ADRENERGIC AND CHOLINERGIC REGULATION OF CARDIOVASCULAR FUNCTION IN EMBRYONIC NEOTROPIC CORMORANTS (*Phalacrocorax brasilianus*)

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Investigations of cholinergic and adrenergic tone on heart rate (fH) and mean arterial pressure (Pm) during embryonic development have been conducted on numerous avian species. While these investigations have documented that adrenergic tone, a continuous stimulation, on fH and Pm is vital to embryonic development in the birds studied to date, development of cholinergic tone on fH has been shown to vary even within species. Further, past studies have been bias to focus primarily on precocial species while altricial species remain poorly understood in this context. The goal of this investigation was to investigate the role of cholinergic and adrenergic tone on fH and Pm of an altricial species, the neotropic cormorant (*P. brasilianus*) to address this bias. The embryonic neotropic cormorant possesses B-and-a adrenergic tone on fH and Pm at 70% and 90% incubation while cholinergic tone on fH occurs at 90% incubation. This pattern of control is similar to that previously reported for several species of precocial birds suggesting the development of tonic cardiovascular regulation may be conserved across avian taxa.

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CHAPTER 1

INTRODUCTION

The study of cardiovascular function in embryonic birds have been the focus of multiple investigations over the past several decades (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Hochel et al., 1998; Crossley and Altimiras, 2000; Altimiras and Crossley, 2000; Crossley et al., 2003a, b, c; Chiba et al., 2004; Andrewartha et al., 2011; Swart et al., 2013; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). Recent works have reported that adrenergic receptor tone, or continuous stimulation, on heart rate and blood pressure during development is a common characteristic during embryonic life in bird species, suggesting that adrenergic tone may be vital to embryonic development (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). However, the occurrence of cholinergic tone, a continuous inhibition of heart rate, during embryonic development differs across species which has led to speculation that cholinergic tone is not critical to embryonic cardiovascular homeostasis (Tazawa et al., 1992; Hochel et al., 1998; Crossley and Altimiras, 2000; Crossley et al., 2003a, b, c; Chiba et al., 2004; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). While prior works have been critical in furthering our understanding of cardiovascular maturation in avian species, questions remain regarding differences between the different categories of birds at hatch.

Avian species can be separated into two categories: precocial species defined at hatch as readily mobile, eyes open, with thermoregulatory capacity and the ability to self-feed or altricial species defined at hatch as immobile, eyes closed with poor thermoregulatory capacity and are feed by their parents (Ar & Yom-Tov, 1978). While the avian classification of altricial species are

noted as the majority avian species, embryonic cardiovascular functional studies have been primarily conducted on precocial birds, with few studies conducted on altricial birds (Van Mierop and Bertuch, 1967; Ar & Yom-Tov, 1978; Girard, 1973; Tazawa et al., 1992; Hochel et al., 1998; Crossley and Altimiras, 2000; Altimiras and Crossley, 2000; Crossley et al., 2003a, b, c; Chiba et al., 2004; Andrewartha et al., 2011; Swart et al., 2013; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). This gap in the knowledge base is important because investigations of developing altricial species may provide insight into whether the presence of specifically cholinergic tone is related to relative maturation of the embryonic bird or timed to coincide with the hatching event. Furthermore, investigating altricial species will clarify if cholinergic tone during development, or its absence, is unique to specific species or a feature that is shared across avian phylogeny. Therefore, the goal of this project was to characterize the maturation of cardiovascular function and the role of adrenergic and cholinergic tone in maintaining cardiovascular homeostasis in neotropic cormorant (*Phalacrocorax brasilianus*) embryos.

1.1 Cardiac Regulation Mechanisms

There are multiple regulating mechanisms that juvenile and adult organisms use to maintain cardiovascular homeostasis including neural control, endocrine control, and local autoregulation (Morgan & Baker, 1991; Lakatta, 1993). However, given the nature of developmental processes, it is conceivable there is a period when these systems differ in the relative importance. In chicken (*Gallus gallus domesticus*) embryos, cholinergic and adrenergic receptors are present on the cardiovascular system early in development along with the anabolic and catabolic enzymes for acetylcholine and catecholamines implying function of these control systems (Berry, 1950; Ignarro and Shideman, 1968). Studies have also shown that embryonic chickens develop parasympathetic

cholinergic efferent nerves that can release acetylcholine by day 12 of incubation (21 day incubation period) while sympathetic efferent nerves are capable of releasing noradrenaline happens on day 21 of incubation (Ignarro and Shideman, 1968; Pappano, 1977; Higgins and Pappano, 1981, Crossley and Altimiras, 2000). While chicken (*Gallus gallus*) embryos have the components of the autonomic nervous system, adrenergic tone is functional at day 12 of incubation which is derived from circulating catecholamines while cholinergic tone on heart rate varies across studies from absent to being present in later stages of embryonic development (Tazawa et al., 1992; Crossley and Altimiras, 2000; Chiba et al., 2004; Crossley and Altimiras, 2012). The presence and origin of cholinergic and adrenergic tone varies with species. Both the Canada goose and the domestic goose (*Branta Canadensis* & *Anser anser domesticus)*, chickens (*Gallus gallus*), as well as emus (*Dromaius novaehollandiae*) have β-and-α – adrenergic tone on the cardiovascular system that originates from circulating catecholamines, (Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014) However, cholinergic receptor tone varies across these species from absent until after hatching, to present in the later portion of embryonic development (Higgins and Pappano, 1981; Crossley and Altimiras, 2000; Andrewartha et al., 2011; Swart et al., 2013; Taylor et al., 2014). From these studies a general theme appears in which adrenergic tone is derived from non-neural catecholamine release while if cholinergic tone is present, it is derived from neural sources in embryonic precocial bird species.

1.1.1 Neural Control

In adult species, central nervous system control of the cardiovascular system can be separated into two components of the autonomic nervous system, the sympathetic and parasympathetic division. The sympathetic nervous system innervates the heart increasing

contraction rate and force of contraction (Levy and Martin, 1984; Morgan & Baker, 1991; Engelhardt and Hein, 2004; Malpas, 2010). Blood pressure is also regulated by sympathetic nerves innervating the vasculature creating an increase in vascular tonus or contraction (Malpas, 2010). However, in embryonic birds, β-and-α – adrenergic tone on arterial pressure and heart rate are primarily derived from circulating catecholamines with little contribution from the autonomic nervous system (Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). The parasympathetic system action differs from the sympathetic system.

The parasympathetic nervous system innervates the adult heart via the vagus which has a negative chronotropic effect depressing heart rate (Brezenoff and Giuliano, 1982; Levy and Martin, 1984; Taylor and Ihmied, 1995). Acetylcholine released from the vagus nerve acts upon muscanaric receptors on the sinoatrial (SA) node (Brezenoff and Giuliano, 1982; Levy and Martin, 1984; Taylor and Ihmied, 1995). Acetylcholine increases the permeability of the resting membrane to potassium and decreases permeability to sodium resulting in the decreased rate of depolarization of the SA node leading to a decrease in heart rate (Brezenoff and Giuliano, 1982; Levy and Martin, 1984; Taylor and Ihmied, 1995). The assumption for embryonic birds has been that if the vagus is present, the negative chronotropic action and the mechanisms of its action are similar to that known for adult species. However, in many embryonic avian species this remains unknown.

1.1.2 Endocrine Control

In addition to the neural regulation, there are key endocrine regulators that modulate acute changes in cardiovascular function. Critical endocrine regulators are the amine hormones, catecholamines, which are briefly outline here. Catecholamines, noradrenaline and adrenaline,

derive from the adrenal medulla which produce positive chronotropic and ionotropic effects on the heart as well as increase vascular contraction (Malpas, 2010). While the source of catecholamines are characterized in adults, the origin of circulating catecholamines in embryonic birds has been suggested to be exclusively from chromaffin tissue (Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). Noradrenaline and adrenaline have both positive chronotropic and ionotropic cardiac affects (Morgan & Baker, 1991; Engelhardt and Hein, 2004; Brodde et al., 2006; Malpas, 2010). Noradrenaline and adrenaline act on the SA node increasing the influx of sodium and calcium during diastolic interval, which leads to an increase in heart rate (Morgan & Baker, 1991; Brodde et al., 2006; Malpas, 2010). Noradrenaline and adrenaline also increases the strength of cardiac contraction via β_1-adrenergic receptors which leads to increasing free sarcoplasmic calcium allowing for more cross-bridges to form and results in an increase in myocyte shortening (Engelhardt and Hein, 2004; Brodde et al., 2006).

In addition to the cardiac actions, noradrenaline and adrenaline also bind β_2-adrenoreceptors and α_1-adrenoreceptors located on the vascular system. Adrenaline in low amounts preferentially binds to β_2-adrenorecptors which cause vasodilation (Barbato, 2009). However, as adrenaline concentrations increase adrenaline will bind to α_1-adrenoreceptors which offsets the β_2-adrenorecptor mediated vasodilation and leads to vasoconstriction (Molinoff, 1984; Engelhardt and Hein, 2004; Barbato, 2009). Because of this, β-adrenergic and α-adrenergic receptors are important factors in maintaining vascular tone in the cardiovascular system.

1.2 Embryonic Avian Cardiovascular Regulation

1.2.1 Adrenergic Tone

Adrenergic tone, or continuous stimulation, on the cardiovascular system is prominent in embryonic bird species. For example, Canada geese (*Branta canadensis*) and domestic geese (*Anser anser domesticus*) possess a significant β-adrenergic receptor stimulatory tone on heart rate at 70% and 90% incubation and α-adrenergic tone on arterial pressure and heart rate in both geese species at 70% and 90% incubation (Swart et al., 2013). This adrenergic tone is found in multiple avian species and has been suggested to be vital to embryonic development in embryos (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). While adrenergic tone on the cardiovascular system has been constantly reported in embryonic species, cholinergic tone varies even within species.

1.2.2 Cholinergic Tone

An inhibitory cholinergic tone on heart rate has been reported in two embryonic geese species at 70% and 90% incubation (Swart et al., 2013). Cholinergic tone on heart rate has also been reported in embryonic emus (*Dromaius novaehollandiae*) (Crossley et al., 2003a) and black sumatran bantam chickens (*Gallus gallus domesticus*) (Crossley et al., 2003c). However, cholinergic tone in the white leghorn chicken varies, with studies reporting that cholinergic tone presence on heart rate varies, ranging from 60% incubation to as late as post hatching (Tazawa et al., 1992; Crossley et al., 2000; Chiba et al., 2004; Crossley and Altimaris, 2012; Taylor et al., 2014). Importantly, cholinergic tone on the cardiovascular system has only been investigated in

precocial birds but is unknown in altricial species. Due to common traits in adrenergic tone and differences in cholinergic tone in embryonic avian species, an investigation conducted on altricial species could give insight and information for new discoveries regarding common traits in the ontogeny of the cardiovascular regulatory elements in birds.

