HYPOXIC AND HYPEROXIC INCUBATION AFFECTS THE DUCTUS ARTERIOSUS IN THE DEVELOPING CHICKEN EMBRYO (*Gallus gallus*)

Jennifer Copeland, B.S.

Dissertation Prepared for the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

December 2009

APPROVED:

Edward Dzialowski, Major Professor
Thomas L. Beitinger, Committee Member
Warren Burggren, Committee Member and Dean of the College of Arts and Sciences
Mark L. Burleson, Committee Member
Pamela Padilla, Committee Member
Art Goven, Chair of the Department of Biological Sciences
Michael Monticino, Dean of the Robert B. Toulouse School of Graduate Studies
Copeland, Jennifer. *Hypoxic and hyperoxic incubation affects the ductus arteriosus in the developing chicken embryo (Gallus gallus).* Doctor of Philosophy (Biology), December 2009, 101 pp., 5 tables, 20 figures, references, 73 titles.

Developing chicken embryos have two ductus arteriosus (DA) that shunt blood away from the lungs and to the chorioallantoic membrane, the embryonic gas exchanger. In mammals, DA closure is stimulated by an increase in blood gas O$_2$ that occurs as the animal begins to breathe with its lungs. The goal of this study was to determine the influence of O$_2$ levels during incubation on the vascular reactivity and morphology of the O$_2$-sensitive DA and to examine the effects of changing O$_2$ levels during late incubation on the morphology of the DA from chicken embryos.

In comparison to normoxia, hypoxia (15%) reduced venous O$_2$ levels in day 16 and day 18 embryos and reduced aircell O$_2$ values in day 16, day 18, and internally pipped (IP) embryos, whereas hyperoxia (30%) increased venous O$_2$ levels and aircell O$_2$ level in day 16, day 18, and IP embryos. In comparison to normoxia, hypoxia delayed closure of the DA, whereas hyperoxia accelerated DA closure.

In comparison to the left DA from externally pipped (EP) normoxic embryos, the left DA from EP hypoxic embryos exhibited a significantly weaker contractile response to O$_2$. The DA from day 18 hypoxic embryos exhibited a significantly weaker contractile response to norepinephrine and phenylephrine when compared with the DA from day 18 normoxic and hyperoxic embryos.

The effect of incubation in hypoxia / hyperoxia during different developmental windows on the DA O$_2$-induced contractile response was observed only in IP embryos that were incubated in normoxia for 16 days and were then moved to hyperoxia.
Incubation in hypoxia / hyperoxia resulted in differences in embryo mass, yolk mass, and heart mass. There is an association between the decreased contractile response to $O_2$ and delayed closure in the proximal portion of the DA from hypoxic embryos; as well as an increased contractile response to $O_2$ and accelerated closure in the proximal portion of the DA from hyperoxic embryos.
Copyright 2009

by

Jennifer Copeland
ACKNOWLEDGEMENTS

I would like to thank Dr. Edward Dzialowski. I would like to thank my lab coworkers, Nicole Palenske, Katie Fosha, Tushar Sirsat, and Henry Greyner. I would like to thank Dr. Thomas Beiting, Dr. Warren Burggren, Dr. Mark Burleson, and Dr. Pamela Padilla for serving on my committee. I would like to thank Dr. Benny Copeland for his helpful comments. I would like thank my husband Blair for his support throughout the years.

Part of this dissertation is published in Experimental Physiology (Copeland and Dzialowski, 2009).

This study was supported by a grant from the American Heart Association South Central Affiliate and National Science Foundation Grant # IOB – 0417205.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapters</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACKNOWLEDGEMENTS</strong> .........................................................................</td>
<td>iii</td>
</tr>
<tr>
<td><strong>LIST OF TABLES</strong> ............................................................................</td>
<td>vi</td>
</tr>
<tr>
<td><strong>LIST OF FIGURES</strong> ..........................................................................</td>
<td>vii</td>
</tr>
<tr>
<td><strong>Chapters</strong></td>
<td></td>
</tr>
<tr>
<td>1. <strong>INTRODUCTION</strong> ...........................................................................</td>
<td>1</td>
</tr>
<tr>
<td>Functional Closure of the Ductus Arteriosus</td>
<td></td>
</tr>
<tr>
<td>Anatomical Closure of the Ductus Arteriosus</td>
<td></td>
</tr>
<tr>
<td>Chicken Embryo as a Model</td>
<td></td>
</tr>
<tr>
<td>The Developing Chicken Embryo in Hypoxia</td>
<td></td>
</tr>
<tr>
<td>The Developing Chicken Embryo in Hyperoxia</td>
<td></td>
</tr>
<tr>
<td>Windows of Development</td>
<td></td>
</tr>
<tr>
<td>Adrenergic Agonists</td>
<td></td>
</tr>
<tr>
<td>The Effects of Hypoxia on the Ductus Arteriosus</td>
<td></td>
</tr>
<tr>
<td>Research Objectives</td>
<td></td>
</tr>
<tr>
<td>2. <strong>MORPHOLOGICAL CHANGES IN THE DUCTUS ATERIOSUS DURING DEVELOPMENT</strong></td>
<td>12</td>
</tr>
<tr>
<td>DISCUSSION AND AS A RESULT OF HYPOXIC OR HYPEROXIC INCUBATION</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>Conclusions</td>
<td></td>
</tr>
<tr>
<td>3. <strong>THE REACTIVITY OF THE DUCTUS ARTERIOSUS TO CATECHOLAMINES</strong></td>
<td>39</td>
</tr>
<tr>
<td>Introduction</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
</tr>
</tbody>
</table>
Conclusions

4. PHYSIOLOGICAL CHANGES THAT OCCUR IN THE DUCTUS ATERIOSUS AS A RESULT OF INCUBATION IN HYPOXIA OR HYPEROXIA DURING DEVELOPMENTAL WINDOWS ......................... 58
   Introduction
   Materials and Methods
   Results
   Discussion
   Conclusions

5. CONCLUSIONS ...................................................................................... 88

APPENDIX: LIST OF ABBREVIATIONS .......................................................... 93

REFERENCES .............................................................................................................. 95
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Blood gas and air cell measurements from <em>Gallus gallus</em> eggs incubated in various levels of oxygen. Measurements taken on day 16, day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia. Blood gas samples were obtained from major chorioallantoic veins or arteries</td>
<td>21</td>
</tr>
<tr>
<td>3.1</td>
<td>Contractile responses of the proximal ductus arteriosus from <em>Gallus gallus</em>. Measurements made on left proximal day 16, and left and right proximal DA in day 19 embryos incubated in hypoxia, normoxia or hyperoxia in response to $10^{-4}$ M phenylephrine or $10^{-5}$ M norepinephrine</td>
<td>47</td>
</tr>
<tr>
<td>4.1</td>
<td>Wet mass, dry mass, wet yolk mass, dry yolk mass and heart mass of day 16, day 18, internally pipped, externally pipped embryos, and hatchlings incubated in normoxia or hypoxia during the first 18 days of incubation, then moved to normoxia</td>
<td>72</td>
</tr>
<tr>
<td>4.2</td>
<td>Wet mass, dry mass, wet yolk mass, dry yolk mass and heart mass of day 16, day 18, internally pipped, externally pipped embryos, and hatchlings incubated in hypoxia or normoxia for either the first 16 or 18 days of development and moved to hypoxia</td>
<td>74</td>
</tr>
<tr>
<td>4.3</td>
<td>Wet mass, dry mass, wet yolk mass, dry yolk mass and heart mass of day 16, day 18, internally pipped, externally pipped embryos, and hatchlings incubated in hyperoxia or normoxia for either the first 16 or 18 days of development and moved to hyperoxia</td>
<td>77</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Page

1.1  Diagram of the right and left ductus arteriosus from *Gallus gallus*......................... 2

2.2 Sections of the left proximal ductus arteriosus from day 18 *Gallus gallus*. (A, B) Day 18 hypoxic left proximal ductus arteriosus (C, D) Day 18 normoxic left proximal ductus arteriosus (E, F) Day 18 hyperoxic left proximal ductus arteriosus. Tissue was stained with Van Gieson (A, C, E) or hematoxylin and eosin (C, D, F).................................................................................................... 22

2.3 Van Geison stained sections of the left proximal ductus arteriosus from *Gallus gallus*. (A, D, G) Left proximal ductus arteriosus hypoxic IP, and EP embryo and hatchling (B, E, H) Left proximal ductus arteriosus normoxic IP, and EP embryo and hatchling (C, F, I) Left proximal ductus arteriosus hypoxic IP, and EP embryo and hatchling. Arrows indicate internal elastic lamina, m indicates media......... 23

2.4 Lumen diameter of the left proximal ductus arteriosus from *Gallus gallus*. Measurements taken from day 16, day 18, internally pipped, and externally pipped embryos, and hatchlings incubated in hypoxia, normoxia, and hyperoxia .................................................................................................................................................. 25

2.5 Mean vessel wall and smooth muscle thickness of the left proximal ductus arteriosus from *Gallus gallus*. (A) Measurements taken of the total vessel thickness of the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, externally pipped, and hatchlings in hypoxia, normoxia, hatch, or hyperoxia. (B) Measurements taken of the smooth muscle thickness of the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, externally pipped, and hatchlings in hypoxia, normoxia, or hyperoxia ... 26

2.6 Proximal and distal length measurements of the ductus arteriosus from *Gallus gallus*. (A) Measurements made on the proximal and distal portions of the left ductus arteriosus from day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia. (B) Measurements made the proximal and distal portions of the right ductus arteriosus from day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia........................................................................................................ 28

2.7 Total length of right and left ductus arteriosus from *Gallus gallus*. Measurements made on day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia................................................................. 29
2.8 Proximal and distal width of the ductus arteriosus from *Gallus gallus*. (A) Measurements made on the left proximal ductus arteriosus from day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia. (B) Measurements made on the right proximal ductus arteriosus from day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia ............................ 31

3.1 Contractile responses of the left and right proximal DA from *Gallus gallus* incubated under different oxygen levels. (A) Response of the left proximal DA to increasing concentrations of norepinephrine. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in norepinephrine. (B) Response of the right proximal DA to increasing concentrations of norepinephrine. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in norepinephrine .................................................................. 45

3.2 Contractile responses of the left and right proximal DA from *Gallus gallus* incubated under different oxygen levels. (A) Response of the left proximal DA to increasing concentrations of phenylephrine. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in phenylephrine. (B) Response of the right proximal DA to increasing concentrations of phenylephrine. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in phenylephrine.................................................................... 48

3.3 The effect of oxygen on the active tension of the left proximal DA from *Gallus gallus* in response to norepinephrine $10^{-4}$ M in 4% and 25% oxygen. Measurements made on day 16, day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia. Vessel response was measured at 4% $O_2$ and 25% $O_2$ ........................................................................................................ 50

3.4 The effect of oxygen on the active tension of the right proximal DA from *Gallus gallus* in response to norepinephrine $10^{-4}$ M in 4% and 25% oxygen. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia. Vessel response was measured at 4% $O_2$ and 25% $O_2$ ............................................................................................................... 51

4.1 Contractile responses to 120mM KCl of the left and right proximal ductus arteriosus from *Gallus gallus*. (A) Measurements made on the left proximal ductus arteriosus from day 16, day 18, internally pipped and externally piped embryos incubated in hypoxia, normoxia, or hyperoxia. (B) Measurements made on the left proximal ductus arteriosus from day 16, day 18, internally pipped and externally pipped embryos incubated in hypoxia, normoxia, or hyperoxia ........................................................................................................ 64
4.2 Oxygen induced contractile response of the right proximal ductus arteriosus. Measurements made on the right proximal ductus arteriosus from day 18, internally pipped, and externally pipped (hypoxic and normoxic) embryos incubated in hypoxia, normoxia, or hyperoxia to an increase in organ bath O$_2$ from 4% to 25% .................................................................................................................. 66

4.3 Oxygen induced contractile response of the left proximal ductus arteriosus from *Gallus gallus*. Measurements made on the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, and externally pipped embryos incubated in normoxia or hypoxia for the first 18 days of incubation and moved to normoxic for the rest of incubation to an increase in organ bath O$_2$ from 4% to 25% .................................................................................................................... 67

4.4 Oxygen induced contractile response of the left proximal ductus arteriosus from *Gallus gallus*. Measurements made on the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, and externally pipped embryos incubated in hypoxia, normoxia for the first 16 days of development and moved to hypoxia, normoxia for the first 18 days of development and moved to hypoxia and normoxia to an increase in organ bath O$_2$ from 4% to 25% ..................................................................................... 68

4.5 Oxygen induced contractile response of the left proximal ductus arteriosus from *Gallus gallus*. Measurements made on the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, and externally pipped embryos incubated in hyperoxia, normoxia for the first 16 days of development and moved to hyperoxia, normoxia for the first 18 days of development and moved to hyperoxia and normoxia for the entire length of incubation to an increase in organ bath O$_2$ from 4% to 25% .................................................................................................................. 69

4.6 Wet mass of day 16, day 18, internally pipped, and externally pipped embryos and hatchlings incubated in normoxia or hypoxia for the first 18 days of development and moved to normoxia ............................................................................................................................... 71

4.7 Wet mass of day 16, day 18, internally pipped, and externally pipped embryos and hatchlings incubated in hypoxia, normoxia for the first 16 days of development and moved to hypoxia, normoxia for the first 18 days of development and moved to hypoxia and normoxia ............................................................... 73

4.8 Wet mass of day 16, day 18, internally pipped, and externally pipped embryos and hatchlings incubated in hyperoxia, normoxia for the first 16 days of development and moved to hyperoxia, normoxia for the first 18 days of development and moved to hyperoxia and normoxia ............................................................... 76
CHAPTER 1
INTRODUCTION

The ductus arteriosus (DA; other abbreviations can be found in the appendix) is an embryonic blood vessel that connects the pulmonary artery to the aorta in mammals, reptiles and birds (Bergwerff et al., 1999). A patent DA during development provides a right-to-left shunt of blood away from the unventilated lungs and to the embryonic gas exchanger; the placenta in mammals and the chorioallantoic membrane (CAM) in birds (Heymann and Rudolph, 1975). In mammals, prostaglandins and nitric oxide maintain DA patency during fetal development (Clyman et al., 1980; Smith, 1998; Baragatti et al., 2007). If the DA fails to close after birth, the animal has a condition known as a patent ductus arteriosus (PDA) which can cause complications in the developing animal (Smith, 1998). An infant with persistent PDA is more likely to develop chronic lung disease (Clyman, 2000).

Functional Closure of the Ductus Arteriosus

In mammals, DA closure is stimulated by an increase in blood gas O₂ that occurs as the animal begins to breathe with its lungs (Heymann and Rudolph, 1975; Berger et al., 1990; Bergwerff, 1996; Smith, 1998). In chickens (Gallus gallus), the increase in DA (Figure 1.1) responsiveness to O₂ corresponds to the onset of lung ventilation (Ågren et al., 2007; Belanger et al., 2008). Closure of the DA that involves the O₂-induced constriction of the smooth muscle cells is termed functional closure (Fay, 1971; Archer et al., 2004). Studies on both mammalian as well as avian species suggest that the DA responsiveness to O₂ is developmentally regulated (Noel and Cassin, 1976; Clyman et
al., 1978; Thebaud et al., 2004; Kajimoto et al., 2007; Ågren et al., 2007; Belanger et al., 2008; Dzialowski and Greyner, 2008). Studies performed on rabbit, guinea pig, and lamb show that DA from preterm animals exhibited a weaker contractile response to O2 when compared to the DA from full term animals (Kajimoto et al., 2007; Thebaud et al., 2004; Clyman et al., 1978; Noel and Cassin, 1976). Similarly, studies performed on the avian DA from older animals exhibit a stronger O2-induced contraction when compared with the response from younger animals (Ågren et al., 2007; Belanger et al., 2008; Dzialowski and Greyner, 2008).

Figure 1.1 Diagram of the right and left ductus arteriosus from Gallus gallus.
Anatomical Closure of the Ductus Arteriosus

Anatomical remodeling of the DA occurs after functional closure (Clyman et al., 1999; Kajino et al., 2000; Sutendra and Michelakis, 2007). In mammals, as the DA constricts in response to an increase in $O_2$ (Heymann and Rudolph, 1975; Berger et al., 1990; Bergwerff, 1996; Smith, 1998), blood flow inside the DA is reduced, which leads to hypoxia within the DA (Clyman et al., 1999; Kajino et al., 2000). During mammalian development, prostaglandins and nitric oxide maintain DA patency (Clyman et al., 1980; Smith, 1998; Baragatti et al., 2007). Upon DA constriction, which leads to extreme hypoxia within the wall of the DA, prostaglandins and nitric oxide no longer induce relaxation (Clyman et al., 1999; Kajino et al., 2000; Clyman, 2006). This helps maintain DA constriction (Clyman et al., 1999; Kajino et al., 2000; Clyman, 2006). A link between the degree of $O_2$-induced constriction in the DA and the ability of the vessel to anatomically remodel has been established (Clyman et al., 1999; Kajino et al., 2000).

Unlike mammals, there are two DA present throughout incubation in the chicken embryo (Bergwerff, 1999). There are two distinct morphologies in both the right and left DA (Bergwerff, 1996; Belanger et al., 2008). A muscular phenotype is present in the portion of the vessel proximal to the pulmonary artery (Bergwerff, 1996; Belanger et al., 2008), while an elastic phenotype is present distal to this (Belanger et al., 2008). There are few morphological differences between a day 19 and internally pipped (IP) proximal DA (Belanger et al., 2008). During these stages the tunica intima of the proximal DA is comprised of a single endothelial layer and a thin internal elastic lamina which surrounds the lumen (Belanger et al., 2008). The tunica media of the proximal DA is composed predominately of smooth muscle cells and is surrounded by the tunica
adventitia, which is comprised of collagen and elastin (Belanger et al., 2008). The proximal portion of the chicken embryo DA begins to close 5 hours after the animal externally pips (EP; Belanger et al., 2008). Closure is characterized by the swelling and migration of endothelial cells into the lumen of the vessel (Belanger et al., 2008). This is followed by the migration of smooth muscle cells into the lumen. The proximal portion of the DA is anatomically closed by day 2 post-hatch (Belanger et al., 2008).

Chicken Embryo as a Model

The chicken embryo (Gallus gallus) provides an excellent model for developmental studies of chronic O₂ effects, because the embryo develops in ovo, allowing the experimenter the ability to easily alter the developmental environment (Sutendra and Michelakis, 2007). Another advantage of using chicken embryo is the transition from embryonic gas exchanger to lung respiration occurs over approximately a 24 hour period whereas, in mammals this transition occurs rapidly (Visschedijk, 1968; Ar et al., 1980; Rahn et al., 1985). This allows a greater opportunity to observe any changes in developmental trajectory that may occur in the DA as a result of hypoxic or hyperoxia incubation.

