THE IMPACT OF DEVELOPMENTAL STRESS ON CARDIOVASCULAR
PHYSIOLOGY OF TWO ARCHOSAUR SPECIES: AMERICAN
ALLIGATOR (Alligator mississippiensis) AND
DOMESTIC CHICKEN (Gallus gallus)

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Crocodilians and birds comprise sister taxa of archosaurs, the development of these vertebrates occurs within an egg case that leaves developing embryos susceptible to fluctuations in the nesting environment. Studies suggest that sub-optimal conditions alter morphological growth and cardiovascular physiology. Regulation of the cardiovascular system is immature in the subjects studied, and embryos may rely on humoral rather than neural control of the cardiovascular system. The primary focus of this dissertation was to assess regulatory mechanisms responsible for maintenance of arterial pressure and heart rate.

Dehydration stress had marked effects on embryo growth, and altered baseline cardiovascular parameters, while leaving the response to humoral regulator, angiotensin II (Ang II), unaffected. However, dehydrated alligator embryos developed cholinergic tone on heart rate. Hypoxic incubated chicken embryos were reduced in embryo mass, and altered response to humoral regulatory components Ang I and adenosine in addition identifying a novel regulatory component of the cardiovascular response to acute hypoxia.

Collectively, these studies add to the existing knowledge of cardiovascular physiology in embryonic archosaurs and suggest that some components of cardiovascular regulation are plastic following developmental stress.
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By

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CHAPTER 1
INTRODUCTION TO TOPIC

Vertebrate physiology has classically focused on organ system function in juvenile or adult animals. This approach has broadened the understanding of physiology across vertebrate taxa, however embryonic development of vertebrate life history has in comparison largely been neglected (Burggren, 1991). This is an important omission given that natural selection may reach its peak during the embryonic/fetal phase of life (Burggren and Warburton, 2005). Recent work has begun to address this deficiency, demonstrating that physiological systems are, not surprisingly, immature in embryonic vertebrates (Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003A; Black and Burggren, 2004; Dzialowski et al., 2007; Copeland and Dzialowski, 2009; Eme et al., 2011A,B; Marks et al., 2013). Given known fluctuations in the developmental environment in both in utero and ex utero developing species, there is the potential for the animal to experience environmental stress during embryogenesis. Thus to construct a more thorough understanding of vertebrate physiology studies must be extended to investigate the maturation of critical organ systems during this vulnerable phase of life.

The first functional organ system in vertebrate development is the cardiovascular system, thus at an early phase of life environmental perturbations may alter the function of this system (Stainier et al., 1995). Therefore, a thorough understanding of cardiovascular development is essential to determine how variations in the developmental environment affect maturation of animal physiology. The current knowledge base of cardiovascular maturation is derived from investigations of a select
number of model vertebrate species: larval zebra fish (*Danio rerio*), larval clawed frogs (*Xenopus laevis*), embryonic chickens (*Gallus gallus*), and fetal sheep (*Ovis aries*) (Iwamoto and Rudolph, 1981; Tazawa, 1981; Alonso et al., 1989; Territo and Burggren, 1998; Altimiras and Crossley, 2000; Bagatto, 2005). However, recent investigations have since expanded this knowledge to include multiple reptilian species (Crossley, 1999; Eme et al., 2011A; Crossley et al., 2013; Eme et al., 2013B). Reptiles are a key clade in the evolution of endothermy; which evolved from a basal reptile approximately 310 million years ago (Kumar and Hedges, 1998). Birds and crocodilians represent sister taxa of the archosaur lineage (Brusatte et al., 2011). Given this evolutionary history, further studies of cardiovascular ontogeny in extant reptiles and birds will provide insight into the common developmental features that may be required for normal maturation of the amniotic vertebrate cardiovascular system.

Non-mammalian amniotes, birds and reptiles, develop in an egg that lacks protective features inherent with *in utero* development. This reproductive strategy could subject the immature animal to fluctuations in environmental conditions due to temporal variation. Environmental conditions vary and may include fluctuations in: gas composition, water availability, and temperature (Ackerman, 1980; Packard et al., 1993; Ackerman and Lott, 2004). While the majority of avian species maintain parental care of embryos during incubation, reptiles exhibit little to no parental care leaving reptilian embryos potentially subjected to fluctuations in the local nest environment (Shine, 2004). However our understanding of this large polyphyletic group remains limited.

American alligators (*A. mississippiensis*) lay eggs within a nest that may
fluctuate tremendously with variations in climate, as suggested for other reptiles. For the American crocodile (*Crocodylus acutus*) the nest PO$_2$ can decrease from 150 torr down to 85 torr during incubation, whereas the broad-shelled river turtle values can decrease to 100 torr following rain fall with an increase in PCO$_2$ (*Chelodina expansa*) (Lutz and Dunbar-Cooper, 1984; Booth, 1998). Low rainfall reduces nest water content, affecting embryo survival to hatching and can result in high embryonic mortality in both the American alligator (*A. mississippiensis*) and the American crocodile (*C. acutus*) (Ferguson, 1982; Mazzotti et al., 1988). Temperature also has a noted impact on development of the alligator embryo, most notably is the effect of temperature on sex determination, also of significance the incubation temperature also has profound effects on post-hatchling growth (Ferguson, 1982; Joanen and Mcnease, 1989). Multiple studies have demonstrated that the incubation environment has a significant effect on embryonic growth and hatching success in natural nests (Packard et al., 1987; Packard, 1991; Mazzotti et al., 1988). Further laboratory-based studies focused on the impact of reduced oxygen demonstrate that growth is significantly affected in multiple species of reptiles and birds (Packard et al., 1987; Dzialowski et al., 2002; Packard et al., 2002; Chan and Burggren, 2005; Crossley et al., 2005; Delmas et al., 2008; Eme et al., 2011A,B; Eme et al., 2013B; Iversen et al., 2014).

Following developmental stress, cardiovascular physiology has been studied in a limited number of birds and reptiles including the American alligator, (*A. mississippiensis*) the common snapping turtle (*C. serpentina*) and the domestic chicken (*G. gallus*) (Crossley et al., 2005; Eme et al., 2011A,B; Crossley and Altimiras, 2012; Eme et al., 2013B; Lindgren et al., 2011; Iversen et al., 2014). Collectively these
studies suggest that the cardiovascular system is altered by developmental stress and recent work begun to explore humoral control of the system.

The adult vertebrate cardiovascular system is controlled through short and long-term regulatory mechanisms. These include the central nervous system (CNS) and endocrine, or humoral, components that maintain blood pressure and heart rate within an optimal range. Short-term regulation includes reflex responses such the baro- and chemoreflex that are activated by short-term disturbances in the cardiovascular system (Macdonald et al., 1980; Ishii and Ishii, 1986; Eme et al., 2011B).

