68$). The harmonic mean thickness of the blood–gas tissue barrier was nearly identical in all four groups (treatment: $F_{1,16}=0.059$, $P=0.810$; age: $F_{1,16}=0.014$, $P=0.908$; no interaction: $F_{1,16}=0.448$, $P=0.513$). The ADFs of 1- and 10-day-old chicks exposed to hypoxia increased by 49.47% and 27.57%, respectively, compared with normoxic chicks (treatment: $F_{1,16}=4.496$, $P=0.050$; age: $F_{1,16}=3.983$, $P=0.063$; no interaction: $F_{1,16}=0.659$, $P=0.429$). Similarly, following the population-based approach, the ADFs were increased by approximately 48.53% and 31.72%, respectively, in the hypoxic groups.

DISCUSSION

Our results indicate that exposure to hypoxia during a main phase of embryonic lung development can induce a morphological remodeling of parabronchial tissue in chicks up to 10 days after hatching. To our knowledge, this is the first demonstration of a direct effect of hypoxia during embryonic development resulting in post-natal lung morphological remodeling. This response was characterized by an increase in the volume densities of air and blood capillaries together with an increase in respiratory surface area, involved in gas exchange, over a decrease in volume densities of air-conducting structures (atria and parabronquial lumen).

The effect of hypoxia favoring structural changes related to gas exchange seems to occur in many species. Young and adult hypoxic rats show heavier lungs (Cunningham et al., 1974) and increased lung volume (Bartlett and Remmers, 1971; Mortola et al., 1986; Sekhon and Thurlbeck, 1996), surface area (Bartlett and Remmers, 1971; Cunningham et al., 1974), number of alveoli (Sekhon and Thurlbeck, 1996), alveolar density (Bartlett and Remmers, 1971; Cunningham et al., 1974; Sekhon and Thurlbeck, 1996; Burri and Weibel, 1971) and diffusion capacity (Howell et al., 2003). Guinea pigs also show greater alveolar surface and lung volume in hypoxia (Lechner and Banchero, 1980). In addition, dogs exposed to hypoxia from a young age possess increased air volume, surface area and pulmonary diffusion capacity (Johnson et al., 1985). Hypoxic chicken embryos show increased parabronchial tissue (Lewallen and Burggren, 2015), and birds evolutionarily adapted to hypoxic exposures (*Anser indicus* and *Chloephaga melanoptera*) show thin blood–gas diffusion barriers (Maina and King, 1982; Maina et al., 2017). Even ectothermic vertebrates, such as tadpoles, show gill hypertrophy, thinner skin (blood–water barrier) and an increased capillary bed in response to hypoxia (Burggren and Mwalukoma, 1983). Moreover, in a rare case of unilateral lung aplasia, an adult one-lunged snapping turtle (*Chelydra serpentina*) specimen showed a marked increase in compensatory pulmonary volume and surface area, as well as a larger surface-area-to-volume ratio in the remaining lung (Schachner et al., 2017).

It is known that hypoxia affects the expression of genes associated with metabolic adaptation to low oxygen (Semenza,

Table 4. Functional anatomical parameters, based on electron transmission microscopy analysis, of the lungs in 1- and 10-day-old chicks after exposure to normoxic (Nx) or hypoxic (Hx; 15% O_2, during days 12–18) incubation

	1-day old chicks		10-day old chicks	
Parameters	Nx	Hx	Nx	Hx
Surface-to-volume ratio (cm^{-1})	4056.4±662.4 (4100.2)	4557.7±432* (4538.2)	4150.4±392.7 (4129.2)	4648.4±273* (4631.2)
Surface area of the air capillaries ($cm^2\ g^{-1}$)	13.81±6 (13.65)	19.08±1.93* (19.09)	10.58±4.13 (10.3)	14.32±3.29* (14.33)
Harmonic mean barrier thickness (μm)	0.354±0.038 (0.349)	0.345±0.065 (0.329)	0.342±0.042 (0.338)	0.362±0.04 (0.357)
Anatomical diffusion factor ($cm^2\ \mu m^{-1}\ g^{-1}$)	38.97±15.57 (39.09)	58.25±19.53* (58.06)	31.19±11.77 (30.45)	39.79±10.1* (40.11)

Data are means±s.d. for the specimen-based approach with values for the population-based approach in parentheses (see Materials and Methods, 'Transmission electron microscopy' for details). *Significant difference between treatments within the same age (two-way ANOVA, $P \leq 0.05$). No interaction between treatment and age was observed in any parameter analyzed. $N=5$.