CHAPTER 2

GOAL AND HYPOTHESIS

The goal of this project was to investigate adrenergic and cholinergic tone at 70% and 90% incubated neotropic cormorant (*Phalacrocorax brasilianus*) embryos (26 day incubation period). While precocial species have adrenergic tone, the onset of cholinergic tone has been shown to vary in chickens which is attributed to the indirect selection of breeds to increase commercial growth (Crossley and Altimaris, 2012). Because altricial species hatch at an earlier stage of development compared to precocial species, they are more vulnerable to fluctuations in the environment (Ar & Yom-Tov, 1978). Therefore, development of the regulatory mechanisms for cardiovascular system may occur earlier in altricial species when compared to precocial species in order to survive environmental stresses. During this project two basic hypotheses were tested:

I. Adrenergic tone maintains baseline blood pressure and heart rate of the neotropic cormorant.

II. Baseline heart rate is maintained by tonic cholinergic stimulation in the neotropic cormorant.

CHAPTER 3

METHODOLOGY

3.1 Species of Study

Eggs of neotropic cormorants (*Phalacrocorax brasilianus*) were collected from Henderson County and Freestone County from the years 2015 and 2016 during the months of May - July with permission from the Texas Fish and Wildlife Scientific Collection permit (SPR-1114-257) and U.S. Fish and Wildlife Migratory Bird Scientific Collecting permit (MB57014B-1). Egg collection in Henderson County took place on Bird Island Wildlife Management Area (WMA) which is located within the Cedar Creek Reservoir. The bird rookery located in Freestone County took place within a wetland in Richland Creek WMA North Sector alongside the banks of Alligator Creek.

A total of 100 eggs from 50 nests during the 2015 breeding season and a total of 150 eggs from 56 nests during the 2016 breeding season were collected from the Henderson County Bird Island WMA. A total of 50 eggs from 16 nests were collected from Freestone County Richland Creek WMA during the 2015 breeding season. Humidity and temperature of nests were recorded with a Smart Sensor AR 847 (Smart Sensor Intel Instruments Plus, Singapore) before eggs were removed. Because of the proximity of large bodies of water near each collection point, the relative humidity of each nest ranged from 90% - 100%. Temperature fluctuated greatly between nests from 26.7 – 36°C. Once the eggs were collected from the nest, they were placed in egg cartons and immediately placed within a Coleman Cooler (Coleman Company, Inc., Wichita, KS, USA). Eggs were transported directly to the University of North Texas by car. Total time between egg collections from nest to placement in an incubator at the University of North Texas was less than 3 hours.

3.2 Incubation

The eggs were placed in a Grumbach incubator (Grumbach model BSS 160 Digital, Grumbach Brugeraete GmbH., Asslar Germany) programmed to maintain a constant temperature (38°C), humidity (60%), and were rotated (45°) every two-hours based on incubation procedures conducted on the double crested cormorant (Powell et al., 1996).

Eggs collected from Bird Island WMA during the 2015 breeding season were set in the incubator at a vertical position with the air sac facing upwards were 49% (n=100 eggs) viable, while eggs collected during the 2016 breeding season were set in the incubator horizontally were 65% (n=150 eggs) viable. The viability of eggs collected from Richland Creek WMA was 80% (n=50 eggs). Difference in egg viability between the two rookery sites could be due to the percentage of incubation the embryos were in during collection. All eggs acquired from Bird Island WMA were incubated for 9 days or less when collected. Eggs collected from Richland Creek WMA were collected when the embryos were incubated for 13 days or more. Embryos in the later stages of incubation may be able to handle external stress greater than embryos in the early stages of incubation.

3.3 Age

Upon collection, the eggs were immediately floated (Westerskov, 1950) for a general estimate of incubation percentage. Once the eggs were transported to the University of North Texas, they were candled and floated again for a more accurate determination of the embryo's incubation period (Westerkov, 1950; Hanson, 1954; Reiter and Andersen, 2008). Because floating and candling are rough estimates of the incubation time, the incubation windows that were studied were days 18-20 (70%) and 23-25 (90%). After the completion of each experiment, neotropic

cormorant embryos were euthanized and dissected to obtain the precise age by comparing the physical features to the previously recorded stages of the embryonic double crested cormorant (*Phalacrocorax auritus*) (Powell et, al. 1998).

3.4 Pharmacological Protocol

For the purpose of this study, blood volume and embryonic body mass of the neotropic ormorant were estimated to determine delivery doses and volumes of drugs. Body mass of the embryonic neotropic cormorant was estimated based on the known masses of altricial embryonic pigeons (*Columba livia*) and crows (*Corvus cornix*) (Romanoff, 1967) from similar sized eggs. The combined average wet weight for each age relative to the post hatching mass versus incubation percent of the embryonic pigeon and crow, was used to establish a relationship between embryonic mass and incubation percentage. A sigmoidal equation was used to predict the average wet weight percent to incubation percent of the neotropic cormorant.

$$y = D + \frac{(A - D)}{1 + (\frac{x}{C})^B}$$

In the above equation, A(3.53), B(5.61), C(91.87) and D(162.42) are all constants identified by Microsoft Excel 2013 (Microsoft Office 2013, Microsoft Corporation, Redmond, WA, USA). The coefficient x is the incubation percentage of the neotropic cormorant embryo during the 26 day incubation period. The coefficient y is the predicted average wet weight percentage of the neotropic cormorant. (Table 1)

Estimate Embryo Wet Weight

Day	Incubation (%)	*n*	Actual Embryo Wet Weight (g)	Estimated Embryo Wet Weight (g)
16	61.5	4	2.67±0.15	3.65
17	65.3	10	3.77±0.17	4.69
18	69.9	21	5.61±0.32	5.95
19	73	24	7.10±0.25	7.41
20	76.9	16	8.70±0.21	9.05
21	80.7	11	10.31±0.22	10.83
22	84.6	9	11.30±0.24	12.68
23	88.4	38	12.75±0.26	14.56
24	92.3	19	16.09±0.55	16.41
25	96.1	10	19.73±0.49	18.18
26	100	5	21.66±0.82	19.83

Table 1. Comparrison of actual avg. embryo wet weight and estimated embryo wet weight of the embryonic neotropic cormorant.

Blood volume of neotropic cormorants were estimated based off of previously reported blood volumes of embryonic chickens (*Gallus gallus*) at similar masses (Crossley and Altimiras, 2000). Plotting embryonic chicken's percent of incubation to the percent of total blood volume of wet mass produced the following equation.

$$y = -0.7138x + 74.53$$

In the above equation, coefficient x is the percentage of incubation of the neotropic cormorant. The coefficient y is the estimated blood volume of the neotropic cormorant.

3.5 Surgical Procedures

For surgical instrumentation, eggs were placed in a custom temperature-controlled (38°C) surgical chamber. Using a dissection microscope (Lieca MZ6 Leica Microsystems, Waukegan, Il, USA) within the chamber, a tertiary chorioallantoic artery was located by candling and then traced with a graphite pencil on the egg shell. Once the chorioallantoic artery was identified, the artery was exposed by a small 10x10 mm opening in the shell. Once the artery was exposed, the artery was catheterized with a heat-pulled polyethylene catheter (PE 50; Clay-Adams, Parsippany, NJ, USA) containing heparinized (100IU)(Sagent Pharmaceuticals, Schaumbug, IL, USA) saline (0.9% NaCl). Once in place, a silk suture was used to secure the catheter to the vessel, and the catheter was glued to the eggshell with cyanoacrylic glue.

After catheterization, the eggs were placed in a temperature-controlled chamber. Each individual chamber is fitted with a steel lid with two 6mm in diameter holes for externalizing the catheter and supply the chamber with air. Air was continuously passing through the chamber after passing through a humidifier and then through a copper coil submerged in a water bath kept at 38°C. Each chamber in the incubation apparatus was continuously supplied with air at a flow rate of 200 mL min^{-1}. The catheter was then passed out of the chamber and attached to a pressure transducer (MLT0670 BP, ADInstruments, Colorado Springs, CO, USA), which was connected to a bridge amplifier (ML224 Quad amp, ADInstruments, Colorado Springs, CO, USA). The amplifier was connected to a Powerlab data acquisition system (Powerlab 16/30, ADInstruments,

Colorado Springs, CO, USA) and the signal was acquired on a computer (Mac Mini, Apple Incorporated, Cupertino, CA, USA) with Chart software (LabChart 7, ADInstruments, Colorado Springs, CO, USA) at a frequency of a 100 hz. Heart rate was derived from the pressure signal through an acquisition software tachometer.

Prior to experimentation, the pressure transducers were calibrated to 0 and 1kPa using a vertical column of saline (0.9% NaCl). The reference zero point is set at the top of the experimental chamber and egg distance from the top of the chamber is measured (cm) to correct for the actual position of each egg.

3.6 Blood Properties and Osmolarities

Prior to pharmacological protocol, 500ul of blood from 70% and 90% incubated embryos were collected and stored in tubes with EDTA (Scientific Products, McCaw Park, IL, USA). Samples were then measured for pH, pCO2, Na^+, Cl^-, Glucose, Lactate with a Nova Biomedical Stat Profile Prime system (Nova Biomedical Corporation, Waltham, MA, USA).

3.7 Interval Blockade

Once embryos were successfully cannulated and attached to the experimental apparatus, baseline P_m and f_H were acquired prior to drug injections from the incubation periods of: 60% (days 16-17), 70% (days 18-20), 80% (days 21-22) and 90% (23-25) (Figure 1). All embryos were monitored without pharmacological manipulation for one hour to ensure values for both arterial pressure and heart rate were stable. After determining that the values were stable, each embryo received a control injection of heparinized (100IU) saline (0.9% NaCl) <5% of the estimated total blood volume of the neotropic cormorant. After the control injection, each embryo either received

atropine (3 mg kg^{-1}, Sigma-Aldric, St. Louis, MO, USA) to assess the response to cholinergic receptor blockade or a series of two drugs to assess the response to β-adrenergic (propranolol 3 mg kg^{-1}) (Sigma-Aldric, St. Louis, MO, USA), and α-adrenergic (phentolamine 3 mg kg^{-1}) (Sigma-Aldric, St. Louis, MO, USA) receptor blockade. The concentration of these drugs have been previously shown to be effective at blocking each of the desired receptor types in other embryonic species (Crossley and Altimiras, 2000; Crossley et al., 2003a; Crossley and Altimiras, 2012; Swart et al., 2013). Total injection volumes were <5% of total blood volume based on comparison of blood volumes of embryonic chicken and average wet weights of embryonic crows and pigeons. (Crossley and Altimiras, 2000; Romanoff 1967). After each injection, a saline flush was injected with double volume. After each injection, arterial pressure and heart rate was monitored for 45-60 minutes to ensure parameter response stabilization before the next drug treatment.

CHAPTER 4

ACUTE TEMPERATURE STRESS

To identify the cardiovascular response to abiotic challenges, a subset of embryos (n=8) at 90% incubation were studied at 32°C. Following stabilization of cardiovascular parameters embryos were injected with atropine (3mg kg^{-1}). Parameters were then recorded for 45-60 minutes to ensure parameter response stabilization.

CHAPTER 5

DATA ANALYSIS

Control P_m and f_H were recorded prior to drug blockade in 5 minute intervals. After each injection of atropine, propranolol, and phentolamine, P_m and f_H were allowed to stabilize before a series of 5 minute intervals was collected. Data from embryos that died from low P_m and f_H without any pharmacological manipulation was discarded.

After the pharmacological studies, all the embryos were euthanized with an overdose of pentobarbital before being able to hatch. neotropic cormorant embryos were dissected to obtain the precise age of incubation determine organ masses.

5.1 Morphological Data

Masses of the right breast muscle, yolk, albumin, embryo, heart, liver, lungs, kidneys, and brain were taken. Masses of collected tissues were obtained by using a balance (0.1mg)(Mettler Toledo XS204, CH-8606 Greifensee, Switzerland). After mass determination, the right breast muscle, heart, liver, lungs, kidneys, and brain were flash frozen in liquid nitrogen and stored at -80°C (Thermo Scientific Forma 900 Series, Waltham, MA, USA). Yolk masses of 90% embryos were only collected do to collection errors with 70% embryos.