The baroreflex serves to maintain arterial pressure within a range with deviations from a set point detected by the stretch sensitive baroreceptors located throughout the vasculature (Canine lupus familiariis, Ito and Scher, 1978; Bufonidae, Smith et al., 1981; Anas platyrhynchos, Jones, 1973; Testudinidae, Ishii and Ishii, 1986). Deviation from the set point of the baroreflex initiates changes in heart rate and peripheral resistance that is inversely related to the arterial pressure change. For example in adult vertebrates, an increase in the arterial pressure produce a bradycardia which decrease cardiac output returning pressure to the set-point (Lucitti and Hedrick, 2005; Hagensen et al., 2010). Pharmacological tools can be used to induce rapid changes in blood pressure to mimic and quantify the functional capacity as well as sensitivity of the baroreflex. Using a pharmacological approach, the capacity for the CNS to regulate cardiovascular function can be quantified; additionally investigations of other mechanisms of cardiovascular control also provide important information regarding the capacity of the animal to regulate cardiovascular function.
Tonic CNS mediated control continuously maintains cardiovascular function at some level in embryonic birds and reptiles, and those species that have been investigated possess limited tonic capacity (Altimiras and Crossley, 2000; Crossley et al., 2003A; Eme et al., 2011A). Studies in archosaurs, such as the chicken and alligator, suggest that cholinergic receptor tonic regulation is absent during embryonic development (Crossley and Altimiras, 2000; Crossley et al., 2005; Eme et al., 2011A). However, cholinergic function is possible as indicated by the presence of a baroreflex mediated bradycardia, which is absent without function cholinergic receptors (Altimiras and Crossley, 2000; Crossley et al., 2003B). Although the onset is variable, ranging from the final 30 to 15% of incubation in alligator and chicken, respectively, the capacity for cholinergic or vagal function is clear (Altimiras and Crossley, 2000; Crossley and Altimiras, 2000; Crossley et al., 2003B; Crossley and Altimiras, 2012; Eme et al., 2011A). An additional similarity between embryonic chickens and alligators is the presence of adrenergic tone, mediated through circulating catecholamines rather than through the CNS throughout ontogeny (Crossley and Altimiras, 2000; Eme et al., 2011A; Crossley and Altimiras, 2012; Eme et al., 2013A). Therefore, during embryonic environmental challenges, humoral regulatory systems may be heavily relied upon.

Environmental challenges during incubation alter the phenotype of the embryo and hatching, with marked impact of function cardiovascular phenotype. Development under environmental challenges produces smaller embryos and hatchlings (Packard et al., 1987; Packard et al., 2002; Dzialowski et al., 2002; Crossley and Altimiras, 2005; Delmas et al., 2008; Eme et al., 2011A,B; Crossley and Altimiras, 2012; Marks et al., 2013; Iversen et al., 2014). Specifically regulatory capacity of the cardiovascular
system is altered under these conditions, both adrenergic and cholinergic regulation of cardiovascular function suggest plasticity. Cholinergic input on heart rate, normally absent during embryonic development in the chicken embryo, is activated in hypoxic incubated embryos, similarly hypoxic incubation increases β-adrenergic tonus on heart rate in the hypoxic American alligator (*A. mississippiensis*) (Eme et al., 2011A; Crossley and Altimiras, 2012). Furthermore, hypoxic incubation of the common snapping turtle (*C. serpentina*) alters the balance of cholinergic and adrenergic tonus of heart rate (Eme et al., 2013B). However, these studies provide only a cursory understanding of developmental cardiovascular physiology. Thus further study is warranted to determine the basis for these differences to isolate source of the deviation. The focus of these studies was to assess regulatory system known to play a role in the cardiovascular response in adult vertebrates, in embryos of two archosaurs the domestic chicken (*G. gallus*) and the American alligator (*A. mississippiensis*). While the function of adrenergic receptors has been investigated, several other regulatory mechanisms have gone unexplored in non-mammalian vertebrates embryos, with the exception of recent studies.

Assessments of alternative regulatory effectors of cardiovascular function have begun to mount. Reptilian studies have documented the response to two components, histamine and adenosine have been both reported to serve in cardiovascular regulation in the red-footed tortoise (*Chelonoidis carbonaria*) and in the common snapping turtle (*C. serpentina*), respectively (Crossley et al., 2013; Eme et al., in prep). Whereas, studies in embryonic chickens (*G. gallus*) have focused on nitric oxide and angiotensin II, elevated plasma concentration of Ang II in normoxic incubated embryos and a
blunted response to nitric oxide donor, sodium nitroprusside in hypoxic incubated embryos, support a regulatory role for both systems in cardiovascular function (Crossley et al., 2010; Iversen et al., 2014). While these studies have provided important information, measurement following developmental hypoxia was only assessed for adenosine in the snapping turtle (C. serpentina) and nitric oxide in the embryonic chicken (G. gallus) embryos. Both of these effectors of cardiovascular function demonstrate an effect of developmental stress on cardiovascular responses. Findings presented in these studies encourage further study of the effects of stress during development.

The renin angiotensin system is critically involved in the cardiovascular response to multiple acute perturbations in the fetal sheep, thus was the primary focus of the first study determining the effects of dehydration during incubation. These were followed by investigations designed to tease apart the different components of the Ang II response and assess baroreflex function in dehydrated embryonic. This study was followed by an investigation of the effects of hypoxic incubation on the cardiovascular response to Ang II. Finally the function of adenosine in cardiovascular function was assessed following to chronic hypoxic incubation was determined.

This dissertation is divided into chapters based on hypotheses presented below.

1.1 Hypotheses Addressed in Chapter 2

Desiccation due to nest drying increases embryo mortality in crocodilians and reduces embryonic and hatchling mass in hole nesting turtles. Based on this alligator eggs exposed to dehydration events will be smaller than control embryos. The
chorioallantoic membrane, which serves as the gas exchange organ is located just beneath the eggshell, this location may promote water loss from circulation thus reducing blood volume in eggs exposed to dehydration events. Reduction in blood volume will increase cardiac work to move blood and increased work by the kidney to counter volume loss would increase the relative heart and kidney masses. Lastly dehydration events during embryonic development would intensify the cardiovascular response to Ang II injection due to increased reliance on this peptide to maintain embryonic cardiovascular function.