Journal of Experimental Biology (2019) 222, jeb197970. doi:10.1242/jeb.197970

2000). In this context, reduction in tissue oxygen supply can often lead to neovascularization to match the needs of the hypoxic tissues (Adair et al., 1990), as can be observed in tissues of mammals and birds, such as the brain, heart (Miller and Hale, 1970), skeletal muscles (Miller and Hale, 1970; Cassin et al., 1971; Snyder et al., 1984), and lungs (Howell et al., 2003; Burri and Weibel, 1971). An angiogenic response is observed in the pulmonary circulation of adult rats exposed to hypoxia for 2 weeks, where lower O_2 concentrations increase both the length of pulmonary vessels and the surface area of the capillary endothelium (Howell et al., 2003). High-altitude birds (*Fulica americana peruviana*), which live under hypobaric hypoxia, show a greater capillarity in flight muscles compared with lowland species (León-Velarde et al., 1993). It is interesting to note that this does not seem to be a response to longtime genetic selection, because Snyder et al. (1984), studying Canadian geese (*Branta canadensis*) and bar-headed geese (*A. indicus*), showed that increases in capillarity in flight muscles can also be stimulated in birds from low altitudes exposed to hypoxia during embryonic/fetal development. Regarding embryos, an increase in blood vessel number likely improves the functional surface for gas exchange under exposure to hypoxia, especially during the late stages of incubation. A 40–60% mass increment and angiogenesis of the chorioallantoic membrane, a critical structure for O_2 uptake and CO_2 release, was in fact demonstrated to occur in avian embryos (Strick et al., 1991; Wagner-Amos and Seymour, 2003; Chan and Burggren, 2005; Azzam and Mortola, 2007; Zhang and Burggren, 2012). Interestingly, an increased expression of vascular endothelial growth factor (VEGF), a protein that stimulates angiogenesis and vasculogenesis, was identified in the lungs of chicken embryos incubated in hypoxia (Lewallen and Burggren, 2015). A similar increase in VEGF may explain the greater volume density of blood capillaries observed by us in 1- and 10-day-old chicks incubated under hypoxia.

What is interesting regarding our results is that even at 10 days after hatching, under normoxic conditions, the morphological remodeling of lungs induced by hypoxia during incubation had been preserved. This indicates that lung development was affected by the low oxygen supply and that these morphological changes remained after termination of the hypoxic exposure. This agrees with the results of Ravikumar et al. (2015), who raised guinea pigs at high altitude for 4 months followed by an intermediary altitude for 7–8 months. In their study, at the end of that phase, the guinea pigs still preserved some lung structure rearrangement, such as increased type-2 cell and interstitial volumes and the reduction of alveolar ducts, without changes in type-1 epithelium volume and alveolar surface. Moreover, Okubo and Mortola (1989) exposed neonate rats to 10% hypoxia from the first to the sixth day of post-natal life, and then returned them to normoxia until 50 days of age, resulting in animals having a larger chest, anteroposterior diameter and diaphragm surface area, as well as heavier and expanded lungs. Taken together, these studies suggest that some structural changes induced during neonatal hypoxia are not reversed after the animal is returned to normoxia. Our study adds information on the direct effect of embryonic hypoxia on lung structures in post-natal life in a bird, which is difficult to demonstrate in mammals as the embryonic development occurs inside the mother's body.

Regardless of age, the respiratory surface area of our normoxia-incubated groups fell within the range of previous estimates for the domestic fowl (10.09 $cm^2\ g^{-1}$ according to Abdalla et al., 1982, and 12.97 $cm^2\ g^{-1}$ according to Vidyadaran et al., 1990; cf. Table 4). Incubation in hypoxia induced comparable increases in respiratory surface area in both ages (39.85% higher than 1-day-old Nx chicks; 39.13% higher than 10-day-old Nx chicks), whereas mass-specific respiratory surface area was always lower in the older groups.

In contrast to the increase in air and blood capillary volumes and respiratory surface area, the volumes of atria and the parabronquial lumen decreased. After normoxic incubation, the volume densities of atria decreased in older animals, a pattern that was not observed in chicks incubated in hypoxia, because they already showed reduced atria on the first day after hatching. As for the parabronchial lumen, it occupied a larger relative space in the lung in older animals incubated in normoxia, but this was not observed in the hypoxia group. Age did not affect air or blood capillaries in chicks incubated in normoxia, but hypoxia changed this pattern, inducing a greater volume of air capillaries in older animals. Thus, it seems that hypoxia not only changes lung structure, but also affects the anatomical design during early development after hatching.

From our results, as the total lung volume was not changed by hypoxia (Table 1), we suggest that the increase in the structures involved in gas exchange, i.e. air and blood capillary volumes (Tables 2 and 3), and air capillary surface (Table 4), are related to the reduction in volumes of the atria and parabronchial lumen in the chicks incubated in hypoxia (Tables 2 and 3). Actually, the volume densities of air and blood capillaries increased by approximately 50% and 56% in 1- and 10-day-old chicks, respectively, whereas atria and parabronchial lumen volume densities decreased by approximately 52% and 50% in the 1-