Under normoxic conditions, the chicken embryo uses the CAM as its sole gas exchanger until approximately incubation day 20 (Rahn et al., 1985). On day 20 the embryo breaks through the CAM with its beak and begins to respire gas in the aircell. This process is known as internal pipping (IP). During this period the embryo begins to use its lungs; however the CAM is still the primary gas exchanger (Rahn et al., 1985). Approximately 12 hours later the embryo breaks through the shell with its beak and
begins to respire air (Visschedijk, 1968; Ar et al., 1980; Rahn et al., 1985). This process is known as externally pipping (EP). During this period, the embryo is still using both CAM and lungs; however the embryo begins to shift predominately to lung ventilation (Rahn et al., 1985). The animal hatches on approximately incubation day 21 and relies only on its lungs (Rahn et al., 1985).

The Developing Chicken Embryo in Hypoxia

Developing in chronic hypoxia produces significant abnormalities in the developing cardiovascular system (Rouwet et al., 2002; Villamor et al., 2004) as well as increased mortality (Rouwet et al., 2002; Villamor et al., 2004) and decreased body weight (Ruijtenbeek et al., 2000; Dzialowski et al., 2002; Rouwet et al., 2002; Villamor et al., 2004; van der Sterren et al., 2009; Tintu et al., 2007). The vascular phenotype of the developing embryo responds to chronic hypoxic exposure (Ruijtenbeek et al., 2000; Rouwet et al., 2002; Villamor et al., 2004; van der Sterren et al., 2009). Chicken embryos developing in chronic hypoxia exhibit reduced pulmonary arterial contractile responses to norepinephrine (NE), endothelin-1, and the thromboxane A2 mimetic U-46619 (Villamor et al., 2004). The DA of day 19 chicken embryos incubated in chronic hypoxia exhibit a reduction in the relaxation response to isoproterenol, sodium nitroprusside, and acetylcholine (van der Sterren et al., 2009). Chronic hypoxic incubation also results in sympathetic hyperinnervation of the arterial system (Ruijtenbeek et al., 2000; Rouwet et al. 2002) and an increase in heart to body mass ratio in chicken embryos (Rouwet et al. 2002).

In addition to causing changes in physiological responses to various drugs,
development in hypoxia causes changes in vascular morphology (Rouwet et al., 2002, Hansen, 2005). Development in hypoxia causes a decrease in the lumen diameter of the aorta in chicken (Rouwet et al., 2002, Hansen, 2005). In addition, incubation in chronic hypoxia causes a decrease in aorta wall thickness (Hansen, 2005). Van der Sterren et al. (2009) found that incubation in hypoxia did not result in any changes in the cross sectional area of the lumen of the day 19 chicken DA.

**The Developing Chicken Embryo in Hyperoxia**

Incubation in hyperoxia has also been shown to cause a number of physiological and morphological changes in the developing chicken embryo including increased embryo mass (McCutcheon et al., 1982; Stock et al., 1983; van Golde et al., 1998), increased heart mass (McCutcheon et al., 1982), increased liver mass (van Golde et al., 1998), increased intestine mass (van Golde et al., 1998), increased adenosine triphosphate (ATP) levels and decreased 2,3 diphosphoglycerate (2,3 DPG) in comparison to control embryos (Ingermann et al., 1983). Stock et al. (1983) reported that embryo mass is different in groups incubated in 40% O₂ than in groups incubated in 70% O₂. Incubation in 40% O₂ results in significantly greater day 18 embryo masses in comparison to normoxic incubated day 18 embryo masses, whereas incubation in 70% O₂ results in day 18 embryo masses that are not significantly different than day 18 normoxic incubated embryo masses (Stock et al., 1983). Additionally, incubation in 40% O₂ results in day 18 embryos with significantly greater heart masses in comparison to day 18 normoxic incubated embryo heart masses, whereas incubation in 70% O₂ results in day 18 embryos with significantly lower heart masses in comparison to day 18
normoxic incubated embryo heart masses (Stock et al., 1983).

Windows of Development

Several studies have focused on incubating chicken embryos in hypoxia or hyperoxia during differing windows of development. The outcomes of these studies depended on what was measured as well as when the embryo was exposed to the hypoxic or hyperoxic environment. Incubation in hypoxia during different developmental windows causes a number of physiological and morphological changes in the chicken embryo (Dzialowski et al., 2002; Chen and Burggren, 2004; Azzam and Mortola 2007). Decreased eye mass (Chen and Burggren, 2004), changes in hemoglobin and hematocrit (Dzialowski et al., 2002), and an increase in chorioallantoic mass are a few examples changes related to development in chronic hypoxia (Chen and Burggren, 2004; Azzam and Mortola 2007).

Incubation in hyperoxia during the final days of development results in an increase in body mass (Stock and Metcalfe, 1984). In addition, incubation in hyperoxia (60%) for 48 hours during day 10-11 or day 14-15 results in larger hatchling mass when compared to the control group (van Golde et al., 1998). Incubation in hyperoxia (60%) during day 18-19 results in an increase in brain, heart, liver and intestine mass, however those animals did not survive to hatch (van Golde et al., 1998).

Adrenergic Agonists

Ågren et al. (2007) hypothesized that catecholamines may play a role the closure of the DA in chicken embryos. Studies have examined the levels of circulating
catecholamines in the chicken embryo during development as well as during acute hypoxia. Wittman and Prechtl (1991) found that circulating catecholamines peak on day 19 of development. Additionally, they reported that administering adrenaline, noradrenaline, and phenylephrine (PE) all significantly increased O₂ saturation in comparison to control values (Wittman and Prechtl, 1991). Mulder et al. (2001) showed that blocking α adrenergic receptors with phentolamine during hypoxic exposure resulted in significant differences in redistribution of embryo cardiac output.

Several studies have examined the effect of chronic hypoxic incubation on the vessel reactivity to norepinephrine (NE) in chicken embryos (Ruitjtenbeek et al., 2000; Rouwet et al., 2002; Villamor et al., 2004; van der Sterren et al., 2009). The mesenteric resistance arteries of day 19 embryos exhibited a similar contractile response to NE in both normoxic and chronic hypoxic embryos (Rouwet et al., 2002). In contrast, chronic hypoxia reduced pulmonary arterial contractile reactivity to NE in day 19 chicken embryos (Ruitjtenbeek et al., 2000; Villamor et al., 2004). The DA from day 19 chronic hypoxic incubated chicken embryos exhibited an increased contractile response to NE and PE (van der Sterren et al., 2009). The effect of chronic hypoxia on the reactivity to adrenergic agonists has also been studied in sheep. There is an increased contractile response to NE of the pulmonary artery of fetal sheep that developed in chronic hypoxic (Xue et al., 2008). Similarly, in the small femoral arteries of newborn sheep that developed in chronic hypoxia, there is an increased contractile response to both NE and PE (Herrera et al., 2007). In contrast, there is a decrease in the contractile response of cerebral arteries to NE in fetal sheep exposed chronic hypoxia (Longo and Pearce, 1998).
The Effects of Hypoxia on the Ductus Arteriosus

A link between human development in chronic hypoxia and the incidence of PDA in preterm infants has been established (Rakza et al., 2007; Chen et al., 2008). Chen et al. (2008) found that the incidence of PDA in children was significantly higher in children born at higher altitudes (3847-4533m) in comparison to children born at lower altitudes (1650-2835m or 2792-4360m). There is association between fetal development in hypoxia and intrauterine growth restriction (IUGR; Carlson, 1988; Pollack and Divon, 1992). Rakza et al. (2007) reported that 60% of preterm infants with IUGR developed PDA, whereas only 15% of preterm infants without IUGR developed PDA. Intrauterine growth restriction is associated with a number of factors including, but not limited to high altitude, asthma, maternal smoking, maternal alcohol use, maternal narcotic use, hypertension, pregnancy weight gain and prepregnancy weight (Scott et al., 1981; Carlson, 1988; Pollack and Divon, 1992). Little is known about how the mammalian or avian DA develops under chronic hypoxia or hyperoxia. Having a better understanding of the underlying mechanisms governing the closure of the ductus arteriosus could provide a foundation for future research on the treatment or prevention of PDA, a potentially life threatening-disease.

Research Objectives

The major objective of this research was to characterize physiological and morphological differences in the DA of chicken embryos incubated in differing O2 levels. Because the DA is a tissue sensitive to O2, changes in environmental O2 levels during development were predicted to significantly alter the developmental trajectory of the
vessels response to O₂ (Heymann and Rudolph, 1975; Berger et al., 1990; Smith, 1998). I hypothesized that under hypoxic conditions the DA would take a longer period of time to close, while under hyperoxic conditions the DA closure would be accelerated.

In Chapter 2, I present an examination of the morphological changes that occur in the proximal portion of the left DA from chicken embryos incubated in hypoxia (15% O₂), normoxia (21% O₂) or hyperoxia (30% O₂) during hatching. This study includes a comparison of length and width measurements of the right and left DA from chicken embryos incubated in hypoxia, normoxia, or hyperoxia during hatching. Additionally, I present a comparison of blood gas and aircell values for day 16, day 18 and IP embryos incubated in hypoxia, normoxia, and hyperoxia.

In Chapter 3, I present a comparison of the response to the adrenergic agonist, NE and PE, of the right (day 18 and IP only) and left DA from day 16, day 18, and IP chicken embryos incubated in hypoxia (15% O₂), normoxia (21% O₂) or hyperoxia (30% O₂). Additionally, I present a comparison of the DA contractile response to 10⁻⁵ M NE in 4% O₂ and 25% O₂.

In Chapter 4, I present an examination of the effect of exposing chicken to differing periods of hypoxic (15%), normoxic (21%) or hyperoxic (30%) incubation on the O₂-induced contraction of the ductus arteriosus. In addition, I present an examination of the effect of exposing chicken embryos (Gallus gallus) to differing periods of hypoxia, normoxia, or hyperoxia on the mass of the embryo and heart.

In Chapter 5, hypoxic (15%) and hyperoxic (30%) incubation effects on the DA maturation of the adrenergic and O₂-induced contractile response were summarized. I summarized the effects of hypoxic and hyperoxic incubation on the morphology and
closure of the DA during hatching. Additionally, the effects of altering O₂ incubation levels during different developmental windows on embryo mass and heart mass were summarized.
CHAPTER 2

MORPHOLOGICAL CHANGES IN THE DUCTUS ARTERIOSUS DURING DEVELOPMENT AND AS A RESULT OF HYPOXIC OR HYPEROXIC INCUBATION

Introduction

Unlike mammals, there are two DA (ductus arteriosus) present throughout incubation in the chicken embryo (Bergwerff, 1999). There are two distinct morphologies in both the right and left DA (Bergwerff, 1996; Belanger et al., 2008). A muscular phenotype is present in the portion of the vessel proximal to the pulmonary artery (Bergwerff, 1996; Belanger et al., 2008), while an elastic phenotype is present the portion distal to this (Belanger et al., 2008). During hatching in birds, DA constrict preferentially in the proximal portion (Belanger et al., 2008), resulting in the disappearance of the right-to-left shunt and increased blood flow through the pulmonary arteries to the lungs (Rahn et al., 1985). In mammals, DA closure is stimulated by an increase in blood gas partial pressure of O₂ (Po₂) that occurs at birth when the animal begins to ventilate its lungs (Heymann and Rudolph, 1975; Berger et al., 1990; Smith, 1998). In chickens, DA closure begins after the embryo externally pips (EP), which corresponds with an increased contractile sensitivity to O₂ and an increased arterial Po₂ (Tazawa et al., 1983; Belanger et al., 2008).

The exact mechanisms that lead to DA closure are still being studied. There are at least two steps of DA closure: functional closure involves the constriction of the smooth muscle cells (Fay, 1971 Archer et al., 2004) which is followed by anatomical remodeling (Clyman et al., 1999; Kajino et al., 2000; Sutendra and Michelakis, 2007). Anatomical remodeling of the mammalian DA during closure has been well characterized. In the DA of many mammals, reorientation of cells and thickening of the
intima occur prior to birth (Yoder et al., 1978; Tada et al., 1990; Slomp et al., 1992). In the chicken embryo, the proximal portion of the DA begins to close 5 hours after the animal EP (Belanger et al., 2008). Anatomical closure is characterized by the swelling and migration of endothelial cells into the lumen of the vessel (Belanger et al., 2008). This is followed by the migration of smooth muscle cells into the lumen (Belanger et al., 2008).

Hypoxic incubation causes a number of morphological changes in the vessels of developing chickens (Rouwet et al., 2002, Hansen, 2005). Rouwet et al. (2002) found that chronic hypoxia led to a decrease in lumen diameter in the ascending aorta. Hansen (2005) reported that chronic hypoxia caused the aortic wall of jungle fowl to be significantly thinner than the aortic wall of controls. Additionally, he found that lumen diameter of white leghorn aortas were significantly smaller in the hypoxic group when compared to the control group (Hansen, 2005).

A link between human development in chronic hypoxia and the incidence of PDA in preterm infants has been established (Rakza et al., 2007; Chen et al., 2008). Chen et al. (2008) observed that the incidence of PDA was significantly higher in children (4-18 years) born at higher altitudes (3847-4533 m at least 3 generations) in comparison to children born at lower altitudes (1650-2835 m or 2792-4360 m at least 3 generations). There is association between fetal development in hypoxia and intrauterine growth restriction (IUGR; Carlson, 1988; Pollack and Divon, 1992). Rakza et al. (2007) found that 60% of preterm infants exposed to intrauterine growth restriction (IUGR) developed PDA, whereas only 15% of preterm infants not exposed to IUGR developed PDA.

In this study, an examination of the effect of chronic hypoxic or hyperoxic
incubation on the morphology of the DA is presented. I hypothesized that the lumen of
the DA will be larger in hypoxic embryos and smaller in hyperoxic embryos throughout
development, with the difference greater in earlier stages of development, i.e. day 16
and day 18. In addition, I hypothesized the initiation of closure of the DA from hyperoxic
embryos will begin earlier in development than the DA from normoxic or hypoxic
embryos. Studying the effects of chronic hypoxia and hyperoxia on the morphology of
the DA will help identify any differences in the timing of critical events, i.e. could the DA
of hyperoxic embryos have migrating endothelial cells a stage sooner than the DA from
control embryos. To ensure the hypoxic/ hyperoxic embryos were exposed to
lower/higher O2 levels I measured arterial and venous Po2 and Pco2 as well as the
aircell levels at different developmental stages. Examining how chronic hypoxia and
hyperoxia affect the morphology and the physiology of the O2-sensitive DA will provide
an understanding of the changes that must occur for proper DA closure (Heymann and
Rudolph, 1975; Noel and Cassin, 1976; Clyman et al., 1978; Berger et al., 1990; Smith,
1998; Thebaud et al., 2004; Kajimoto et al., 2007 Ågren et al., 2007; Belanger et al.,
2008; Dzialowski and Greyner, 2008).

Materials and Methods

Eggs and Incubation

White leg-horn chicken (Gallus domesticus; layers) eggs were obtained from
Texas A & M University and incubated at about 37.5 °C and a relative humidity of 40-
75%. Eggs were incubated in Hova-Bator™ incubators (G.Q.F. Manufacturing,
Savannah, GA, USA), model 1502 circulated air incubators (G.Q.F. Manufacturing,
Savannah, GA, USA), or RC1 & 2 reptile incubators (Lyon Technologies, Inc., Chula Vista, CA, USA) and maintained at three levels of O₂, 15% O₂ (hypoxia), 21% O₂ (control) or 30% O₂ (hyperoxia). Hypoxic and hyperoxic incubators were maintained at a relative humidity of 70-75% whereas normoxic incubators were maintained at either 40-45% or 70-75%. The O₂ level in the incubators was maintained using model 1600 Engineered Systems & Designs oxygen controllers (Newark, DE, USA) or a Sable Systems Roxy-4 four channel gas regulator (Las Vegas, NV, USA). Embryos were euthanized with isoflurane or by decapitation. Preliminary experiments found that the method of euthanasia did not influence the response of the DA to O₂. All experiments were approved by the University of North Texas Institutional Animal Care and Use Committee.

**Blood Gas**

Blood gases were measured on day 16, day 18, and IP (internally pipped) embryos incubated at the three O₂ levels. On the day of measurement, eggs were moved to a hypoxia/normoxia/hyperoxia chamber maintained at 37.5°C and the appropriate incubation O₂ level. Eggs were allowed to equilibrate for 2 hours in the hypoxia/normoxia/hyperoxia chamber prior to sampling. The O₂ level in the sampling chamber was regulated by a Porter Instrument gas mixer (Hatfield, PA, USA) and monitored with a Sable Systems FOX O₂ analyzer (Las Vegas, NV, USA). The embryos were candled to locate major chorioallantoic veins and arteries. A small portion of the shell and outer member over the vessel was removed and 150 μl of blood was withdrawn from a major CAM vein or artery using a heparanized 31-gauge needle and
syringe. Blood $\text{Po}_2$ and $\text{Pco}_2$ were measured with a Radiometer ABL® 5 blood gas meter (A/S Corp., Copenhagen, Denmark).

**Air Cell**

Air cell $\text{Po}_2$ was measured using the technique described by Tazawa et al. (1980). Air cell measurements were made on day 16, day 18, and IP eggs. A small hole was drilled in the shell, using a 18 gauge needle, over the air cell and a port for a syringe was fixed into the shell, using J-B Kwik® epoxy (J-B Weld Co., Sulphur Springs, TX, USA). A glass syringe was attached to the port and the embryo was placed back into the incubator for at least 12 hours. This allowed the gas in the syringe to equilibrate with the air cell gas. The syringe was removed and the content of the syringe was injected into an $\text{O}_2$ electrode (Microelectrodes Inc., Bedford, NH, USA) maintained at 37.5°C. The $\text{O}_2$ content of the syringe was recorded using Powerlab® 8SP data acquisition system (ADInstruments Pty Ltd., Colorado Springs, CO, USA) and Chart® data acquisition and analysis software (ADInstruments Pty Ltd., version 5.4.2, Colorado Springs, CO, USA).

