The Effects of Chronic Hypoxia on Cardiac Function Measured by Pressure-Volume Catheter in Fetal Chickens

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Abstract

Hypoxia is a common component of many developmental insults and has been studied in early-stage chicken development. However, its impact on cardiac function and arterial-ventricular coupling in late-stage chickens is relatively unknown. To test the hypothesis that hypoxic incubation would reduce baseline cardiac function but protect the heart during acute hypoxia in late-stage chickens, white Leghorn eggs were incubated at 21% O₂ or 15% O₂. At 90% of incubation (19 days), hypoxic incubation caused growth restriction (-20%) and increased the LV/body ratio (+41%). Left ventricular (LV) pressure-volume loops were measured in anesthetized chickens in normoxia and acute hypoxia (10% O₂). Hypoxic incubation lowered the maximal rate of pressure generation ($\Delta P/\Delta t_{\text{Max}}$ ; -22%) and output (-57%), while increasing end systolic elastance ($E_{\text{LV}}$; +31%) and arterial elastance ($E_{\text{A}}$; +122%) at similar heart rates to normoxic incubation. Both hypoxic incubation and acute hypoxia lengthened the half-time of relaxation ($\tau$; +24%). Acute hypoxia reduced heart rate (-8%) and increased end diastolic pressure (+35%). Hearts were collected for mRNA analysis. Hypoxic incubation was marked by decreased mRNA expression of sarco(endo)plasmic reticulum Ca²⁺-ATPase 2, Na⁺/Ca²⁺ exchanger 1, phospholamban and ryanodine receptor. In summary, hypoxic incubation reduces LV function in the late-stage chicken by slowing pressure generation and relaxation, which may be driven by altered intracellular excitation-contraction coupling. Cardiac efficiency is greatly reduced following hypoxic incubation. In both incubation groups acute hypoxia reduced diastolic function.

Keywords: heart development, animal model, embryo, IUGR, prenatal hypoxia
Introduction

Prenatal hypoxia affects about 12% of pregnancies in the United States and often results in intrauterine growth restriction and premature delivery (20, 55). Chronic fetal hypoxia is linked to depressed cardiac function in humans and animal models (15, 18, 32, 37). Some effects of hypoxia in the mammalian fetus are mediated by the placenta or by maternal factors (54), independent of the direct influence of oxygen restriction on the fetal cardiovascular system (16). To understand how cardiac development is directly altered by hypoxia, it is necessary to separate placental and maternal influences from primary fetal responses (3, 23, 47).

The developing chicken is a model that uniquely allows the investigator to isolate the effects of reduced oxygen from maternal and placental mediators. Arterial pressure and heart rate have been successfully measured throughout the majority of chicken ontogeny (9, 13, 14, 43, 44, 53), while quantification of cardiac function has been restricted to early stages of development (24, 41, 42, 49, 50) and lung-ventilating chickens prior to hatching (48). Function of the late-stage fetal chicken heart in which ventilation has not begun (internal pipping occurs at about day 19.5) is less well understood due to technical difficulties such as loss of transparency of embryonic tissues and reduced ease of access to the heart. Thus, in vivo cardiac function and arterial-ventricular coupling in the late-stage, non-ventilating fetal chicken are not well understood.

Studies of isolated hearts, and of hearts of lung-ventilating fetal chickens, indicate that chronic hypoxia leads to cardiac chamber dilation and reduced cardiac function (38, 48). However, we previously found that hypoxia-incubated fetuses exhibit less arterial pressure and heart rate depression in response to acute hypoxic challenge (21). Based on this discrepancy, we hypothesized that fetal chicken cardiac adaptation to hypoxic incubation would reduce baseline cardiac function but precondition the animal to maintain cardiac function during acute hypoxia. We further sought to investigate the transcriptional regulation underlying
functional changes, focusing on altered mRNA expression of excitation-contraction coupling and growth-
controlling genes.

Methods

Ethical Approval

All protocols were conducted with the approval of the University of North Texas Animal Care and Use
Committee under protocol #11007.

Animals

White Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from the Department of Poultry
Science at Texas A&M University. Upon delivery, fertilized eggs were randomly separated into two groups:
normoxic incubation (21% O₂) or hypoxic incubation (15% O₂). The degree of hypoxia was selected to
allow comparison with previous studies (12, 21, 27, 38, 39, 48). Both groups were incubated at 38ºC with a
relative humidity of 60% and hourly rotation in chicken egg incubators (BSS 160 Grumbach, Asslar,
Germany). Oxygen concentrations were set using rotameters or a Sechrist Air-Oxygen mixer (Model
3500HL, Sechrist Industries, Inc., Anaheim, CA, USA), downstream of compressed N₂ and air, or air alone,
with the mixtures being bubbled through water to ensure appropriate humidity. Gas compositions within
both incubation conditions were monitored with an oxygen analyzer (S-3AII, Ametek Applied
Electrochemistry, Pittsburgh, PA, USA) via a PowerLab data recording system (ADInstruments, Colorado
Springs, CO, USA) connected to a computer running LabChart software (v7.2, ADInstruments). Fetuses
from day 19 of the 21-day incubation period (i.e. 90% of development) were included in the study.