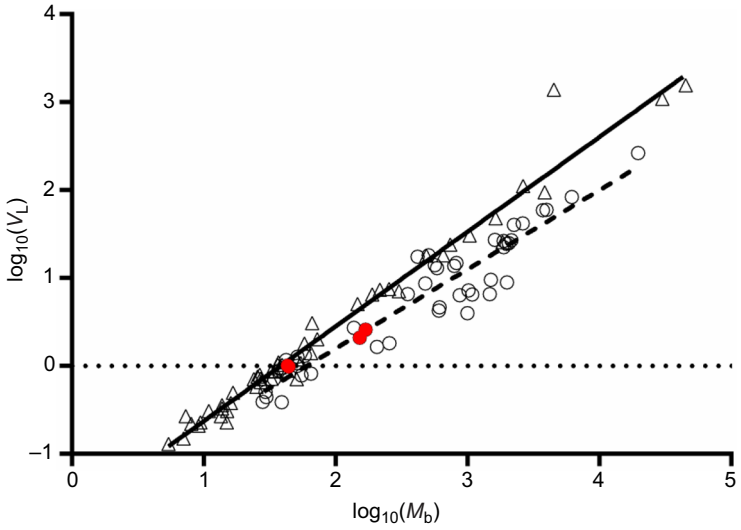

Fig. 3. Relationship between lung volume (V_L; cm^3) and body mass (M_b; g) in birds. Open triangles indicate V_L and M_b in non-galliform birds (N=51; data from Maina, 2005, Maina et al., 2017) and the corresponding linear ordinary least squares (OLS) regression [continuous line: $\log_{10}(V_L)=1.077.\log_{10}(M_b)–\log_{10}(1.701)$; $R^2=0.9730$]. The upper outlier in this relationship with a much larger V_L for its M_b represents *Spheniscus humboldti*. Circles indicate V_L and M_b in galliform birds [open circles, N=54; data from Burton and Smith, 1968 (as estimated by Lasiewski and Calder, 1971); Abdalla et al., 1982; Timmwood et al., 1987; Julian, 1989; Vidyadaran et al., 1988; Vidyadaran et al., 1990; Owen et al., 1995; Hassanzadeh et al., 2005; and from the present study (red circles)] and the corresponding linear OLS regression [dashed line: $\log_{10}(V_L)=0.9005\times\log_{10}(M_b)–\log_{10}(1.600)$; $R^2=0.9082$]. Using OLS linear regression analyses, the slopes of the non-galliform and galliform regressions have been found to be significantly different (P<0.001, ANCOVA).

and 10-day-old animals, respectively. This scenario may corroborate the idea of a remodeling of lung tissues based on an anatomical limitation related to body size (keeping the same total lung volume), similar to what happens with post-natal guinea pigs acclimated to hypoxia (Lechner and Banchero, 1980). Moreover, it would indicate a functional tissue rearrangement in the lung favoring gas exchange structures at the expense of airflow conduction regions. Indeed, a reduced atrial volume, as found in efficient flying birds, particularly in small forms (Duncker, 1972), is considered of functional importance for reducing dead space. Moreover, the changes observed in our hatchlings became more pronounced in 10-day-old chicks, which might be related to physiological changes induced by hypoxia during embryonic development. Increased metabolic rate, possibly related to catch-up growth, is indeed observed in 10-day-old chicks submitted to the same hypoxia treatment as that of the present study (Amaral-Silva et al., 2017).

In contrast to the differences found in parabronchial structure volumes and respiratory surface area, the thickness of the tissue barrier did not differ between treatments at either age (Table 4). The morphological diffusing capacity of the tissue barrier for oxygen correlates inversely with its thickness, but directly with the surface area (e.g. Hills, 1974). Birds present the thinnest tissue barrier among vertebrates (Maina and West, 2005), and the values found in our chicks (0.33–0.36 µm) are well within the range of variation found in chickens (Maina and West, 2005). Regarding the chicks incubated in hypoxia, it seems that the reduced O_2 supply to the embryonic lung tissue at days 12–18 was not enough to affect the thickness of the tissue barrier. Interestingly, the blood–gas (tissue) barrier of the Andean goose, a bird that is adapted to fly at high altitudes, is not considered remarkably thin compared with that of other birds (Maina et al., 2017). The thinnest barriers are generally found in small, metabolically demanding birds (Maina and West, 2005; Maina et al., 2017).

The relative volumes of air and blood capillaries in our chicks (Table 3) were somewhat smaller than those (55.5–64.8% for air capillaries and 21.3–33.8% for blood capillaries) demonstrated in other studies in chickens (Abdalla, 1977, as cited in Vidyadaran et al., 1988; Abdalla et al., 1982; Duncker, 1972; Duncker, 1973, as cited in Maina, 2005; Vidyadaran et al., 1987, as cited in Maina, 2005; Abdalla and Maina, 1981, as cited in Maina, 2005). These differences are because the relative volumes were calculated in the earlier studies considering only the gas exchange structures (air capillaries, blood capillaries, blood–gas barrier) as a reference volume, whereas in the present work, the volumes are all relative to the total lung volume.