5.2 Statistical Design

Dependent t-test was used to compare P_m and f_H before and after each drug treatment at all stages of incubation and temperature manipulation. A one-way ANOVA with Fisher's Least Significant Difference (LSD) post hoc test was used to determine differences between baseline P_m and f_H values between the incubation periods. A one-way ANOVA with LSD post hoc test was

also be used to compare baseline P_m and $f_{H,}$ between embryos exposed to 38°C to embryos exposed to acute decrease in temperature (32°C). One-way ANOVA on log transformed data fractions was used to determine differences between drug response intensity across different incubation periods and between embryos that underwent pharmacological manipulation at 38°C and 32°C. Log transformed data was used to create variance independent of the mean (Sokal and Rohlf, 1995; Mirkovic and Rombough, 1998). An independent t-test was used to analyze mass change over incubation periods. Differences in the right breast muscle, heart, liver, lungs, kidneys, and brain masses were compared in both incubation periods using an ANCOVA with embryo mass as the covariate. An independent t-test was used analyze pH, pCO2, Na^+, Cl^-, glucose, and lactate between ages.

Data analysis was completed with Statistica version 12 (Dell StatSoft Inc., Round Rock, TX, USA). All statistical significance was determined based on $a = 0.05$.

CHAPTER 6

RESULTS

All embryos were monitored without pharmacological manipulation for one hour to ensure values for both arterial pressure and heart rate were stable. After determining that the values were stable, each embryo received a control injection of heparinized (100IU) saline (0.9% NaCl) <5% of the estimated total blood volume of the neotropic cormorant. After the control injection, each embryo either received atropine (3 mg kg^{-1}, Sigma-Aldric, St. Louis, MO, USA) to assess the response to cholinergic receptor blockade or a series of two drugs to assess the response to β-adrenergic (propranolol 3 mg kg^{-1}) (Sigma-Aldric, St. Louis, MO, USA), and α-adrenergic (phentolamine 3 mg kg^{-1}) (Sigma-Aldric, St. Louis, MO, USA) receptor blockade. (Table 2)

Neotropic Cormorant

Incubation (%)	Drug	*n*	Pre P_m	Post P_m	Δ P_m	Pre f_H	Post f_H	Δ f_H
70	S	37	0.32 ± 0.03	0.38 ± 0.04	0.05 ± 0.01*	224 ± 2	223 ± 2	1 ± 0
	A	8	0.38± 0.07	0.39 ± 0.08	0.01 ± 0.01	209± 9	191 ± 15	18 ± 6
	Pr	20	0.27 ± 0.04	0.56 ± 0.07	0.56± 0.03*	227 ± 2	178 ± 5	49 ± 3*
	Ph	13	0.62 ± 0.07	0.31 ± 0.06	0.31 ± 0.01*	183 ±5	171±8	12 ± 3
90	S	31	0.92 ± 0.07	0.99 ± 0.07	0.07± 0.01*	228±3	228±3	0 ± 0
	A	8	0.81 ± 0.17	0.89 ± 0.18	0.08 ± 0.01	234± 6	241 ± 5	7 ± 1*
	Pr	20	0.87 ± 0.07	1.45 ± 0.11	0.58 ± 0.04*	231 ±2	164±4	67 ± 2*
	Ph	14	1.49 ± 0.08	0.49 ± 0.06	1.00 ± 0.02*	159 ± 4	153 ± 5	6 ± 1

Table 2. Data are presented as mean ± SE. An asterisk indicates a significant response to drug response (P<0.05). S=Saline, A=Atropine, Pr = Propranolol, Ph= Phentolamine, n= number of animals tested on each stage of incubation, P_m = mean arterial pressure (kPa), f_H = mean heart rate (beats·min^{-1}), Pre = before injection of each drug, Post = after injection of each

6.1 Baseline P_m and f_H across 60% - 90%

As development progressed, P_m increased from 0.10 ± 0.04 kPa at 60% to 0.92 ± 0.07 kPa at 90% (Figure 1). P_m significantly increased when comparing 60% and 80% incubated embryos, 0.10 ± 0.04 kPa to 0.50 ± 0.10 kPa, respectively ($P<0.001$). P_m in 90% incubated embryos (0.92 ± 0.07 kPa) were significantly higher when compared to 60% (0.10 ± 0.04 kPa, $P<0.001$), 70% (0.32 ± 0.03 kPA, $P<0.001$), and 80% (0.50 ± 0.10 kPa, $P<0.001$) incubated embryos (Figure 1). f_H rose slightly during each stage of development from 211 ± 8 beats min^{-1} at 60% to 229 ± 4 beats min^{-1} at 90% (Figure 2).

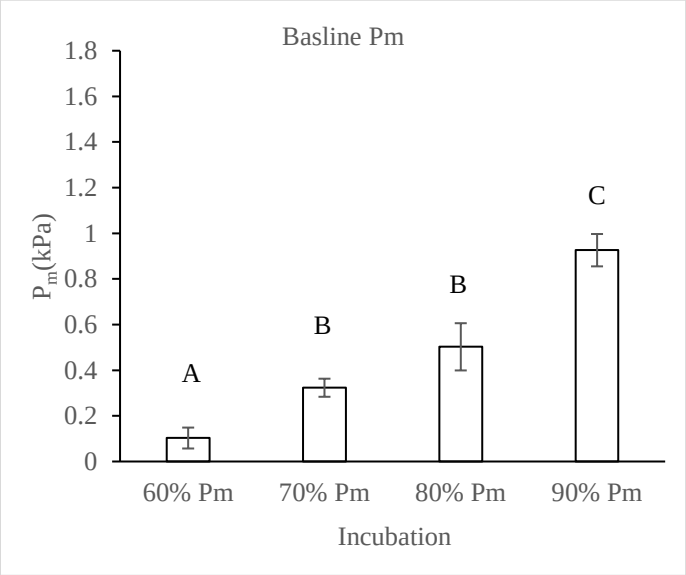

Figure 1. Embryonic neotropic cormorant baseline P_m (A) and f_H (B). Data are presented as mean ± SE. One-way ANOVA with LSD post hoc. Letters identify significant differences between the different percentages of incubation($p<0.001$). n = 11 at 60%, n = 37 at 70%, n = 7 at 80%, n = 31 at 90%.

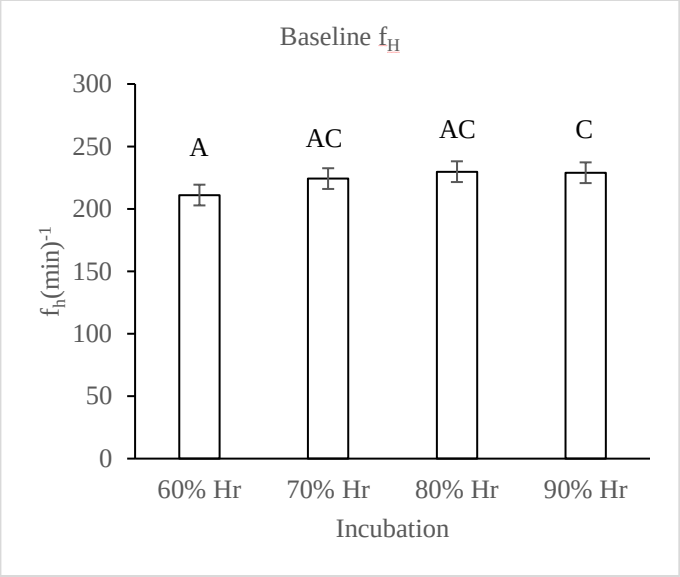

Figure 2. Embryonic neotropic cormorant baseline f_H. Data are presented as mean ± SE. One-way ANOVA with LSD post hoc demonstrated f_H significantly increased across incubation between 60% and 90% incubation, as indicated by dissimilar letters ($p<0.01$). n = 11 at 60%, n = 37 at 70%, n = 7 at 80%, n = 31 at 90%.

Saline injections to incubated embryos resulted in a significant increase in P_m with no significant changes to f_H (Figure 3). P_m of 70% incubated embryos rose from 0.32 ± 0.03 to 0.38 ± 0.24 kPa ($P<0.001$) after being injected with saline (Figure 3 A). P_m of 90% incubated embryos rose from 0.92 ± 0.07 to 0.99 ± 0.001 ($P<0.001$). However, while injection of saline caused significant rise in 70% and 90% incubated embryos, P_m would return to baseline values within 10 minutes of injection.

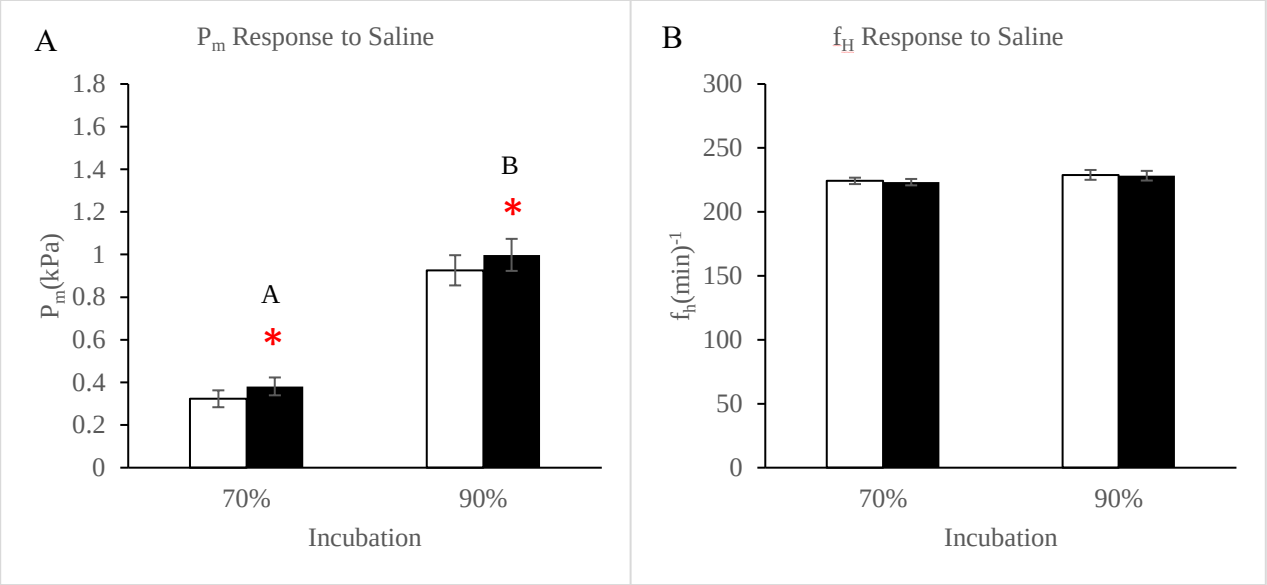

Figure 3. Embryonic neotropic cormorant P_m (A) and f_H (B) response to 3mg kg^{-1} saline. Data are presented as mean ± SE. An asterisk indicates a significant response to drug response (P<0.001). Dissimilar letters indicate significant differences in the intensity of the response across age (P<0.001). P_m = mean arterial pressure, f_H = mean heart rate, White = pre-injection, black = post-injection, n = 35 at 70%, n = 31 at 90%.

6.2 Cholinergic Blockade

Injection of 3 mg kg^{-1} of atropine had no effect on P_m in 70% and 90% embryos (Figure 4 A). Cholinergic blockade in 90% embryos resulted in a significant increase in f_H from 236 ± 6 beats min^{-1} to 242 ± 7 beats min^{-1} (Figure 4 B). However, two out of the eight 90 % incubated neotropic cormorants injected with 3 mg kg^{-1} of atropine 90% incubation experienced no change in f_H.