**Histology**

Morphological changes in the DA during development were examined histologically. Because the chicken DA has two distinct morphological regions along its length (Belanger et al. 2008), the proximal and distal portions of the DA were separated based on visual inspection of their vessel morphology and diameter. A clear transition between the proximal portion and distal portion can be seen in the middle of each
vessel (Ågren et al. 2007; Belanger et al. 2008). The animal was decapitated and the left proximal portion of the DA was removed from day 16, day 18, IP, EP (2-4 hours), and day 0 hatchlings (2-24 hours) and placed in 4% buffered paraformaldehyde (pH 7.4) for 11-24 hours at 4°C. After fixation, the vessels were dehydrated in ethanol (15-18 min) and isopropyl alcohol (16-19 min) and impregnated in paraffin under a vacuum using a Milestone RHS1 Microwave Rapid Histoprocessor (Kalamazoo, MI, USA). Vessels were embedded with Shandon Histocentre™ 3 embedding center (Thermo Fisher Scientific Inc., Waltham, MA, USA). Paraffin blocks were serial sectioned at 5 µm using a model RM2245 Leica™ microtome (Leica Microsystems, Wetzelar, Germany). Coverslips were mounted using Cytoseal™ XYL (Richard-Allan Scientific now sold under Thermo Scientific brand, Waltham, MA, USA). Morphological changes were assessed during development on tissue stained with hematoxylin and eosin or Van Gieson using an Eclipse E200 microscope (A. G. Heinz Inc., Lake Forest, CA, USA) using Nikon® optics (Nikon Corp., Melville, NY). Digital images were taken with Olympus® DP70 camera (Olympus Optical Co. Ltd., Tokyo, Japan). Measurements were made on images of the serial sections of stained tissues using Image-Pro® Plus image analysis software (Media Cybernetics, version 5.0.0.39, Bethesda, MD, USA). Vessel wall thickness and smooth muscle thickness were calculated by using the mean of eight measurements from a cross-sectional view. In vessels with no lumen, the thickness was calculated as half of the total diameter. To measure inner lumen diameter, the inner lumen circumference was measured from the digital images. The lumen diameter was then calculated assuming the circumference of the vessel was circular (Rouwet et al., 2002; Belanger et al., 2008).
In situ Lengths and Widths of the Ductus Arteriosus

The lengths and widths of both the left DA and right DA during development were measured in situ in day 18, IP, and EP embryos and day 0 hatchlings. The animals were euthanized with isoflurane or decapitated, the chest cavity was opened and the esophagus, stomach, liver, gall bladder, and heart were removed. The connective tissue surrounding the DA was then removed. A Nikon® SMZ 1000 dissecting scope (Nikon Corp., Melville, NY, USA) was used to assist during the dissection and to magnify the image for analyses. Digital images were made using an Olympus® DP70 camera (Olympus Optical Co. Ltd., Tokyo, Japan). Image-Pro® Plus image analysis software (Media Cybernetics, version 5.0.0.39, Bethesda, MD, USA) was used to measure lengths and widths of the vessel. The proximal portion was determined to be from half way between the intermediate zone of the proximal and distal sections to where the tissue began to increase in diameter to connect with the pulmonary artery. The distal portion was halfway between the intermediate zone between the proximal and distal sections to where the two distal portions met at the aorta (Figure 1.1).

Statistical Analyses

The blood gas and air cell O₂ level, lumen diameter, DA wall thickness, smooth muscle layer thickness, and lengths and widths were tested for differences among means using a two-way ANOVA with age and incubation O₂ levels as factors. Overall length of right DA and left DA were tested using a two-way ANOVA with age/incubation O₂ levels and right or left side as factors. All statistically significant ANOVAs were followed by a Sidak–Holm posthoc test of pairwise comparisons. Data are reported as
the means ± S.E.M. The level of significance for all tests was p<0.05. Statistics were run by Sigmastat® 3.5 software (SyStat Software Inc., Chicago, IL, USA).

Results

Blood Gas and Air Cell

Arterial and venous Po\textsubscript{2} were dependent on the incubation O\textsubscript{2} level. Hyperoxic embryos had significantly higher arterial and venous Po\textsubscript{2} levels than normoxic embryos (Table 2.1). Day 16 and IP hypoxic embryos had significantly lower arterial levels than normoxic embryos (p<0.05; Table 2.1). Day 16 and day 18 hypoxic embryos had significantly lower venous levels than normoxic embryos (p<0.05; Table 2.1). Within a given incubation O\textsubscript{2} treatment, there were no significant differences in arterial Po\textsubscript{2} between the different stages of development. In contrast, venous Po\textsubscript{2} decreased as the embryos aged, with the exception of the hypoxic embryos, in which Po\textsubscript{2} remained constant. Internally pipped normoxic embryos had significantly lower venous Po\textsubscript{2} than day 16 and day 18 embryos, whereas venous Po\textsubscript{2} decreased significantly at each stage in hyperoxic embryos (p<0.05).

Arterial and venous Pco\textsubscript{2} were dependent on the incubation O\textsubscript{2} level. Hyperoxic and normoxic embryos had significantly greater arterial Pco\textsubscript{2} levels than hypoxic embryos (Table 2.1). Day 16 hyperoxic embryos had significantly greater arterial Pco\textsubscript{2} levels than normoxic embryos (Table 2.1). Within each incubation O\textsubscript{2} treatment, the only significant difference in arterial Pco\textsubscript{2} was found in the IP hyperoxic embryos, which had a significantly greater arterial Pco\textsubscript{2} than day 18 hyperoxic embryos (p<0.05). Hyperoxic embryos had significantly greater venous Pco\textsubscript{2} levels than normoxic embryos except on
day 18 (p<0.05). Normoxic embryos had a significantly greater venous Pco₂ level than hypoxic embryos (p<0.05). In hypoxic embryos, there were no significant differences in venous Pco₂ between the different stages of development. In normoxic embryos, IP embryos had a significantly greater venous Pco₂ than day 16 embryos (p<0.05). Internally pipped hyperoxic embryos had a significantly greater venous Pco₂ than day 16 or day 18 embryos (p<0.05; Table 2.1).

Air cell O₂ tension was greatest in embryos from the hyperoxic treatment and lowest in eggs in the hypoxic treatment (Table 2.1). Internally pipped hypoxic eggs had significantly lower O₂ air cell content than day 16 or day 18 hypoxic eggs (p<0.05). Oxygen air cell content of normoxic eggs remained constant. Day 18 hyperoxic eggs had a significantly lower O₂ air cell content than day 16 eggs (p<0.05). Internally pipped hyperoxic eggs had significantly lower O₂ air cell content than day 16 or day 18 hyperoxic eggs (p < 0.05).

**Morphological Changes in the Ductus Arteriosus**

Significant morphological changes in the DA occur at different stages of development in each of the incubation O₂ groups. In all incubation O₂ groups, day 16 and day 18 vessels exhibit a similar morphology (Figure 2.2; day 16 not shown). The outer tunica adventitia, which is comprised of elastin, surrounds the smooth muscle containing tunic media (Figure 2.3D; marked with m). At this stage the tunica intima is comprised of a single layer of endothelial cells and an internal elastic lamina (IEL; Figure 2.3A). In the DA of the hyperoxic embryo the smooth muscle cells swell and begin to migrate toward the lumen after the embryo IP (Figure 2.3C). This does not
occur until EP in normoxic embryos (Figure 2.3E). Additionally, the swelling of the endothelial cells appears to be greater in the hyperoxic vessels during EP compared with the normoxic EP vessels. The initiation of closure in the DA from hypoxic animals occurs either during the late stages of EP or during hatching (Figure 2.3D, G). During hatching the IEL fragments (Figure 2.3G). Based in a visual inspection, there do not appear to be any differences in the DA morphology between incubation groups once the embryos hatch (Figure 2.3G, H, I). In day 0 hatchlings the lumen is not completely occluded at any incubation O₂ group (Figure 2.3G, H, I).

Table 2.1: Blood gas and air cell measurements from Gallus gallus eggs incubated in various levels of oxygen. Measurements taken on day 16, day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia. Blood gas samples were obtained from major chorioallantoic veins or arteries. Values are mean ± standard error. n is the sample size. * indicates a significant difference compared with the age matched normoxic values at p<0.05. † indicates a significant difference from the day 16 value within a given incubation treatment at p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Arterial Po2 (mmHg)</th>
<th>Venous Po2 (mmHg)</th>
<th>Arterial Pco2 (mmHg)</th>
<th>Venous Pco2 (mmHg)</th>
<th>Air cell (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>22.6 ± 1.2*</td>
<td>48.4 ± 3.0*</td>
<td>30.1 ± 0.9*</td>
<td>21.7 ± 1.5*</td>
<td>72.9 ± 1.9*</td>
</tr>
<tr>
<td></td>
<td>n = 14</td>
<td>n = 11</td>
<td>n = 14</td>
<td>n = 11</td>
<td>n = 15</td>
</tr>
<tr>
<td>Day 18</td>
<td>20.5 ± 1.3</td>
<td>38.9 ± 3.1*</td>
<td>33.4 ± 1.3*</td>
<td>26.2 ± 2.2*</td>
<td>70.7 ± 2.0*</td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
<td>n = 9</td>
<td>n = 12</td>
<td>n = 9</td>
<td>n = 13</td>
</tr>
<tr>
<td>IP</td>
<td>18.7 ± 1.4*</td>
<td>45.4 ± 3.9</td>
<td>32.4 ± 1.8*</td>
<td>19.9 ± 1.1*</td>
<td>57.0 ± 2.5*</td>
</tr>
<tr>
<td></td>
<td>n = 10</td>
<td>n = 9</td>
<td>n = 10</td>
<td>n = 9</td>
<td>n = 11</td>
</tr>
<tr>
<td><strong>Normoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>28.0 ± 0.7</td>
<td>63.9 ± 1.8</td>
<td>42.8 ± 1.4</td>
<td>28.2 ± 1.2</td>
<td>119.3 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>n = 13</td>
<td>n = 17</td>
<td>n = 13</td>
<td>n = 17</td>
<td>n = 8</td>
</tr>
<tr>
<td>Day 18</td>
<td>23.6 ± 0.9</td>
<td>57.3 ± 2.8</td>
<td>47.1 ± 1.1</td>
<td>33.1 ± 1.9</td>
<td>118.1 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>n = 22</td>
<td>n = 11</td>
<td>n = 22</td>
<td>n = 11</td>
<td>n = 8</td>
</tr>
<tr>
<td>IP</td>
<td>25.9 ± 1.5</td>
<td>46.6 ± 2.1†</td>
<td>48.8 ± 2.7</td>
<td>34.6 ± 1.5†</td>
<td>107.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>n = 13</td>
<td>n = 10</td>
<td>n = 13</td>
<td>n = 10</td>
<td>n = 12</td>
</tr>
<tr>
<td><strong>Hyperoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>36.6 ± 2.5†</td>
<td>85.7 ± 3.1†</td>
<td>47.7 ± 2.3*</td>
<td>34.9 ± 1.8*</td>
<td>217.5 ± 2.7†</td>
</tr>
<tr>
<td></td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 16</td>
</tr>
<tr>
<td>Day 18</td>
<td>35.1 ± 2.1†</td>
<td>76.3 ± 3.1†</td>
<td>44.5 ± 1.4</td>
<td>32.8 ± 1.5</td>
<td>198.9 ± 5.6†</td>
</tr>
<tr>
<td></td>
<td>n = 15</td>
<td>n = 20</td>
<td>n = 15</td>
<td>n = 20</td>
<td>n = 12</td>
</tr>
<tr>
<td>IP</td>
<td>32.6 ± 2.1†</td>
<td>67.9 ± 3.0†</td>
<td>51.8 ± 3.3</td>
<td>41.1 ± 2.0†</td>
<td>180.3 ± 6.5†</td>
</tr>
<tr>
<td></td>
<td>n = 11</td>
<td>n = 13</td>
<td>n = 11</td>
<td>n = 13</td>
<td>n = 18</td>
</tr>
</tbody>
</table>
Figure 2.2: Sections of the left proximal ductus arteriosus from day 18 *Gallus gallus*. (A, B) Day 18 hypoxic left proximal ductus arteriosus (C, D) Day 18 normoxic left proximal ductus arteriosus (E, F) Day 18 hyperoxic left proximal ductus arteriosus. Tissue was stained with Van Gieson (A, C, E) or hematoxylin and eosin (C, D, F).
Figure 2.3: Van Geison stained sections of the left proximal ductus arteriosus from *Gallus gallus*. (A, D, G) Left proximal ductus arteriosus hypoxic IP, and EP embryo and hatchling (B, E, H) Left proximal ductus arteriosus normoxic IP, and EP embryo and hatchling (C, F, I) Left proximal ductus arteriosus hypoxic IP, and EP embryo and hatchling. Arrows indicate internal elastic lamina, m indicates media.
Lumen diameter was dependent on both age and incubation O₂ levels (Figure 2.4). DA lumen diameter of hyperoxic day 16 embryos was significantly smaller than DA lumen diameter of normoxic day 16 embryos (p<0.05). Lumen diameter of the DA from hypoxic day 16 embryos was significantly larger than lumen diameter of the DA from normoxic day 16 embryos (p<0.05). DA from IP hyperoxic embryos had a significantly smaller lumen diameter than DA from hypoxic and normoxic embryos (p<0.05). At EP, DA from hyperoxic embryos had the smallest lumen diameter and hypoxic embryos had the largest lumen diameter (p<0.05). In all treatments after day 18, DA lumen diameter decreased with age (Figure 2.4). DA lumen diameter of hyperoxic embryos changed significantly at each developmental stage until EP (p<0.05). DA lumen diameter of the hyperoxic embryo increased significantly from day 16 to day 18 and decreased significantly from day 18 to IP and IP to EP (p<0.05). DA lumen diameter from normoxic embryos decreased significantly at each developmental stage after day 18, however there was not a significant difference between DA lumen diameter of day 16 and IP embryos (p<0.05). DA lumen diameter from hypoxic embryos decreased significantly at hatch (p<0.05).

DA vessel wall thickness was dependent on age (Figure 2.5A). The only significant difference due to incubation O₂ level was vessel wall thickness of the DA of EP hyperoxic embryos, which was significantly thicker than the vessel wall thickness of the DA from hypoxic embryos (p<0.05). After day 18 DA thickness of hyperoxic embryos increased significantly at each developmental stage (p<0.05). Thickness of normoxic DA increased significantly at each stage after IP (p<0.05). There was a significant increase in DA thickness from hypoxic day 18 to IP embryos and EP embryos to hatchlings (p<0.05).
Figure 2.4: Lumen diameter of the left proximal ductus arteriosus from *Gallus gallus*. Measurements taken from day 16, day 18, internally pipped, and externally pipped embryos, and hatchlings incubated in hypoxia, normoxia, and hyperoxia. Values are mean ± standard error. n = 7-16. * denotes a group significantly different from normoxia at that age. Open symbols denote a significant difference between hyperoxic and hypoxic groups within a given age.

Smooth muscle thickness was dependent on age (Figure 2.5B). The only significant difference due to incubation O₂ level was between the DA from hyperoxic and hypoxic embryos during EP and hatchlings. In comparison to DA smooth muscle thickness of EP hyperoxic embryos, smooth muscle thickness of the DA from EP hypoxic embryos was significantly thinner (p<0.05). In comparison to DA smooth muscle thickness of hyperoxic hatchlings, smooth muscle thickness of the DA from hypoxic hatchlings was significantly thicker (p<0.05).
Figure 2.5: Mean vessel wall and smooth muscle thickness of the left proximal ductus arteriosus from *Gallus gallus*. (A) Measurements taken of the total vessel thickness of the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, externally pipped, and hatchlings in hypoxia, normoxia, hatch, or hyperoxia. Values are mean ± standard error. n = 7-16. Open symbols denote a significant difference between hyperoxic and hypoxic groups at a given age. (B) Measurements taken of the smooth muscle thickness of the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, externally pipped, and hatchlings in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 2-15. Open symbols denote a significant difference between hyperoxic and hypoxic groups at a given age.
Smooth muscle thickness of the DA from hyperoxic embryos increased significantly from day 18 to IP and IP to EP (p< 0.05). Smooth muscle thickness of the normoxic DA increased significantly every developmental stage after IP (p<0.05; Figure 2.5B). Smooth muscle thickness of the hypoxic DA increased significantly at hatch (p<0.05).

**In situ Lengths of the Ductus Arteriosus**

The *in situ* total length of left DA was dependent on incubation O₂ levels (Figure 2.7). The total length of the left hypoxic DA was significantly shorter than the normoxic left DA on day 18 and IP (p<0.05). The significant differences observed between treatments in the total lengths were a result of significant differences in either the proximal portion of the DA or a combination of proximal and distal portions. Both the proximal and distal portions of the left DA in hypoxic day 18 embryos were significantly shorter than the left DA of normoxic (p<0.05; Figure 2.6A). The proximal portions of the left DA in hypoxic IP embryos and hatchlings were significantly shorter than the proximal portions of the left DA from normoxic IP embryos and hatchlings (p<0.05).

Total length of the right DA was dependent on incubation O₂ levels (Figure 2.7). Total length of the hypoxic right DA was significantly shorter than the normoxic right DA on day 18 (p<0.05). The significant differences observed in the total lengths were a result of significant differences in both proximal and distal portions. The length of both the proximal and distal portions of the right DA from hypoxic day 18 embryos were significantly shorter than the right DA of normoxic embryos (p<0.05). The lengths of the right distal DA from hyperoxic day 18 and EP embryos and hatchlings were significantly longer than the lengths of age matched normoxic right DA (p<0.05). The lengths of the
Figure 2.6: Proximal and distal length measurements of the ductus arteriosus from *Gallus gallus*. (A) Measurements made on the proximal and distal portions of the left ductus arteriosus from day 18, internally pipped, and externally pipped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 5-12. * denotes a group significantly different from normoxia at that age. (B) Measurements made the proximal and distal portions of the right ductus arteriosus from day 18, internally pipped, and externally pipped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 4-8. * denotes a group significantly different from normoxia at that age.
Figure 2.7: Total length of right and left ductus arteriosus from *Gallus gallus*. Measurements made on day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 3-10. * denotes a group significantly different from normoxia at that age.

right proximal DA from EP hypoxic embryos and hatchlings were significantly shorter than the age matched lengths of the right proximal DA from EP normoxic embryos and hatchlings (p<0.05; Figure 2.6B). The overall lengths of the left DA were significantly longer than the overall lengths of right DA at every developmental stage (p<0.05; Figure 2.7).
In situ Widths of the Ductus Arteriosus

The widths of the proximal portions of the left DA were dependent on both age as well as incubation O$_2$ levels (Figure 2.8A). The width the proximal portion of the left DA in EP hypoxic embryos was significantly larger than the left DA from normoxic embryos (p<0.05). The width of the proximal portion of the left DA in day 18 normoxic embryos was significantly larger than the left DA from normoxic EP embryos and hatchling (p<0.05). The width of the proximal portion of the left DA in day 18 hyperoxic embryos was significantly larger than the DA from IP hyperoxic embryos and hatchlings (p<0.05). There were no age dependent differences observed in the hypoxic embryos (Figure 2.8A).

The widths of the distal portion of the left DA were dependent on both age as well as incubation O$_2$ levels (Figure 2.8A). The only incubation O$_2$ level dependent difference observed was the width of the left DA from EP normoxic embryos was significantly smaller than the width of the left DA from hyperoxic EP (p<0.05). The only age dependent differences were observed in the hyperoxic embryos. The width of distal left DA from hyperoxic hatchlings was significantly smaller than day 18, IP, and EP embryos (p<0.05).