1.2 Hypotheses Addressed in Chapter 3

Embryonic alligators do not possess functional cholinergic tone on heart rate during incubation, while hypoxic incubation alters β-adrenergic tone, based on these characteristics the bradycardia in dehydrated embryos observed in chapter 2, is likely due to β-adrenergic withdrawal on the heart given plasticity evident in hypoxic incubated embryos. In adult vertebrates Ang II elicits the pressor response through both direct and indirect actions through secondary α-adrenergic receptor stimulation without changing heart rate due to Ang II inhibitory action on the vagal output. The findings from chapter 2 suggest that embryonic cardiovascular responses to Ang II are distinct from the adults, Ang II increases blood pressure similar to the adult response however, a reduction in heart rate is observed. Given Ang II has no direct chronotropic actions on the heart; this decrease is likely due to vagal stimulation, given alligator embryos possess a hypertensive baroreflex. Finally water loss without concurrent ion loss
increases blood osmolality, and hyperosmolality in rats decreases baroreflex sensitivity, thus dehydration will reduce sensitivity of baroreflex compared to control embryos.

1.3 Hypotheses Addressed in Chapter 4

Embryonic chickens maintain high circulating levels of Ang II, and fetal sheep increase circulating Ang II during acute hypoxia. Given the heavy reliance on Ang II in embryonic chickens and increased plasma concentration in fetal sheep following acute hypoxia, hypoxic incubation will decrease the pressor response to exogenous Ang I and II in chicken embryos due to higher circulating levels. Adult vertebrates and birds elicit α-adrenergic contribution to the Ang II pressor response, and hypoxic incubation increases plasma catecholamines. Normoxic but not hypoxic incubated embryos will augment the direct actions of Ang II through α-adrenergic stimulation, likely due to α-adrenergic receptor saturation. High plasma concentrations of Ang II in chicken embryos suggest an important role in cardiovascular regulation; inhibition of Ang II production through angiotensin converting enzyme inhibition will significantly reduce arterial pressure in embryos incubated in both normoxia and hypoxia.

1.4 Hypotheses Addressed in Chapter 5

Adenosine is a potent nucleoside that increases in ischemic or hypoxic tissues. Adenosine reduces vascular resistance and has direct actions on the sinoatrial and atrioventricular nodes to reduce heart rate. Additionally isolated atrial tissue from chicken embryos is responsive to adenosine as early as 30% of incubation presence through incubation. Further studies support an active role in the chicken embryo in
angiogenesis in the chorioallantoic membrane suggesting adenosine serves an active role in the cardiovascular system during embryonic development. Given the actions of adenosine on the cardiovascular system in embryonic chickens and adult vertebrates, adenosine will reduce heart rate and arterial pressure in embryonic chickens. Given the early chronotropic actions of adenosine, non-selective adenosine receptor blockade with theophylline will reveal a tonic contribution of adenosine to the baseline heart rate in normoxic embryos, and will be reduced in hypoxic embryos.

Techniques and experimental protocols have been discussed within each chapter. In addition, each chapter has been prepared as individual units to contain all components of published manuscripts.
CHAPTER 2

EFFECTS OF DEHYDRATION ON CARDIOVASCULAR DEVELOPMENT IN THE EMBRYONIC AMERICAN ALLIGATOR (*Alligator mississippiensis*)

2.1 Introduction

Water balance and regulation of water fluxes are critical during *in ovo* or fetal development, and environmental perturbations can significantly impact morphological and physiological maturation of the organism (Ross and Desai, 2005). Dehydration in adult amniotic vertebrates initiates numerous homeostatic mechanisms in an attempt to maintain osmotic concentration and blood volume, such as arginine vasopressin (vasotocin), mineral corticosteroids, and angiotensin II (Ang II) (Nouwen, 1984). These components have been identified in numerous adult vertebrate taxa; however, limited information is available on their function during the prenatal or embryonic period of life (Robilliard et al., 1979; Takei, et al., 1993; Oudit and Butler, 1995). Environmental stressors such as chronic hypoxic or dehydration during incubation have been shown to alter embryonic cardiovascular physiology, reduce embryo mass and increase relative heart mass in both American alligator (*Alligator mississippiensis*) and common snapping turtle embryos (*Chelydra serpentina*) (Packard et al., 2002; Crossley and Altimiras, 2005; Eme et al., 2011A). However the specifics of how periods of egg water loss change key parameters such as cardiovascular function and blood properties remain unknown.

Reptiles differ from avian species due to nesting strategies of the females; with reptilian eggs frequently placed within detritus or soil, as well as the relatively increased

shell porosity of reptilian eggs that increases water flux rates (Packard et al., 1982; Mazzotti et al., 1988). In addition, previous studies have demonstrated that rigid shelled reptile eggs, like crocodilian eggs, do not recover water following its removal, and that water necessary for development is present upon laying (Packard et al., 1982; Grigg and Beard, 1985). This has been previously recognized for the American crocodile (Crocodylus acutus) egg, which may be exposed to both acute and chronic desiccating conditions as a consequence of oviposition in underground or mound nests (Mazzotti, et al., 1988; Packard, 1991; Ackerman and Lott, 2004). Given these characteristics, during periods of environmental drought and associated nest water loss, the developing embryo may experience periods of reduced water availability. Previous investigations have focused on morphological changes associated with these periods of water stress in the C. serpentina embryos, which show lowered embryonic body mass and increased residual yolk mass (Packard et al., 1987; Packard et al., 2002). However, the physiological and morphological consequences of drying in embryonic American alligator are unknown.

Given the anatomical changes previously reported for embryonic C. serpentina, it is conceivable that homeostatic mechanisms in American alligators attempt to buffer the impact of dehydration, particularly those mechanisms that regulate cardiovascular function. Angiotensin II circulates in embryonic chickens (Gallus gallus) in higher concentrations than adults, and also plays a role in the response to hemorrhage in fetal sheep (Gomez and Robillard, 1984; Crossley et al., 2010). If Ang II activity during development is conserved in vertebrates, it may also aid embryonic alligators in maintaining cardiovascular function during water loss. Currently, our understanding of
Ang II function in egg laying amniotes is based on chickens, whose embryos display a typical vertebrate pressor response to bolus injections of Ang II that increase in responsiveness with incubation (Crossley et al., 2010). This peptide may also be critical in embryonic American alligator, given the potential for natural hydration stress, limited cardiovascular regulation capacity, and close phylogenetic relationship to birds.

We examined morphological and physiological responses of American alligator embryos to dehydration, and we predicted that water loss from eggs would compromise normal embryonic development and physiology. Our first objective was to determine if dehydration alters embryonic growth in the alligator, and our second objective was to assess the response to arterial Ang II injection. We hypothesized that exposure to dehydration events during embryonic alligator development would intensify the response to Ang II injection due to increased reliance on this peptide to maintain embryonic cardiovascular function. In addition, we hypothesized that dehydration events would reduce embryonic mass and blood volume and increase relative heart and kidney masses.