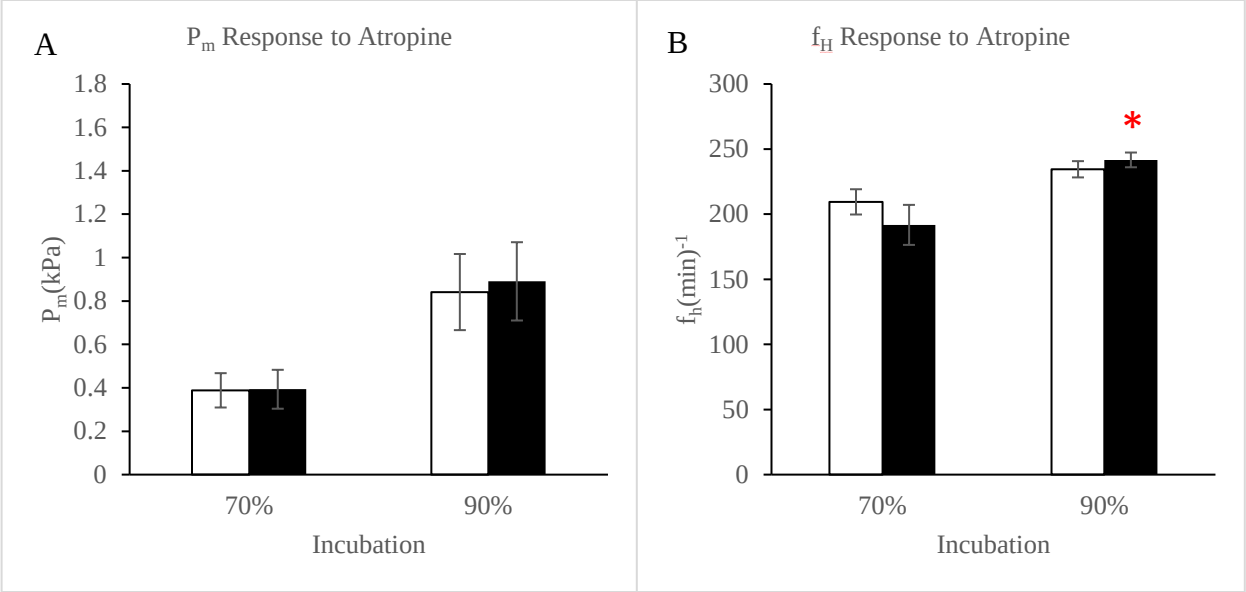

Figure 4. Embryonic neotropic cormorant P_m (A) and f_H (B) response to 3mg kg^{-1} atropine. Data are presented as mean ± SE. An asterisk indicates a significant response to drug response (P<0.01). P_m = mean arterial pressure, f_H = mean heart rate, White = pre-injection, black = post-injection, n = 8 at 70%, n = 8 at 90%.

6.3 Adrenergic Blockade

β - adrenergic blockade with 3 mg kg^{-1} of propranolol increased P_m and decreased f_H of 70% and 90% neotropic cormorant embryos (Figure 5). 70% incubated embryo P_m increased from 0.27 ± 0.04 kPa to 0.56 ± 0.07 kPa (Figure 5 A) while f_H decreased from 227 ± 3 beats min^{-1} to 178 ± 6 beats min^{-1} (Figure 5 B). At 90%, P_m increased from 0.87 ± 0.07 kPa to 1.45 ± 0.11 kPa (Figure 5 A) and f_H decreased from 231 ± 3 beats min^{-1} to 165 ± 5 beats min^{-1} (Figure 5 B). f_H response to propranolol had a significant intensity change from 70% to 90% incubation (P<0.01) with no significant intensity change in regards to P_m (Figure 5)

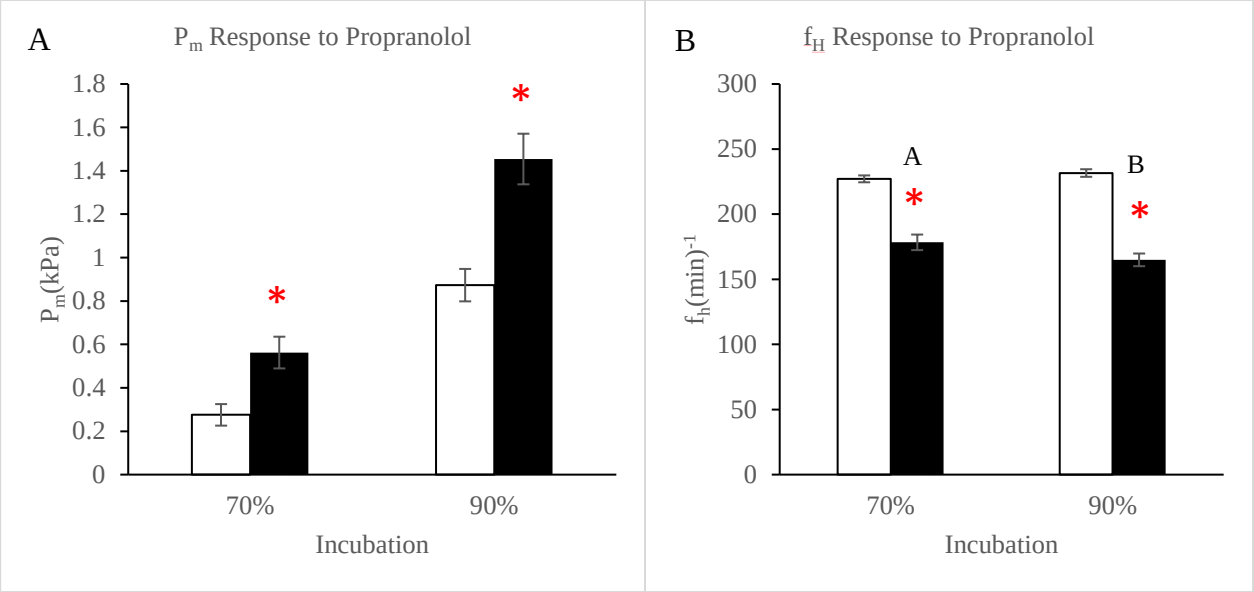

Figure 5. Embryonic neotropic cormorant P_m (A) and f_H (B) response to 3mg kg^{-1} propranolol. Data are presented as mean ± SE. An asterisk indicates a significant response to drug response (P<0.001). Dissimilar letters indicate significant differences in the intensity of the response across age (P<0.001). P_m = mean arterial pressure, f_H = mean heart rate, White = pre-injection, black = post-injection, n = 20 at 70%, n = 20 at 90%.

α – adrenergic blockade with 3 mg kg^{-1} of phentolamine decreased P_m and f_H of both 70% and 90% incubated embryos (Figure 6). Injection of phentolamine decreased the P_m of 70% embryos from 0.62 ± 0.07 kPa to 0.31 ±0.06 kPa (Figure 6 A) and 90% embryos from 1.49 ± 0.08 kPa to 0.49 ± 0.06 kPa (Figure 6 A). f_H of both 70% and 90% embryos were unaffected by α – adrenergic receptor blockade (Figure 6 B). There was no significant change in intensity to P_m or f_H in 70% and 90% incubated embryos (Figure 6 A&B).

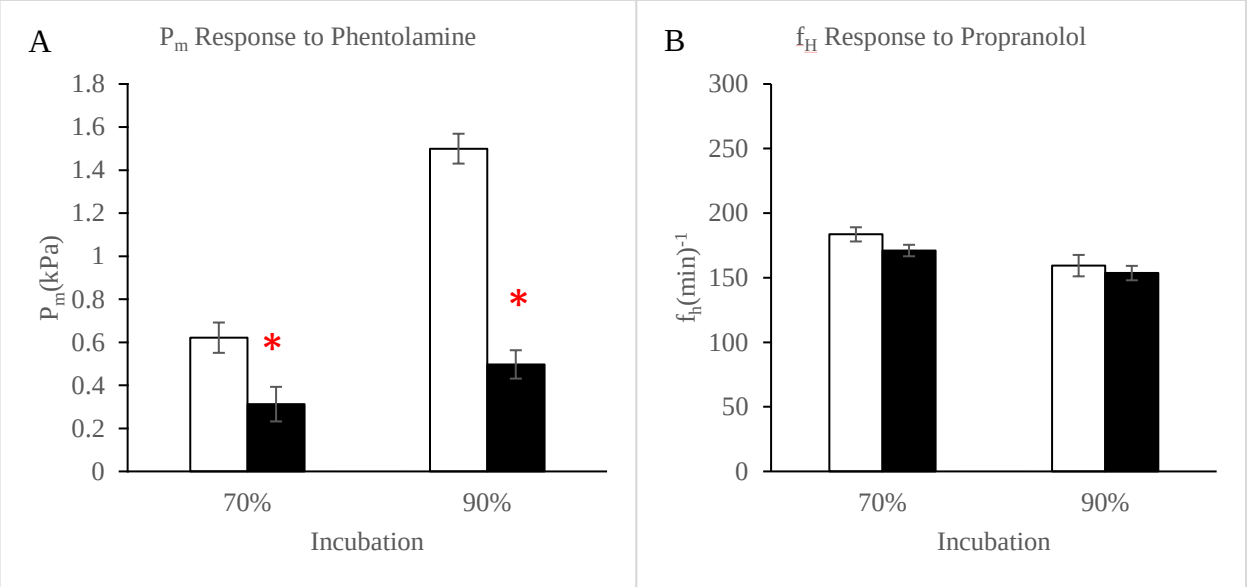

Figure 6. Embryonic neotropic cormorant P_m (A) and f_H (B) response to 3mg kg^{-1} phentolamine. Data are presented as mean ± SE. An asterisk indicates a significant response to drug response (P<0.001). P_m = mean arterial pressure, f_H = mean heart rate, White = pre-injection, black = post-injection, n = 13 at 70%, n = 14 at 90%.

6.4 Acute Temperature Stress

To identify the cardiovascular response to abiotic challenges, a subset of embryos (n=8) at 90% incubation were studied at 32°C. Following stabilization of cardiovascular parameters embryos were injected with atropine (3mg kg^{-1}). Results were then compared to 90% embryos kept at 38°C who underwent cholinergic blockade (Table 3).

Cardiovascular Response to Acute Temperature Reduction

Temperature	Drug	*n*	Pre P_m	Post P_m	Δ P_m	Pre f_H	Post f_H	Δ f_H
38°C	S	31	0.92 ± 0.07	0.99 ± 0.07	0.07± 0.01	228±3	228±3	0 ± 0
	A	8	0.81 ± 0.17	0.89 ± 0.18	0.08 ± 0.01	234± 6	241 ± 5	7 ± 1
32°C	S	8	1.17 + 0.10	1.21 + 0.11	0.04 + 0.01	150 + 3 *	149 + 2	1 + 1
	A	8	1.16 + 0.12	1.08 + 0.17	0.08 + 0.05	150 + 2	155 + 5	5 + 3

Table 3. Comparison of 90% incubated embryonic neotropic cormorant P_m (A) and f_H (B) while being exposed to either 38°C or 32°C. A 1-way ANOVA demonstrated heart rate significantly decreased when 38°C compared to embryos exposed to 32°C, as indicated by asterisk (P<0.001). Data are plotted as mean ± SE. S = Saline, A = Atropine, Pre = before injection of each drug, Post = after injection of each drug, n=31 at 38°C, n=8 at 32°C.

6.4.1 Base P_m and f_H

Control saline injections had no effect on P_m or f_H in 90% embryos studied at 32°C experienced (fig 7A&B).