Cardiac pressure-volume recordings

Surgical preparation: Both incubation groups had cardiac studies performed in room air and in hypoxia.
Thus, four conditions were compared: 1) normoxic incubation (21% O₂) in normoxia, 2) normoxic
incubation in acute hypoxia (10% O₂), 3) hypoxic incubation (15% O₂) in experimental normoxia, and 4)
hypoxic incubation in acute hypoxia. Eggs were removed from incubation and candled to locate the fetus. Eggs were then placed in a custom made, heated (38°C) surgical chamber at 21% O₂ under a dissecting microscope (Leica M60, Leica Microsystems, Waukegan, IL, USA) and a small section of eggshell was removed. The underlying shell membrane and chorioallantoic membrane were opened to isolate the fetus. The amniotic membrane was opened and the fetus was located. Temperature was continuously monitored with a probe placed inside the egg (BAT-12, Physitemp Instruments, Clifton, NJ, USA). Pentobarbital (0.75-1.0 mg subcutaneous) was injected to achieve a surgical plane of anesthesia. Movement was monitored to assess anesthesia; additional incremental doses were provided as needed. The chest was opened through a midline incision above the keel. The muscle was blunt dissected away from the keel, and the bone was cut to provide access to the thoracic cavity. The ventral aspect of the pericardium was opened and a pericardial sling was created to support the heart. A loop of silk suture (6-0) was passed around the aorta to intermittently constrict the aorta and increase load on the left ventricle (LV). Heparinized saline (2-2.5 units in 20-25μl) was given with a fine needle (25-30 gauge) through the LV apex. A calibrated, pre-soaked, zeroed Scisense 1.2Fr ADVantage admittance catheter (Transonic Scisense Inc, Toronto, ON, Canada; 46) was introduced into the LV through the 25-30 gauge needle hole in the LV apex and oriented with the tip pointed at the aortic valve. The position of the admittance catheter was adjusted to minimize the phase signal (a high phase indicates interference by the myocardium), to maximize the magnitude range, and to obtain a pressure-volume loop free of artifacts. Once a satisfactory signal was obtained, the preparation was allowed to stabilize for about 10 minutes.

**Experimental protocol:** Three recordings of LV pressure and volume were made in normoxia, starting at baseline pressure and during increasing aortic occlusion until maximum LV systolic pressure was achieved. Heart rate and LV pressure were allowed to return to normal between each pressure “ramp”. Ramps were compared to ensure a consistent response, but only one ramp was analyzed from each fetus (Fig. 1).
Acute experimental hypoxia was achieved by mixing nitrogen gas with room air to achieve 10% O₂, warmed to 38°C, and passed into the surgical chamber at a flow rate of 2 L min⁻¹. Chamber gas percentage was continuously monitored with an Oxygen analyzer (S-3AII, Ametek Applied Electrochemistry, Pittsburgh, PA, USA). Recordings of LV pressure and volume starting at baseline and during increasing aortic occlusions were repeated as described beginning 2 minutes after chamber gas reached 10% O₂ and concluded by 5 minutes after chamber gas reached 10% O₂. This level of acute hypoxia was chosen because it was lower than the developmental oxygen concentration for both groups, but all fetuses could maintain normal LV systolic pressure during the data collection period. Catheter data (pressure, phase, magnitude) and oxygen percentage of the chamber were continuously recorded at 100 Hz using a Powerlab and computer (Apple, Cupertino, CA, USA) running LabChart. Heart rate was calculated from LV pulse pressure frequency.

**Blood resistivity**

Calculation of chamber volume by the Scisense system requires input of a blood resistivity constant. Chicken fetuses were anesthetized and heparinized as described above. Approximately 200-300μl of blood was withdrawn into a syringe from the LV, aorta or main pulmonary artery. Blood was placed within a small plastic container within a small airtight bag and resistivity was immediately measured with a Scisense reference probe. The gas mixture was changed within the bag to 10% O₂, the blood gently but thoroughly mixed, and resistivity measured again. Blood resistivity at 21% O₂ was 1.6 ± 0.1 Ωm for normoxic incubation (n=8) and 2.5 ± 0.1 Ωm for hypoxic incubation (n=6). In 10% O₂, blood resistivity was 1.8 ± 0.1 Ωm (n=5) for normoxia-incubated and 2.4 ± 0.2 Ωm for hypoxia-incubated (n=3) fetuses. The oxygen gas concentration at the time of measurement did not have a statistically significant effect on blood resistivity, but incubation oxygen concentration did (P<0.0001). Rheological changes alter blood resistivity (30, 33), and may explain differences between normoxia- and hypoxia-incubated fetuses (21).

**Stroke volume**
Calculation of chamber volume by the Scisense system requires input of a baseline stroke volume constant. Chicken fetuses were anesthetized and heparinized as described, and their thoracic cavities were opened. The heart was exposed and a 1.5 mm flow probe (Transonic Inc., Ithaca, NY, USA) was placed sequentially around each of the brachiocephalic arteries and aorta proximal to the ductus arteriosus, and stable flow was recorded for several minutes in each of the vessels. Due to size and anatomical limitations, simultaneous flow measurements could not be conducted. Blood flows from these vessels were summed to obtain LV stroke volumes used to calibrate the Scisense system.

Cardiac pressure-volume calculations

Regions of interest in the LabChart record were converted to text format with no loss of fidelity, and analyzed in the data post-processor software Advol (Transonic Scisense Inc) using the measured constants, a “heart type” value of 1 (personal communication from A. Kottam of Transonic Scisense Inc.), and the phase and magnitude channels (35), to obtain LV volume. Data files were then opened in LabChart v7.3.5 and pressure-volume loops were analyzed using the Pressure-Volume Loop module. End diastole was taken to be inflection between low-pressure ventricular filling and isovolumetric contraction.