One could argue that the morphological changes in the lung induced by hypoxia could be caused by differences in the time for air capillaries to fill with air during the first breaths after internal piping. Although we have not analyzed the internal and external piping time, the total duration of incubation was not different between the Nx and Hx groups (Table 1). In addition, exposure to 15% O_2 from day 12 to 18 of incubation did not change internal (Nx=476.1 h versus Hx=476.7 h) and external (Nx=488.8 h versus Hx=490.8 h) piping times in another group of embryos (N=45 and 48 for Nx and Hx, respectively; K.C.B. and P. A. Toro-Velasquez, unpublished data).

Hypoxia during incubation did not affect body mass in either group of chicks (Table 1), corroborating results from previous studies on hatchlings (Azzam et al., 2007; Lourens et al., 2007; Ferner and Mortola, 2009), but differing from others showing smaller (Hassanzadeh et al., 2004; Dzialowski et al., 2002; Amaral-Silva et al., 2017) or larger chicks (Bahadoran et al., 2010). It is known that hypoxia decreases yolk consumption (Chan and Burggren, 2005), which may be the reason for the absence of changes in body mass in our hatchlings.

Finally, lung volume was not affected by hypoxic incubation, but by age. Total lung volume was greater in older chicks, regardless of treatment, but V_L/M_b was significantly greater in younger chicks. When comparing our results with data from the literature (Fig. 3), we observed that non-galliform birds increase lung volume proportionally with body mass, following the scaling relationship of $V_L=0.0199M_b^{1.077}$. On a smaller number of species, Lasiewski and Calder (1971) found a similar relationship with lung volume, scaling as $M_b^{0.94}$, and Maina et al. (1989) found a regression for birds, including Galliformes, of $V_L=0.0218M_b^{1.048}$. Galliform birds, in contrast, show an allometric relationship of $V_L=0.0251.M_b^{0.9005}$, the slope being significantly different from that of non-galliform birds. Therefore, the reduction in relative lung volume in 10-day-old chicks (Table 1) can be explained by the non-proportionality of lung volume to body mass in Galliformes (Fig. 3).

In conclusion, our results indicate that a reduction in O_2 supply during embryonic lung development and maturation induces a morphological adjustment of parabronchi that persists through post-hatching life, for at least 10 days, in chickens. The changes indicate a parabronchial remodeling favoring pulmonary structures involved in gas exchange (increased blood and air capillary volume densities and respiratory surface area) at the expense of air-conducting structures (reduced atria and parabronchial lumen volume densities). Suitable follow-up studies could be designed to evaluate the physiological role of this remodeling in a possible increase in lung oxygen extraction in young birds that have been exposed to low O_2 concentrations during embryonic development.

Acknowledgements
This study was part of the activities developed by L.A.-S. during her Master's at the Animal Science Graduate Program, FCAV-UNESP, Jaboticabal, SP, Brazil. We would like to thank Marcia F. Mataqueiro for support with light and electron microscopy preparations. We would also like to thank the Electron Microscopy Laboratory from the FCAV-UNESP, Jaboticabal Facility for technical support. We thank Steven F. Perry for discussions about estimating ADF. M.L. would like to thank Michael H. Hofmann for supporting his research. Finally, we appreciated all the comments by the two anonymous reviewers, which resulted in the improvement of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.A.-S., K.C.B.; Methodology: L.A.-S., F.J.Z., W.K., K.C.B.; Formal analysis: L.A.-S., M.L., F.J.Z., K.C.B.; Investigation: K.C.B.; Writing - original draft: L.A.-S., W.K., L.H.G., K.C.B.; Writing - review & editing: L.A.-S., M.L., F.J.Z., W.K., L.H.G., K.C.B.; Visualization: L.A.-S., M.L., F.J.Z., W.K., L.H.G., K.C.B.; Supervision: K.C.B.; Project administration: K.C.B.; Funding acquisition: F.J.Z., K.C.B.

Funding
This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo [FAPESP; 13/13386-4 and 08/57712-4 (INCT Fisiologia Comparada) to K.C.B.] and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; CIMAR II 1989/2014 to F.J.Z.). L.A.-S. was the recipient of a FAPESP master fellowship (2014/01861-2).

Data availability
Lara do Amaral Silva's Masters thesis is available from the Sao Paulo State University-UNESP: https://repositorio.unesp.br/handle/11449/136303.