2.2 Materials and Methods

2.2.1 Alligator Embryo Acquisition and Incubation

American alligator eggs (Alligator mississippiensis; N = 50 eggs from 7 clutches) were collected from wild nests at the Rockefeller Wildlife Refuge in Grand Chenier, LA, USA by Dr. Ruth Elsey. Two eggs from each clutch were used for staging, according to Ferguson (1985), to accurately establish the initial percentage of incubation (total incubation period 72 days at 30 °C). Eggs were weighed, numbered, and transported by
car to the laboratory. Upon arrival, eggs were randomly distributed into Ziploc® boxes (1L, containing fine vermiculite mixed with water at a 2 g vermiculite: 1 g water ratio; Crossley and Altimiras, 2005). Each box was placed inside a large Ziploc® bag (100 L) with fully water-saturated air passed through the bag at a rate of 1-2 L min⁻¹. Water content of the vermiculite, determined by mass at the beginning of the study, was maintained by weighing the box twice weekly, with water added as needed. Eggs were maintained in an environmental chamber at 30 °C (Percival model #: I-66LLVL, Perry, IA), ensuring that all embryos were developing as females (Ferguson, 1985; Ferguson and Joanen, 1982).

2.2.2 Alligator Embryo Dehydration Protocol

Eggs were randomly divided between dehydration and control groups. Dehydrated eggs (n = 25) were exposed to dehydration condition, which consisted of individual acute dehydration events at 30%, 40%, 50% and 60% of embryonic development/incubation based on initial staging (Ferguson, 1985). During each acute dehydration event, egg mass was reduced by 5%, with the 5% loss calculated prior to each dehydration event. For each dehydration event, eggs were placed on plastic racks above Drierite (W.A. Hammond Drierite Co., Ltd., Xenia, OH, USA) and returned to the 30 °C chamber (Percival model #: I-66LLVL, Perry, IA). Egg mass was determined every 12 hours until 5% pre-dehydration egg mass was lost. Following each dehydration event, eggs were returned to their respective box, containing vermiculite mixed with water at a 2:1 ratio, as above. Control embryos (n = 25) did not undergo any
dehydration events and remained in control conditions until removed for experimentation.

At 70% and 90% of incubation, eggs were removed from incubation and candled to locate an accessible tertiary chorioallantoic membrane (CAM) artery. Eggs were placed in a temperature-controlled surgical chamber (30 °C), and a portion of the eggshell removed under a dissection microscope (Leica MZ6 or MZ3; Leica Microsystems, Waukegan, IL, USA). A tertiary CAM artery was isolated for arterial pressure monitoring and drug injection see below. The isolated artery was catheterized using heat-pulled, heparinized and saline-filled PE-50 tubing, and fixed to the eggshell with cyanoacrylic glue, as previously described (e.g. Crossley and Altimiras, 2005; Eme et al., 2011A). Following catheterization, embryos were transferred to a four-chamber (730 ml chamber volume, with one embryo per chamber placed on cotton), water-jacketed, stainless steel experimental apparatus and allowed to recover for at least 1 hr. Temperature (30 °C) was maintained throughout the apparatus’ chambers by a circulating water bath (VWR 1165; VWR International, LLC, West Chester, PA, USA). Each chamber had a stainless steel lid, with three small holes that allowed for the catheter line (1x 5 mm³) and air-lines (2 x 3 mm³) to enter each chamber. Room air warmed to 30 °C was delivered into each chamber from an aquarium pump (each chamber, 200 ml·min⁻¹).

The arterial catheter was attached to a pressure transducer (ADInstruments disposable transducer, Colorado Springs, CO, USA) connected to a bridge amplifier (4-Octal bridge, AD Instruments) and calibrated against a vertical saline column between 0 kPa, level with the transducer, and 1 kPa, set 10 cm above the transducer. Chart
acquisition software (v 5.4.2 ADInstruments) was used to record arterial pressure. Voltage output signals were converted with a data acquisition system (ML 785, Powerlab/8SP) and stored in a computer for analyses. Absolute blood pressure was corrected by adding the measured position of the egg below the transducer to record pressure (kPa).

Pressure transducers were calibrated for pressure prior to each measurement period, and heart rate was calculated from the arterial pressure trace using Chart acquisition software. Total injection volumes were normalized for each embryonic age to an estimate of <5% of total blood volume, with drugs administered through a T connector in the arterial catheter line (~35 μl, 70%; ~50 μl, 90%), and each drug injection was flushed with saline (~70 μl, 70%; ~100 μl, 90%) to ensure the drug had entered the CAM artery (Crossley and Altimiras, 2005). Following recovery, embryos received a control injection of heparinized saline into their catheter, with the volume identical to each injection volume (drug plus saline flush).

2.2.3 Blood Volume in Response to Dehydration

In the first groups of embryos, blood volume was determined at 70% (n = 6, each) and 90% (n = 7 control, n = 5 dehydrated) of development, using a modified method previously described (Hillman et al., 2010). In each embryo following catheterization, a 20 μl control blood sample was taken by allowing the animal to bleed back through the catheter into a heparinized 40 μl hematocrit tube (Drummond Scientific, Broomall, PA). Subsequent to the control sample, adult alligator plasma bound to Evans Blue Dye (‘EB’; 2.0 mg ml⁻¹; Sigma, St. Louis, MO) was injected through
the catheter into the CAM circulation. The volume injected (100 ± 1 µl) was followed with enough saline (20 µl) to completely flush the dye into circulation. At successive, 2 min intervals after dye injection, 20 µl blood samples (n = 8) were collected as above. Blood samples were centrifuged at 11,500 rpm (IEC MB Centrifuge, model# M.B., Needham HTS, Mass). Hematocrit was estimated (± 0.1%) using Mitutoyo digital calipers (± 0.01 mm; Aurora, IL, USA), and the absorbance of dye in the blood sample determined using a ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, DE) at 620 nm. Following completion of a protocol, the embryo was euthanized with an overdose of isoflurane (Isoflo; Abbott Laboratories, North Chicago, IL, USA).

Blood volume was calculated for each individual embryo using the mean of triplicate absorbance readings converted into dye concentration using a standard curve generated using a 2 mg ml⁻¹ dye plasma solution (adult alligator plasma; Gibson and Evans, 1937). Plasma volume was determined using the known amount of dye injected (mg), divided by the dye concentration in the plasma (mg ml⁻¹) to produce the plasma volume (ml) according to equation 1. Total blood volume (ml) was calculated by dividing plasma volume (ml) by one minus the hematocrit (Hct), according to equation 2.

\[
1) \text{Plasma volume (ml)} = \frac{EB \text{ injected (mg)}}{[EB]_{\text{plasma}}}
\]

\[
2) \text{Blood volume (ml)} = \frac{\text{Plasma volume (ml)}}{(1 - \text{Hct})}
\]

**CAM mean arterial pressure response to exogenous, native Angiotensin II**

In a second group of embryos, after the 1 hr recovery period from catheterization (above) blood pressure and heart rate reached stable control values. Control (no dehydration events) and dehydrated embryos were sampled at 70% (n = 6, both groups) and 90% (n = 7 control; n = 6 dehydrated) of incubation. The experimental
protocol (identical for both 70% and 90% of incubation) consisted of five sequential bolus injections of synthetic [Asp1, Val5]-Angiotensin II ('Ang II'; synthesized by J.M. Conlon; Takei et al., 1993) in ascending order at 100 ng kg⁻¹, 250 ng kg⁻¹, 500 ng kg⁻¹, 1000 ng kg⁻¹ and 2000 ng kg⁻¹. Before any successive dose of Ang II was administered embryos were allowed to recover from the previous dosage for at least 1 hr.