Masses and tissue collection

Prior to setting eggs, egg mass was measured. At the completion of the hemodynamic measurements, fetuses were euthanized with an overdose of the anesthetic isoflurane. Yolk-free fetal and heart (including proximal great vessels) masses were measured. A separate set of chicken fetuses incubated as described under normoxic and hypoxic conditions, but that did not undergo surgery, were euthanized and their hearts collected for mRNA analysis. After blotting, hearts were dissected into LV freewall, right ventricular (RV) freewall, septum, and atria (combined left and right). Samples were immediately immersed in RNAlater (Life Technologies, Grand Island, NY) and then stored at -20°C.

mRNA expression analysis
Tissue processing, RNA isolation and first strand cDNA synthesis. RNA from ventricular tissue was isolated by homogenizing with a TissueLyser LT (Qiagen Valencia, CA, USA) in TRIzol (Ambion Grand Island, NY). The RNA samples were cleaned using the RNeasy Mini Kit (Qiagen, Germantown, MD) and analyzed (Synergy H1 Hybrid Multi-Mode Microplate Reader Gen5, data analysis software from BioTek Winooski, VT, USA) to ensure integrity of RNA prior to first strand cDNA synthesis. Total first strand cDNA was synthesized using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies) per manufacturer's protocol (10 min at 25°C, 120 min at 37°C, 5 min at 85°C, hold at 4°C), with the addition of 2.5 uM oligo dT (Table 1).

Quantitative PCR. Candidate genes from genomic databases were identified by analysis of sequence alignments of chicken (Gallus gallus) target sequences. Genes assessed in the excitation-contraction coupling pathway include Na+/Ca2+ exchanger 1 (NCX1), sarco(endo)plasmic reticulum Ca2+-ATPase 2 (SERCA2), phospholamban (PLN), cardiac ryanodine receptor (RYR). Growth-associated genes assessed include collagen alpha-2(I) chain (COL1A2), kinase insert domain receptor (KDR, also known as VEGFR-2), vascular endothelial growth factor A (VEGF-A), Notch1, and Cyclin D1. B-type natriuretic peptide (BNP)-family gene chicken cardiac natriuretic peptide B (chNP) was also assessed. qPCR primer design was accomplished using Primer 3 software program (San Diego Biology Workbench 3.2, http://workbench.sdsc.edu/). All primers (Eurofins MWG Operon Inc, Huntsville, AL, USA) were tested to optimize annealing temperatures and ensure single product formation. Each primer pair produced a single PCR product as evidenced by melt curve analysis and gel electrophoresis (Table 1). Amplification products were sequenced at Eurofins MWG Operon Inc, and the sequences were compared to other DNA sequences using BLAST in The Gene Index database (TGI, http://compbio.dfci.harvard.edu/tgi/ncbi/blast/blast.html) to confirm amplification of the target gene. The BLAST queries resulted in correct gene hits with BLAST e-values <10–15. As part of our validation strategy, we also compared similar sequences with lesser e-values before assigning identities to the target genes.
After PCR product validation and assay optimization, qPCR assays were conducted in a 96-well format under the optimal PCR conditions with Applied Biosystems Power SYBR Green Master Mix (Life Technologies) using the relative standard curve method on a Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). Reactions for each cDNA sample were performed in triplicate, including the standard curve, which was generated from pooled samples and quantified before serial dilution. PCR amplifications were performed for 1 cycle at 95 °C for 10 min, 42 cycles with denaturation at 94 °C for 20 s, annealing at optimum temperature for primers (55–58 °C) for 30 s, and extension at 72 °C for 30 s followed by melt curve analysis. All samples were normalized against 18S ribosomal RNA (18S) quantities.

Statistics

Physiological data from chronic and acute hypoxia were analyzed by repeated measures analysis of variance (ANOVA; Prism 6.0d, Graphpad Software Inc, San Diego, CA). If the F-statistic justified further comparisons based on either incubation condition, or oxygen at the time of measurement, Bonferroni-corrected comparisons between groups were performed within Prism and the multiplicity-adjusted P value is reported (57). If the F-statistic justified further comparisons based on both incubation condition and oxygen at the time of measurement, comparisons were carried out in the GraphPad QuickCalcs Post test calculator using the Bonferroni method (31). Heart mass, body mass and gene expression values were compared by unpaired two-tailed t-test. Physiological data, mass, and gene expression are shown as mean ± standard error of the mean (SEM). Intercepts and slopes relating LV pressure to volume were compared between incubation groups and between experimental conditions using mixed models with unstructured autoregressive covariance matrices (SAS version 9.3, SAS Inc., Cary, NC, USA). To facilitate these comparisons, we incorporated interaction between LV volume and incubation condition, and between LV volume and experimental condition. These regression data are presented as estimate ± SEM. A P value of less than 0.05 was taken to be significant.
Results

Fetal and heart mass

Hypoxia-incubated chicken fetuses were 20% smaller than normoxia-incubated fetuses at 19 days of incubation, indicating developmental growth restriction (P<0.0001; Table 2). Heart mass was similar between the groups; consequently the heart to body mass was 17% greater in hypoxia-incubated fetuses (P<0.02; Table 2). LV mass relative to body mass was 41% greater in hypoxia-incubated fetuses (P<0.002; Table 2). Absolute LV, RV and septal masses were similar in both groups (Table 2).