References
Abdalla, M. A. (1977). Morphometry of the avian lung. *J. Anat.* **123**, 262.
Abdalla, M. A. and Maina, J. N. (1981). Quantitative analysis of the exchange tissue of the avian lung (Galliformes). *J. Anat.* **134**, 677-680.
Abdalla, M. A., Maina, J. N., King, A. S., King, D. Z. and Henry, J. (1982). Morphometrics of the avian lung. 1. The domestic fowl, *Gallus domesticus*. *Respir. Physiol.* **47**, 267-278. doi:10.1016/0034-5687(82)90057-3
Adair, T. H., Gay, W. J. and Montani, J. P. (1990). Growth regulation of the vascular system: evidence for a metabolic hypothesis. *Am. J. Physiol.* **259**, 393-404. doi:10.1152/ajpregu.1990.259.3.R393

Amaral-Silva, L., Scarpellini, C. S., Toro-Velasquez, P. A., Fernandes, M. H. M. R., Gargaglioni, L. H. and Bícego, K. C. (2017). Hypoxia during embryonic development increases energy metabolism in normoxic juvenile chicks. *Comp. Biochem. Physiol. A* **207**, 93-99. doi:10.1016/j.cbpa.2017.03.002

Azzam, M. A. and Mortola, J. P. (2007). Organ growth in chicken embryos during hypoxia: implications on organ 'sparing' and 'catch-up growth'. *Respir. Physiol. Neurobiol.* **159**, 155-162. doi:10.1016/j.resp.2007.06.003

Azzam, M. A., Szdzuy, K. and Mortola, J. P. (2007). Hypoxic incubation blunts the development of thermogenesis in chicken embryos and hatchlings. *Am. J. Physiol.* **292**, 2373-2379. doi:10.1152/ajpregu.00885.2006

Bahadoran, S., Hassanzadeh, M. and Zamanimoghaddam, A. K. (2010). Effect of chronic hypoxia during the early stage of incubation on prenatal and postnatal parameters related to ascites syndrome in broiler chickens. *Iran. J. Vet. Res.* **11**, 64-71. doi:10.1080/03079450400013188

Bartlett, D., Jr. and Remmers, J. E. (1971). Effects of high altitude exposure on the lungs of young rats. *Respir. Physiol.* **13**, 116-125. doi:10.1016/0034-5687(71)90068-5

Baumann, R., Padeken, S., Haller, E. A. and Brilmayer, T. (1983). Effects of hypoxia on oxygen affinity, hemoglobin pattern, and blood volume of early chicken embryos. *Am. J. Physiol.* **244**, 733-741. doi:10.1152/ajpregu.1983.244.5.R733

Bjønnes, P. O., Aulie, A. and Høiby, M. (1987). Effects of hypoxia on the metabolism of embryos and chicks of domestic fowl. *J. Exp. Zool.* **1**, 209-212.

Burggren, W. and Mwalukoma, A. (1983). Respiration during chronic hypoxia and hyperoxia in larval and adult bullfrogs *(Rana catesbeiana)*. Morphological responses of lungs, skin and gills. *J. Exp. Biol.* **105**, 191-203.

Burri, P. H. and Weibel, E. R. (1971). Morphometric estimation of pulmonary diffusion capacity II. Effect of PO_2 on the growing lung adaption of the growing rat lung to hypoxia and hyperoxia. *Respir. Physiol.* **11**, 247-264. doi:10.1016/0034-5687(71)90028-4

Burton, R. R. and Smith, A. H. (1968). Blood and air volumes in the avian lung. *Poult. Sci.* **47**, 85-91. doi:10.3382/ps.0470085

Cassin, S., Gilbert, R. D., Bunnell, C. E. and Johnson, E. M. (1971). Capillary development during exposure to chronic hypoxia. *Am. J. Physiol.* **220**, 448-451. doi:10.1152/ajplegacy.1971.220.2.448

Chan, T. and Burggren, W. W. (2005). Hypoxic incubation creates differential morphological effects during specific developmental critical windows in the embryo of the chicken (*Gallus gallus*). *Respir. Physiol. Neurobiol.* **145**, 251-263. doi:10.1016/j.resp.2004.09.005

Copeland, J. and Dzialowski, E. M. (2008). Effects of hypoxic and hyperoxic incubation on the reactivity of the chicken embryo (*Gallus gallus*) ductus arteriosi in response to catecholamines and oxygen. *Exp. Physiol.* **94**, 152-161. doi:10.1113/expphysiol.2008.044214

Cunningham, E. L., Brody, J. S. and Jain, B. P. (1974). Lung growth induced by hypoxia. *J. Appl. Physiol.* **37**, 362-366. doi:10.1152/jappl.1974.37.3.362

Duncker, H.-R. (1972). Structure of avian lungs. *Respir. Physiol.* **14**, 44-63. doi:10.1016/0034-5687(72)90016-3

Duncker, H. R. (1973). Der quantitative Aufbau des Lungen-Luftsacksystems der Vögel. *Verh. Anat. Ges.* **67**, 197-204.