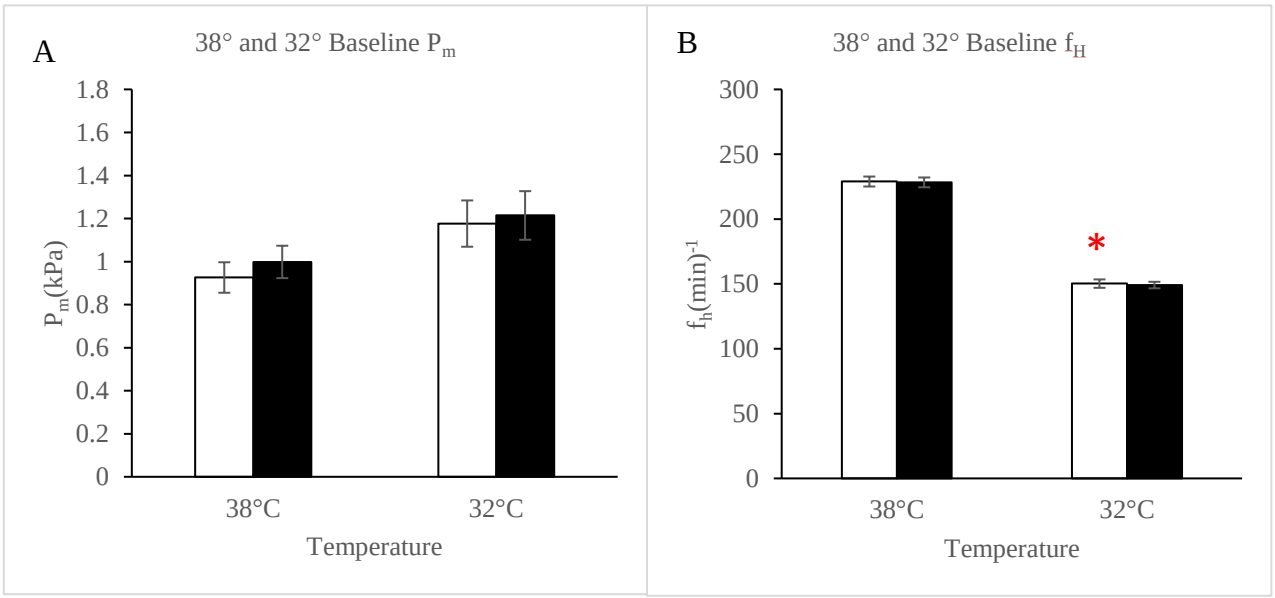

Figure 7. Comparison of 90% incubated embryonic neotropic cormorant P_m (A) and f_H (B) response to saline while being exposed to either 38°C or 32°C. A 1-way ANOVA demonstrated heart rate significantly decreased when 38°C compared to embryos exposed to 32°C, as indicated by dissimilar letters (P<0.001). Asterisk indicates significant change in response to temperature decrease (P<0.001). Data are plotted as mean ± SE. White = pre-injection, black = post-injection, n=31 at 38°C, n=8 at 32°C.

6.4.2 Cholinergic Blockade

An injection of atropine to 90% embryos experiencing 32°C acute temperature stress had no effect on P_m or f_H (Fig 8 A&B).

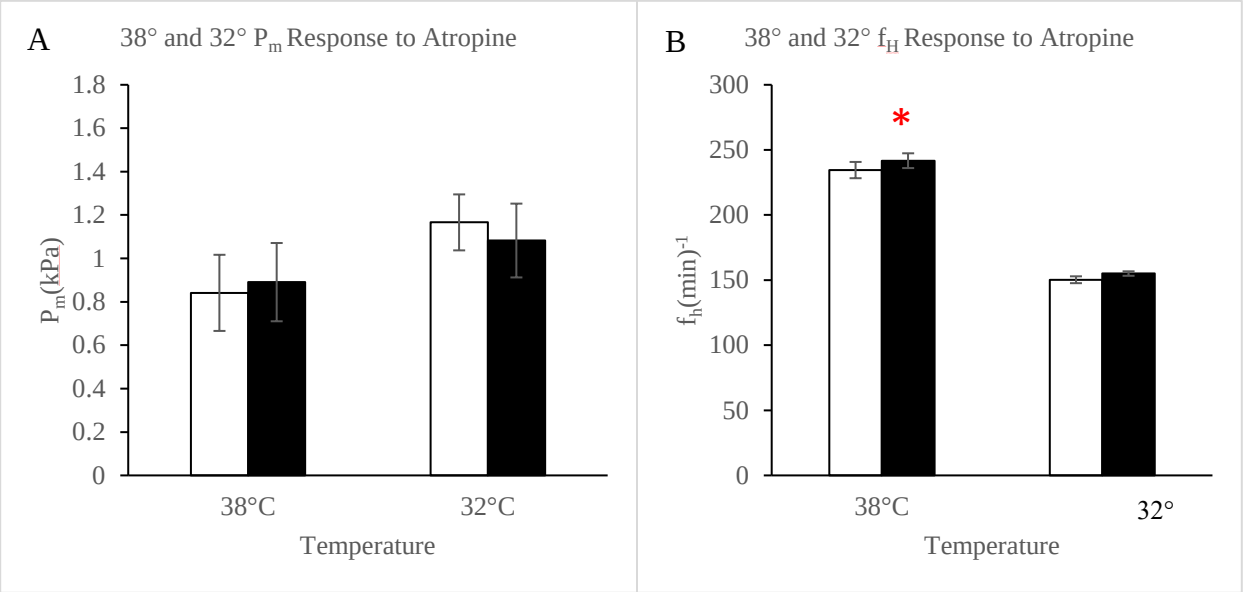

Figure 8. Comparison of 90% incubated embryonic neotropic cormorant P_m (A) and f_H (B) response to 3mg kg^{-1} atropine while being exposed to either 38°C or 32°C. Asterisk indicates significant drug response (P<0.001). Data are plotted as mean ± SE. White = pre-injection, black = post-injection, n=8 at 38°C, n=8 at 32°C.

6.4.3 Temperature Differences

Embryos kept at 38°C had a non-significant lower P_m when compared to embryos at 32°C at 0.92 ± 0.07 kPa to 1.17 ± 0.10 kPa, respectively (Figure 7 A, Table 2). In regards to f_H, embryos exposed to 32°C experienced a significant decrease at 150 ± 3 beats min^{-1} when compared to embryos kept at 38°C at 229 ± 4 beats min^{-1} (Figure 7 B, Table 3).

Embryos at both 38°C and 32°C experienced an increase in f_H when injected with atropine. However, while the increase in f_H in 38°C embryos was significant, the increase in f_H in 32°C embryos was non-significant. Furthermore, when comparing intensity of response to atropine, there was no significant change in intensity between 38°C and 32°C embryos (P=0.49)(Figure 8 A, Table 3).

6.5 Morphological Features

Embryonic mass increased from 5.61 ± 0.32 g to 12.75 ± 0.26 g from 70% (Day 18) to 90%, respectively (Day 23) (Table 3). Albumin was present at 70% embryos but absent at 90% (Table 3). The average yolk mass collected from 90% embryos was 5.21±0.21 g (Table3).

Mass of the right breast muscle significantly increased from 0.05 ± 0.01 g to 0.14 ± 0.02 g from 70% to 90% respectively. Masses of the heart, liver, lungs, kidneys, and brain also increased from 70% to 90%. However, changes of all recorded vital organs and right breast muscle were attributed to an increase in embryonic mass (P<.001) (Table 4).

Morphological Masses

Day	Incubation (%)	*n*	Embryo (g)	Yolk (g)	Albumin (g)	Heart (g)	Liver (g)	Lung (g)	Kidney (g)	Brain (g)	Right Breast Muscle (g)
16	61.5	4	2.67±0.15	-	5.37±0.41	0.02±0.01	0.03±0.01	0.04±0.05	0.01±0.01	0.17±0.01	0.02+0.01
17	65.3	10	3.77±0.17	-	5.36±0.27	0.02±0.01	0.04±0.01	0.06±0.01	0.02±0.01	0.22±0.01	0.03+0.01
18	69.9	21	5.61±0.32	-	4.07±0.35	0.03±0.01	0.07±0.01	0.09±0.01	0.04±0.01	0.28±0.01	0.05+0.01
19	73	24	7.10±0.25	-	3.11±0.32	0.04±0.01	0.11±0.01	0.11±0.01	0.09±0.03	0.31±0.01	0.09+0.01
20	76.9	16	8.70±0.21	-	-	0.06±0.01	0.15±0.01	0.15±0.01	0.10±0.01	0.38±0.01	0.10+0.01
21	80.7	11	10.31±0.22	-	-	0.06±0.01	0.18±0.01	0.16±0.01	0.12±0.01	0.42±0.01	0.12+0.01
22	84.6	9	11.30±0.24	4.52±0.31	-	0.08±0.01	0.21±0.01	0.15±0.01	0.14±0.01	0.44±0.01	0.13+0.01
23	88.4	38	12.75±0.26*	5.21±0.21	-	0.08±0.01*	0.23±0.01*	0.18±0.01*	0.16±0.01*	0.47±0.01*	0.14+0.01*
24	92.3	19	16.09±0.55	4.94±0.31	-	0.09±0.01	0.29±0.01	0.19±0.01	0.21±0.01	0.53±0.01	0.16+0.01
25	96.1	10	19.73±0.49	4.34±0.33	-	0.11±0.01	0.37±0.02	0.18±0.01	0.26±0.02	0.51±0.02	0.19+0.01
26	100	5	21.66±0.82	4.45±0.45	-	0.14±0.01	0.42±0.05	0.21±0.02	0.35±0.04	0.61±0.02	0.20+0.01

Table 4. Masses of the embryo, yolk, albumin, and all vital organs of the neotropic cormorant. Statistical analysis was only completed between days 18 and 23. Asterisks indicate a significant (p<0.001) change in mass. n= number of samples. Data are presented as mean values (g) ± SE.

6.6 Blood Properties

There was a significant increase in lactate when comparing 70% and 90% embryos, 1.26 ± 0.13 mmol L^{-1} to 1.66 ± 0.07 mmol L^{-1} respectively (Figure 9). All other blood parameters were similar between 70% and 90% embryos (Table 5).

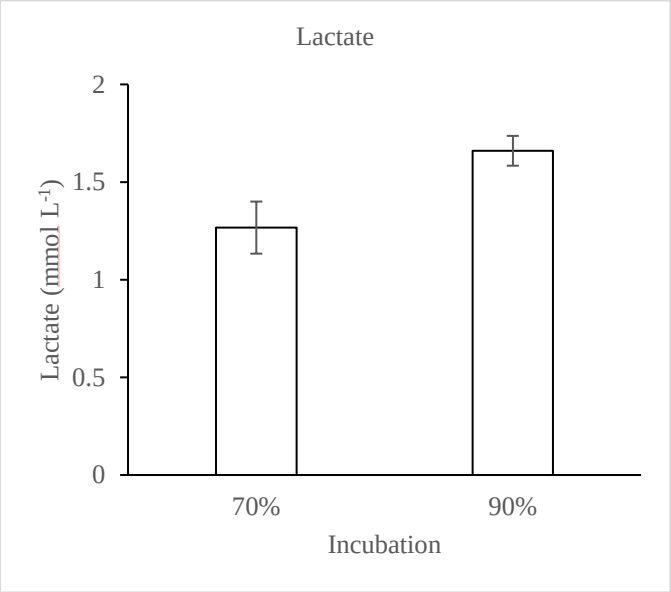

Figure 9. Differences in blood Lactate levels between 70% and 90% incubation. Data are plotted as mean ± SE. An independent t-test indicates there was a significant difference between 70% and 90% incubation, as indicated by an asterisk ($p<0.01$). 70% n = 6, 70%, 90% n = 10.