The widths of the proximal portions of the right DA was dependent on age (Figure 2.8B). The only incubation O$_2$ level dependent difference was observed in hatchlings (Figure 2.8B) The width of the proximal portion of the right DA of hypoxic hatchlings were significantly larger than the width of the proximal portion of the right DA from normoxic hatchlings (p<0.05). The only age dependent differences were observed in normoxic embryos. The width of the proximal portion of the right DA of normoxic
Figure 2.8: Proximal and distal width of the ductus arteriosus from *Gallus gallus*. (A) Measurements made on the left proximal ductus arteriosus from day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 4-12. * denotes a group significantly different from normoxia at that age. (B) Measurements made on the right proximal ductus arteriosus from day 18, internally pipped, and externally piped embryos, and hatchlings incubated in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 4-10. * denotes a group significantly different from normoxia at that age.
day 18 embryos was significantly larger than the right DA from both hatchlings and EP embryos (p<0.05). The width of the proximal portion of the right DA of normoxic IP embryos was significantly larger than the width of the right DA from hatchlings (p<0.05). The widths of the distal portions of the right DA were dependent on incubation O\textsubscript{2} levels (Figure 2.8B). The only incubation O\textsubscript{2} level dependent difference observed in hatchlings (Figure 2.8B). The width of the distal portion of the right DA of normoxic hatchlings was significantly smaller than the width of the DA from hyperoxic hatchlings (p<0.05). The only age dependent differences were observed in the normoxic embryos. The width of distal right DA of hatchlings was significantly smaller than the right DA from day 18 and IP embryos (p<0.05).

Discussion

Incubation in chronic hypoxia is known to cause significant cardiovascular abnormalities in the developing chicken embryo (Rouwet et al., 2002; Villamor et al., 2004). Here I have shown that this includes an alteration in the developmental trajectory of the closure of the DA. Embryos incubated in hypoxia had decreased blood gas Po\textsubscript{2} and air cell Po\textsubscript{2}, whereas the reverse was true for embryos incubated in hyperoxia. The DA from hypoxic embryos began closing at a later developmental stage, whereas the DA from hyperoxic embryos began closing at an earlier developmental stage. Once the embryos hatched, there were no significant differences in the lumen diameter among the O\textsubscript{2} incubation groups. The overall length of the hypoxic and hyperoxic right and left DA differed significantly at every developmental stage, whereas the overall length of the right and left DA from normoxic embryos differed significantly from the DA of hypoxic
and hyperoxic embryos only in a few groups. The widths of the proximal and distal portions of the vessels differed significantly in only a few groups.

**Blood Gas and Air Cell**

Blood gas values were dependent upon both age and incubation O$_2$ level. The blood gas values obtained from normoxic embryos were similar to those obtained by Tazawa et al. (1983). As expected, chorioallantoic arterial Po$_2$ and Pco$_2$ were lowest in hypoxic embryos and greatest in hyperoxic embryos. Chorioallantoic venous Po$_2$ in both normoxic and hyperoxic embryos decreased with development; however in hypoxic embryos venous Po$_2$ remained relatively constant. At IP both the normoxic and hypoxic oxygenated venous Po$_2$ levels were similar, even though they were exposed to markedly different levels of O$_2$ in the incubators. Tintu et al. (2007) obtained arterial Po$_2$ values from hypoxic (15% O$_2$) and normoxic embryos that were markedly different than the values obtained in the present study; however it is unclear from where the blood was obtained. The values obtained in the Tintu et al. (2007) study are similar to the venous Po$_2$ values obtained in the present study. Just before chicken embryos internally pip, they experience an increase in circulating catecholamines (Wittman and Prechtl, 1991). This increase in catecholamines corresponds with a period of increased hypoxia in the embryo and aids the embryos by decreasing 2,3 DPG levels which facilitates an increase in O$_2$ saturation of hemoglobin (Wittman and Prechtl, 1991). Higher levels of circulating catecholamines may help hypoxic embryos maintain venous Po$_2$ throughout development.

Oxygen content of the aircell was dependent upon both age and incubation O$_2$
Within hypoxic and normoxic O₂ treatments, O₂ content of day 16 and day 18 eggs were similar; however, hyperoxic day 18 eggs had a significantly lower O₂ content than day 16 embryos. Once embryos began to breathe the gas in the aircell during IP, the O₂ content significantly decreased in all treatments. Hypoxic incubated eggs had the lowest aircell O₂ content, whereas hyperoxic incubated eggs had the highest aircell O₂ content. Normoxic values obtained in this study were consistent with both Tazawa et al. (1980) and Wangensteen and Rahn (1970/71). Hypoxic values were similar to those obtained by Wangensteen et al. (1974) from chicken embryos that were incubated at high altitude. Animals with higher aircell O₂ contents typically have higher venous Po₂. An exception is IP hypoxic embryos, which had significantly lower aircell O₂ content than IP normoxic embryos, but similar venous Po₂ levels. This suggests hypoxic IP embryos are more efficient in taking O₂ from the air cell and this may be due to an increase in lung ventilation rate of the hypoxic exposed embryo when compared with the normoxic exposed embryo. In response to acute hypoxia during IP, normoxic embryos have been shown to increase tidal volume (Sbong and Dzialowski, 2007).

**Morphological Changes that Occur in the Ductus Arteriosus During Hatching**

Anatomical remodeling of the DA during closure has been well characterized in mammals. In the DA of many mammals, reorientation of cells and thickening of the intima occur prior to birth (Yoder et al., 1978; Tada et al., 1990; Slomp et al., 1992). The proximal portion of the chicken embryo DA begins to close 5 hours after the animal EP (Belanger et al., 2008). Closure is characterized by the swelling and migration of endothelial cells into the lumen of the vessel, followed by the migration of smooth
muscle cells into the lumen (Belanger et al., 2008). Similarly, the present study showed that the DA from normoxic embryos began to close at EP. Closure of the DA was accelerated in the hyperoxic DA and delayed in the hypoxic DA. Belanger et al. (2008) found that the lumen was occluded by day 0 (<8 hours post-hatch), which is not consistent with my findings. Differences could be due to location in the proximal portion from which the sample was obtained or variability in the species.

Incubation in hypoxia and hyperoxia had significant effects on lumen diameter of the left DA during incubation. The IP hyperoxic left DA lumen diameter was significantly smaller than both normoxic and hypoxic left DA, indicating closing of the DA was accelerated in hyperoxic embryos. Van der Sterren et al. (2009) reported that the cross sectional area of the lumen of day 19 hypoxic right proximal DA did not differ significantly from the cross sectional area of the lumen of day 19 normoxic right proximal DA. Similarly, the present study found that the lumen diameter of day 18 and IP hypoxic proximal DA did not differ significantly from the lumen diameter of day 18 and IP normoxic proximal DA. In contrast, Rouwet et al. (2002) observed that chronic hypoxia lead to a decrease in lumen diameter in the ascending aorta of day 19 chicken embryos. Hansen (2005) found that the lumen diameter of aortas from day 19 white leghorn embryos were significantly smaller in the hypoxic group (14% O₂) when compared to the control group (21% O₂). Observed differences could be due to differences in vessel function.

Once the embryos hatched there was no difference in the lumen diameter among the three groups. Coughlin and Husson (1960) exposed hatching chicken embryos to hypoxia and reported that hypoxia was associated with a delay in DA closure. The
incidence of PDA in children is significantly higher in children born at higher altitudes (3847-4533m) in comparison to children born at lower altitudes (1650-2835m or 2792-4360m; Chen et al., 2008). The DA from normoxic embryos begins to close after the embryo becomes EP (Belanger et al., 2008), which corresponds with the timing of decreased blood flow to the CAM and increased blood flow to the lungs (Rahn et al., 1985). During the transition to lung respiration metabolic demand increases (Rahn et al., 1974). If the DA fails close, the embryo may not be able meet the increased metabolic demand, resulting in death. Several studies have reported that incubation in hypoxia leads to an increase in mortality (Rouwet et al., 2002; Villamor et al., 2004; Tintu et al., 2007). Since tissue samples were only obtained from live embryos / hatchlings it is possible that the average lumen diameter of DA from hypoxic embryos may have been larger. During the course of this study it was not unusual for an embryo to die after EP (personal observation). If the present study measured DA lumen diameter from hypoxic embryos that died, there might have been a larger average lumen diameter.

As the embryo developed, overall thickness and smooth muscle thickness of the vessel increased. In the DA from normoxic incubated embryos, significant increases in overall thickness and smooth muscle thickness occurred at developmental stage after IP, indicating closure of the DA. The overall thickness of the hyperoxic DA increased significantly every developmental stage after day 18, indicating closure was accelerated. The changes in the thickness of the DA from hypoxic embryos appeared to occur rapidly at the end of incubation. Hansen (2005) found that chronic hypoxia (14% O₂) caused the aortic wall of the day 19 jungle fowl to be significantly thinner than
the aortic wall of the controls (21% O₂). In contrast, chronic hypoxia (simulated altitude 4,570m) resulted in thicker pulmonary artery vessel walls in calves (Davie et al., 2003). Goldberg et al. (1971) observed that the media to external diameter ratio of pulmonary arteries of newborn rats from hypoxic (13% at term) mothers were significantly larger in comparison to newborn rats from normoxic (room air) or hyperoxic (40% at term) mothers. It is possible that hypoxic incubation causes an increase in vessel wall thickness of the DA, but since the vessel remodels under normal situations, the difference goes undetected.

*In situ Length and Width of the Ductus Arteriosus*

The lengths and widths of the DA were dependent on both age and incubation O₂ level. On day 18 the total length of both the right and left hypoxic DA were significantly shorter than day 18 hyperoxic and normoxic DA. Similarly, van der Sterren et al. (2009) found that the day 19 right and left DA from hypoxic embryos was significantly shorter than the right and left DA from normoxic embryos. Van der Sterren et al. (2009) reported the difference in length was due to the proximal portion of the vessel, whereas the present study observed differences in both the proximal and distal portions. Embryo size could account for differences in length. Hypoxic embryos were smallest in size, whereas hyperoxic embryos are largest in size.

There were few differences in widths of the DA. Van der Sterren et al. (2009) reported no significant differences between the width of the day 19 hypoxic and normoxic DA. The width of the left DA tended to decrease as the embryo aged in all
incubation O_{2} groups. Since measurements are made externally, observed differences in widths could be due to the immature vessel flattening.

Conclusions

The present study examined the effect of O_{2} incubation level on the histology of the chicken DA. Blood gas and air cell data followed the expected pattern, with hypoxic embryos having the lowest O_{2} levels and hyperoxic embryos having the highest O_{2} levels. Incubation in hyperoxic resulted in an acceleration of the closure of the DA, while incubation in hypoxic resulted in a delay of the closure of the DA. There were no significant differences in the DA once the animal hatched.

In humans, development in hypoxia is linked with a higher incidence of patent DA (Rakza et al., 2007; Chen et al., 2008). I expected to find differences in the lumen diameter of hypoxic, normoxic, and hyperoxic DA; however once the embryos hatched there were no differences. Hypoxic incubation leads to an increase in mortality in chicken embryos (Rouwet et al., 2002; Villamor et al., 2004; Tintu et al., 2007). It is possible that embryo mortality toward the end of incubation was a result of a patent DA. There appeared to be rapid increase in thickness and decrease in lumen diameter in the DA from hypoxic embryos towards the end of incubation. Future study can determine if the DA from hypoxic embryos rapidly remolds at end of incubation or if embryos with a patent DA die before they hatch.
CHAPTER 3

REACTIVITY OF THE DUCTUS ARTERIOSUS TO CATECHOLAMINES

Introduction

Development in chronic hypoxia produces significant abnormalities in the developing cardiovascular system as well as increased mortality in chicken embryos (Rouwet et al. 2002; Villamor et al. 2004). Additionally, the vascular phenotype of the developing chicken embryo responds to chronic hypoxic exposure (Rouwet et al., 2002; Ruijtenbeek et al., 2000; van der Sterren et al., 2009). Van der Sterren et al. (2009) found that the ductus arteriosus (DA) from day 19 embryos incubated in hypoxia (15% O₂) exhibited a significantly greater contractile response to norepinephrine (NE) and phenylephrine (PE) than the DA from day 19 normoxic incubated embryos. Villamor et al. (2004) reported that chicken embryos developing in chronic hypoxia exhibited reduced pulmonary artery contractile responses to norepinephrine (NE), endothelin-1, and the thromboxane A₂ mimetic U-46619. Chronic hypoxic incubation also produces sympathetic hyperinnervation of the arterial system (Rouwet et al., 2002; Ruijtenbeek et al., 2000).

Under certain conditions such as developing at high altitude or intrauterine growth restriction, the mammalian fetus develops in hypoxic conditions (Carlson, 1988; Pollack and Divon, 1992). The influence of altered oxygen levels on the development and physiology of the O₂-sensitive DA is unknown (Heymann and Rudolph, 1975; Noel and Cassin, 1976; Clyman et al., 1978; Berger et al., 1990; Smith, 1998; Thebaud et al., 2004; Kajimoto et al., 2007 Ågren et al., 2007; Belanger et al., 2008; Dzialowski and Greyner, 2008). It has been suggested that catecholamines play a role in the closure of
the DA (Ågren et al., 2007). The goal of this study was to use the chicken DA as a model to characterize the effects of developing in chronic hypoxia or hyperoxia on DA reactivity to catecholamines. Because the DA response to catecholamines is developmentally regulated in chickens (Ågren et al., 2007), the DA from day 16 and day 18 non-lung ventilating embryos as well as IP embryos, breathing with their lungs, were tested to examine how chronic incubation O₂ levels influenced development of the adrenergic response in the DA. In addition, since there are two DA present throughout incubation the reactivity of both the right and left DA of day 18 and IP embryos were examined to see if there are any contractile differences between the two DA (Bergwerff, 1999). I hypothesized that incubation in chronic hypoxia would delay the maturation of the adrenergic response of the DA, while incubation in chronic hyperoxia would accelerate development.

Materials and Methods

Eggs

Eggs were incubated as stated in chapter 2.

In vitro Vessel Physiology

The right and left DA was removed from day 16 (left only), day 18, IP, and EP embryos and placed in a physiological saline solution (PSS composed of 120.5 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.6 mM CaCl₂, 1.2 mM NaH₂PO₄, 20.4 mM NaHCO₃, and 10 mM glucose) equilibrated with 95% N₂ and 5% CO₂ and any remaining connective tissue was removed.
Because the chicken DA has two distinct morphological regions along its length (Belanger et al. 2008), the proximal and distal portions of the DA were separated based on visual inspection of their vessel morphology and diameter. A clear transition between the proximal portion and distal portion can be seen in the middle of each vessel (Ågren et al., 2007; Belanger et al., 2008). In this study, only the muscular proximal portion of the DA was used.

In vitro contraction was measured on the isolated vessels using a 4 chamber 610M Danish Myo Technologies myograph (Aarhus, Denmark). The excised vessels ranging in length from 0.3 to 2 mm were mounted into myograph chambers by threading two 40 µm diameter stainless steel wires through the lumen of the vessel. One wire was attached to a force transducer and the other to a micromanipulator that allowed for the adjustment of vessel tension. The force generated by each vessel was recorded as the active wall tension (mN mm\(^{-1}\)) using Powerlab® 8SP data acquisition system (ADInstruments Pty Ltd., Colorado Springs, CO) and Chart® data acquisition and analysis software (ADInstruments Pty Ltd., version 5.4.2, Colorado Springs, CO). Vessel contractile data are presented as the net active tension generated in response to the contractile agent.

Each chamber was filled with 5 ml of PSS and bubbled with a 95% N\(_2\), and 5% CO\(_2\) gas mixture resulting in a Po\(_2\) of 30 mmHg (4%) and a Pco\(_2\) of 40 mmHg. The gas mixture in the chambers was regulated by a Brooks® Instrument model 0154 (Brooks Instrument, LLC, Hatfield, PA, USA). Bath Po\(_2\) and Pco\(_2\) were monitored with a Radiometer ABL® 5 blood gas meter (A/S Corp., Copenhagen, Denmark). The vessels were allowed to equilibrate for 30 minutes and the baseline tension for all vessels was
set to a tension that produced the largest contraction in response to 120 mM KCl as determined in preliminary experiments. During this time the vessels were contracted once or twice with 120 mM KCl-PSS followed by three PSS rinses. All in vitro measurements were made under low light conditions.

**Contractile Response**

In day 18 (prepipped) and internally pipped (IP) embryos, DA contraction of isolated vessels was measured in response to cumulative stepwise increases in PE (10^{-8} to 10^{-4} M) and NE (10^{-8} to 10^{-5} M). Because the hypoxic embryo IP approximately 1 day later than normoxic embryos, the day 19 hypoxic DA contractile response to stepwise increases of PE and NE was measured. After the addition of each contractile agent, the vessel was allowed to stabilize before the next concentration was added to the chamber. The contractile response of the left DA from day 16 embryos was measured only in response to the highest concentrations, 10^{-4} M PE and 10^{-5} M NE.

To see if O_2 augments the DA’s response to NE on day 16, day 18, and IP, NE was added to the organ bath after an increase in O_2. The O_2-induced contraction was measured in response to an increase in organ bath O_2 from 4% to 25%, followed by the addition of 10^{-5} M NE to the organ bath.

**Chemicals**

Phenylephrine and NE were obtained from Sigma-Aldrich® (Sigma-Aldrich Biotechnology, St Louis, MO, USA). Initial stock solutions of PE and NE were made daily with distilled water followed by further dilutions in PSS.
Statistical Analyses

The effect of NE and PE on vessel tension of day 18 and IP embryos was tested for differences among means using two-way repeated measures ANOVAs with age/incubation O₂ level and concentration as factors. The effect of 10⁻⁵ M NE and 10⁻⁴ M PE on vessel tension of the DA from day 16 embryos was tested along with the day 18 and IP DA contractile response using two-way ANOVAs with age incubation O₂ level as factors. The effect of 10⁻⁵ M NE and 10⁻⁴ M PE on vessel tension of the DA from day 19 hypoxic embryos was compared with the DA response from hypoxic day 18 and IP (and day 16 for left DA) using one way ANOVAs with age as the factor. The effect of 10⁻⁵ M NE on vessel tension of the DA from day 19 hypoxic embryos was compared with the DA response from normoxic and hyperoxic IP using an independent t-test. An independent t-test was used to test for differences between the right and left DA in response to NE and PE on vessel tension of day 18, day 19 (hypoxic only), and IP embryos. The effect of organ bath O₂ level on the DA response to NE was tested using a two-way ANOVA with age/incubation O₂ level and organ bath O₂ level as factors. All statistically significant ANOVAs were followed by a Sidak-Holm posthoc to test pairwise comparisons. Data are reported as the mean ± SE. The level of significance for all tests was p<0.05. Statistics were run by Sigmastat® 3.5 software (SyStat Software Inc., Chicago, IL, USA).

Results

Contractile Response to Adrenergic Agonists

The left DA response to NE was dependent on both age and incubation O₂ level
(Figure 3.1A). There were no differences in any incubation group on the left DA contractile response to NE in day 16 embryos (Figure 3.3). The left DA from normoxic and hyperoxic embryos had a significant increase in the contractile response to NE from day 16 to day 18 (p<0.05). In the left DA of hypoxic embryos, there was not a significant increase in the contractile response to NE from day 16 and day 18 embryos, however there was a significant increase between day 16 to IP (p<0.05). There were significant effects of incubation O₂ on the left DA contractile response to NE in day 18 embryos. The DA from both normoxic and hyperoxic day 18 embryos exhibited a significant dose dependent contractile response to NE. In contrast, the DA from hypoxic day 18 embryos did not exhibit a significant dose dependent contractile response to NE (Figure 3.1A). The left DA from IP embryos from all incubation O₂ treatments exhibited a significantly stronger contractile response to NE than day 18 embryos within the same incubation treatment (Figure 3.1A). The left DA from day 19 hypoxic embryos exhibited a significantly stronger contractile response to NE than the left DA from day 18 hypoxic embryos (p<0.05). The contractile response of the left DA from day 19 hypoxic embryos to NE was similar to the response of the DA from hypoxic IP embryos.