Dzialowski, E. M., von Plettenberg, D., Elmonoufy, N. A. and Burggren, W. W. (2002). Chronic hypoxia alters the physiological and morphological trajectories of developing chicken embryos. *Comp. Biochem. Physiol. A* **131**, 713-724. doi:10.1016/S1095-6433(02)00009-0

Ferner, K. and Mortola, J. P. (2009). Ventilatory response to hypoxia in chicken hatchlings: a developmental window of sensitivity to embryonic hypoxia. *Respir. Physiol. Neurobiol.* **165**, 49-53. doi:10.1016/j.resp.2008.10.004

Freeman, B. M. and Misson, B. H. (1970). PH, pO_2, and pCO_2, of blood from the foetus and neonate of *Gallus domesticus*. *Comp. Biochem. Physiol.* **33**, 763-772. doi:10.1016/0010-406X(70)90025-3

Hassanzadeh, M., Fard, M. H. B., Buyse, J., Bruggeman, V. and Decuypere, E. (2004). Effect of chronic hypoxia during embryonic development on physiological functioning and on hatching and post-hatching parameters related to ascites syndrome in broiler chickens. *Avian Pathol.* **33**, 558-564. doi:10.1080/03079450400013188

Hassanzadeh, M., Gilanpour, H., Charkhkar, S., Buyse, J. and Decuypere, E. (2005). Anatomical parameters of cardiopulmonary system in three different lines of chickens: further evidence for involvement in ascites syndrome. *Avian Pathol.* **34**, 188-193. doi:10.1080/03079450500096372

Hills, B. A. (1974). *Gas Transfer in the Lung*. Cambridge: Cambridge University Press.

Hislop, A. and Reid, L. (1976). New findings in pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. *Br. J. Exp. Pathol.* **57**, 542-554.

Howard, C. V. and Reed, M. G. (2005). *Unbiased Stereology: Three Dimensional Measurement in Microscopy Advanced Methods*, 2nd edn. Oxford, UK: Oxford University Press.

Howell, K., Preston, R. J. and Mcloughlin, P. (2003). Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. *J. Physiol.* **547**, 133-145. doi:10.1113/jphysiol.2002.030676

Johnson, R. L., Jr., Cassidy, S. S., Grover, R. F., Schutte, J. E. and Epstein, R. H. (1985). Functional capacities of lungs and thorax in beagles after prolonged residence at 3,100 m. *J. Appl. Physiol.* **59**, 1773-1782. doi:10.1152/jappl.1985.59.6.1773

Julian, R. J. (1989). Lung volume of meat-type chickens. *Avian Diseases* **33**, 174-176. doi:10.2307/1591084

Lasiewski, R. C. and Calder, W. A., Jr. (1971). A preliminary allometric analysis of respiratory variables in resting birds. *Respir. Physiol.* **11**, 152-166. doi:10.1016/0034-5687(71)90020-X

Lechner, A. J. and Banchero, N. (1980). Lung morphometry in guinea pigs acclimated to hypoxia during growth. *Respir. Physiol.* **42**, 155-169. doi:10.1016/0034-5687(80)90112-7

León-Velarde, F., Sanchez, J., Bigard, A. X., Brunet, A., Lesty, C. and Monge, C. (1993). High altitude tissue adaptation in Andean coots: capillarity, fiber area, fibre type and enzymatic activities of skeletal muscle. *J. Comp. Physiol. B.* **163**, 52-58. doi:10.1007/BF00309665

Lewallen, M. A. and Burggren, W. W. (2015). Chronic hypoxia and hyperoxia modifies morphology and VEGF concentration of the lungs of the developing chicken (*Gallus gallus* variant *domesticus*). *Respir. Physiol. Neurobiol.* **219**, 85-94. doi:10.1016/j.resp.2015.08.004

Lourens, A., van den Brand, H., Heetkamp, M. J. W., Meijerhof, R. and Kemp, B. (2007). Effects of eggshell temperature and oxygen concentration on embryo growth and metabolism during incubation. *Poult. Sci.* **86**, 2194-2199. doi:10.1093/ps/86.10.2194

Maina, J. N. (1984). Morphometrics of the avian lung. 3. The structural design of the passerine lung. *Respir. Physiol.* **55**, 291-307. doi:10.1016/0034-5687(84)90052-5

Maina, J. N. (2003). A systematic study of the development of the airway (bronchial) system of the avian lung from days 3 to 26 of embryogenesis: a transmission electron microscopic study on the domestic fowl, *Gallus gallus* variant *domesticus*. *Tissue Cell.* **35**, 375-391. doi:10.1016/S0040-8166(03)00058-2

Maina, J. N. (2005). Quantitative morphology (morphometry). In *The Lung-Air Sac System of Birds: Development, Structure, and Function* (ed. J. N. Maina), pp. 125-157. Heidelberg-Berlin: Springer-Verlag.