Measured cardiac function

LV output, as determined by stroke volume and heart rate, was affected both by incubation group (P<0.0001) and by acute hypoxia (P<0.02; Table 3). LV output of hypoxia-incubated fetal chickens was 58% lower than the normoxic incubation group in room air (P<0.0001; Table 3), and 55% lower in acute hypoxia (P<0.001). We could not detect a difference in cardiac output within incubation groups between normoxia and acute hypoxia. LV stroke volume in the hypoxia-incubated group was less than half that of the normoxia-incubated group (P<0.001; Table 3). In room air, LV output normalized to body mass in room air was 0.25 ± 0.01 ml min⁻¹ g⁻¹ in the normoxia-incubated group and 0.12 ± 0.01 ml min⁻¹ g⁻¹ in the hypoxia-incubated group (P<0.0001). In acute hypoxia, normalized LV output was 0.20 ± 0.02 ml min⁻¹ g⁻¹ in the normoxia-incubated group and 0.11 ± 0.01 ml min⁻¹ g⁻¹ in the hypoxia incubated group (P<0.001).

Acute hypoxia reduced heart rate by 8% (P<0.02), but differences were not detectable between incubation groups. Incubation oxygen concentration did not affect heart rate at 19 days of incubation. Neither hypoxic incubation nor acute hypoxia significantly altered the period spent in systole (120 ± 2 ms). Hypoxic incubation did not significantly affect diastolic period, but it was significantly lengthened by acute hypoxia (164 ± 5 ms in room air, 191 ± 9 ms in 10% O₂).
End systolic pressure was not different between incubation groups, and did not differ with acute hypoxia (4.28 ± 0.09 kPa, equivalent to 32.1 ± 0.1 mmHg; Table 3). Maximum end systolic pressure generated during aortic occlusions also did not differ by incubation or acute hypoxia (normoxic incubation and experimental normoxia: 7.8 ± 0.4 kPa, normoxic incubation and acute hypoxia: 7.9 ± 0.4 kPa; hypoxic incubation and experimental normoxia: 7.4 ± 0.5 kPa, hypoxic incubation and acute hypoxia: 6.9 ± 0.4 kPa; equivalent to 51-59 mmHg). End diastolic pressure was affected by acute hypoxia (P<0.0001) but not by incubation oxygen concentration. No significant differences were found in end diastolic volume (normoxic incubation and experimental normoxia: 74.6 ± 4.8 μl, normoxic incubation and acute hypoxia: 77.2 ± 9.9 μl; hypoxic incubation and experimental normoxia: 68.4 ± 6.8 μl, hypoxic incubation and acute hypoxia: 67.2 ± 5.3 μl). During acute hypoxia, end diastolic pressure increased by 21% in the hypoxia-incubated group (P<0.02) and 50% in the normoxia-incubated group (P<0.0005).

Calculated cardiac function
Stroke work, the product of change in ventricular pressure and stroke volume, was affected by incubation oxygen concentration (P=0.0003) but not acute hypoxia. In both room air and during acute hypoxia, stroke work more than 50% less in the hypoxia-incubation group compared to the normoxia-incubation group (P<0.0004; Table 3). The maximum derivative of change in systolic pressure over time (ΔP/Δt_{Max}) was affected by incubation oxygen concentration (P<0.02). ΔP/Δt_{Max} was about 22% lower in hypoxia-incubated chicken hearts in both room air (P<0.0001) and in acute hypoxia (P<0.001; Table 3 and Fig. 3). ΔP/Δt_{Max} was 6% lower during acute hypoxia compared to ΔP/Δt_{Max} in room air (P<0.04), but there were no significant differences between specific groups by post-test. LV relaxation was also affected by both incubation oxygen (P<0.03) and acute hypoxia (P=0.0003). The minimal derivative of change in systolic pressure over time (ΔP/Δt_{min}) was not affected by incubation or acute hypoxia (Table 3). The time constant of relaxation (τ) in hypoxia-incubated fetuses was 22% greater than in the normoxia-incubated group, indicating a slower rate of relaxation (P<0.01 for both experimental oxygen concentrations; Fig. 3). Acute
hypoxia increased $\tau$ by 20% over control values for hypoxia-incubated chicken fetuses (P<0.01) and for normoxia-incubated fetuses (P<0.05).

End systolic elastance ($E_{LV}$), which is the slope of LV pressure versus LV volume, was significantly steeper in the hypoxia-incubated group compared to normoxia-incubated group (P=0.003; Table 3). The regression intercepts at 0 μl LV volume were significantly less in the hypoxic incubation fetuses (P=0.03). The end systolic pressure-volume relationships (ESPVR) intercept at 0 kPa pressure ($V_0$) was calculated from the regression intercept at 0 μl LV volume and $E_{LV}$. $V_0$ was right-shifted by hypoxic incubation (Table 3). The ESPVR was not affected by acute experimental oxygen conditions. Effective arterial elastance ($E_A$), defined as end systolic pressure divided by stroke volume, was affected by incubation oxygen concentration (P<0.0001) but not acute hypoxia (Table 3). $E_A$ was ~121% greater in the hypoxia- compared to normoxia-incubated group (P<0.05 in both experimental oxygen conditions). The ratio $E_A / E_{LV}$ was higher in hypoxia- than normoxia-incubated chicken hearts in room air (44%; P=0.0007), and in acute hypoxia (38%; P<0.002; Table 3).