The right DA response to NE was dependent on both age and incubation O₂ levels (Figure 3.1B). There were significant effects of incubation O₂ on right DA contractile response to NE in day 18 embryos. The DA from both normoxic and hyperoxic day 18 embryos exhibited a significant dose dependent contractile response to NE (p<0.05). In contrast, the right DA from hypoxic day 18 embryos did not exhibit a significant dose dependent contractile response to NE (Figure 3.1B). The right DA from day 18 hyperoxic embryos exhibited a significantly stronger contractile response to NE
Figure 3.1 Contractile responses of the left and right proximal DA from *Gallus gallus* incubated under different oxygen levels. (A) Response of the left proximal DA to increasing concentrations of norepinephrine. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in norepinephrine. Values are mean ± standard error. n = 9-13. Letters denote significantly different groups at p<0.05. (B) Response of the right proximal DA to increasing concentrations of norepinephrine. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in norepinephrine. Values are mean ± standard error. n = 6-13. Letters denote significantly different groups at p<0.05.

than the right DA from normoxic embryos (p<0.05). Internally pipped embryos from all incubation O₂ treatments exhibited a significantly stronger contractile response to NE
than day 18 embryos within the same incubation treatment (Figure 3.1B). The right DA from day 19 hypoxic embryos exhibited a significantly stronger contractile response to NE than the right DA from day 18 embryos (p<0.05; Table 3.1). The contractile response of the right DA from day 19 hypoxic embryos to NE was similar to the response of the DA from IP hypoxic embryos. The right DA from IP hyperoxic embryos exhibited a significantly stronger contractile response to NE than the right DA from IP normoxic embryos (p<0.05). There were no significant differences between the contractile response of the left DA and right DA to NE.

There were significant effects of incubation O$_2$ and age on left DA contractile response to PE (Figure 3.2A). There were no differences in contractile response of the DA from day 16 embryos to PE (Table 3.1). The left DA from normoxic and hyperoxic embryos had a significant increase in the contractile response to PE from day 16 to day 18 (p<0.05). The left DA from hypoxic embryos did not exhibit a significant increase in the contractile response to PE from day 16 to day 18 embryos, however there was a significant increase from day 16 to IP (p<0.05). In day 18 embryos, PE produced a significantly stronger contractile response in the hyperoxic left DA than normoxic and hypoxic left DA (p<0.05). The left DA from hypoxic embryos exhibited a significantly weaker contractile response to PE than age-matched day 18 normoxic and hyperoxic embryos (p<0.05). Internally pipped embryos from all incubation O$_2$ treatments exhibited significantly stronger contractile responses than day 18 embryos within the same incubation treatment (Figure 3.2A). The left DA from day 19 hypoxic embryos exhibited a significantly stronger contractile response to PE than the left DA from day 18 hypoxic embryos (p<0.05; Table 3.1). The contractile response of the left DA from day 19
hypoxic embryos to PE was similar to the contractile response of the DA from IP hypoxic embryos (Table 3.1). The left DA from IP hypoxic embryos exhibited a significantly weaker contractile response to PE in comparison to the contractile response of the left DA from normoxic and hyperoxic embryos (Figure 3.2A). The contractile responses of normoxic and hyperoxic IP left DA to PE were not significantly different (Figure 3.2A).

Table 3.1: Contractile responses of the proximal ductus arteriosus from Gallus gallus. Measurements made on left proximal day 16, and left and right proximal DA in day 19 embryos incubated in hypoxia, normoxia or hyperoxia in response to $10^{-4}$ M phenylephrine or $10^{-5}$ M norepinephrine. Values are mean ± standard error. n is the sample size.

<table>
<thead>
<tr>
<th></th>
<th>Hypoxic</th>
<th>Normoxic</th>
<th>Hyperoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 9</td>
<td>n = 9</td>
</tr>
<tr>
<td>Day 19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0.77 ± 0.12</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 7</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>0.71 ± 0.17</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 4</td>
<td>n/a</td>
</tr>
<tr>
<td>Day 19</td>
<td>NE</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0.94 ± 0.12</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 8</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>0.97 ± 0.13</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 8</td>
<td>n/a</td>
</tr>
</tbody>
</table>

There were significant effects of incubation O$_2$ and age on right DA contractile response to PE (Figure 3.2B). The right DA from day 18 and IP hypoxic embryos exhibited a significantly weaker contractile response to PE than age matched normoxic and hyperoxic embryos (p<0.05). Internally pipped embryos from all incubation O$_2$ treatments exhibited a significantly stronger contractile response than day 18 embryos within the same incubation treatment (Figure 3.2B). The right DA from day 19 hypoxic embryos exhibited a significantly stronger contractile response to PE than the right DA
**Figure 3.2** Contractile responses of the left and right proximal DA from *Gallus gallus* incubated under different oxygen levels.  
(A) Response of the left proximal DA to increasing concentrations of phenylephrine. Measurements made on day 18 and internally piped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in phenylephrine. Values are mean ± standard error.  
(B) Response of the right proximal DA to increasing concentrations of phenylephrine. Measurements made on day 18 and internally piped embryos incubated in hypoxia, normoxia, or hyperoxia to a stepwise increase in phenylephrine. Values are mean ± standard error.  

Letters denote significantly different groups at p<0.05.
from day 18 hypoxic embryos (p<0.05). The DA contractile response to PE from day 19 and IP hypoxic embryos was not significantly different. There were no significant differences between the contractile response of the left DA and right DA to PE.

**Contractile Response to Norepinephrine in the Presence of 25% Oxygen**

An acute increase in O₂ augmented the adrenergic response of the left DA in all incubation O₂ treatments. On day 16, the left DA contractile response to NE in 4% O₂ and NE in 25% O₂ were not different in any treatment (Figure 3.3). In the left day 18 and IP, NE produced a significantly greater contractile response in the presence of 25% O₂ than 4% O₂ in all treatments (p< 0.05). The left DA from day 18 hypoxic embryos exhibited a significantly weaker response to NE in the presence of 25% O₂ than normoxic and hyperoxic day 18 embryos; however, the left DA from IP hypoxic embryos exhibited a significantly stronger contractile response to NE in the presence of 25% O₂ than their normoxic and hypoxic counterparts (p<0.05).

An acute increase in O₂ augmented the adrenergic response of the right DA in all incubation O₂ treatments. In the right DA from day 18 and IP normoxic and hypoxic embryos, NE produced a significantly greater contractile response in the presence of 25% O₂ than 4% O₂ (p< 0.05; Figure 3.4). In the right DA from day 18 hyperoxic embryos, NE produced a significantly greater contractile response in the presence of 25% O₂ than 4% O₂ (p< 0.05; Figure 3.4). The right DA from day 18 hypoxic embryos exhibited a significantly weaker response to NE in the presence of 25% O₂ than the DA from day 18 normoxic and hyperoxic embryos; however, the right DA from IP hypoxic embryos exhibited a significantly greater response to NE in the presence of 25% O₂.
than the DA from IP normoxic and hypoxic embryos (p<0.05). There were no significant differences between the contractile response of the left DA and right DA to NE in the presence of 25% O₂.

Figure 3.3: The effect of oxygen on the active tension of the left proximal DA from *Gallus gallus* in response to norepinephrine 10⁻⁴ M in 4% and 25% oxygen. Measurements made on day 16, day 18 and internally piped embryos incubated in hypoxia, normoxia, or hyperoxia. Vessel response was measured at 4% O₂ and 25% O₂. Values are mean ± standard error. 4% group n = 8-13. 25% group n = 8-12. *indicates a significant difference in net active tension of the 4% O₂ group compared with the 25% O₂ group for a given age p<0.05.
Figure 3.4: The effect of oxygen on the active tension of the right proximal DA from *Gallus gallus* in response to norepinephrine $10^{-4}$ M in 4% and 25% oxygen. Measurements made on day 18 and internally pipped embryos incubated in hypoxia, normoxia, or hyperoxia. Vessel response was measured at 4% O$_2$ and 25% O$_2$. Values are mean ± standard error. 4% group n = 9-13. 25% group n = 8-11. * indicates a significant difference in net active tension of the 4% O$_2$ group compared with the 25% O$_2$ group for a given age p<0.05.

**Discussion**

Under certain conditions such as developing at high altitude or intrauterine growth restriction, the mammalian fetus develops under hypoxic conditions (Carlson, 1988; Pollack and Divon, 1992). During embryonic development, the influence of
altered oxygen levels on the physiology of the O₂-sensitive DA is unknown (Heymann and Rudolph, 1975; Noel and Cassin, 1976; Clyman et al., 1978; Berger et al., 1990; Smith, 1998; Thebaud et al., 2004; Kajimoto et al., 2007 Ågren et al., 2007; Belanger et al., 2008; Dzialowski and Greyner, 2008). Incubation in chronic hypoxia is known to cause significant cardiovascular abnormalities in the developing chicken embryo (Rouwet et al., 2002; Villamor et al., 2004). Here I have shown that hypoxic/hyperoxic incubation alters the developmental trajectory of the maturation of the adrenergic response of the DA. Incubation in hypoxia produced a developmental delay in the contractile response of the chicken DA to catecholamines. Oxygen augmented the adrenergic response of the left DA in day 18 and IP embryos in all treatments and the right DA from day 18 and IP normoxic and hypoxic embryos, as well as day 18 hyperoxic embryos.

**Adrenergic Agonist**

Both age and incubation O₂ level affected the DA contractile response to adrenergic agonists. In comparison to day 16 normoxic and hyperoxic embryos, the DA from day 18 normoxic and hyperoxic embryos exhibited a significantly stronger contractile response to NE and PE. This is consistent with the work done by Å gren et al. (2007), who found that the DA from day 19 embryos exhibited a stronger contractile response to PE than day 15 embryos. The DA from hypoxic day 18 embryos exhibited a similar response to NE and PE as the DA from day 16 embryos, suggesting a developmental delay of the maturation of the adrenergic pathway in hypoxic incubated embryos. The DA from day 18 embryos incubated in hyperoxia and normoxia exhibited
a significantly stronger contractile response to NE and PE than the DA from day 18 embryos incubated in hypoxia. Van der Sterren et al. (2009) reported that in comparison to DA from day 19 normoxic embryos, the DA from day 19 hypoxic embryos exhibited a significantly stronger contractile response to both PE and NE. Differences in the findings may be due to age of the embryo. Van der Sterren et al. (2009) only studied day 19 embryos, whereas the present study used day 18 and IP normoxic embryos and day 18, day 19 and IP hypoxic embryos. The present study shows that the DA contractile response to PE and NE was significantly stronger in the DA from hypoxic day 19 embryos than the DA from day 18 hypoxic embryos; however no measurements were made on day 19 normoxic or hyperoxic embryos. The DA from IP embryos in all treatments exhibited a significantly stronger contractile response to NE and PE than the DA from day 18 embryos, suggesting further maturation of the adrenergic pathway. Similarly, Ågren et al. (2007) found that there was a significant increase in the response of the DA to NE from day 19 to day 21 (EP embryos). There was also an increase in the response of the DA to PE from day 19 to EP; however the increase was not significant (Ågren et al., 2007). The DA from day 19 hypoxic embryos exhibited a similar contractile response to NE as the DA from IP normoxic and hyperoxic embryos, suggesting that any developmental delays due to incubation in hypoxia disappeared by day 19, which is about the time normoxic and hyperoxic embryos IP. The DA from IP hypoxic incubated embryos exhibited a significantly weaker contractile response to PE in comparison to the DA from IP normoxic incubated embryos. This suggests at IP there is still a developmental delay of the maturation of the adrenergic response in the DA from hypoxic embryos.
Studies have examined the effect of incubation in chronic hypoxia on vessel reactivity to NE from developing chicken embryos (Ruitjtenbeek et al., 2000; Rouwet et al., 2002; Villamor et al., 2004; van der Sterren et al., 2009). Rouwet et al. (2002) showed that mesenteric resistance arteries of day 19 chicken embryos exhibited a contractile response to NE that was similar in normoxic and chronic hypoxic (15% O₂) embryos. In contrast, Villamor et al. (2004) reported that chronic hypoxia (15% O₂) reduced pulmonary arterial contractile reactivity to NE in day 19 embryos. After spending the last two days of incubation in normoxia, the arterial reactivity of the hypoxic embryos was similar to the control pulmonary arteries (Villamor et al. 2004).

Several studies have examined the effect of chronic hypoxia as a result of development at high altitude on vessel reactivity to NE and PE in newborn and fetal sheep (Hu et al., 1996; Longo and Pearce, 1998; Zhang et al., 1998; Herrera et al., 2007; Xue et al., 2008. An increased contractile response to NE was observed in the pulmonary artery of fetal sheep that developed in chronic hypoxia (altitude 3,801 m for ~110 days; Xue et al., 2008). An increased contractile response to both NE and PE was observed in the small femoral arteries of newborn sheep that developed in chronic hypoxia (altitude 3,600 m for 50 generations; Herrera et al., 2007). In contrast, Longo and Pearce (1998) found that development in chronic hypoxia (altitude 3,380 m for ~110 days) caused a decrease in the cerebral arteries contractile response to NE, and decreased α₁ adrenergic receptor density in common carotid and intracranial arteries in fetal sheep. Similarly, Zhang et al. (1998) reported that development in chronic hypoxia (altitude of 3820m ~110 days) resulted in a decrease in sheep umbilical vein reactivity to NE, and decreased α₁ adrenergic receptor density in both the umbilical vein and
umbilical artery. Hu et al. (1996) found that development in chronic hypoxia (altitude of 3820 m ~110 days) resulted in a decrease in sheep main and fourth branch uterine artery reactivity to NE and decreased $\alpha_1$ adrenergic receptor density in those arteries. The DA from hypoxic IP embryos exhibited a significantly weaker contraction to PE than the DA from IP normoxic or hyperoxic embryos. Several studies have shown that development in hypoxia leads to a decrease in $\alpha_1$ adrenergic receptors in various vessels (Hu et al., 1996; Longo and Pearce, 1998; Zhang et al., 1998). This could be the reason the DA from IP hypoxic incubated embryos exhibited a significantly weaker response to PE in comparison to the DA from IP normoxic and hyperoxic embryos. If catecholamines play a role in the closure of the DA (Ågren et al., 2007), hypoxic incubation could result in a decrease in adrenergic receptor density, which could ultimately delay closure of the DA.

Hyperoxia has also been shown to affect vessel contractile responses to catecholamines. Lakshminrusimha et al. (2006) showed that ventilation of newborn sheep with 100% $O_2$ for 24 hours significantly increased the pulmonary artery contractile response to NE. In contrast, the DA from day 19 hyperoxic (60%) embryos exhibited a similar contractile response to PE as the DA from day 19 normoxic embryos (Villamor et al, 2008). In the present study, the right DA from day 18 hyperoxic embryos did not exhibit a significantly greater contractile response to PE in comparison to the DA from day 18 normoxic embryos, whereas the left DA from day 18 hyperoxic embryos did exhibit a significantly greater contractile response to PE in comparison to the DA from day 18 normoxic embryos. The differences between the findings of Villamor et al. (2008) and the present study could be due to the age of the embryos used. The left DA
from day 18 hyperoxic embryos exhibited a significantly greater contractile response to PE; however, in comparison to the left DA from normoxic embryos, no further differences were observed between the DA contractile response to PE in normoxic and hyperoxic embryos. This suggests that if hyperoxic incubation caused an acceleration of the maturation of the adrenergic response, it was only for a short period of time.

**Contractile Response to Norepinephrine in the Presence of 25% Oxygen**

Oxygen augmented the response of the DA to NE (Figure 3.3, 3.4). The left DA from day 18 and IP embryos in all treatments produced a significantly greater contractile response to NE in 25% O₂ compared with 4% O₂. The right DA from day 18 and IP normoxic and hypoxic embryos as well as day 18 hyperoxic embryos produced a significantly greater contractile response to NE in 25% O₂ compared with 4% O₂. The DA from IP hypoxic embryos in NE in 25% O₂ exhibited the greatest contractile response in both the right and left vessel groups, in comparison to DA from IP normoxic and hyperoxic embryos. In contrast, Ågren et al. (2007) showed that pretreatment with NE did not have an effect on the O₂ response. This difference between studies may be due to the portions of the DA used. Ågren et al. (2007) used both proximal and distal tissues, whereas the current study used only proximal tissue. Smith and McGrath (1988) found that NE did not augment the DA response to O₂ in rabbits. It has been suggested that catecholamines play a role in the closure of the DA (Ågren et al., 2007). Unlike certain mammalian species (Boréus et al., 1969; Ikeda, 1970; Bodach et al., 1980), the DA from chicken embryos is not innervated (Ågren et al., 2007). A maturation of the adrenergic response of the DA during development in combination with O₂
augmenting the adrenergic response would suggest that circulating catecholamines may augment the O₂-induced constriction in the DA of the chicken embryo (Ågren et al., 2007). However, the plasma concentrations of NE and epinephrine have begun to decrease by the time the DA becomes most sensitive to NE in the chicken embryo (Mulder et al., 2002; Wittman and Prechtl, 1991).

Conclusions

The present study examined the effect of O₂ incubation level on the vasoreactivity of the chicken DA. Incubation O₂ level had an effect on the maturation of the DA vasoreactivity to catecholamines. Embryos incubated in hypoxia experienced a delayed response to both NE and PE. The DA response to NE was similar in IP hypoxic, normoxic and hyperoxic embryos, whereas the DA response to PE was significantly weaker in the DA from IP hypoxic embryos, in comparison to the DA from IP normoxic and hyperoxic embryos. Oxygen augmented the DA response to NE in the left DA from day 18 and IP embryos in all treatments. Oxygen augmented the DA response to NE in the right DA from day 18 and IP normoxic and hypoxic embryos, as well as the right DA from day 18 hyperoxic embryos.

If catecholamines play a role in DA closure, incubation in hypoxia could lead to delays in DA closure. The DA from IP hypoxic embryos had a significantly weaker contractile response to PE in comparison to the DA from IP normoxic and hyperoxic embryos. Future study could discover if the delayed maturation of the adrenergic response in the DA of hypoxic embryos continues through EP.
CHAPTER 4

PHYSIOLOGICAL CHANGES THAT OCCUR IN THE DUCTUS ATERIOSUS AS A RESULT OF INCUBATION IN HYPOXIA OR HYPEROXIA DURING DEVELOPMENTAL WINDOWS

Introduction

Incubation in hypoxia has been shown to cause a number of physiological and morphological changes in the developing chicken embryo, including decreased embryo mass (Dzialowski et al., 2002; Azzam and Mortola 2007; Tintu et al., 2007), decreased eye mass (Chan and Burggren 2004), changes in hematocrit (Dzialowski et al., 2002), and an increase in chorioallantoic mass in comparison to control embryos (Chan and Burggren 2004; Azzam and Mortola 2007). Incubation in hyperoxia has also been shown to cause a number of physiological and morphological changes in the developing chicken embryo, including increased embryo mass (McCutcheon et al., 1982; Stock et al., 1983; van Golde et al., 1998), increased heart mass (McCutcheon et al., 1982), increased liver mass (van Golde et al., 1998), increased intestine mass (van Golde et al., 1998), increased ATP levels and decreased 2,3 DPG in comparison to control embryos (Ingermann et al., 1983).