Blood Properties

Incubation (%)	*n*	Glucose (mg dl^{-1})	Lactate (mmol L^{-1})	pH	pCO2 (mmHg)	Na^{+} (mmol L^{-1})	Cl^{-} (mmol L^{-1})
70	6	134.66 ± 4.07	1.26 ± 0.13	7.17 + 0.07	62.61 ± 10.31	125.13 ± 0.18	106.03 + 1.11
90	10	140.9 ± 7.85	1.66 ± 0.07*	7.12 ± 0.05	73.56 ± 8.00	0.35 ± 0.02	105.38 + 0.80

Table 5. Blood levels of glucose, lactate, pH, pCO2, Na^{+}, and Cl^{-} of the 70% and 90% neotropic cormorant embryos. Asterisks indicate a significant change in blood levels ($p<0.001$). n= 6 at 70, n = 10 at 90%. Data are presented as mean values (g) ± SE.

CHAPTER 7

DISCUSSION

These findings are the first data collection on altricial embryonic birds demonstrating commonalities across the taxa of avian species studied to date. This investigation identified that the neotropic cormorant embryo possessed β-adrenergic tone on f_H and P_m at both 70% and 90% incubation as well as α-adrenergic tone on P_m at both 70% and 90% incubation. Further, neotropic cormorant embryos showed an increase in intensity of β-adrenergic tone on f_H from 70% to 90% incubation. The findings of both β-adrenergic and α-adrenergic tones in the embryonic neotropic cormorant are similar to other investigations that have found adrenergic tone on f_H and P_m in other avian ontogeny (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). Cholinergic tone was absent in 70% incubated embryos while weakly present at 90% f_H, however given the limited strength cholinergic tone on f_H remains in question.

With multiple investigations identifying that adrenergic tone on f_H and P_m are found in multiple precocial avian species and now in the altricial neotropic cormorant, adrenergic tone may be shared across the avian ontogeny. With the onset of cholinergic tone on f_H now identified within 90% incubated neotropic cormorants and multiple of investigations identifying that cholinergic tone on f_H varies across avian species from absent to being present in later stages; cholinergic tone may not be vital to embryonic development and may be timed to coincide with the hatching event. (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014).

7.1 Changes in P_m and f_H

Resting P_m and f_H was compared across the developmental stages of 60% (days 16-17), 70% (days 18-20), 80% (days 21-22) and 90% (days 23-25). Resting P_m increased gradually from 60%-90%, 80% incubated embryos were significantly higher than 60% and significantly lower than 90% (Figure 1). Embryos at 90% incubation had a significant P_m when compared to 60%, 70%, and 80% incubated embryos. This increase in resting P_m in the neotropic cormorant is similar to resting P_m's in the Canada goose (*Branta Canadensis*) and the domestic Goose (*Anser anser domesticus*), in that resting P_m in both geese nearly doubled from 70% to 90%(Swart et al., 2013). The increase in P_m from 60%-90% may be attributed to the development of adrenergic tone on the vasculature.

In regards to resting f_H, the neotropic cormorant resting f_H was relatively constant throughout incubation (Figure 1). This finding is similar to what other studies have found in domestic chickens and geese (Crossley et al., 2000; Swart et al., 2013). neotropic cormorant resting f_H did increase from 60%-80% incubation with a slight decrease from 80% to 90%, however these changes were non-significant. The relative steady f_H of the neotropic cormorant could be attributed to the lack of parasympathetic regulation on the heart till late development. In emus (*Dromiceius novaehollandiae*) f_H initially falls between the incubation stages of 60% and 70% (Crossley et al., 2003a), while in embryonic white leghorn chickens f_H is maintained throughout development (Crossley et al., 2000). These differences may be attributed with cholinergic tone on f_H, with findings of cholinergic tone on embryonic emus beginning at 70% of development while embryonic white leg horn chickens development of cholinergic tone on f_H has been shown to vary with investigations showing development of vagal tone at 60% to not developing until post hatch. (Tazawa et al., 1992; Chiba et al., 2004; Crossley et al., 2000: Crossley et al., 2003a).

7.2 Vagal and β-Adrenergic Tones on f_H

This investigation suggests that there is an absence of vagal tone during embryonic cardiac regulation up till 90% incubation. My finding is in agreement with other investigations that cholinergic tone is not detectible until close to or after hatching, this can be due to start of respiratory rhythmicity or movements. (Crossley et al., 2003b; Chiba et al., 2004; Swart et al., 2013; Taylor et al., 2014). Avian species that gain vagal tone during embryonic development could be based on multiple of factors; years of selective breeding, higher demand of nutrients for growth of tissues, or due to the degree of maternal care with embryos that are more exposed to environmental fluctuations (Altimiras and Crossley, 2000; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). Eggs that are exposed to the environment have shown that f_H reduces to decreases in temperature to reduce heat loss (Andrewartha et al., 2011; Tazawa, 1986; Tazawa, 1985). However, while the ontogeny of vagal tone was identified within 90% incubated neotropic cormorants, the minimal increase in f_H induced by cholinergic blockade may indicate that vagal tone on cardiac regulation may not be vital to embryonic development. Therefore, vagal tone on cardiac regulation of the neotropic cormorant may only be necessary for post hatch survivability.

The absence of cholinergic tone till 90% incubation in the neotropic cormorant may be due to maternal care of the embryos during incubation or to increase post-hatch survivability. Because both rookery sites are located on wildlife management areas with strict rules regarding to how close the public can come into proximity of the rookeries, the cormorants and other avian species within the rookery experienced little to no disturbance from human interaction. With little to no disturbance, adult neotropic cormorants are able to incubate their eggs in a continuous manner preventing eggs from getting exposed from environmental stresses (Weseloh & Ewins, 1994). As well as little to no disturbance from humans, the neotropic cormorant is identified in an

avian family in which both male and female take turns incubating eggs. (Snow, 1963; Schwagmeyer et al., 1999). Co-incubation allows eggs to incubate for longer periods of time and help protect against predators. (Cockburn, 1998).

Additionally, the finding of neotropic cormorants not developing vagal tone on f_H till 90% is similar to fetal sheep (Giussani et al., 1993). This late development of vagal tone in fetal sheep has been contributed to help increase late-term survival (Giussani et al., 1993). Because of maternal care during incubation and the susceptibility to environmental stresses that altricial species experience post hatch, the ontogeny of vagal tone occurring within 90% incubated neotropic cormorant embryos may only be necessary for post hatch survivability. Because of the conflicting findings of, or lack of, vagal tone on cardiac regulation leads to possibility that cholinergic tone is not vital to embryonic development.

The findings from β-adrenergic blockade produced a pronounced decrease in f_H in both 70% and 90% incubated embryos with a significant change in intensity to the response of propranolol. This finding agrees with multiple studies that have also discovered β-adrenergic tone on the basal chronotropic activity across multiple embryonic avian species suggesting that β-adrenergic tone is vital to embryonic cardiovascular regulation (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Altimiras and Crossley, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). Because sympathetic neuron contribution on f_H is minimal, the β-adrenergic tone on the f_H is derived from circulating catecholamines (Higgins and Pappano, 1981; Crossley and Altimiras, 2000; Andrewartha et al., 2011; Swart et al., 2013; Taylor et al., 2014). The increase in β-adrenergic tone on f_H with increased development has been shown in geese and domestic chicken embryos and may be attributed to an increase in circulating levels of catecholamines with increase

development (Crossley and Altimiras, 2000; Swart et al., 2013). However, the source of these catecholamines were not investigated and further study must be needed to find the origin.

7.3 β-Adrenergic and α-Adrenergic Tones on P_m

β-and-α – adrenergic antagonist blockade both showed prominent changes in P_m. When embryonic neotropic cormorants were injected with β-adrenergic antagonist propranolol, a hypertensive response occurred at both 70% and 90% incubation with no change in intensity in response to drug dose indicating the dependence of β-adrenergic receptors for vascular regulation.

Blockade of the α-adrenergic receptors with the antagonist phentolamine produced a hypotensive response that was seen in 70% and 90% incubation with no change in the response intensifying as embryo development increased. Due to neotropic cormorant embryos having a relative low P_m, a strong α-adrenergic tone could be used to offset this. Because of the absence of vagal tone until 90% incubation, α-and-β adrenergic tone are the main factors of vascular regulation.

The findings of both β-and-α – adrenergic tones on P_m within the neotropic cormorant coincides with multiple investigations showing adrenergic tone within a number of studied embryonic avian species (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014). Given the similarity of adrenergic tone in multiple embryonic birds, adrenergic tone may be essential to maintain cardiovascular function during embryonic development.

Even though we did not locate the origin of the stimulus, previous investigations indicate that β-and-α – adrenergic stimuli derive from catecholamines circulating in the blood while

cardiovascular system stimuli from autonomic innervation is absent from the chorioallantoic vasculature (Higgins and Pappano, 1981; Crossley and Altimiras, 2000; Andrewartha et al., 2011; Swart et al., 2013).

7.4 Acute Temperature Stress

7.4.1 Base P_m and f_H

Embryos placed in 32°C had a higher P_m than embryos placed in 38°C. While this change in P_m between eggs placed in either 32°C or 38°C was non-significant, this rise in P_m in embryos placed in 32°C should be addressed. This moderate increase in P_m could be attributed to prevent heat loss through convection by limiting the amount of blood in chorioallantoic vessels (Sun, 2011). The cause of the increase in the P_m of embryos exposed to 32°C may be caused by the renin angiotensin system. Even though investigations of the renin angiotensin system in embryonic avian species is understudied, cold exposure has shown to increase plasma renin activity and angiotensin II formation in adult rats (Sun, 2011). Investigations on embryonic chickens have identified angiotensin II as a trophic factor and tonic regulator of cardiovascular function (Crossley et al., 2010; Mueller et al., 2014). Further study is required to see how the renin angiotensin system is used for environmental stresses in avian embryonic development.

Conversely, f_H of embryos in 32°C was significantly lower than embryos in 38°C. This finding agrees with other investigations that acute exposure to low temperatures induce bradycardia in avian embryos (Tazawa and Nakagawa 1985; Tazawa and Rahn 1986; Bennett, 1981). Bradycardia and vasoconstriction induced by colder temperatures may be used to decrease blood flow to the chorioallantoic membrane to prevent heat loss through convection to the external environment (Tazawa and Nakagawa 1985; Tazawa and Rahn 1986; Bennet, 1981).

90% incubated embryos placed 32°C did not experience any significant changes when injected with saline. While embryos in 38°C experienced a significant rise in P_m after saline injections, embryos in 32°C experienced no significant change. This change in response to saline in 32°C embryos can be attributed to the fact of that the CAM was already in a constricted state due to acute temperature change.

7.4.2 Cholinergic Tone

While 90% embryos kept at 38°C had significant increase in f_H after an injection from atropine, 90% embryos experiencing acute temperature stress at 32°C during pharmacological protocol had a non-significant increase when injected with atropine. This finding is similar to white leghorn chickens exposed to acute hypoxic conditions, identifying that cholinergic tone is not used to compensate for environmental stressors (Crossley, 2000). While vagal tone has been identified in 90% neotropic cormorant embryos kept in 38°C to maintain a resting f_H, this investigation shows that cholinergic tone is not used in response to environmental stresses.