Maina, J. N. and King, A. S. (1982). Morphometrics of the avian lung. 2. The wild mallard (*Anas platyrhynchos*) and greylag goose (*Anseranser*). *Respir. Physiol.* **50**, 299-310. doi:10.1016/0034-5687(82)90025-1

Maina, J. N. and West, J. B. (2005). Thin and strong! The bioengineering dilemma in the structural and functional design of the blood-gas barrier. *Physiol. Rev.* **85**, 811-844. doi:10.1152/physrev.00022.2004

Maina, J. N., King, A. S. and Settle, G. (1989). An allometric study of pulmonary morphometric parameters in birds, with mammalian comparisons. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **326**, 1-57. doi:10.1098/rstb.1989.0104

Maina, J. N., McCracken, K. G., Chua, B., York, J. M. and Milsom, W. K. (2017). Morphological and morphometric specializations of the lung of the Andean goose, *Chloephaga melanoptera*: a lifelong high-altitude resident. *PLoS ONE* **12**, e0174395. doi:10.1371/journal.pone.0174395

Makanya, A. N. and Djonov, V. (2009). Parabronchial angioarchitecture in developing and adult chickens. *J. Appl. Physiol.* **106**, 1959-1969. doi:10.1152/japplphysiol.91570.2008

Michel, R. P. and Cruz-Orive, L. M. (1988). Application of the Cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. *J. Microsc.* **150**, 117-136. doi:10.1111/j.1365-2818.1988.tb04603.x

Miller, A. T., Jr. and Hale, D. M. (1970). Increased vascularity of brain, heart, and skeletal muscle of polycythemic rats. *Am. J. Physiol.* **219**, 702-704. doi:10.1152/ajplegacy.1970.219.3.702

Mortola, J. P. (2009). Gas exchange in avian embryos and hatchlings. *Comp. Biochem. Physiol. A* **153**, 359-377. doi:10.1016/j.cbpa.2009.02.041

Mortola, J. P. and Cooney, E. (2008). Cost of growth and maintenance in chicken embryos during normoxic or hypoxic conditions. *Respir. Physiol. Neurobiol.* **162**, 223-229. doi:10.1016/j.resp.2008.07.015

Mortola, J. P. and Labbè, K. (2005). Oxygen consumption of the chicken embryo: interaction between temperature and oxygenation. *Respir. Physiol. Neurobiol.* **146**, 97-106. doi:10.1016/j.resp.2004.10.011

Mortola, J. P., Morgan, C. A. and Virgona, V. (1986). Respiratory adaptation to chronic hypoxia in newborn rats. *J. Appl. Physiol.* **61**, 1329-1336. doi:10.1152/jappl.1986.61.4.1329

Nielsen, B. S., Lund, L. R., Christensen, I. J., Johnsen, M., Usher, P. A., Wulf-Andersen, L., Frandsen, T. L., Danø, K. and Gundersen, H. J. G. (2001). A precise and efficient stereological method for determining murine lung metastasis volumes. *Am. J. Physiol.* **158**, 1997-2003. doi:10.1016/S0002-9440(10)64671-8

Okubo, S. and Mortola, J. P. (1989). Respiratory mechanics in adult rats hypoxic in the neonatal period. *J. Appl. Physiol.* **66**, 1772-1778. doi:10.1152/jappl.1989.66.4.1772

Owen, R. L., Wideman, R. F., Jr., Barbato, G. F., Cowen, B. S., Ford, B. C. and Hattel, A. L. (1995). Morphometric and histologic changes in the pulmonary system of broilers raised at simulated high altitude. *Avian Pathol.* **24**, 293-302. doi:10.1080/03079459508419070

Perry, S. F. (1978). Quantitative anatomy of the lungs of the red-eared turtle, *Pseudemys scripta elegans*. *Respir. Physiol.* **35**, 245-262. doi:10.1016/0034-5687(78)90001-4