During embryonic development there are windows in which the embryo is more susceptible to a change in its environment (Burggren, 1998). Studies have shown that exposing chicken embryos to hypoxic or hyperoxic conditions during different developmental windows results in physiological and morphological changes (van Golde et al., 1998; Dzialowski et al., 2002). Dzialowski et al. (2002) found that incubation in hypoxia during day 6-12 of development resulted in hatchlings with a decreased Vo$_2$, whereas earlier or later incubation in hypoxia did not result in hatchlings with Vo$_2$
significantly different from controls. Van Golde et al. (1998) reported that incubation in hyperoxia during day 14-15 of development resulted in hatchlings with larger brains, liver and intestine in comparison to control embryos, whereas incubation in hyperoxia during days 10-11 of development resulted in hatchlings with brain, liver and intestine masses not significantly different than controls.

Altering O$_2$ incubation levels during development could affect the O$_2$-sensitive ductus arteriosus (DA; Noel and Cassin, 1976; Clyman et al., 1978; Thebaud et al., 2004; Kajimoto et al., 2007; Ågren et al., 2007; Belanger et al., 2008; Dzialowski and Greyner, 2008). The DA is an embryonic blood vessel that connects the pulmonary artery to the aorta in mammals, reptiles and birds (Bergwerff et al., 1999). The DA provides a right-to-left shunt of blood away from the unventilated lungs and to the embryonic gas exchanger; the placenta in mammals and the chorioallantoic membrane (CAM) in birds (Heymann and Rudolph, 1975). In mammals DA closure is stimulated by an increase in blood gas O$_2$ that occurs as the animal begins to breathe with its lungs (Heymann and Rudolph, 1975; Berger et al., 1990; Bergwerff, 1996; Smith, 1998). In chickens, the increase in DA responsiveness to O$_2$ corresponds to the onset of lung ventilation (Ågren et al., 2007; Belanger et al., 2008).

In this study, I examined the effect of chronic hypoxic or hyperoxic incubation during different periods of development on the O$_2$-induced contractile response of the DA and embryo mass. Since the DA is an O$_2$-sensitive tissue (Noel and Cassin, 1976; Clyman et al., 1978; Thebaud et al., 2004; Kajimoto et al., 2007; Ågren et al., 2007; Belanger et al., 2008; Dzialowski and Greyner, 2008), changes in environmental O$_2$ levels during development are predicted to have significant effects on the
developmental trajectory of the O₂-induced contractile response of the DA. I hypothesize that altering incubation O₂ levels will affect both mass of the embryo as well as O₂-induced contraction of the DA.

Materials and Methods

Eggs

Eggs were incubated as stated in chapter 2. To study of incubation in varying O₂ levels during development, eggs were incubated in one of a number of treatments: (hypo18)norm - incubation in hypoxia until day 18 and moved to normoxia for the rest of incubation; (norm16)hypo - incubation in normoxia until day 16 and moved to hypoxia for the rest of incubation; (norm18)hypo - incubation in normoxia until day 18 and moved to hypoxia for the rest of incubation; (norm16)hyper - incubation in normoxia until day 16 and moved to hyperoxia for the rest of incubation; (norm18)hyper - incubation in normoxia until day 18 and moved to hyperoxia for the rest of incubation.

In vitro vessel physiology

The left and right DA was removed from day 16, day 18, IP, and EP (normoxic and hypoxic only) embryos and placed in a physiological saline solution (PSS composed of 120.5 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.6 mM CaCl₂, 1.2 mM NaH₂PO₄, 20.4 mM NaHCO₃, and 10 mM glucose) equilibrated with 95% N₂ and 5% CO₂ and any remaining connective tissue was removed.

Because the chicken DA has two distinct morphological regions along its length (Belanger et al. 2008), the proximal and distal portions of the DA were separated based
on visual inspection of their vessel morphology and diameter. A clear transition between the proximal portion and distal portion can be seen in the middle of each vessel (Ågren et al. 2007; Belanger et al. 2008). In this study, only the muscular proximal portion of the DA was used.

*In vitro* contraction was measured on the isolated vessels using a 4 chamber 610M Danish Myo Technologies myograph (Aarhns, Denmark). The excised vessels ranging in length from 0.3 to 2 mm were mounted into myograph chambers by threading two 40 µm diameter stainless steel wires through the lumen of the vessel. One wire was attached to a force transducer and the other to a micromanipulator that allowed for the adjustment of vessel tension. The force generated by each vessel was recorded as the active wall tension (mN mm⁻¹) using Powerlab® 8SP data acquisition system (ADInstruments Pty Ltd., Colorado Springs, CO, USA) and Chart® data acquisition and analysis software (ADInstruments Pty Ltd., version 5.4.2, Colorado Springs, CO, USA). Vessel contractile data is presented as the net active tension generated in response to the contractile agent.

Each chamber was filled with 5 ml of PSS and bubbled with a 95% N₂, and 5% CO₂ gas mixture resulting in a Po₂ of 32 ± 8 mmHg (~ 4%) and a Pco₂ of 40 mmHg. The gas mixture in the chambers was regulated by a Brooks® Instrument model 0154 (Brooks Instrument, LLC, Hatfield, PA, USA) and a Sable Systems mass flow controller (Las Vegas, NV, USA). Bath Po₂ and Pco₂ were monitored with a Radiometer ABL® 5 blood gas meter (A/S Corp., Copenhagen, Denmark). The vessels were allowed to equilibrate for 30 minutes and the baseline tension for all vessels was set to a tension that produced the largest contraction in response to 120 mM KCl as determined in
preliminary experiments. During this time the vessels were contracted two or three times with 120 mM KCl-PSS followed by three PSS rinses.

Day 16, day 18, IP, and EP embryos DA contractile response was measured in response to an increase in organ bath O₂ from 4% to 25%. The contractile response from the EP hyperoxic DA was not measured because it was functionally closed by this point in hatching. Following the initial 30 minute equilibration to 4% O₂, the gas mixture bubbling the organ bath was then increased to achieve an organ bath of 25% O₂ and the vessel tension was measured once the vessel had reached a plateau.

To test for differences in O₂-induced contraction of the DA due to incubation in hypoxia or hyperoxia during windows of development, the O₂-induced contraction of the DA was measured as described above. The O₂-induced contraction of the DA was measured from (hypo18) norm IP and EP, (norm16) hypo 18, IP and EP, (norm18) hypo IP and EP, (norm16) hyper 18 and IP, and (norm18) hyper IP and EP embryos.

**Mass**

To test for differences in embryo, yolk and heart mass due to incubation in hypoxia or hyperoxia during different windows of development, masses were obtained from day 16, day 18, IP, and EP embryos and hatchlings incubated in normoxia, hypoxia, or hyperoxia during the entire length of incubation. In addition, masses were obtained from (hypo18) norm IP, EP and hatch, (norm16) hypo 18, IP, EP, hatch, (norm18) hypo IP, EP, and hatch, (norm16) hyper 18, IP, EP, and hatch, (norm18) hyper IP, EP, and hatch. Eggs were weighed, the embryo was then removed and the yolk free mass was recorded. The yolk and heart were then weighed. For hatchlings,
the yolk free mass was obtained. The yolk and heart were then weighed. The embryo/hatchling and yolk were then dried in an oven at 78 ± 3°C for at least 96 hours and dry embryo and dry yolk mass was recorded.

Statistical Analyses

The effect of KCl and O₂ on DA vessel tension was tested for differences among means using a one way ANOVA with age/incubation O₂ level as the variable. The effect of different O₂ incubation levels on mass of day 16, day 18, IP and EP embryos was tested using an ANCOVA. Embryo wet mass, embryo dry mass, yolk wet mass, and yolk dry mass was corrected for egg mass. Heart mass was corrected for embryo wet mass. The effect of different O₂ incubation levels on embryo and yolk mass for hatchlings was tested using a one way ANOVA. The effect of different O₂ incubation levels on heart mass was tested using an ANCOVA. Heart mass was corrected for hatchling mass. The differences between the O₂-induced contractile response of right DA and left DA were tested using a t-test. All statistically significant ANOVAs were followed by a Sidak-Holm posthoc to test pairwise comparisons. Statistics were run by SAS® software (SAS Institute Inc., version 9.1, Cary, NC, USA) and Sigmastat® 3.5 software (SyStat Software Inc., Chicago, IL, USA).

Results

Contractile Response to Potassium Chloride

The left DA contractile response to KCl was dependent on age (Figure 4.1A). The left DA from day 18 hyperoxic embryos had a significantly greater contractile response to
Figure 4.1 Contractile responses to 120mM KCl of the left and right proximal ductus arteriosus from *Gallus gallus*. (A) Measurements made on the left proximal ductus arteriosus from day 16, day 18, internally pipped and externally pipped embryos incubated in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 6-14. (B) Measurements made on the left proximal ductus arteriosus from day 16, day 18, internally pipped and externally pipped embryos incubated in hypoxia, normoxia, or hyperoxia. Values are mean ± standard error. n = 8-13.
KCl than the left DA from hypoxic day 18 embryos (p<0.05). The contractile response of the left DA in normoxic and hypoxic embryos increased significantly at every developmental stage until IP (p<0.05). The contractile response of the left DA from hyperoxic embryos increased significantly at every developmental stage (p<0.05).

The right DA response to KCl was dependent on age (Figure 4.1B). The contractile response of the DA from normoxic, hypoxic, and hyperoxic embryos did not differ significantly at any developmental stage. The contractile response of the right DA in normoxic, hypoxic and hyperoxic embryos increased significantly from day 18 to IP (p<0.05). The only significant difference between the contraction of the left DA and right DA to KCl was normoxic in the EP embryo (p<0.05).

Altering Oxygen Incubation Levels During Developmental Windows and the Ductus Arteriosus Contractile Response to Oxygen

The right DA contractile response to an acute increase in O₂ increased with age and incubation O₂ level (Figure 4.2). The right DA from EP normoxic embryos exhibited a significantly stronger contractile response to an acute increase in O₂ in comparison to the contractile response of the right DA from day 18 normoxic embryos (p<0.05). The response of the right DA from hypoxic embryos to an acute increase in O₂ was not significantly different between the developmental stages (Figure 4.2). The contractile response of the right DA from hyperoxic embryos to an acute increase in O₂ increased significantly from day 18 to IP (p<0.05). During the IP stage, an acute increase in O₂ produced a significantly greater contractile response in the right DA from hyperoxic embryos than in the right DA of hypoxic or normoxic embryos (p<0.05). The right DA contractile response to an acute increase in O₂ was not significantly different for
hyperoxic IP and normoxic EP embryos; however the right DA from hyperoxic IP embryos exhibited a significantly stronger contractile response in comparison to the contractile response of the right DA from EP hypoxic embryos (Figure 4.2). The contractile response of the DA from EP hyperoxic embryos was not measured because it was functionally closed.

![Graph showing contractile response of the right proximal ductus arteriosus in hypoxic, normoxic, and hyperoxic conditions.](image)

Figure 4.2: Oxygen induced contractile response of the right proximal ductus arteriosus. Measurements made on the right proximal ductus arteriosus from day 18, internally pipped, and externally pipped (hypoxic and normoxic) embryos incubated in hypoxia, normoxia, or hyperoxia to an increase in organ bath O₂ from 4% to 25%. Values are mean ± standard error. n = 10-16. * indicates a significant difference compared with the age matched normoxic values at p<0.05.

In embryos that ended incubation in normoxia, the left DA contractile response to an acute increase in O₂ increased with age (Figure 4.3). The left DA from normoxic IP
embryos exhibited a significantly stronger contractile response to an acute increase in O₂ than the DA from day 16 normoxic embryos (p<0.05). The left DA from EP normoxic embryos exhibited a significantly stronger response to an acute increase in O₂ than day 16, day 18 and IP embryos (p<0.05). There were no significant differences in the O₂-induced contraction of the DA from (hypo18) norm IP and (hypo18) norm EP and age matched normoxic incubated embryos (Figure 4.3).

Figure 4.3: Oxygen induced contractile response of the left proximal ductus arteriosus from Gallus gallus. Measurements made on the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, and externally pipped embryos incubated in normoxia or hypoxia for the first 18 days of incubation and moved to normoxic for the rest of incubation to an increase in organ bath O₂ from 4% to 25%. Values are mean ± standard error. n = 6-18.
In the embryos that ended incubation in hypoxia, the left DA contractile response to an acute increase in O\textsubscript{2} was dependent on age and incubation O\textsubscript{2} (Figure 4.4). The contractile response of the left DA from EP hypoxic embryos to an acute increase in O\textsubscript{2} was significantly stronger than the contractile response of the left DA from day 16 hypoxic embryos (p<0.05). The left DA from EP hypoxic embryos exhibited a significantly weaker contractile response to an acute increase in O\textsubscript{2} in comparison to the left DA from EP normoxic embryos (p<0.05).

![Graph showing the relationship between development stage and active tension](image)

**Figure 4.4:** Oxygen induced contractile response of the left proximal ductus arteriosus from *Gallus gallus*. Measurements made on the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, and externally pipped embryos incubated in hypoxia, normoxia for the first 16 days of development and moved to hypoxia, normoxia for the first 18 days of development and moved to hypoxia and normoxia to an increase in organ bath O\textsubscript{2} from 4% to 25%. Values are mean ± standard error. n = 6-18. * indicates a significant difference compared with the age matched normoxic values at p<0.05.
In the embryos that ended incubation in hyperoxia, the left DA response to an acute increase in $O_2$ was dependent on age and incubation $O_2$ (Figure 4.5). The left DA from IP hyperoxic embryos exhibited a significantly stronger contractile response to an acute increase of $O_2$ in comparison to the left DA from day 18 embryos ($p<0.05$).

Figure 4.5: Oxygen induced contractile response of the left proximal ductus arteriosus from Gallus gallus. Measurements made on the left proximal portion of the ductus arteriosus from day 16, day 18, internally pipped, and externally pipped embryos incubated in hyperoxia, normoxia for the first 16 days of development and moved to hyperoxia, normoxia for the first 18 days of development and moved to hyperoxia and normoxia for the entire length of incubation to an increase in organ bath $O_2$ from 4% to 25%. Values are mean ± standard error. $n = 7-18$. In comparison to the proximal ductus arteriosus from normoxic IP embryos, the oxygen induced contraction of the left proximal ductus arteriosus from hyperoxic IP and (norm16) hyper IP embryos are significantly different ($p<0.05$), which is indicated by *. 
The left DA from IP hyperoxic embryos exhibited a significantly stronger contractile response to an acute increase in O\textsubscript{2} in comparison to the left DA from IP normoxic embryos (p<0.05). The left DA from (norm16) hyper IP embryos exhibited a significantly stronger contractile response to O\textsubscript{2} in comparison to the left DA from IP normoxic embryos (p<0.05).

The only significant differences between right DA and left DA contractile response to O\textsubscript{2} was from normoxic day 18 embryos and hyperoxic IP embryos. The DA contractile response to an acute increase in O\textsubscript{2} was significantly stronger in the right DA of day 18 normoxic embryos in comparison to the DA contractile response from the left DA from day 18 normoxic embryos (p<0.05). The DA contractile response to an acute increase in O\textsubscript{2} was significantly stronger in the right DA of IP hyperoxic embryos in comparison to the DA contractile response from the left DA from IP hyperoxic embryos (p<0.05).

*The Effects of Altering Oxygen Incubation Levels During Windows of Development on Embryo Mass*

In eggs that ended incubation in normoxia, embryo wet mass was dependent on age (Figure 4.6). Normoxic embryo wet mass increased significantly at each development stage until EP (p<0.05). In eggs that ended incubation in normoxia, embryo dry mass was dependent on incubation O\textsubscript{2} (Table 4.1). Normoxic IP and EP embryo dry masses were significantly greater than day 18 embryo dry mass (p<0.05). Embryo dry mass of (hypo18) norm IP embryos was significantly lower than embryo dry mass of normoxic IP embryos (p<0.05). Embryo dry mass of (hypo18) norm EP embryos was significantly lower than dry mass of normoxic EP embryo (p<0.05).
Figure 4.6: Wet mass of day 16, day 18, internally pipped, and externally pipped embryos and hatchlings incubated in normoxia or hypoxia for the first 18 days of development and moved to normoxia. Values reported for embryo mass are corrected for egg mass. Values are mean ± standard error. n = 6-37.

In eggs that ended incubation in normoxia, yolk wet mass was dependent on incubation O₂ (Table 4.1). Normoxic IP and EP yolk wet mass were significantly lower than day 18 embryo wet mass (p<0.05). Yolk masses from (hypo18) norm EP were significantly greater than yolk wet masses from normoxic EP (p<0.05). In eggs that ended incubation in normoxia, there were no differences in yolk dry mass (Table 4.1).

In eggs that ended incubation in normoxia, heart mass was dependent on incubation O₂ (Table 4.1). Normoxic EP heart mass was significantly greater than day 18 and IP heart mass (p<0.05). Heart mass from (hypo18) norm IP embryos were significantly greater than heart masses from normoxic IP heart masses (p<0.05).
Table 4.1: Wet mass, dry mass, wet yolk mass, dry yolk mass and heart mass of day 16, day 18, internally pipped, externally pipped embryos, and hatchlings incubated in normoxia or hypoxia during the first 18 days of incubation, then moved to normoxia. Values reported for embryo and yolk mass are corrected for egg mass. Values reported for heart mass are corrected for embryo/hatchling mass. Values are mean ± standard error. n is sample size. * indicates a significant difference compared with the age matched normoxic values at p<0.05.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Wet mass (g)</th>
<th>Dry mass (g)</th>
<th>Wet yolk mass (g)</th>
<th>Dry Yolk mass (g)</th>
<th>Heart mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norm Day 16</td>
<td>14.2 ± 0.5</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Norm Day 18</td>
<td>23.3 ± 0.6</td>
<td>4.0 ± 0.2</td>
<td>10.0 ± 1.0</td>
<td>4.6 ± 0.3</td>
<td>0.17 ± 0.008</td>
</tr>
<tr>
<td>Norm IP</td>
<td>31.2 ± 0.4</td>
<td>5.8 ± 0.2</td>
<td>7.9 ± 0.4</td>
<td>4.0 ± 0.2</td>
<td>0.17 ± 0.005</td>
</tr>
<tr>
<td>Norm EP</td>
<td>33.3 ± 0.6</td>
<td>6.1 ± 0.2</td>
<td>7.0 ± 0.5</td>
<td>3.4 ± 0.3</td>
<td>0.19 ± 0.006</td>
</tr>
<tr>
<td>Norm Hatch</td>
<td>33.4 ± 1.5</td>
<td>6.8 ± 0.2</td>
<td>6.2 ± 0.6</td>
<td>3.2 ± 0.3</td>
<td>0.23 ± 0.009</td>
</tr>
<tr>
<td>(hypo18) norm IP</td>
<td>30.6 ± 0.8</td>
<td>5.1 ± 0.2*</td>
<td>8.7 ± 0.5</td>
<td>4.6 ± 0.3</td>
<td>0.20 ± 0.006*</td>
</tr>
<tr>
<td>(hypo18) norm EP</td>
<td>31.7 ± 1.0</td>
<td>5.4 ± 0.2*</td>
<td>8.6 ± 0.6*</td>
<td>4.2 ± 0.3</td>
<td>0.18 ± 0.007</td>
</tr>
<tr>
<td>(hypo18) norm hatch</td>
<td>33.1 ± 1.2</td>
<td>6.4 ± 0.4</td>
<td>6.1 ± 0.6</td>
<td>2.9 ± 0.3</td>
<td>0.28 ± 0.012*</td>
</tr>
</tbody>
</table>

In hatchlings that ended incubation in normoxia, wet mass, dry mass, yolk wet mass, and yolk dry mass did not significantly differ from (hypo18) norm hatch (Table 4.1). Heart masses from (hypo18) norm hatchlings were significantly greater than heart masses from normoxic hatchlings (p<0.05; Table 4.1).