7.5 Cholinergic and Adrenergic Tone within Precocial and Altricial Species

The findings within this investigation identify that adrenergic tone is a main factor in maintaining baseline f_H and P_m within 70% and 90% incubated neotropic cormorants. This finding of adrenergic tone within the altricial neotropic cormorant coincides with studies conducted on precocial species (Van Mierop and Bertuch, 1967; Girard, 1973; Tazawa et al., 1992; Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003a, b; Andrewartha et al., 2011; Crossley and Altimiras, 2012; Swart et al., 2013; Taylor et al., 2014), even though altricial species hatch at a relatively early stage of development when compared to precocial species (Table 6).

With the identification of adrenergic tone on the cardiovascular system at 70% and 90% incubation within multiple precocial embryonic birds and now within the altricial neotropic cormorant, this leads to the possibility that the maturation of adrenergic tone on f_H and P_m must occur prior to hatching.

7.6 Morphological Features

The albumin, embryonic mass, muscle, and all vital organs of the neotropic cormorant all experienced a significant change in mass from 70% (day 18) incubation to 90% (day 23). Yolk and albumin masses were compared to multiple precocial and altricial species, while vital organs can only be compared to precocial species due to the lack of information on collected organs from altricial avian embryos (Table 3.).

Adrenergic Regulation Comparison

Incubation (%)	Species	Pre Propranolol f_H	Post Propranolol f_H	Propranolol f_H % Δ	Pre Propranolol P_m	Post Propranolol P_m	Propranolol P_m % Δ	Pre Phentolamine P_m	Post Phentolamine P_m	Phentolamine P_m % Δ
70	*P. brasilianus*	227	178	-21.59	0.28	0.56	104.23	0.62	0.31	-49.69
	G. Gallus	210	166	-20.95	1.60	1.63	1.88	1.62	1.27	-21.60
	A. anser domesticus	264	243	-7.95	1.25	1.38	10.40	1.38	1.22	-11.59
	B. candadensis	250	225	-10.00	1.30	1.46	12.31	1.46	1.25	-14.38
	D. novaehollandiae	177	144	-18.64	1.90	2.05	7.89	2.05	1.70	-17.07
90	*P. brasilianus*	232	165	-28.88	0.87	1.45	66.56	1.49	0.49	-67.11
	G. Gallus	215	183	-14.88	2.60	2.95	13.46	2.95	1.75	-40.68
	A. anser domesticus	265	220	-16.98	2.40	2.49	3.75	2.49	1.98	-20.48
	B. candadensis	284	224	-21.13	2.55	2.65	3.92	2.65	2.23	-15.85
	D. novaehollandiae	200	154	-23.00	2.55	3.45	35.29	3.45	1.95	-43.48

Table 6. Comparrison of f_H and P_m response to propranolol and phentolamine within the 70% and 90% incubated neotropic cormorant (*P. basilianus*), white leghorn chicken (*G. Gallus*), Domestic Goose (*A. anser domesticus*), Canada goose (*B. candadensis*), and Emu (*D. novaehollandiae*).

Yolk of the neotropic cormorant would begin to be engulfed by the embryo at days 21-22 and would be completely engulfed by day 26. Although the yolk would be engulfed by the embryo, the yolk could still be retrieved intact within its membrane. The large amount of residual yolk is

similar to the altricial Australian pelican (*Pelecanus conspicillatus*) which may be important for nutrition during the first days of post-hatch when sibling rivalry may limit food intake (Pearson et al., 2002). The slow rate of consumption of the yolk could be due to metabolic rate. When comparing precocial and altricial eggs who have the same mass, the precocial species incurs higher total energy costs because the embryo is larger for a greater proportion of the incubation period than an altricial embryo (Starck, 1998).

Albumin was collected from the neotropic cormorant embryos. From days 20 to 26, albumin was absent from eggs. At days 18 and 19, albumin was present and showed a decrease in mass respectively. Out of the 16 eggs that were dissected during day 20, five eggs contained albumin and by day 21, albumin was absent from the egg. The fast rate of albumen losing mass and eventually disappearing is comparable to other avian species (Romanoff, 1967). Although we did not complete a makeup of the albumen in this investigation, in chickens, albumen is primarily made up of water, proteins, carbohydrates, enzymes and vitamins all of which is poured into the amniotic cavity for development of the embryo (Romanoff, 1967). Albumin at 90% incubation of neotropic cormorants was similar to 90% domestic chicken in that albumin was non-existent in the egg. However, when comparing 70% mass specific-ratio of albumin between the neotropic cormorant and domestic chicken, the neotropic cormorant had a specific mass ratio of 0.72 while the domestic chicken had a smaller specific mass ratio of 0.44. This difference has been attributed for the larger amount of water needed by altricial hatchlings since only food stuff and not water is brought by feeding parents (Ar and Yom-Tov, 1978).

The neotropic cormorant heart at both 70% and 90% were smaller in mass when compared to other previously studied precocial avian species; chickens, domestic goose, and Canada goose (Table 7). In regards to mass-specific ratio of the heart, 70% heart mass of the neotropic cormorant

(.007) was similar to chicken (.007), domestic goose (.006), and Canada goose (.006). 90% mass-specific ratio of the neotropic cormorant heart (.007) was similar to chickens (.007) and greater than domestic goose (.004) and Canada goose (.005). Showing that there is little difference between in mass specific ratio of the heart during early development among these avian species, this may indicate that cardiac output does not vary. In 90%, the neotropic cormorant and the domestic chicken mass specific ratio of the heart stayed the same while the two geese mass specific ratio decreased. Because altricial embryos show an increase in metabolic rate continually throughout incubation (Vleck and Vleck, 1996; Starck, 1998), an increase in cardiac output may be needed during the late stages of development.

Morphological Mass Comparisons

Incubation (%)	Species	Embryo (g)	Yolk (g)	Albumen (g)	Heart (g)	Liver (g)	Lung (g)	Kidney (g)	Brain (g)
70	P. brasilianus	5.61	-	4.07	0.03	0.07	0.09	0.04	0.28
	G. Gallus	12.5	13.69	5.56	0.09	0.29	0.08	0.02	0.51
	A. anser domesticus	39.8	44.59	-	0.23	0.59	-	0.24	1.12
	B. candadensis	36.24	56.86	-	0.19	0.6	-	0.24	1.05
90	P. brasilianus	12.75	5.21	-	0.08	0.23	0.18	0.16	0.47
	G. Gallus	25.79	10.46	-	0.19	0.71	0.18	0.01	0.86
	A. anser domesticus	75.33	30.77	-	0.35	1.34	-	0.24	1.96
	B. candadensis	80.59	36	-	0.4	1.59	-	0.48	1.88

Table 7. Comparrison of masses between the embryonic species neotropic cormorant (*P. basilianus*), white leghorn chicken (*G. Gallus*), domestic goose (*A. anser domesticus*), and the Canada goose (*B. candadensis*). Data is presented in grams.

The liver of the neotropic cormorant increased in mass from 0.06g at 70% to 0.24g at 90%. Mass specific ratio of the liver did not differ between 70% and 90% incubation across embryonic avian species that have been studied: neotropic cormorant (0.02), domestic chicken (0.02),

domestic goose (0.02), and Canada goose (0.02). This may indicate that the demands of the liver are similar across avian species.

The neotropic cormorant kidneys increased from 0.03g at 70% incubation to 0.49g at 90% incubation with a mass specific ratio of 0.006 and 0.015 respectively. At 70% the kidney mass specific ratio of the neotropic cormorant were similar to geese (0.006) and greater than the domestic chickens (0.002). At 90% the neotropic cormorant kidney mass specific ratio (0.015) was greater than the domestic chicken (0.0003), domestic goose (0.003), and the Canada goose (0.005). Because neotropic cormorants breed in areas with high humidity, the kidneys may have to be more developed for osmoregulation. (Doneen and Smith, 1981)

7.7 Blood Properties

90% incubated embryos experienced a non-significant rise in glucose levels when compared to 70% incubated embryos. When comparing glucose levels of embryonic neotropic cormorants to the precocial Canada geese and domestic geese, glucose levels of embryonic neotropic cormorants were higher than both species of geese at both 70% (Canada goose 87.29 mg dl^{-1}; domestic goose 109.8 mg dl^{-1}) and 90% (Canada goose 122.54 mg dl^{-1}; domestic goose 94.80 mg dl^{-1}) incubation (Swart, 2013). Swart identified that the glucose levels in the wild Canada geese were higher than the domestic geese, and attributed this rise to the fact that the domesticated geese incubated eggs in a more a stable environment when compared to the wild Canada geese. Since altricial species are more susceptible to the environment when compared to precocial species, altricial species such as the neotropic cormorant may require higher glucose levels to increase survival from environmental stresses (Swart, 2013).

While non-significant, there was a rise of pCO2 blood levels from 70% to 90% incubated neotropic cormorant embryos. This rise in pCO2 was expected as previous investigations have

shown that oxygen consumption in altricial embryos continually increases with increased development (Tazawa, 1980; Hoiby et al., 1986). While the oxygen consumption in precocial species plateau during the last 20% of incubation, the finding of the rise in pCO2 in the neotropic cormorant embryo is similar to findings in chicken embryos (*Gallus gallus*) where this rise in blood pCO2 levels is attributed to increase metabolism with increased development and due to the restriction of diffusive ventilation (Tazawa, 1980).

The significant rise in lactate from 70% to 90% incubated embryos is similar to what has been seen in embryonic fowl (Hoiby et al., 1986; Vleck & Vleck 1996). Altricial embryos have been identified to increase embryo mass and growth rate continuously throughout incubation (Tazawa, 1980; Hoiby et al., 1986; Vleck & Vleck 1996). After external pipping, avian embryos see an increase in metabolic rate which has been partially attributed to the initiation of ventilation and removal of the egg shell and chorioallantoic membrane (Hoiby et al., 1986; Vleck & Vleck 1996). This rise in blood lactate from 70% to 90% can be attributed with the increase in energy demand with increase development while still being limited in gas exchange using the chorioallantoic (Tazawa, 1980; Hoiby et al., 1986; Vleck & Vleck 1996). Previous investigations conducted on embryonic chickens identified an increase in blood lactate from days 18 to 19 (21 day incubation period) which was attributed to energy demand with the increase maturation of hatching muscles and start of the withdrawal of the yolk. During external pipping, blood lactate levels reach the highest values shortly after hatching which has been attributed to hatching muscles using anaerobic glycolysis (Hoiby et al., 1986; Vleck & Vleck 1996). While the ratio of aerobic to anaerobic metabolism was not quantified, the findings of the significant rise in blood lactate seen in neotropic cormorant embryos from 70% to 90% incubation may be attributed to a continual rise in embryo mass and growth rate.

Despite seeing a significant rise in blood lactate and non-significant rise in blood pCO2, blood pH was relatively unchanged from 70% to 90%. This can be attributed to the increase in O2 affinity due to hypoxia caused by the restraint of gas diffusion through the shell against the continually increasing metabolism (Tazawa, 1980; Hoiby et al., 1986).

CHAPTER 8

PERSPECTIVE

The neotropic cormorant at 70% and 90% incubation possessed β-adrenergic tone and f_H on P_m which increased in intensity with development. Neotropic cormorant embryos also showed a continuous α-adrenergic tone on P_m. Therefore, the findings support the hypothesis regarding adrenergic tone on f_H and P_m. In regards cholinergic tone, our hypothesis that baseline f_H is maintained by tonic cholinergic stimulation is partially correct. While cholinergic tone was not shown in 70% embryos, cholinergic tone on f_H was identified in 90% incubated embryos. Our findings help establish that during embryonic development adrenergic regulation on the cardiovascular system is vital to embryonic development while cholinergic receptors may not be vital to embryonic development.