**Excitation-contraction coupling gene expression**

Cardiac ryanodine receptor (RYR) mRNA levels were more than 50% lower in hypoxia-incubated fetal chickens compared to the normoxia incubated group in both LV (P<0.02) and RV (P<0.02; Fig. 2). Hypoxic incubation also decreased Na$^+$/Ca$^{2+}$ exchanger 1 (NCX1) mRNA by 40% in the RV (P<0.05), and although there was a similar tendency in the LV it was not significant (P<0.07; Fig. 2). Sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase 2 (SERCA2) mRNA levels were 37% lower in the hypoxia-incubated LV (P<0.02; Fig. 2).

Phospholamban (PLN) mRNA levels were 44% lower in both ventricles in the hypoxia-incubated fetus compared to the normoxia-incubated control (LV: P<0.02; RV: P<0.03; Fig. 2).

**Expression of growth-associated genes**
Expression of growth regulating genes kinase insert domain receptor (KDR, also known as VEGFR-2) and vascular endothelial growth factor A (VEGF-A) were unchanged, although they tended to be lower in the LV of hypoxia-incubated fetuses (both P<0.06; Fig. 2). Expression of cyclin D1 was 40% lower in the hypoxia-incubated LV (P<0.003), although proliferation-associated Notch1 expression was not different (Fig. 2). Neither gene was different in the RV (Fig. 2). The LV (but not RV) mRNA relative concentrations of collagen alpha-2(1) chain (COL1A2), which encodes one of the chains for type 1 collagen, was 45% lower in hypoxic incubation group (P<0.04; Fig. 2). Expression of the chicken cardiac natriuretic peptide B (chNP) was similar between incubation conditions (Fig. 2).

Discussion

This study represents the first direct measurements of cardiac pressure-volume relationships in the intact late-stage chicken prior to ventilation. Further, although LV end systolic elastance, $E_{LV}$ (41, 42), and effective arterial elastance, $E_A$ (59), have been described separately in the stage 21-24 chicken embryo, this is the first description of simultaneous in ovo cardiac pressure-volume measurements in a non-mammalian embryonic or fetal organism. The primary findings of this study were that at 90% of incubation (19 day) hypoxic incubation led to greatly increased $E_A$ (an index of afterload; 58), slower LV contraction, and slower LV relaxation (Table 3, Fig. 3). During acute hypoxia (10% O2), the time constant of relaxation ($\tau$), a diastolic parameter of function, was increased in both normoxic and hypoxic incubation groups. These functional changes were concurrent with decreased expression of genes involved in cardiac calcium handling. Together, the data suggest that chronic developmental hypoxia reduces cardiac function and decreases cardiac energy efficiency.

Arterial-cardiac coupling

The $E_A / E_{LV}$ ratio captures the interaction between LV performance and arterial properties that affect energetic efficiency (7). Just prior to lung ventilation (90% of incubation, or day 19) the $E_A / E_{LV}$ ratio is 1 in the normoxia-incubated chicken fetus (Table 3), which is similar to normal values in healthy adult...
mammals at rest and in fetal lambs (7, 25). This suggests that mechanical efficacy and energetic efficiency are balanced similarly in fetal chickens as in adult and fetal mammals.

Hypoxia-incubated fetal chickens had a greater $E_A$ than control fetuses. End systolic and end diastolic pressures were similar between incubation groups at similar end diastolic pressures; $E_A$ is different because stroke volume in the hypoxic incubation group is less than half that in the normoxic incubation group.

Although $E_A$ was greater in hypoxia-incubated fetuses, $E_{LV}$ did not increase proportionally. The ESPVR relationship is steeper in hypoxia-incubated fetuses, but $E_{LV}$ and $V_0$ must be interpreted together, and the picture they provide is one of reduced contractility (4). Supporting this conclusion is our finding that the maximal LV rate of pressure generation was 22% lower in hypoxic-incubated chicken fetuses. Furthermore, LV stroke work in the hypoxic-incubated group was less than half the value in the control group, despite similar LV masses.

Increased $E_A$ may be due to thicker, stiffer arteries (7, 40), resulting from differences in arterial anatomy and resting vascular tone (38). These vascular changes may be specific to certain vascular beds. For instance, aortic wall thickness is increased, lumen diameter is reduced, and peripheral vascular sympathetic innervation increased in chronically hypoxic fetal chickens (38). In contrast, hindlimb arterial resistance is not different between fetal chickens from hypoxic and normoxic incubation (21). Further, our prior studies indicate that vascular adrenergic pathways in the chicken fetus are stimulated mainly by catecholamines of adrenomedullary rather than sympathetic origin (9, 10, 28). The impact of a higher $E_A$ may affect post-hatching physiology given that it has been linked to exercise intolerance and systolic pressure sensitivity (8, 19). Indeed, rats subjected to intra-uterine growth restriction have impaired cardiovascular performance in response to dobutamine challenge as adults (60). Further, adult chickens that were incubated under hypoxic conditions have enlarged hearts and systolic dysfunction (27).