In eggs that ended incubation in hypoxia, embryo wet mass was dependent on incubation O2 (Figure 4.7). Hypoxic embryo wet mass increased significantly at each development stage until IP (p<0.05). Day 18, IP, and EP embryos incubated in hypoxia had significantly lower wet masses than age matched embryos incubated in normoxia.
Embryo wet mass from (norm16) hypo IP and EP embryos were significantly lower than wet masses from age matched normoxic embryos (p<0.05).

<table>
<thead>
<tr>
<th>Development (day / stage)</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Hypoxic (Norm16) Hypo (Norm18) Hypo Normoxic

* indicates a significant difference compared with the age matched normoxic values at p<0.05.

Figure 4.7: Wet mass of day 16, day 18, internally pipped, and externally pipped embryos and hatchlings incubated in hypoxia, normoxia for the first 16 days of development and moved to hypoxia, normoxia for the first 18 days of development and moved to hypoxia and normoxia. Values reported for embryo mass are corrected for egg mass. Values are mean ± standard error. n = 6-37. * indicates a significant difference compared with the age matched normoxic values at p<0.05.

In eggs that ended incubation in hypoxia, embryo dry mass was dependent on incubation O₂ (Table 4.2). Internally pipped and EP embryos incubated in hypoxia had significantly lower dry masses than normoxic embryos (p<0.05). Embryo dry masses of (norm16) hypo 18, IP and EP embryos were significantly lower than the dry masses of age matched normoxic embryos (p<0.05). Embryo dry masses of (norm18) hypo IP and
EP embryos were significantly lower than the dry masses of age matched normoxic embryos (p<0.05).

Table 4.2: Wet mass, dry mass, wet yolk mass, dry yolk mass and heart mass of day 16, day 18, internally pipped, externally pipped embryos, and hatchlings incubated in hypoxia or normoxia for either the first 16 or 18 days of development and moved to hypoxia. Values reported for embryo and yolk mass are corrected for egg mass. Values reported for heart mass are corrected for embryo / hatching mass. Values are mean ± standard error. n is sample size. * indicates a significant difference compared with the age matched normoxic values at p<0.05.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Wet mass (g)</th>
<th>Dry mass (g)</th>
<th>Wet yolk mass (g)</th>
<th>Dry Yolk mass (g)</th>
<th>Heart mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypo Day 16</td>
<td>14.5 ± 0.5 n = 27</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.18 ± 0.007 n = 8</td>
</tr>
<tr>
<td>Hypo Day 18</td>
<td>20.8 ± 0.6* n = 27</td>
<td>3.4 ± 0.2 n = 8</td>
<td>8.4 ± 0.6 n = 7</td>
<td>4.2 ± 0.3 n = 7</td>
<td>0.15 ± 0.005 n = 8</td>
</tr>
<tr>
<td>Hypo IP</td>
<td>26.9 ± 0.5* n = 23</td>
<td>4.5 ± 0.2* n = 11</td>
<td>9.8 ± 0.4* n = 11</td>
<td>4.9 ± 0.2* n = 11</td>
<td>0.15 ± 0.008* n = 11</td>
</tr>
<tr>
<td>Hypo EP</td>
<td>27.3 ± 0.6* n = 18</td>
<td>4.4 ± 0.2* n = 5</td>
<td>9.7 ± 0.7* n = 5</td>
<td>4.7 ± 0.3* n = 5</td>
<td>0.15 ± 0.008* n = 5</td>
</tr>
<tr>
<td>Hypo Hatch</td>
<td>32.2 ± 0.6 n = 15</td>
<td>5.3 ± 0.3* n = 4</td>
<td>8.5 ± 0.6 n = 4</td>
<td>4.6 ± 0.4 n = 4</td>
<td>0.22 ± 0.016 n = 4</td>
</tr>
<tr>
<td>(norm16) hypo 18</td>
<td>21.9 ± 1.0 n = 6</td>
<td>3.3 ± 0.2* n = 6</td>
<td>8.6 ± 0.6 n = 6</td>
<td>4.0 ± 0.3 n = 6</td>
<td>0.18 ± 0.008 n = 6</td>
</tr>
<tr>
<td>(norm16) hypo IP</td>
<td>27.5 ± 1.0* n = 7</td>
<td>4.5 ± 0.2* n = 7</td>
<td>8.7 ± 0.6 n = 6</td>
<td>4.1 ± 0.3 n = 6</td>
<td>0.16 ± 0.007 n = 7</td>
</tr>
<tr>
<td>(norm16) hypo EP</td>
<td>29.3 ± 1.1* n = 6</td>
<td>4.6 ± 0.2* n = 6</td>
<td>8.8 ± 0.6* n = 6</td>
<td>4.3 ± 0.3* n = 6</td>
<td>0.17 ± 0.007* n = 6</td>
</tr>
<tr>
<td>(norm16) hypo hatch</td>
<td>32.5 ± 0.3 n = 6</td>
<td>6.2 ± 0.3 n = 6</td>
<td>7.7 ± 1.0 n = 6</td>
<td>4.1 ± 0.6 n = 6</td>
<td>0.27 ± 0.013* n = 6</td>
</tr>
<tr>
<td>(norm18) hypo IP</td>
<td>30.0 ± 1.0 n = 7</td>
<td>4.9 ± 0.2* n = 7</td>
<td>8.6 ± 0.6 n = 6</td>
<td>4.3 ± 0.3 n = 6</td>
<td>0.15 ± 0.007 n = 7</td>
</tr>
<tr>
<td>(norm18) hypo EP</td>
<td>32.0 ± 0.8 n = 9</td>
<td>5.4 ± 0.2* n = 9</td>
<td>8.6 ± 0.5* n = 8</td>
<td>4.8 ± 0.3* n = 8</td>
<td>0.15 ± 0.006* n = 9</td>
</tr>
<tr>
<td>(norm18) hypo hatch</td>
<td>32.1 ± 1.7 n = 6</td>
<td>6.0 ± 0.4 n = 6</td>
<td>7.4 ± 0.4 n = 5</td>
<td>3.8 ± 0.4 n = 5</td>
<td>0.23 ± 0.013 n = 6</td>
</tr>
</tbody>
</table>

In eggs that ended incubation in hypoxia, yolk wet mass was dependent on incubation O₂ (Table 4.2). Internally pipped and EP hypoxic incubated embryos had significantly greater yolk wet mass than age matched normoxic incubated embryos.
Yolk wet masses of (norm16) hypo EP and (norm18) hypo EP embryos were significantly greater than the yolk wet masses of EP normoxic embryos (p<0.05). This same pattern of significance was observed in the dry yolk mass (Table 4.2).

In eggs that ended incubation in hypoxia, heart mass was dependent on incubation O$_2$ (Table 4.2). Externally pipped hypoxic incubated embryos had significantly lower heart masses than normoxic incubated embryos (p<0.05). Heart masses of (norm16) hypo EP and (norm18) hypo EP embryos were significantly lower than the heart masses of EP normoxic embryos (p<0.05).

In hatchlings that ended incubation in hypoxia, wet masses, yolk wet masses, and yolk dry masses did not significantly differ from hatchlings incubated in normoxia (Table 4.2). Hypoxic hatchling dry masses were significantly lower than normoxic hatchling dry masses (p<0.05). Heart masses from (norm16) hypo hatch were significantly greater than heart masses from normoxic hatchlings (p<0.05).

In eggs that ended incubation in hyperoxia, embryo wet and dry mass was dependent on incubation O$_2$ (Figure 4.8). Day 16 and IP hyperoxic incubated embryos had significantly greater wet masses than age matched normoxic incubated embryos (p<0.05). Wet masses of (norm18) hyper IP and EP embryos were significantly greater than wet masses of IP and EP normoxic embryos (p<0.05). Day 18 and IP hyperoxic incubated embryos had significantly greater dry masses than normoxic incubated embryos (p<0.05; Table 4.3).

In eggs that ended incubation in hyperoxia, wet and dry yolk mass was dependent on incubation O$_2$ (Table 4.3). Internally pipped and EP hyperoxic incubated embryos had significantly lower yolk wet masses than age matched normoxic incubated embryos.
embryos (p<0.05). Internally pipped and EP hyperoxic incubated embryos had significantly lower yolk dry masses than age matched normoxic incubated embryos (p<0.05; Table 4.3).

<table>
<thead>
<tr>
<th>Development (day / stage)</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Hyperoxic (Norm16) Hyper (Norm18) Hyper Normoxic

Figure 4.8: Wet mass of day 16, day 18, internally pipped, and externally pipped embryos and hatchlings incubated in hyperoxia, normoxia for the first 16 days of development and moved to hyperoxia, normoxia for the first 18 days of development and moved to hyperoxia and normoxia. Values reported for embryo mass are corrected for egg mass. Values are mean ± standard error. n = 3-37. * indicates a significant difference compared with the age matched normoxic values at p<0.05.

In eggs that ended incubation in hyperoxia, heart mass was dependent on incubation O₂ (Table 4.3). Internally pipped and EP hyperoxic incubated embryos had significantly greater heart masses than age matched normoxic incubated embryos (p<0.05). Heart masses of (norm16) hyper 18 and IP embryos were significantly greater
than heart masses of day 18 and IP normoxic embryos (p<0.05). Heart masses of (norm16) hyper EP and (norm18) hyper EP embryos were significantly greater than heart masses of EP normoxic embryos (p<0.05).

Table 4.3: Wet mass, dry mass, wet yolk mass, dry yolk mass and heart mass of day 16, day 18, internally pipped, externally pipped embryos, and hatchlings incubated in hyperoxia or normoxia for either the first 16 or 18 days of development and moved to hyperoxia. Values reported for embryo and yolk mass are corrected for egg mass. Values reported for heart mass are corrected for embryo / hatchling mass. Values are mean ± standard error. n is sample size. * indicates a significant difference compared with the age matched normoxic values at p<0.05.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Wet mass (g)</th>
<th>Dry mass (g)</th>
<th>Wet yolk mass (g)</th>
<th>Dry Yolk mass (g)</th>
<th>Heart mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyper Day 16</td>
<td>15.7 ± 0.5*</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>n = 27</td>
<td></td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyper Day 18</td>
<td>23.8 ± 0.5</td>
<td>5.0 ± 0.2*</td>
<td>10.6 ± 0.5</td>
<td>5.6 ± 0.3</td>
<td>0.18 ± 0.007</td>
</tr>
<tr>
<td>n = 31</td>
<td>n = 7</td>
<td>n = 7</td>
<td>n = 7</td>
<td>n = 7</td>
<td>n = 7</td>
</tr>
<tr>
<td>Hyper IP</td>
<td>33.9 ± 0.4*</td>
<td>6.9 ± 0.2*</td>
<td>5.5 ± 0.1*</td>
<td>2.6 ± 0.3*</td>
<td>0.20 ± 0.007</td>
</tr>
<tr>
<td>n = 46</td>
<td>n = 11</td>
<td>n = 11</td>
<td>n = 11</td>
<td>n = 11</td>
<td>n = 11</td>
</tr>
<tr>
<td>Hyper EP</td>
<td>33.3 ± 1.3</td>
<td>6.0 ± 0.3</td>
<td>5.1 ± 0.7*</td>
<td>2.4 ± 0.4*</td>
<td>0.23 ± 0.009*</td>
</tr>
<tr>
<td>n = 4</td>
<td>n = 4</td>
<td>n = 4</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 4</td>
</tr>
<tr>
<td>Hyper Hatch</td>
<td>35.6 ± 0.7</td>
<td>7.0 ± 0.2</td>
<td>5.1 ± 0.5</td>
<td>2.5 ± 0.2</td>
<td>0.28 ± 0.011*</td>
</tr>
<tr>
<td>n = 14</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 9</td>
</tr>
<tr>
<td>(norm16) hyper 18</td>
<td>25.2 ± 0.8</td>
<td>4.1 ± 0.2</td>
<td>8.6 ± 0.5</td>
<td>3.7 ± 0.2*</td>
<td>0.19 ± 0.006*</td>
</tr>
<tr>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>(norm16) hyper IP</td>
<td>31.9 ± 0.9</td>
<td>5.5 ± 0.2</td>
<td>6.7 ± 0.5</td>
<td>3.4 ± 0.3</td>
<td>0.19 ± 0.006*</td>
</tr>
<tr>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 9</td>
</tr>
<tr>
<td>(norm16) hyper EP</td>
<td>34.7 ± 1.8</td>
<td>5.8 ± 0.3</td>
<td>6.8 ± 0.8</td>
<td>3.1 ± 0.4</td>
<td>0.24 ± 0.010*</td>
</tr>
<tr>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
<td>n = 3</td>
</tr>
<tr>
<td>(norm16) hyper hatch</td>
<td>35.9 ± 1.2</td>
<td>7.4 ± 0.2</td>
<td>4.8 ± 0.5</td>
<td>2.5 ± 0.3</td>
<td>0.32 ± 0.011*</td>
</tr>
<tr>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 8</td>
</tr>
<tr>
<td>(norm18) hyper IP</td>
<td>32.9 ± 0.8*</td>
<td>6.0 ± 0.2</td>
<td>7.3 ± 0.5</td>
<td>3.7 ± 0.2</td>
<td>0.18 ± 0.006</td>
</tr>
<tr>
<td>n = 11</td>
<td>n = 11</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 9</td>
<td>n = 11</td>
</tr>
<tr>
<td>(norm18) hyper EP</td>
<td>36.9 ± 1.0*</td>
<td>6.7± 0.2</td>
<td>6.3 ± 0.6</td>
<td>3.1 ± 0.3</td>
<td>0.22 ± 0.008*</td>
</tr>
<tr>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td>(norm18) hyper hatch</td>
<td>35.2 ± 1.0</td>
<td>6.9 ± 0.2</td>
<td>5.8 ± 0.5</td>
<td>2.9 ± 0.2</td>
<td>0.25 ± 0.010</td>
</tr>
<tr>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
</tbody>
</table>

In hatchlings that ended incubation in hyperoxia, wet mass, dry mass, yolk wet
mass, and yolk dry mass did not significantly differ from hatchlings incubated in normoxia (Table 4.3). Hyperoxic hatchlings heart mass was significantly greater than heart mass of normoxic hatchlings (p<0.05). Heart mass of (norm16) hyper hatchlings were significantly greater than the heart mass of normoxic hatchlings (p<0.05).

Discussion

Incubation in hypoxia or hyperoxia during the entire length of incubation or during certain developmental windows is known to cause a number of physiological and morphological changes in the developing chicken embryo (McCutcheon et al., 1982; Ingermann et al., 1983; Stock et al., 1983; van Golde et al., 1998; Dzialowski et al., 2002; Azzam and Mortola 2007). Here I have shown this includes alteration in O$_2$-induced contraction of the DA and changes in embryo and heart mass. The left DA from embryos incubated in hypoxia for the entire length of incubation exhibited weaker O$_2$-induced contractile responses in comparison to the DA from normoxic embryos, though the only significant difference observed was the left DA from EP embryos. The DA from embryos incubated in hyperoxia for the entire length of incubation exhibited stronger O$_2$-induced contractile responses in comparison to the DA from normoxic embryos. The DA from embryos incubated in hypoxia or hyperoxia for different development windows typically did not have different O$_2$-induced contractile responses in comparison to age matched DA from normoxic incubated embryos. Incubation in hypoxia resulted in smaller embryo mass, whereas incubation in hyperoxia resulted in greater embryo mass, however differences were no longer present once the animals
hatched. Heart mass was affected by hypoxic and hyperoxic incubation. Differences in heart mass were still present once the animal hatched.

The Contractile Response of the Ductus Arteriosus to Potassium Chloride

Only age affected the receptor-independent left DA contractile response to KCl. The DA from day 16 embryos exhibited a weak contractile response to KCl which increased throughout development. Similarly, Ågren et al. (2007) found that the DA from day 21 embryos exhibited a greater contractile response to KCl than the DA from day 15 or day 19 embryos. The DA from hypoxic and normoxic embryos responded in a similar manner throughout development. Van der Sterren et al. (2009) reported that the DA from day 19 hypoxic and normoxic embryos exhibited a similar response to KCl. This suggests that the DA from hypoxic embryos does not experience a developmental delay in the receptor-independent contraction, but a developmental delay in the pathways involved in the response to O₂.

The Oxygen Induced Contractile Response of the Ductus Arteriosus

There is a clear maturation of the O₂-induced contractile response of the left DA during hatching (Figure 4.3, 4.4, 4.5). The O₂-induced contractile response of the left DA from hypoxic and normoxic EP embryos was significantly greater than the O₂-induced contractile response of the left DA from hypoxic and normoxic day 16 embryos. The O₂-induced contractile response of the left DA from hyperoxic IP embryos was significantly greater than the O₂-induced contractile response of the DA from hyperoxic day 16 embryos. Ågren et al. (2007) showed the DA from day 15 embryos did not
respond to $O_2$, whereas the DA from day 19 and EP chicken embryos produced contractile response to $O_2$. Belanger et al. (2008) observed that the proximal portion of the DA from EP embryos exhibited a significantly stronger contractile response to $O_2$ than the proximal portion of the DA from day 19 embryos. A similar increase in contractile response to $O_2$ as the animal ages has been observed in emu embryos (Dzialowski and Greyner, 2008) and a number of mammalian species, such as rabbits and guinea pigs (Noel and Cassin, 1976; Thebaud et al., 2004).

There was a delay in the maturation of the $O_2$-induced contractile response of the left DA of hypoxic embryos. In contrast, the $O_2$-induced contractile response of the left DA matured earlier in the embryos incubated in hyperoxia throughout development. The response of the DA from EP hyperoxic embryos was not studied because the vessel was almost completely closed at this stage, presumably in response to the increased $O_2$ levels experienced during hyperoxic incubation. The left DA from EP normoxic embryos had a significantly greater $O_2$-induced contraction than the left DA from IP normoxic embryos, which corresponds with the timing of the $P_{O_2}$ increase in the embryos as they respire normoxic gas (Tazawa et al. 1983; Meena and Mortola, 2002).