The implications of this research will provide insight and help improve our understanding of the importance of adrenergic and cholinergic regulation in embryonic avian species. Most investigations on embryonic cardiovascular regulation are performed on precocial bird species while cardiovascular regulation on embryonic altricial birds are relatively unknown. This investigation on neotropic cormorants could provide a better understanding of the altricial bird species.

REFERENCES

Altimiras, J., Aissaoui, A., Tort, L., & Axelsson, M. (1997). Cholinergic and adrenergic tones in the control of heart rate in teleosts. How should they be calculated?. Comparative Biochemistry and Physiology, Part A: Physiology, 118(1), 131-139.

Altimiras, J., & Crossley II, D. A. (2000). Control of blood pressure mediated by baroreflex changes of heart rate in the chicken embryo (*Gallus gallus*). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 278(4), R980-R986.

Andrewartha, S. J., Tazawa, H., & Burggren, W. W. (2011). Embryonic control of heart rate: examining developmental patterns and temperature and oxygenation influences using embryonic avian models. Respiratory Physiology & Neurobiology, 178(1), 84-96.

Ar, A., & Yom-Tov, Y. (1978). The evolution of parental care in birds. Evolution, 655-669.

Bennett, A. F., Dawson, W. R., & Putnam, R. W. (1981). Thermal environment and tolerance of embryonic Western Gulls. Physiological Zoology, 146-154.

Brezenoff, H. E., & Giuliano, R. (1982). Cardiovascular control by cholinergic mechanisms in the central nervous system. Annual Review of Pharmacology and Toxicology, 22(1), 341-381.

Brodde, O. E., Bruck, H., & Leineweber, K. (2006). Cardiac adrenoceptors: physiological and pathophysiological relevance. Journal of Pharmacological Sciences, 100(5), 323-337.

Chiba, Y., Fukuoka, S., Niiya, A., Akiyama, R., Tazawa, H. (2004) Development of cholinergic chronotropic control in chick (Gallus gallus domesticus) embryos. Comparative Biochemistry Physiology, Part A: Molecular Integrative Physiology, 137(1), 65–73.

Cockburn, A. (1998). Evolution of helping behavior in cooperatively breeding birds. Annual Review of Ecology and Systematics, 141-177.

Cohen, H. L. (1986). Development of autonomic innervation in mammalian myocardium. In

Developmental neurobiology of the autonomic nervous system (pp. 159-191). Humana Press.

Crossley II, D., & Altimiras, J. (2000). Ontogeny of cholinergic and adrenergic cardiovascular regulation in the domestic chicken (Gallus gallus). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 279(3), R1091-R1098.

Crossley II, D. A., Bagatto, B. P., Dzialowski, E. M., & Burggren, W. W. (2003a). Maturation of cardiovascular control mechanisms in the embryonic emu (Dromiceius novaehollandiae). Journal of Experimental Biology, 206(15), 2703-2710.

Crossley II, D. A., Burggren, W. W., & Altimiras, J. (2003b). Cardiovascular regulation during hypoxia in embryos of the domestic chicken (Gallus gallus). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 284(1), R219-R226.

Crossley II, D. A., Hicks, J.W., Thornburg, K., (2003c). A comparison of cardiovascular regulatory mechanisms present during fowl ontogeny; white leghorn vs. bantam chickens. Abstract at the Annual Meeting of the Society of Integrative and Comparative Biology, 147.

Crossley II, D. A., Jonker, S. S., Hicks, J. W., & Thornburg, K. L. (2010). Maturation of the angiotensin II cardiovascular response in the embryonic white leghorn chicken (Gallus gallus). Journal of Comparative Physiology B, 180(7), 1057-1065.

Crossley II, D. A., & Altimiras, J. (2012). Effect of selection for commercially productive traits on the plasticity of cardiovascular regulation in chicken breeds during embryonic development. Poultry Science, 91(10), 2628-2636.

Doneen, B. A., & Smith, T. E. (1982). Ontogeny of endocrine control of osmoregulation in chick embryo: II. Actions of prolactin, arginine vasopressin, and aldosterone. General and Comparative Endocrinology, 48(3), 310-318.

Eme, J., Altimiras, J., Hicks, J. W., & Crossley II, D. A. (2011a). Hypoxic alligator embryos: Chronic hypoxia, catecholamine levels and autonomic responses of in ovo alligators. Comparative Biochemistry and Physiology, Part A: Molecular & Integrative Physiology, 160(3), 412-420

Engelhardt, S., & Hein, L. (2004). Adrenergic system. In Transgenic Models in Pharmacology (pp. 33-63). Springer Berlin Heidelberg.

Van Mierop, L. H., & Bertuch, C. J. (1967). Development of arterial blood pressure in the chick embryo. American Journal of Physiology, 212, 43-48.

Girard, H. (1973). Arterial pressure in the chick embryo. American Journal of Physiology, 224(2), 454-460.

Giussani, D. A., Spencer, J. A., Moore, P. J., Bennet, L., & Hanson, M. A. (1993). Afferent and efferent components of the cardiovascular reflex responses to acute hypoxia in term fetal sheep. The Journal of Physiology, 461(1), 431-449.

Guyton, A. C., Coleman, T. G., & Granger, H. J. (1972). Circulation: overall regulation. Annual Review of Physiology, 34(1), 13-44.

Hamburger, V., & Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. Journal of Morphology, 88(1), 49-92.

Higgins, D., & Pappano, A. J. (1981). Developmental changes in the sensitivity of the chick embryo ventricle to beta-adrenergic agonist during adrenergic innervation. Circulation Research, 48(2), 245-253.

Höchel, J., Akiyama, R., Masuko, T., Pearson, J. T., Nichelmann, M., & Tazawa, H. (1998). Development of heart rate irregularities in chick embryos. American Journal of Physiology-Heart and Circulatory Physiology, 275(2), H527-H533.

Hanson, H. C. (1954). Criteria of age of incubated mallard, wood duck, and bob-white quail eggs. The Auk, 71(3), 267-272.

Høiby, M., Aulie, A., & Bjonnes, P. O. (1987). Anaerobic metabolism in fowl embryos during normal incubation. Comparative Biochemistry and Physiology Part A: Physiology, 86(1), 91-94.

Molinoff, P. B. (1984). α-and β-Adrenergic Receptor Subtypes. Drugs, 28(2), 1-15.

Oberg, B. (1976). Overall cardiovascular regulation. Annual Review of Physiology, 38(1), 537-570.

Mueller, C. A., Crossley, D. A., & Burggren, W. W. (2014). The actions of the renin–angiotensin system on cardiovascular and osmoregulatory function in embryonic chickens (Gallus gallus domesticus). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 178, 37-45.

Pearson, J. T., Seymour, R. S., Baudinette, R. V., & Runciman, S. (2002). Respiration and energetics of embryonic development in a large altricial bird, the Australian pelican (Pelecanus conspicillatus). Journal of Experimental Biology, 205(18), 2925-2933.

Phoon, C.K.L. (2001). Circulatory physiology in the developing embryo. Current Opinion Pediatrics, 13(5), 456–464.

Powell, D. C., Aulerich, R. J., Balander, R. J., Stromborg, K. L., & Bursian, S. J. (1998). A photographic guide to the development of double-crested Cormorant embryos. Colonial Waterbirds, 348-355.

Powell, D. C., Aulerich, R. J., Napolitano, A. C., Stromborg, K. L., & Bursian, S. J. (1996). Incubation of double-crested Cormorant eggs (Phalacrocorax auritus). Colonial Waterbirds, 256-259.

Quintana, F., Yorio, P., & Borboroglu, P. (2002). Aspects of the breeding biology of the neotropic cormorant (Phalacrocorax olivaceus) at Golfo San Jorge, Argentina. Marine Ornithology, 30, 25-29.

Reiter, M. E., & Andersen, D. E. (2008). Comparison of the egg flotation and egg candling techniques for estimating incubation day of Canada goose nests. Journal of Field Ornithology, *79*(4), 429-437.

Romanoff, A.L., (1967). Biochemistry of the Avian Embryo. A Quantitative Analysis of Prenatal Development. Interscience Publishers, New York.

Schwagmeyer, P. L., St. Clair, R. C., Moodie, J. D., Lamey, T. C., Schnell, G. D., & Moodie, M. N. (1999). Species differences in male parental care in birds: a reexamination of correlates with paternity. The Auk, 487-503.

Snow, B. K. (1963). The behavior of the shag. British Birds, 56(3-5), 77-103.

Sokal, R. R., & Rohlf, F. J. (1995). Biometry: the principles and practice of statistics in biological research. WH. Freeman& Co., San Francisco. Sokal Biometry: the principles and practice of statistics in biological research1995.

Sun, Z. (2010). Cardiovascular responses to cold exposure. Frontiers in bioscience (Elite edition), *2*, 495-503.

Swart, J., Tate, K., & Crossley II, D. A. (2014). Development of adrenergic and cholinergic receptor cardiovascular regulatory capacity in the Canada goose (Branta canadensis) and domestic goose (Anser anser domesticus). Comparative Biochemistry and Physiology, Part A: Molecular & Integrative Physiology, 167, 59-67.

Starck, J. M. (1998). Avian growth and development: evolution within the altricial-precocial spectrum (No. 8). Oxford University Press on Demand.

Tazawa, H. (1980). Oxygen and CO2 exchange and acid-base regulation in the avian embryo. American Zoologist, 20(2), 395-404.

Tazawa, H., Hashimoto, Y., Doi, K., (1992). Blood pressure and heart rate of the chick embryo (Gallus domesticus) within the egg: responses to autonomic drugs. In: Hill, R.B., Kuwasawa, K., McMahon, B.R., Kuramoto, T. (Eds.), Phylogenetic models in functional coupling of the CNS and the cardiovascular system. Karger, Basel, pp. 86–96.1-September 2, 1991 (Vol. 11, p. 86). Karger Medical and Scientific Publishers.

Tazawa,H., & Rahn, H. (1986). Tolerance of chick embryos to low temperatures in reference to the heart rate. Comparative Biochemistry and Physiology Part A: Physiology, 85(3), 531-534.

Tazawa, H., & Nakagawa, S. (1985). Response of egg temperature, heart rate and blood pressure in the chick embryo to hypothermal stress. Journal of Comparative Physiology B, 155(2), 195-200.

Tazawa, H., Wakayama, H., Turner, J. S., & Paganelli, C. V. (1988). Metabolic compensation for gradual cooling in developing chick embryos. Comparative Biochemistry and Physiology Part A: Physiology, 89(2), 125-129.

Taylor, E. W., Leite, C. A., Sartori, M. R., Wang, T., Abe, A. S., & Crossley, D. A. (2014). The phylogeny and ontogeny of autonomic control of the heart and cardiorespiratory interactions in vertebrates. The Journal of Experimental Biology, 217(5), 690-703.

Vleck, C. M., & Vleck, D. (1996). Embryonic energetics. In Avian energetics and nutritional ecology (pp. 417-454). Springer US.

Weseloh, D. C., & Ewins, P. J. (1994). Characteristics of a rapidly increasing colony of double-crested Cormorants (Phalacrocorax auritus) in Lake Ontario: population size, reproductive

parameters and band recoveries. Journal of Great Lakes Research, 20(2), 443-456.

Westerskov, Kaj. (1950) Methods for determining the age of game bird eggs, The Journal of Wildlife Management, 14(1), 56-67.

Zuscik, M. J., Chalothorn, D., Hellard, D., Deighan, C., McGee, A., Daly, C. J. & Thomas, J. D. (2001). Hypotension, autonomic failure, and cardiac hypertrophy in transgenic mice overexpressing the α1B-adrenergic receptor. Journal of Biological Chemistry, 276(17), 13738-13743.