Following completion of a protocol, the embryo was euthanized with an overdose of isoflorane and embryonic wet mass, wet heart mass (combined atria and ventricles), wet kidney mass (combined left and right kidney) and yolk mass were determined ± 0.001 g using an analytical balance (Mettler Toledo XS204). Tissues were subsequently flash frozen and stored at -80°C.

2.2.4 SDS PAGE and Western Blot Analysis

Frozen 70% (n = 6 control; n = 6 dehydrated) and 90% of incubation (n = 6 control; n = 6 dehydrated) embryonic alligator hearts were assessed for presence of the Ang II type one receptor (AT1), via western blot analysis in a protocol adapted from Darland and D’Amore (2001). Frozen alligator hearts were disrupted in ice cold PBS (0.5 ml ph = 7.2), Protease inhibitor (6 μl; Protease inhibitor cocktail; Sigma Aldrich, St. Louis, MS) and 5X lysis buffer (25 μl; 25 mM CHAPS, 1 M HEPES, 0.5 M DTT) with a mechanical homogenizer. The sample lysate protein concentration was determined using a modified Bradford colorimetric assay according manufacturer’s microassay protocol (Bio-Rad, RC DC protein assay kit II, model #500-0122, Hercules, CA). 12 μl (20 μg of each sample) samples were loaded into 4-20% Tris HCl precast gel (Bio-Rad, Hercules, CA). Sizes of proteins were verified with Precision plus protein standard (Bio-
Rad, Hercules, CA). Gels were electrophoresed for 2.5 hr. at 75 V, and separated proteins were transferred to an immuno-blot polyvinylidene fluoride (PVDF; Millipore, Billerica, MA) membrane at approximately 250 mA for 70 min.

The primary antibody AT1 rabbit polyclonal IgG (SC-1173, lot# J2909, Santa Cruz Biotechnology, Santa Cruz, CA), was applied to the membrane at a 1:500 dilution for one hour. The secondary antibody alkaline phosphatase conjugated Donkey-α-rabbit polyclonal IgG (# 711-055-152, lot # 77662, Jackson ImmunoResearch, West Grove, PA) was applied using a 1:15000 dilution. Loading control followed the previously stated procedure, with mouse -α-tubulin monoclonal IgG (# T5168 Sigma Aldrich, St. Louis, MO) at a 1:10,000 dilution. The secondary antibody was applied at a dilution of 1:20,000 alkaline phosphatase conjugated Donkey-α–mouse monoclonal IgG (#715-055-150, lot# 78244, Jackson ImmunoResearch, West Grove, PA). Membranes were rinsed in Tris MgCl₂ followed by incubation in CDPstar (Applied Biosystems, Carlsbad, CA). Immunolabeled membranes were imaged using Biospectrum® imaging system (Upland, CA), the resulting images were imported into Adobe Photoshop and densitometric analysis was conducted using NIH Image J software.

2.2.5 Data Analyses and Presentation

We used a two-way analysis of variance (ANOVA) for incubation percentage and dehydration effects on morphological and physiological traits. Significant ANOVA results were followed by Student Newman Keuls (SNK) post hoc comparisons to identify groups that differed significantly from each other. A two-way Analysis of covariance (ANCOVA) was used for incubation percentage and dehydration to compare protein
expression for AT1 with alpha tubulin protein expression levels as the covariate. Results were deemed significant when the p-value for a statistic was less than or equal to 0.05 \((\alpha < 0.05)\).

For comparisons of the magnitude of a change in heart rate or blood pressure between incubation percentage and dehydration condition in response to delivery of Ang II; individual data were arcsine square root transformed. Transformations of data for individual embryos consisted of calculating the magnitude of change in heart rate or pressure from baseline as the corresponding decimal proportion of the percentage change (equation 3; i.e., 0.2 from 20%). The square root of the proportion, from equation 3, was arc sin transformed (equation 4) and then converted into degrees (not shown). Separate two-way repeated measures ANOVA (RM-ANOVA) were used to assess the response for arc sin transformed changes in arterial pressure \((P_m)\) and \(f_H\) to Ang II, and dehydration and incubation percentage served as the independent variables with \(P_m\) and \(f_H\) as the dependent variables respectively followed by SNK. Throughout the manuscript, all statistical decisions are made based on \(\alpha = 0.05\), and means are presented \pm standard error of the mean (SEM). All statistical inferences were determined with using Statistica v.9.0 (StatSoft, Tulsa, OK, USA).

3) Proportional change = mean Δ from pre-injection value / mean pre-injection value.

4) Radians = arcsine * (square root (proportional change))

2.3 Results

2.3.1 Morphological Responses to Exposure to Dehydration Events
Eggs exposed to dehydration lost a total of 14.43 ± 0.37 g, approximately 20.56% of the original egg mass from 20% to 70% of incubation, whereas control eggs lost a total 4.28 ± 1.41 g, approximately 6.7% of original egg mass. Embryonic mass was significantly reduced by dehydration exposure and in both groups increased with incubation percentage, (F_{1,46} = 49.69 or 182.13, respectively, P < 0.001). In addition the groups grew differently, with those exposed to the dehydration events changing less from 70 to 90% compared to the controls (Table 2.1). Embryos ranged from 26% (70% of incubation) to 36% (90% of incubation) smaller than control embryos on average.

Residual yolk mass significantly decreased with progressive incubation (F_{1,46} = 155.00, P < 0.001) but was unaffected by dehydration condition and the interaction between the two (Table 2.1). Relative heart mass (mg g\(^{-1}\)) significantly decreased across incubation percentage (F_{1,46} = 9.68, P < 0.05) and did not differ between the experimental groups but at 90% of incubation the dehydration group's relative heart mass showed a trend towards increased size compared to the control at 90% of incubation (Table 2.1). Relative kidney mass (mg g\(^{-1}\)) was significantly affected by dehydration (F_{1,46} = 4.6, P < 0.05), but did not differ between groups; however further delineation with SNK post hoc comparison failed to reveal distinct groups (Table 2.1).