Cardiac function
Indices of depressed cardiac function in this study parallel previously reported consequences of
developmental hypoxia or intrauterine growth restriction in the sheep and human heart (6, 22, 29),
suggesting that placental and maternal factors are not primarily responsible for cardiovascular adaptations to
this stress. We found that developmental hypoxia decreased $\Delta P/\Delta t_{\text{Max}}$ and increased $\tau$, similar to findings
from prior studies of the isolated, Langendorff-perfused fetal chicken heart at 90% of incubation (day 19; 38), and isolated muscle bundles from the 95% incubation (day 20) hypoxia-incubated chicken heart (48).

RYR is responsible for calcium-induced calcium release from the sarcoplasmic reticulum, and decreased
$\Delta P/\Delta t_{\text{Max}}$ may be a result of the more than 50% reduction in expression of this gene (Fig. 2). Similarly, slow
relaxation in this study is likely linked to the substantial reduction in expression of NCX, which moves
cytosolic calcium to the extracellular space during diastole, and SERCA2, which concentrates cytosolic
calcium in the sarcoplasmic reticulum during diastole. These genes, together with the SERCA2-regulating
PLN, are critically important for cardiac function and were profoundly regulated by developmental hypoxia.
Although mRNA expression levels do not directly predict functional protein levels, the mRNA expression
levels of genes involved in excitation-contraction coupling parallel the functional changes.

Cardiac growth

Although stroke volume after hypoxic incubation was halved, heart mass and end diastolic volume were
similar between incubation conditions. Hypoxia-incubated chickens had low yolk-free body mass for their
developmental stage, but their heart masses were similar to the normoxia-incubated group. Similar mass
does not necessarily indicate normal cardiac morphology, and altered cardiac geometry resulting from
altered growth may affect cardiac performance. Differences in wall stress between the LV and RV freewalls,
at the equal arterial pressures of the fetal circulation (34), may account for differences in gene expression
patterns between the two ventricles. Interestingly, there was a large reduction in LV (but not RV) Col1A2
mRNA expression in hypoxia-incubated hearts (Fig. 2). The product of Col1A2 is a component of type 1
collagen, and mutations in this gene are associated with Ehlers–Danlos syndrome. If this indicates abnormal
development of the fibrous myocardial scaffolding, hypoxic incubation may sensitize the heart to diastolic
load. Alternatively, as heart weights were similar between incubation groups, translational and post-
translational regulation may support growth at normal levels despite reduced mRNA expression.

Heart failure or adaptation?
It has been suggested that the cardiac changes seen in the chronically hypoxic fetal chicken are due to
failure (38, 48). This contradicts our own findings that hypoxic incubation may protect the fetal chicken
during acute hypoxia by limiting arterial pressure and heart rate reductions (21). In this study, we found τ
was longer in hypoxia-incubated fetuses as well as lengthened by acute hypoxia in both groups. However,
we also found that cardiac output was similarly preserved during acute hypoxia in both hypoxia-incubated
and normoxia-incubated fetuses. Furthermore, expression for the BNP-family gene chNP, which is
analogous to mammalian BNP (1, 2, 36), did not increase with hypoxic incubation. It is important to note
that, whereas increased cardiac natriuretic peptide expression is a hallmark of failure in mammals (56), birds
lack the atrial natriuretic peptide gene (51), and the correlation between increased chNP expression during
cardiac failure in birds has not been investigated. Therefore, further investigations regarding the correlation
between cardiac failure and chNP gene expression need to be completed to establish the predictive value of
the level chNP reported here in chicken hearts. Expression changes for the excitation-contraction coupling
and growth genes assessed also only partially resembles the pathophysiology described in adult heart failure
(5, 52). While heart failure certainly can occur in the fetus (45), it is clear that the fetal chicken response to
chronic hypoxia cannot be characterized as classic failure.

Limitations of the study
While fetal chickens are sensitive to halogenated ether anesthetics such as isoflurane, which are preferred
for pressure-volume studies in rodents, it is not practical to use inhalation anesthetics prior to lung
ventilation. In this study chicken fetuses were studied under a barbiturate anesthetic and consequently heart
rates were lower than previously reported (11, 21). Furthermore, the heart type parameter, which contributes
to calculation of volume for the Scisense system, could not be directly measured in chicken fetuses due to
their small heart size and rapid heart rate. Consequently, the function of the admittance catheter was essentially equivalent to that of a conductance catheter. To compensate, cardiac output and stroke volume were measured in a separate set of fetuses, allowing calculation of LV volume. While these shortcomings are acknowledged, both experimental groups used the same experimental equipment, and were subjected to the same experimental manipulations and surgical procedures. Therefore, comparisons of functional differences between the groups in this study reflect how developmental hypoxia affects function of the heart.

Conclusions

These findings support our hypothesis that late-stage fetal chickens develop systolic and diastolic dysfunction during chronic hypoxia. We found hypoxic incubation led to less energetically favorable coupling of the heart to the systemic circulation. However, these hypoxia-incubated fetal chickens had a similar response to normoxia-incubated fetuses to severe acute hypoxic challenge as measured by change in LV output. This study demonstrates that late-stage chicken fetuses can be used to study \textit{in vivo} cardiac function, allowing developmental studies isolating hypoxic and nutritional restriction from placental and maternal effects.