The proximal portion of the DA in chicken embryos begins to close during EP (Belanger et al., 2008). An association can be seen between an increased contractile response to $O_2$ and accelerated closure in the proximal portion of the left DA in hyperoxic embryos. When compared with the left DA of normoxic embryos, the left DA of the hyperoxic embryos exhibited an accelerated maturation of the $O_2$-induced constriction, whereas the left DA of the hypoxic embryos experienced a developmental delay in maturation of the $O_2$-induced constriction. As shown in Figure 2.4 the lumen
diameter of the DA from IP and EP hyperoxia embryos was significantly smaller than the lumen diameter of the DA from IP and EP normoxic embryos, whereas the lumen diameter of the DA from EP hypoxia embryos is significantly larger than the lumen diameter of the DA from EP normoxic embryos.

Coughlin and Husson (1960) exposed hatching chicken embryos to hypoxia and found that hypoxia was associated with a delay in DA closure. Ductus arteriosus closure in mammals is stimulated by an increase in blood Po\textsubscript{2} that occurs as the animal begins to breathe with its lungs (Heymann and Rudolph, 1975; Smith, 1998). As shown in Table 2.1, IP hyperoxic embryos have significantly lower venous Po\textsubscript{2} than day 16 hyperoxic embryos; however, this value is greater than that of the normoxic and hypoxic embryos. This suggests that in hyperoxic embryos, the pathway governing the O\textsubscript{2}-induced contraction of the left DA matured with O\textsubscript{2} levels that would normally constrict the mature vessel from normoxic embryos, and closure during IP might not need a subsequent increase in O\textsubscript{2} above the already high levels needed to stimulate contraction.

The maturation of O\textsubscript{2}-induced contractile response of the right DA during hatching followed the same trend as the left DA, however there were fewer significant differences between the groups (Figure 4.2). There were no differences between the O\textsubscript{2}-induced contractile response of the right DA from normoxic and hypoxic embryos; however the O\textsubscript{2}-induced contractile response of the right DA from IP hyperoxic embryos was significantly stronger than the O\textsubscript{2}-induced contractile response of the right DA from IP normoxic and hypoxic embryos. Observed differences in the O\textsubscript{2}-induced contractile response of the right and left DA could be a result of sample variability.
Incubation in hypoxia or hyperoxia during different windows of development had an effect on the O$_2$-induced contractile response of the left DA in only one group. The O$_2$-induced contractile response of the left DA from (norm16) hyper IP exhibited a significantly stronger response than the DA from IP normoxic incubated embryos. The left DA from (norm18) hyper IP did not exhibit a significantly stronger O$_2$-induced contractile response in comparison to IP normoxic incubated embryos. This may indicate the left DA is sensitive to hyperoxic incubation between day 16 and day 18.

*The Effects of Altering Oxygen Incubation Levels During Windows of Development on Embryo Mass*

During embryonic development there are developmental windows during which the embryo is more susceptible to a change in its environment (Burggren, 1998). Incubation in hypoxia and hyperoxia has been shown to cause a number of physiological and morphological changes in the developing chicken embryo (McCutcheon et al., 1982; Stock et al., 1983; Dzialowski et al., 2002; Chan and Burggren, 2005; Azzam and Mortola 2007; Tintu et al., 2007). Similar to previous studies, the present study found that incubation in hypoxia produced significantly smaller embryos (Azzam and Mortola, 2007; Dzialowski et al., 2002; Rouwet et al., 2002; Villamor et al., 2004; Tintu et al., 2007), whereas incubation in hyperoxia produced significantly larger embryos (McCutcheon et al., 1982; Stock et al., 1983). In the present study, the period of development in which the embryo was exposed to hypoxic/hyperoxic incubation significantly affected embryo, yolk and heart mass.

Several studies have examined the effects of hypoxic/hyperoxic incubation during different developmental windows in chicken embryos. Dzialowski et al. (2002)
found day 18 embryos incubated in hypoxia (15% O₂) during middle (days 6-12), or late (days 12-18) periods of development experienced a decrease in embryo mass in comparison to normoxic embryos. The current study focused on changing O₂ incubation levels during the later stages of embryonic development after day 16. Embryos that were incubated in hypoxia for the first 18 days and moved to normoxia did not have significant different embryo mass in comparison to normoxic embryo mass. This suggests that the embryo is able to accelerate growth since the mass of day 18 hypoxic embryos is significantly less than the mass of day 18 normoxic embryos. Embryos that were incubated in normoxia for the first 18 days and moved to hypoxia did not have significant different embryo masses in comparison to normoxic embryo mass; however, embryos that were incubated in normoxia for the first 16 days and moved to hypoxia had significant lower embryo mass in comparison to normoxic embryo mass. This suggests that incubation in hypoxia between days 16-18 is a critical period of development for the embryos mass. Van Golde et al. (1998) reported that incubation in 48 hours of hyperoxia (60% O₂) between days 14-15 and 18-19 resulted in day 19 and day 20 embryos with significantly greater masses in comparison to normoxic incubated embryos. Similarly, the present study showed that incubation in hyperoxia (30% O₂) after day 18 of development resulted in IP and EP embryos with significantly greater masses in comparison to normoxic incubated embryos. Embryos that were moved to hyperoxia on day 16 did not have significantly different wet masses than normoxic embryos. This suggests that incubation in hyperoxia between days 18-19 has a significant effect on embryo mass.
Incubation in hypoxia/hyperoxia resulted in significantly different embryo weights during development, however once the animals hatched there were no longer significant differences between the groups. Dzialowski et al. (2002) found that incubation in hypoxia (15% O₂) days 1-6, 6-12, or 12-18 did not result in differences in yolk free hatchling mass in comparison to yolk free normoxic hatchling mass. Ferner and Mortola (2009) showed that incubation in hypoxia (15% O₂) during the 1st, 2nd, or 3rd week of incubation did not result in differences in hatchling mass in comparison to normoxic hatchling mass. In addition, they reported that incubation in hypoxia during the entire length of incubation did not result in differences in hatchling mass in comparison to normoxic hatchling mass. The present study observed no significant differences in hatchling mass between yolk free normoxic hatchlings and yolk free hatchlings that spent either part or the entire incubation period in hypoxia. Van Golde et al (1998) found that incubation in 48 hours of hyperoxia (60% O₂) between days 10-11 and 14-15 resulted yolk free hatchlings with significantly greater masses in comparison to yolk free normoxic hatchlings. The present study observed no significant differences in hatchling mass between yolk free normoxic hatchlings and yolk free hatchlings that spent either part or all of incubation in hyperoxia. Observed differences could be due to either the period of time in which the embryos were exposed to hyperoxic incubation or the degree of hyperoxia during incubation.

The heart mass of embryos was significantly affected by O₂ incubation level. Dzialowski et al. (2002) found that incubation day 12 embryos incubated in hypoxia (15% O₂) between days 6-12 had significantly greater heart masses than day 12 embryos incubated in normoxia. In addition, they found that day 18 embryos that
incubated in hypoxia between days 1-6, 6-12, or 12-18 did not have significantly different heart masses in comparison to normoxic embryos (Dzialowski et al., 2002). Chan and Burggren (2005) reported that incubation in hypoxia (15% O<sub>2</sub>) between days 1-6, 6-12, or 12-18 did not have an effect on heart mass when compared with hearts from normoxic embryos. In addition, they observed that day 18 embryos incubated in hypoxia throughout development did not have significantly different heart masses in comparison to heart masses normoxic embryos. Similarly this study showed that there were no significant differences between the heart masses of day 18 hypoxic and normoxic embryos. Heart masses of EP hypoxic embryos, (norm16) hypo EP embryos, and (norm18) hypo EP embryos were significantly lower than heart mass of EP normoxic embryos. McCutcheon et al. (1982) showed that day 18 embryos incubated in chronic hyperoxia (40% O<sub>2</sub>) had significantly greater heart masses in comparison to the heart masses of day 18 normoxic embryos. Van Golde et al (1998) found that incubation in hyperoxia for 48 hours during days 18-19 resulted in day 19 and day 20 embryos with significantly greater heart masses in comparison to the heart masses of age matched normoxic embryos. The present study observed that incubation in hyperoxia (30% O<sub>2</sub>) throughout development resulted in IP and EP embryos having significantly greater heart masses in comparison to the heart masses of age matched normoxic embryos. Heart mass was most affected in the (norm16) hyper embryos. Day 18, IP and EP heart masses in this group were significantly greater than heart masses from age matched normoxic embryos. This indicates the heart is sensitive to hyperoxic incubation between days 16 and 18.
Unlike embryo mass, there were still significant differences in heart mass present after the animal hatched. Dzialowski et al. (2002) found that incubation in hypoxia between days 1-6, 6-12, or 12-18 did not result in hatchlings with significantly different heart masses in comparison to heart masses of normoxic hatchlings. The present study observed that (hypo18) norm hatchlings had significantly greater heart masses than normoxic hatchlings. In addition, (norm 16) hypo hatchlings also had significantly greater heart masses than normoxic hatchlings. Van Golde et al (1998) reported that incubation in hyperoxia for 48 hours during days 10-11 or 14-15 did not result in hatchlings with significantly different heart masses in comparison to heart masses of normoxic hatchlings. The present study showed that incubation in hyperoxia throughout development as resulted in hatchlings with significantly greater heart masses in comparison to heart mass of normoxic hatchlings. In addition (norm16) hyper hatchlings had significantly greater heart masses in comparison to heart mass of normoxic hatchlings. Heart mass is significantly affected by altering O₂ incubation levels towards the end of development.

Studies have focused on incubating embryos in differing O₂ levels during different developmental windows and examining other morphological and physiology parameters. Dzialowski et al. (2002) found that hypoxic (15% O₂) incubation during day 6-12 resulted in hatchlings with a decreased Vo₂. Chan and Burrgren (2005) showed that hypoxic (15% O₂) incubation during day 12-18 resulted in a significantly greater chorioallantoic membrane mass (CAM). Van Golde et al. (1998) reported that incubation in hyperoxia (60% O₂) during various 48 hour periods during development had the largest effect on liver and intestine mass. The present study found that
incubation in hypoxia between days 16–18 had significant affects on embryo mass, whereas incubation in hyperoxia between days 18–19 had significant affects on embryo mass. Heart mass was significantly affected when the embryos were moved to hypoxia or hyperoxia on day 16. Incubation hypoxia/hyperoxia during late incubation has a significant effect on both embryo and heart mass.

Conclusions

Incubation O₂ levels affect the maturation of the O₂-induced contractile response of the DA. The left DA from IP hyperoxic embryos exhibited a significantly stronger O₂-induced contractile response than the left DA from IP normoxic and hypoxic embryos, which indicated an accelerated maturation of the O₂-induced contractile response. The left DA from EP embryos incubated in hypoxia had a significantly weaker O₂-induced contractile response than the left DA from EP embryos incubated in normoxia.

Hypoxic and hyperoxic incubation had significant effects on embryo and heart mass. Incubation in hypoxia/hyperoxia during windows of development also had significant effects on embryo and heart mass. Hypoxic incubation between days 16–18 had significant effects on embryo mass, whereas hyperoxic incubation between days 18–19 had significant effects on embryo mass. Once the animals hatched there were no longer differences in mass. Heart mass was most effected when the embryos were moved to hypoxia or hyperoxia on day 16. Heart mass was significantly affected during development, as well as once the animal hatched. Hypoxia/hyperoxia during late incubation had significant effects on both embryo and heart mass; however, there was a greater impact on the heart.
CHAPTER 5

CONCLUSIONS

Understanding of the influence of altered oxygen levels on the development and physiology of the O$_2$-sensitive ductus arteriosus (DA) is limited. The major objective of this research was to characterize physiological and morphological differences in the DA of chicken embryos incubated in differing O$_2$ levels. I hypothesized that under hypoxic conditions the DA would take a longer period of time to close, while under hyperoxic conditions the DA closure would be accelerated.

Incubation in hyperoxia resulted in an acceleration of the closure of the DA, while incubation in hypoxia resulted in a delay of the closure of the DA. The DA from IP hyperoxic embryos had a significantly smaller lumen diameter, significantly thicker vessel wall, and significantly thicker smooth muscle layer than the DA from day 18 hyperoxic embryos, indicating the initiation of closure. The DA from normoxic embryos did not increase vessel wall thickness or smooth muscle layer thickness significantly until they were externally pipped (EP). Closure of the DA from hypoxic embryos appeared to begin after EP. The changes in the DA from hypoxic embryos at the end of incubation occurred rapidly. The smooth muscle layer thickness of the DA was significantly thicker in EP hyperoxic embryos than hypoxic embryos, however once the animals hatched the reverse was true.

There were no significant differences in the DA lumen diameter once the animal reached day 0 (hatchling, 2-24 hours). An increased mortality in the hypoxic group towards the end of incubation could account for the lack of changes in vessel lumen diameter in the DA of day 0 hatchlings. Since tissue samples were only obtained from
live embryos/hatchlings, it is possible that the average lumen diameter of the DA from hypoxic embryos may be larger. The DA from normoxic embryos begins to close after the animal EP (Belanger et al., 2007), which results in deceased blood flow to the CAM and increased blood flow to the lungs (Rahn et al., 1985). During the transition to lung respiration metabolic demand increases (Rahn et al., 1974). I hypothesize that if the DA fails to begin to close at EP the embryos may not be able to meet metabolic demand, which leads to mortality of the embryo.

Oxygen incubation level and age had a significant effect on the vasoreactivity of the chicken DA in response to adrenergic agonists. The DA from day 16 embryos in all treatments exhibited a similar weak response to NE and PE. The DA from day 18 hypoxic embryos exhibited a significantly lower contractile response to both NE and PE in comparison to the DA from day 18 normoxic and hyperoxic embryos. In all treatments there was a significant increase in the contractile response of the DA to NE and PE as the embryo aged from day 18 to IP. The DA from IP hypoxic embryos had a similar response to NE as the DA from IP normoxic and hyperoxic embryos; however, the DA from IP hypoxic embryos had a significantly weaker response to PE in comparison to the DA from IP normoxic and hyperoxic.

Incubation O₂ levels affect the maturation of the O₂-induced contractile response of the DA. The left DA from EP embryos incubated in hypoxia had a significantly weaker O₂-induced contractile response than the left DA from EP embryos incubated in normoxia, indicating a delay in the maturation of the O₂-induced contractile response. The left DA from IP hyperoxic embryos exhibited a significantly stronger O₂-induced
contractile response than the left DA from IP normoxic and hypoxic embryos, which indicated an accelerated maturation of the O$_2$-induced contractile response.

Incubation in hypoxia or hyperoxia during different late windows of development only affected the DA contractile response to O$_2$ in one group. The DA from (norm16) hyper IP embryos exhibited a significantly stronger O$_2$-induced contractile response in comparison to the O$_2$-induced contractile response from the DA from IP normoxic embryos. This suggests there was an acceleration of the maturation of the DA O$_2$-induced contractile response in (norm16) hyper IP embryos.

An association can be seen between an increased contractile response to O$_2$ and accelerated closure in the proximal portion of the DA in hyperoxic embryos. When compared to the DA of normoxic embryos, the DA of the hyperoxic embryo had an accelerated maturation of the O$_2$-induced constriction, whereas the DA of the hypoxic embryo experienced a developmental delay in maturation of O$_2$-induced constriction. There are two steps of DA closure: functional closure involves the O$_2$-induced constriction of the smooth muscle cells (Fay, 1972; Archer et al., 2004) which is followed by anatomical remodeling (Clyman et al., 1999; Kajino et al., 2001; Sutendra and Michelakis, 2007). The O$_2$-induced constriction of the DA is necessary for the anatomical remodeling of the vessel (Clyman et al., 1999; Clyman et al., 2002). The DA from hyperoxic IP embryos exhibited a large contractile response which corresponds to the initiation of closure \textit{in vivo}. The DA from normoxic embryos produced a similar contractile response to O$_2$ at EP, which also corresponds with the initiation of closure \textit{in vivo}. The DA from EP hypoxic embryos exhibited a significantly weaker contractile
response to O$_2$ in comparison to the DA from normoxic embryos. This may explain the delayed closure in the hypoxic embryos.

Incubation in hypoxia or hyperoxia resulted in changes in blood gas parameters and air cell O$_2$ content. Blood gas and air cell data followed the expected pattern, with hypoxic embryos having the lowest O$_2$ levels and hyperoxic embryos having the highest O$_2$ levels. Venous Po$_2$ in both normoxic and hyperoxic embryos decreased as the embryos aged, however in hypoxic embryos venous Po$_2$ remained relatively constant. Once the embryos internally pipped (IP) the venous Po$_2$ values for hypoxic and normoxic embryos were similar, even though the hypoxic embryos were respiring a gas with a 53% lower O$_2$ content than normoxic embryos. Increased levels of circulating catecholamines in hypoxic embryos potentially facilitate maintenance of venous Po$_2$ throughout development.

Hypoxic and hyperoxic incubation had significant effects on embryo and heart mass. Incubation in hypoxia/hyperoxia during windows of development had significant effects on embryo and heart mass. Hypoxic incubation between days 16–18 had significant effects on embryo mass, whereas hyperoxic incubation between days 18–19 had significant effects on embryo mass. Once the animals hatched there were no longer differences in yolk free hatchling masses. Heart mass was most affected when the embryos were moved to hypoxia or hyperoxia on day 16. Heart mass was significantly affected during development as well as once the animal hatched.

In summary, incubation in differing O$_2$ levels caused a number of morphological and physiological changes in the developing embryo. Hyperoxic incubation during part or all of development resulted in higher venous Po$_2$, accelerated DA closure,
accelerated maturation of the DA contractile response to O$_2$, increased embryo mass, and increased embryo and hatchling heart mass. Hypoxic incubation during part or all of development resulted in lower venous Po$_2$ during periods of development, delayed DA closure, delayed maturation of the DA contractile response to O$_2$, delayed maturation of the DA adrenergic response, decreased embryo mass decreased embryo heart mass, and increased hatchling heart mass. Closure of the DA in normoxic and hyperoxic groups corresponded with the DA responsiveness to O$_2$. Incubation hypoxia/hyperoxia during late incubation had a significant effect on both embryo and heart mass; however there was a greater impact on the heart. A future study could examine the physiological and morphological effects of hyperoxic exposure during late development on the DA from mammalian fetuses.
APPENDIX

LIST OF ABBREVIATIONS
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3 DPG</td>
<td>2,3 Diphosphoglycerate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>DA</td>
<td>Ductus arteriosus</td>
</tr>
<tr>
<td>EP</td>
<td>Externally pip</td>
</tr>
<tr>
<td>IP</td>
<td>Internally pip</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PDA</td>
<td>Patent ductus arteriosus</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>Po₂</td>
<td>Partial pressure of oxygen</td>
</tr>
</tbody>
</table>
REFERENCES


Hansen M. (2005). Effects of hypoxia on the mechanical properties of the aortic wall in the 19 days old chicken embryo (Gallus gallus). Linköpings Universitet. p. 15