Hematocrit of dehydrated embryonic alligators was not significantly altered by dehydration condition or incubation percentage (Table 2.2). Mass specific blood volume was significantly increased by dehydration condition and decreased with incubation percentage (F_{1,20} = 13.32 and 10.46, respectively P < 0.01), however, the pattern of change in blood volume from 70 to 90% of incubation did not differ between groups. Dehydration had a significant effect on mass-specific blood volume, with dehydrated
embryos at 70% of incubation having a greater volume (Table 2.2), whereas at 90% of incubation embryos’ blood volumes were statistically similar values.

2.3.2 Resting Cardiovascular Parameters

Arterial pressure $P_m$ significantly increased with incubation percentage ($F_{1,21} = 21.87, P < 0.001$) but dehydration did not have a significant impact on resting arterial pressure (Fig. 2.1A). Resting heart rate was unchanged across the period of incubation studied however by dehydration resulted in a significant bradycardia at 90% of incubation ($F_{1,21} = 24.87, P < 0.001$) (Fig. 2.1B).

2.3.3 CAM Mean Arterial Pressure Response to Native Angiotensin II

Incubation percentage ($F_{1,21} = 22.15, P < 0.001$), but not dehydration had a significant effect on the $P_m$ response to Ang II. Increasing concentrations of Ang II injection resulted in significant increases in $P_m$ from both dehydration and incubation percentage ($F_{5,105} = 2.39$ or $5.04$ respectively, $P < 0.05$). Injection of Ang II caused a rise in $P_m$ followed by a return to baseline in both 70% and 90% embryos (Fig. 2.2). At 70% of incubation, this response was only elicited by 2000 ng kg$^{-1}$ (Fig. 2.2, 2.3A), whereas at 90% of incubation 250 ng kg$^{-1}$ elicited a significant increase in $P_m$ (Fig. 2.3A) with increasing intensities until the 1000 ng kg$^{-1}$ dose. At all doses at and above 500 ng kg$^{-1}$ in both 90% groups only, a significant bradycardia was caused by Ang II injections just prior to the peak pressure response (Fig. 2.2B, 2.3B).

SDS PAGE and Western blot analysis
AT1 receptor protein concentration did not differ with dehydration, incubation percentage (P > 0.1; Table 2.3). Quantification of α-tubulin assured consistent protein loading (P > 0.1). Presence of AT1 receptor in alligator embryonic cardiac tissue was verified as early as 70% of incubation.

2.4 Discussion

Adult vertebrates possess the ability to regulate blood volume in response to dehydration, and the typical dehydration response consists of multiple regulatory mechanisms that aid in water conservation and replenish body water (McCormick and Bradshaw, 2005). During embryonic development, control of the cardiovascular system is immature (Crossley and Altimiras, 2000; Fritsche et al., 2000; Crossley et al., 2010), and environmental challenges like dehydration could alter the development of this system, which could decrease fitness following hatching. Our findings demonstrate that embryonic dehydration reduces embryonic alligator size, increases blood volume and decreases resting f_H. However, the response to Ang II and the abundance of the AT1 receptor appear unaffected by this developmental stress.

Dehydration dramatically reduced embryonic body mass, consistent with previous findings from both in ovo and placental vertebrates, and demonstrated that embryonic growth is greatly hindered by water stress (Packard et al., 2002; Ross and Desai, 2005). Dehydration events severely reduced embryo mass at both 70% (≈26%) and 90% (≈36%) of incubation (Table 2.1), findings similar to those reported for embryonic snapping turtles incubated in dry nesting conditions (Packard et al., 2002). A reduction in embryo mass leading to decreased hatchling mass (not measured here)
could greatly affect animal fitness, potentially reducing future reproductive success. There were trends for increased relative heart and kidney masses; however, statistical post hoc comparisons did not identify a clear trend for increased relative mass with dehydration. Dehydration stress could have adversely affected whole embryo growth in several ways, including combinations of the following. Water stress could prevent normal nutrient extraction from the yolk leading to reduce embryonic growth (Packard and Packard, 1985), or it could limit overall cellular growth in a constant fashion, as suggested by the relatively unchanged mass-specific organ masses of dehydrated embryos (Ditmarová, et al., 2009). Preferential water loss from the fluid compartments within the egg may have buffered the water stress experienced by embryonic alligators.

The egg is composed of several fluid compartments that could be depleted of water – including the allantois, amnion, and the embryonic intracellular and extracellular spaces including the blood (Hoyt, 1979). Fluid loss from blood volume undoubtedly occurs during the drying events due to the close proximity of the nest environment to the CAM, a structure that serves an osmoregulatory and respiratory function during in ovo development (Tazawa, 1980). During normal embryonic development, the CAM can draw water stored in the allantois (Hoyt, 1979). The allantois actively transports sodium ions into the plasma present in the CAM circulation, creating a relatively hypotonic allantoic fluid and promoting water flux into the plasma (Stewart and Terepka, 1969). This mechanism serves to mobilize water stores for normal embryonic development and may allow blood volume to remain unchanged in dried eggs (Hoyt, 1979). We hypothesized that overall egg water loss would be reflected in a reduction in blood volume. However, embryos at 70% of incubation responded to dehydration stress by
increasing blood volume approximately two fold compared to control embryos (Table 2.2). This may suggest that embryos are volume loading in response to dehydration stress, increasing plasma ions (not measured here) and facilitating water movement into the plasma.

Increasing blood volume was most likely offset by changes in vascular resistance, given that $P_m$ was the same between experimental groups (Fig 2.1A). This mechanism may be maximized at 70% of incubation, given that no volume difference was found at 90% suggesting an early compensatory response had reached an upper limit. Local hypoxia during development increases vascular proliferation in the alligator CAM; suggesting environmental perturbations have the capacity to alter the size of the vascular tree (Corona and Warburton, 2000). Increasing the size of the CAM vasculature could promote increased water movement from the allantois into the CAM circulation. Alternatively, the increased replacement of plasma volume caused by dehydration may have induced an overcompensation of water uptake, thus requiring expanding the vascular tree to accommodate the increase in volume without altering $P_m$.

Dehydrated alligator embryos were smaller, but maintain mass-specific blood volume and Hct despite having ≈20% of the egg mass removed as water. This finding is similar to that reported for embryonic chickens when 20% of egg mass is depleted through dehydration resulting in an elevation of plasma ion levels (Hoyt, 1979; Davis et al., 1988). While we did not measure the allantoic fluid within eggs, alligator embryos could maintain blood volume through a similar mechanism as chickens (Hoyt, 1979). In addition, embryonic chickens experiencing plasma disturbance quickly returned to pre-
disturbance levels suggesting very tightly regulated control of blood volume (Adolph, 1967). Our blood volume data for alligator embryos are similar to those of previously documented in embryonic chickens (Yosphe-Purer et al., 1953) and both species exhibit elevated levels compared to adult vertebrates. As incubation progresses, blood volume falls to values more similar of typical 5%-9% body mass estimates of adult reptiles and birds (Reynolds, 1953; Huggins and Percoco, 1963; Chien et al., 1973; Carmena-Sureo et al., 1978; Smits and Kozubowski, 1984).