Perspectives and Significance

Hypoxic stress during development has been investigated in a number of vertebrate models (6, 18, 32, 46). Chronic fetal hypoxia has been linked to depressed fetal cardiac function in mammals (15, 18, 32, 37), and is predictive of cardiovascular disease states later in life (16, 26, 27). However, the role of maternal and placental mediating factors on the fetal cardiac response to hypoxia is not well understood. In this study, we found depressed cardiac function in chronically hypoxic fetal chickens lacking placental and maternal mediators, suggesting the primary importance of low oxygen in the fetus for that cardiac response. Gene expression changes and preservation of LV output during acute hypoxia in this study, though, suggest that the depressed function in chronically hypoxic fetal chickens may not be best characterized as cardiac failure.
This new finding may be due to the lack of maternal and placental factors contributing to the fetal response to hypoxia, as they are absent in the fetal chicken. Alternatively the difference between the mammalian and the chicken fetus may be attributable to evolutionary divergence. Phenotypic plasticity of the developing heart may increase the ability of birds to maintain function during hypoxic bouts in peri-hatching (or perinatal) life, suggesting a fetal adaptive response to environmental stress in chickens as has been suggested in humans and other mammals (17).

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


Table 1. Primers for quantitative PCR studies in ventricular freewalls of chickens at 90% of development (19 day) following incubation in 21% O2 or 15% O2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>GenBank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S ribosomal RNA (18S)</td>
<td>Sense: TGTCACCCCTGATCTCTC</td>
<td>AF17361.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: CGCGGATCCAGAAATTCAC</td>
<td></td>
</tr>
<tr>
<td>Na⁺/Ca²⁺ exchanger 1 (NCX1)</td>
<td>Sense: CCTGCTGATCCCTCTTTTTC</td>
<td>NM_001079473.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: CCACTTCTGCTGTAATTCT</td>
<td></td>
</tr>
<tr>
<td>Cardiac ryanodine receptor (RYR)</td>
<td>Sense: CCGATCTGTCGCTGTAATTCT</td>
<td>XM_419553.4 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: TTGAGTGGGTTGTTCTCTCT</td>
<td></td>
</tr>
<tr>
<td>Sarco(endo)plasmic reticulum Ca²⁺-ATPase 2 (SERCA2)</td>
<td>Sense: AGGATCGGGTGATAGGATCAG</td>
<td>M66385.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: GGCAGTTGGAGACAAGGTTC</td>
<td></td>
</tr>
<tr>
<td>Phospholamban (PLN)</td>
<td>Sense: CCTCTTCTGCTGCTCTTCT</td>
<td>NM_205410.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: CAGACACTGGCATTGGAAG</td>
<td></td>
</tr>
<tr>
<td>Collagen alpha-2(I) chain (COL1A2)</td>
<td>Sense: GCACTTCTGCTGCTGCTGCT</td>
<td>NM_001079714.2 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: GTCTCCTGCTGCTGCTGCT</td>
<td></td>
</tr>
<tr>
<td>Chicken cardiac natriuretic peptide B (chNP)</td>
<td>Sense: GCTCATATGTGCACTGCTGCT</td>
<td>NM_004945971.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: CTCAACTGCGATACCAACC</td>
<td></td>
</tr>
<tr>
<td>Notch1</td>
<td>Sense: GAGACGAACTGAGAACAGAGG</td>
<td>NM_205381.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: CGTCACTCTGCTGCTGCTGCTGTG</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Sense: GAAGAGGAGGACGCTGGTTC</td>
<td>NM_000104368.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: GGGAGGAGGACGCTGGTTC</td>
<td></td>
</tr>
<tr>
<td>Kinase insert domain receptor (KDR, or VEGFR-2)</td>
<td>Sense: ATGAGATGTGCGGTTGCTG</td>
<td>NM_001110355.1 (Gallus gallus)</td>
</tr>
<tr>
<td></td>
<td>Antisense: TGGCGCTATGTGCGACTCTGT</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Chicken egg, body and heart masses of chickens at 90% of development (19 day) following incubation in 21% O2 or 15% O2.

<table>
<thead>
<tr>
<th></th>
<th>Control Incubation</th>
<th>Hypoxic Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg (g)</td>
<td>51.2 ± 0.9 (12)</td>
<td>52.9 ± 1.0 (14)</td>
</tr>
<tr>
<td>Body (g)</td>
<td>26.5 ± 0.5 (13)</td>
<td>21.2 ± 0.4 (14) *</td>
</tr>
<tr>
<td>Heart (μg)</td>
<td>149.2 ± 4.0 (13)</td>
<td>138.1 ± 5.4 (14)</td>
</tr>
<tr>
<td>Relative to body (μg g⁻¹)</td>
<td>5.63 ± 0.16 (13)</td>
<td>6.60 ± 0.34 (14) *</td>
</tr>
<tr>
<td>Left Ventricle (μg)</td>
<td>33.7 ± 1.8 (9)</td>
<td>35.9 ± 1.9 (8)</td>
</tr>
<tr>
<td>Relative to body (μg g⁻¹)</td>
<td>1.27 ± 0.07 (9)</td>
<td>1.78 ± 0.12 (8) *</td>
</tr>
<tr>
<td>Septum (μg)</td>
<td>32.9 ± 1.6 (9)</td>
<td>29.7 ± 2.8 (8)</td>
</tr>
<tr>
<td>Relative to body (μg g⁻¹)</td>
<td>1.24 ± 0.07 (9)</td>
<td>1.46 ± 0.13 (8)</td>
</tr>
<tr>
<td>Right Ventricle (μg)</td>
<td>32.0 ± 1.8 (9)</td>
<td>28.8 ± 1.8 (8)</td>
</tr>
<tr>
<td>Relative to body (μg g⁻¹)</td>
<td>1.21 ± 0.07 (9)</td>
<td>1.42 ± 0.08 (8)</td>
</tr>
</tbody>
</table>