Perry, S. F. (1981). Morphometric analysis of pulmonary structure: methods for evaluation and comparison of unicameral lungs. *Mikroskopie* **38**, 178-193.
Rabinovitch, M. (2012). Molecular pathogenesis of pulmonary arterial hypertension. *J. Clin. Invest.* **122**, 4306-4313. doi:10.1172/JCI60658
Rabinovitch, M., Gamble, W., Nadas, A. S., Miettinen, O. S. and Reid, L. (1979). Rat pulmonary circulation after chronic hypoxia: hemodynamic and structural features. *Am. J. Physiol.* **236**, 818-827. doi:10.1152/ajpheart.1979.236.6.H818
Ramirez, J.-M., Folkow, L. P. and Blix, A. S. (2007). Hypoxia tolerance in mammals and birds: from the wilderness to the clinic. *Annu. Rev. Physiol.* **69**, 113-143. doi:10.1146/annurev.physiol.69.031905.163111
Ravikumar, P., Bellotto, D. J. and Hsia, C. C. W. (2015). Persistent structural adaptation in the lungs of guinea pigs raised at high altitude. *Respir. Physiol. Neurobiol.* **208**, 37-44. doi:10.1016/j.resp.2014.12.011
Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208-212. doi:10.1083/jcb.17.1.208
Ruijtenbeek, K., Le Noble, F. A. C., Janseen, G. M. J., Kessels, C. G. A., Fazzi, G. E., Blanco, C. E. and De Mey, J. G. R. (2000). Chronic hypoxia stimulates periarterial sympathetic nerve development in chicken embryo. *Circulation* **102**, 2892-2897. doi:10.1161/01.CIR.102.23.2892
Schachner, E. R., Sedlmayr, J. C., Schott, R., Lyson, T. R., Sanders, R. K. and Lambertz, M. (2017). Pulmonary anatomy and a case of unilateral aplasia in a common snapping turtle (*Chelydra serpentina*): developmental perspectives on cryptodiran lungs. *J. Anat.* **231**, 835-848. doi:10.1111/joa.12722
Sekhon, H. S. and Thurlbeck, W. M. (1996). Lung morphometric changes after exposure to hypobaria and/or hypoxia and undernutrition. *Respir. Physiol.* **106**, 99-107. doi:10.1016/0034-5687(96)00049-7
Semenza, G. L. (2000). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J. Appl. Physiol.* **88**, 1474-1480. doi:10.1152/jappl.2000.88.4.1474
Semenza, G. L. (2012). Hypoxia-inducible factors in physiology and medicine. *Cell* **148**, 399-408. doi:10.1016/j.cell.2012.01.021
Snyder, G. K., Byers, R. L. and Kayar, S. R. (1984). Effects of hypoxia on tissue capillarity in geese. *Respir. Physiol.* **58**, 151-160. doi:10.1016/0034-5687(84)90144-0
Stenmark, K. R. and Mcmurtry, I. F. (2005). Vascular remodeling versus vasoconstriction in chronic hypoxic pulmonary hypertension a time for reappraisal? *Circ. Res.* **97**, 95-98. doi:10.1161/01.RES.00000175934.68087.29
Strick, D. M., Waycaster, R. L., Montani, J.-P., Gay, W. J. and Adair, T. H. (1991). Morphometric measurements of chorioallantoic membrane vascularity: effects of hypoxia and hyperoxia. *Am. J. Physiol.* **260**, 385-389. doi:10.1152/ajpheart.1991.260.4.H1385
Szdzuy, K. and Mortola, J. P. (2007). Ventilatory chemosensitivity of the 1-day-old chicken hatchling after embryonic hypoxia. *Am. J. Physiol.* **293**, 1640-1649. doi:10.1152/ajpregu.00422.2007
Szdzuy, K., Fong, L. M. and Mortola, J. P. (2008). Oxygenation and establishment of thermogenesis in the avian embryo. *Life Sci.* **82**, 50-58. doi:10.1016/j.lfs.2007.10.007
Tazawa, H., Mikami, T. and Yoshimoto, C. (1971). Effect of reducing the shell area on the respiratory properties of chicken embryonic blood. *Respir. Physiol.* **13**, 352-360. doi:10.1016/0034-5687(71)90039-9
Timmwood, K. I., Hyde, D. M. and Plopper, C. G. (1987). Lung growth of the turkey, *Meleagris gallopavo*: II. Comparison of two genetic lines. *Am. J. Anat.* **178**, 158-169. doi:10.1002/aja.1001780207
Venable, J. H. and Coggeshall, R. (1965). A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**, 407-408. doi:10.1083/jcb.25.2.407
Vidyadaran, M. K., King, A. S. and Kassim, H. (1987). Deficient anatomical capacity for oxygen uptake of the developing lung of the female domestic fowl when compared with red-jungle fowl. *Schweiz. Arch. Tierheilkd.* **129**, 225-237. doi:10.1002/aja.1001780207
Vidyadaran, M. K., King, A. S. and Kassim, H. (1988). Quantitative studies of the lung of the domestic fowl *(Gallus gallus* var. *domesticus)*. *Pertanika* **11**, 229-238.
Vidyadaran, M. K., King, A. S. and Kassim, H. (1990). Quantitative comparisons of lung structure of adult domestic fowl and red jungle fowl, with reference to broiler ascites. *Avian Pathol.* **19**, 51-58. doi:10.1080/03079459008418655
Visschedijk, A. H. J. (1968). The air space and embryonic respiration. *Br. Poult. Sci.* **9**, 197-210. doi:10.1080/00071668808415709
Wagner-Amos, K. and Seymour, R. S. (2003). Effect of local shell conductance on the vascularisation of the chicken chorioallantoic membrane. *Respir. Physiol. Neurobiol.* **134**, 155-167. doi:10.1016/S1569-9048(02)00209-4
Weibel, E. R. and Knight, B. W. (1964). A morphometric study on the thickness of the pulmonary air-blood barrier. *J. Cell Biol.* **21**, 367-384. doi:10.1083/jcb.21.3.367
Weibel, E. R., Hsia, C. C. W. and Ochs, M. (2007). How much is there really? Why stereology is essential in lung morphometry. *J. Appl. Physiol.* **102**, 459-467. doi:10.1152/japplphysiol.00808.2006
Xu, L. and Mortola, J. P. (1989). Effects of hypoxia or hyperoxia on the lung of the chick embryo. *Can. J. Physiol. Pharmacol.* **67**, 515-519. doi:10.1139/y89-081
Zhang, H. and Burggren, W. W. (2012). Hypoxic level and duration differentially affect embryonic organ system development of the chicken (*Gallus gallus*). *Poult. Sci.* **91**, 3191-3201. doi:10.3382/ps.2012-02449