Egg mass was measured when eggs were set. Data are presented as mean ± SEM (n). *P<0.05 compared to control by unpaired t-test.
Table 3. Left ventricular hemodynamic and end systolic pressure-volume parameters for chickens at 90% of development (19 day) following incubation in 21% O₂ or 15% O₂.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxic Incubation (n=4)</th>
<th>Hypoxic Incubation (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Acute Hypoxia</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>216 ± 6</td>
<td>193 ± 12</td>
</tr>
<tr>
<td>End Systolic Pressure (kPa)</td>
<td>4.29 ± 0.15</td>
<td>4.48 ± 0.28</td>
</tr>
<tr>
<td>End Diastolic Pressure (kPa)</td>
<td>0.34 ± 0.03</td>
<td>0.51 ± 0.07 †</td>
</tr>
<tr>
<td>Output (ml min⁻¹)</td>
<td>6.5 ± 0.2</td>
<td>5.3 ± 0.6</td>
</tr>
<tr>
<td>Stroke Volume (µl)</td>
<td>30.0 ± 0.5</td>
<td>28.2 ± 4.5</td>
</tr>
<tr>
<td>Stroke Work (kPa µl)</td>
<td>118 ± 4</td>
<td>113 ± 24</td>
</tr>
<tr>
<td>$E_A$ (kPa µl⁻¹)</td>
<td>0.1433 ± 0.0057</td>
<td>0.1681 ± 0.0216</td>
</tr>
<tr>
<td>$E_{LV}$ (kPa µl⁻¹) §</td>
<td>0.1414 ± 0.009</td>
<td>0.1431 ± 0.009</td>
</tr>
<tr>
<td>$E_A$ / $E_{LV}$</td>
<td>1.01 ± 0.04</td>
<td>1.17 ± 0.15</td>
</tr>
<tr>
<td>$V_0$ (µl)</td>
<td>14.11</td>
<td>15.48</td>
</tr>
<tr>
<td>$\Delta P/\Delta t_{\text{max}}$ (kPa s⁻¹)</td>
<td>132 ± 10</td>
<td>122 ± 9</td>
</tr>
<tr>
<td>$\Delta P/\Delta t_{\text{min}}$ (kPa s⁻¹)</td>
<td>−123 ± 4</td>
<td>−126 ± 4</td>
</tr>
<tr>
<td>$\tau$ (ms)</td>
<td>17.3 ± 0.8</td>
<td>21.2 ± 1.6 †</td>
</tr>
</tbody>
</table>

Effective arterial elastance, $E_A$; Left ventricular end systolic elastance, $E_{LV}$; End systolic pressure-volume relationship intercept at 0 kPa pressure, $V_0$; Maximal pressure generation rate, $\Delta P/\Delta t_{\text{max}}$; Minimal pressure generation rate, $\Delta P/\Delta t_{\text{min}}$; Time constant of relaxation, $\tau$. Data are presented as mean ± SEM. *P<0.05 hypoxic incubation different from normoxic incubation in same acute oxygen concentration; †P<0.05 acute hypoxia different from normoxia within incubation group. § Hypoxic incubation different from normoxic incubation by mixed models with unstructured autoregressive covariance matrices.
Figure 1. Pressure-volume loop obtained from fetal chickens incubated for 19 days at 21% O₂ (black row header) or 15% O₂ (grey row header), under the acute influence of either 21% O₂ (black column header) or 10% O₂ (grey column header). Shown also are end systolic and end diastolic points (circles) and the end systolic pressure-volume relationship (straight line).

Figure 2. mRNA expression of select genes in left and right ventricles of normoxia-incubated (n = 9; black bars) and hypoxia-incubated (n = 8; grey bars) chicken fetuses at 90% of incubation (19 day). 18S ribosomal RNA, 18S; Na⁺/Ca²⁺ exchanger 1, NCX1; Cardiac ryanodine receptor, RYR; Sarco(endo)plasmic reticulum Ca²⁺-ATPase 2, SERCA2; Phospholamban, PLN; Collagen alpha-2(I) chain, COL1A2; Chicken cardiac natriuretic peptide B, chNP; Kinase insert domain receptor, KDR (or VEGFR-2); Vascular endothelial growth factor A, VEGF-A. Data are presented as mean ± SEM. Different than normoxia: *P<0.05, **P<0.005.

Figure 3. Cardiac function in the 90% of incubation (19 day) chicken fetus is altered by chronic and acute hypoxia. A) ΔP/Δt_max, the maximal rate of LV contraction, is reduced by hypoxic incubation but not acute hypoxia. B) τ, the half-time of relaxation, is lengthened both by hypoxic incubation and by acute hypoxia. Data are presented as mean ± SEM. *P<0.05 different than normoxic incubation in same experimental oxygen concentration; †P<0.05 different than experimental normoxia of same incubation group.
A

$\Delta P/\Delta t_{\text{Max}}$ (kPa·s$^{-1}$)

![Bar chart showing $\Delta P/\Delta t_{\text{Max}}$ for different incubation and experimental oxygen concentrations.

Incubation $O_2$: 21% 21% 15% 15%
Experimental $O_2$: 21% 10% 21% 10%

B

$\tau$ (ms)

![Bar chart showing $\tau$ for different incubation and experimental oxygen concentrations.

21% 21% 15% 15%