Dehydrated alligators were bradycardic at 90% of incubation, and this heart rate depression could represent increase in vagal tone or a withdrawal of β-adrenergic tone. Depression of embryonic heart rate in dehydrated embryos due to increasing cholinergic activity is not likely, given alligator embryos do not respond to atropine injection and lack a vagally mediated influence on heart rate through 90% of incubation (Eme et al., 2011A). Embryonic chickens increase β-adrenoreceptor mediated relaxation in CAM arteries in response to chronic hypoxia, and alligators also increase β-adrenoreceptor tone on heart rate following chronic embryonic hypoxia (Eme et al., 2011A; Lindgren et al., 2011). Therefore, it is possible that dehydration altered normal β-adrenergic development. The observed bradycardia could be related to an interaction between altered β tone on the vasculature and heart (Eme et al., 2011A), or possibly chronic overstimulation of cardiac β-adrenoceptors was necessary to maintain perfusion of an increased vascular tree and this led to chronic bradycardia due to acquired receptor insensitivity.

Ang II produced an increase in $P_m$ that increased in intensity from 70 to 90% of incubation (Fig. 2.2A, 2.3A). The renin-angiotensin system (RAS) is a critical component
of blood volume regulation in adults, primarily acting through Ang II to mitigate the impact of blood volume loss. Blood volume loss is buffered by decreased sodium excretion via stimulation of aldosterone secretion from the adrenal cortex, as well as through increased water reabsorption by constriction of the glomerular efferent arteriole (Hall et al., 1977). Recent work in embryonic chickens demonstrated that components of the RAS are functional at 50% of development, a characteristic that may be shared by archosaurs (i.e., birds and crocodilians; Crossley et al., 2010). Embryonic American alligators exhibit a response similar to chickens, with a dose dependent increase in the intensity of the P$_m$ to Ang II (Fig. 2.3A). This pressor response at the highest dose was similar in intensity to that seen in adult alligators, spectacled caimans (Caiman crocodilus), quail (Coturnix japonica) and rats (Sprague-Dawley) (Takei et al., 1993; Butler, 2005). However, while the response to Ang II was present, the intensity of the physiological response was unaffected by dehydration. While additional studies targeted at quantifying the Ang II tone on resting cardiovascular function are needed, this finding does suggest that unlike other aspects of cardiovascular regulation in embryonic American alligators, the response to Ang II is relatively non-plastic in response to severe dehydration stress.

The lack of physiological plasticity of the Ang II response extends to the translational level for the AT-1 receptor and resulting protein concentration, which was constant in cardiac tissue under both dehydrated and control conditions (Table 2.3). But, AT-1 receptor protein was also constant across the final 30% of incubation, which contradicts the observed age-dependent increase in intensity of the Ang II response (Fig. 2.3). This finding is similar to that reported for embryonic chickens, which maintain
constant AT1 mRNA expression as the physiological response to Ang II matures with incubation age (Crossley et al., 2010). As previously suggested, this lack of correlation may indicate significant changes in Ang II intracellular signaling mechanisms or maturation of the contractile apparatus (Crossley et al., 2010). The increasing intensity of the Ang II response suggests an increasing role in cardiovascular regulation prior to hatching in the embryonic American alligator, although further investigation is necessary to determine the role in the cardiovascular regulation during embryonic development.

2.5 Summary

This study represents the first assessment of the morphological and physiological effects of desiccation during development of the American alligator. Dehydrated embryos were markedly smaller, bradycardic, and exhibited a transient increase in blood volume. The precise basis for the reduced body mass in response to dehydration could include a whole body limit on cellular growth, altered transcriptome or insufficient yolk mobilization (Packard and Packard, 1985; Ditmarová, et al., 2009). Dry masses were not measured in embryos; however, the difference between wet embryo masses was similar to the water lost during drying events. While embryos demonstrated the ability to control blood volume early in incubation in response to dehydration, neither the response to Ang II nor AT1 quantification demonstrated increased reliance on the main product of the RAS, Ang II. Embryos may rely on other blood volume regulatory control components, such as AVT to maintain blood volume. Although the regulatory response to dehydration was not altered, embryos were able to maintain blood volume, but endured a cost of reduced embryo mass.
Table 2.1: Morphological measurements at 70 and 90% of incubation (Devo) in response to dehydration in embryonic alligator for control (C) and dehydrated (D) embryos, including embryo wet mass, residual yolk mass, heart mass (mg), mass specific heart mass (mg g\(^{-1}\)), kidney mass (mg), and mass specific kidney mass (mg g\(^{-1}\)). Similar Latin letters indicate values that are not significantly different according to post-hoc comparison. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Devo (%)</th>
<th>Condition (n)</th>
<th>Embryo mass (g)</th>
<th>Yolk mass (g)</th>
<th>Heart mass (mg)</th>
<th>Heart mass (mg g(^{-1}))</th>
<th>Kidney mass (mg)</th>
<th>Kidney mass (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 C (13)</td>
<td>14.17 ± 0.85(^{A})</td>
<td>23.15 ± 0.73(^{A})</td>
<td>69.3 ± 4.5</td>
<td>4.94 ± 0.22(^{A})</td>
<td>113.4 ± 11.5</td>
<td>8.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>70 D (13)</td>
<td>10.49 ± 0.91(^{B})</td>
<td>23.04 ± 0.91(^{A})</td>
<td>49.2 ± 4.2</td>
<td>4.82 ± 0.30(^{A})</td>
<td>96.8 ± 14.1</td>
<td>9.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>90 C (13)</td>
<td>32.31 ± 1.27(^{C})</td>
<td>11.64 ± 1.00(^{B})</td>
<td>122.7 ± 3.6</td>
<td>3.82 ± 0.10(^{B})</td>
<td>264.2 ± 26.6</td>
<td>8.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>90 D (11)</td>
<td>21.04 ± 1.37(^{D})</td>
<td>14.05 ± 0.84(^{B})</td>
<td>93.3 ± 7.5</td>
<td>4.42 ± 0.16(^{A,B})</td>
<td>264.7 ± 60.3</td>
<td>12.3 ± 2.3</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2: Hemodynamic variables at 70 and 90% of incubation in response to dehydration in the embryonic alligator for control (C) and dehydrated (D) embryos, including hematocrit, blood volume and embryo mass specific blood volume. Latin letters denote SNK post-hoc comparisons within the mass specific blood volume column. Similar letters indicate values that are not significantly different, and dissimilar letters indicate values are significantly different according to post-hoc comparison.

Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Devo (%)</th>
<th>Condition (n)</th>
<th>Hematocrit (%)</th>
<th>Blood Volume (ml)</th>
<th>Mass Specific Blood Volume (ml g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>C (7)</td>
<td>34.6 ± 1.0</td>
<td>3.47 ± 0.47</td>
<td>0.221 ± 0.031⁴A</td>
</tr>
<tr>
<td>70</td>
<td>D (6)</td>
<td>37.3 ± 2.6</td>
<td>3.29 ± 0.39</td>
<td>0.386 ± 0.044⁸</td>
</tr>
<tr>
<td>90</td>
<td>C (7)</td>
<td>30.9 ± 1.8</td>
<td>5.93 ± 0.66</td>
<td>0.178 ± 0.017⁴A</td>
</tr>
<tr>
<td>90</td>
<td>D (5)</td>
<td>33.4 ± 2.9</td>
<td>5.91 ± 1.07</td>
<td>0.236 ± 0.040⁴A</td>
</tr>
</tbody>
</table>

Table 2.3: AT1 quantification in dehydrated (D) and control (C) embryonic alligator hearts at 70% and 90% of incubation. Data for AT1 and α-tubulin levels represent the number of pixels generated from the gel image in the Image J densitometric analysis. The densitometric analysis indicated no difference in AT1 levels, with α-tubulin as the co-variable. Data presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Devo (%)</th>
<th>Condition (n)</th>
<th>AT1</th>
<th>α -tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>C (6)</td>
<td>2848 ± 371</td>
<td>6306 ± 331</td>
</tr>
<tr>
<td>70</td>
<td>D (6)</td>
<td>3261 ± 607</td>
<td>6830 ± 218</td>
</tr>
<tr>
<td>90</td>
<td>C (6)</td>
<td>2685 ± 602</td>
<td>4892 ± 654</td>
</tr>
<tr>
<td>90</td>
<td>D (6)</td>
<td>3851 ± 838</td>
<td>6059 ± 385</td>
</tr>
</tbody>
</table>
Fig. 2.1: CAM mean arterial pressure ($P_m$; kPa) (A) and heart rate ($f_H$; beats min$^{-1}$) (B) for control (open bars) and dehydrated embryos (closed bars) at 70% and 90% of incubation. Similar Latin letters indicate values are not significantly different, and dissimilar letters indicate values are significantly different according to post-hoc comparison. Data are presented as mean ± SEM.
Fig. 2.2: Representative arterial pressure (kPa) and heart rate (beats min$^{-1}$) traces in response to Ang II (2000 ng kg$^{-1}$) in control embryos at 70% (a) and 90% (b) of incubation. Arrows represent the injection of Ang II.
Fig. 2.3: Ang II induced change in arterial pressure ($P_m$; kPa) (A) and heart rate ($f_H$; beats min$^{-1}$) (B) for control (open symbols) and dehydrated (closed symbols) embryos at 70% (triangles) and 90% (diamonds) of incubation. Similar Latin letters indicate values that are not significantly different. Data are presented as mean ± SEM.
CHAPTER 3

DEHYDRATION DURING INCUBATION INDUCES CHOLINERGIC TONE ON HEART RATE IN AMERICAN ALLIGATOR (*Alligator mississippiensis*) EMBRYOS

3.1 Introduction

Dehydration or low water availability during fetal/embryonic life has a major impact on whole organism growth as evident in birth mass of newborns from dehydrated rats (Desai et al., 2005). An animal that develops *in ovo* lacks the maternal buffer of placental mammals and therefore may have increased susceptibility to changes in environmental conditions, (Packard et al., 1982; Packard et al., 1987; Mazzotti et al., 1988). This may be particularly important in reptilian species such as the crocodilian, that lay eggs that are less resistant to water flux compared to birds, (Packard et al., 1982; Mazzotti et al., 1988; Packard, 1991). Multiple investigations of different turtle species during development have reported smaller sized animals from lower water content soils (Packard et al., 1987; Packard et al., 2002; Delmas et al., 2008). These studies were conducted on species with flexible shelled eggs, which allow water to readily move in and out of the egg, thus water can be regained (Packard et al., 1982). In rigid shelled eggs, like that of crocodilians, water reabsorption occurs at a very low rate and embryo mass is significantly reduced following water loss (Packard et al., 1982; Tate et al., 2012). A prior study demonstrated that egg water-loss causes morphological changes of embryonic alligators, however, this impact on cardiovascular regulation in American alligator embryos is unknown.

Dehydration in adult vertebrates elicits several mechanisms to restore volume lost, and to maintain blood pressure (Burnier et al., 1983; Brooks et al., 2005).
Regulatory control of cardiovascular function following water loss to maintain adequate blood flow to developing organs in embryonic/fetal life is undoubtedly important. Dehydration in adult rats increases plasma renin, arginine vasopressin, and norepinephrine contribution to the maintenance of arterial pressure (Burnier et al., 1983; Brooks et al., 2005). However a systematic investigation of cardiovascular regulation during water loss in egg laying vertebrates, including alligators, have just recently been undertaken.

The autonomic nervous system is critical for acute regulation of cardiovascular function during bouts on fluctuating blood volume and the associated blood pressure, varying the strength of parasympathetic and sympathetic tone on the cardiovascular system as well as altering renal function to restore blood volume during dehydration (Korner et al., 1967; Clement et al., 1972; Batchinsky et al., 2007). Rapid reduction in blood pressure due to volume loss elicits regulatory systems to correct the change, including the renin angiotensin system (RAS) activation and baroreflex. As an example dehydration or hemorrhage in adult birds decreases arterial pressure increasing the RAS and initiates other volume retention mechanisms (Ploucha et al., 1981; Ploucha and Fink, 1986; Gray and Erasmus, 1989).

In adult and fetal vertebrates the RAS acts primarily through the action of Ang II, an octapeptide produced through a series of cleavage events, serving to maintain arterial pressure and return blood volume to pre-volume loss conditions. Fetal sheep respond to hemorrhage with an increase in plasma Ang II causing an increase in arterial pressure (Iwamoto and Rudolph, 1981; Robillard et al., 1982). Further, embryonic chickens (Gallus gallus) maintain high levels of circulating Ang II during incubation
Interestingly chicken embryos may be able to tolerate up to 20% decreases in egg water content suggesting that the initial water volume when laid is sufficient to buffer changes that can occur during incubation (Davis et al., 1988). However, eggs of the American alligators are more porous than birds and may be more susceptible to changes in the hydric conditions of the nest (Packard et al., 1982).

Dehydration during embryonic development of the alligator embryos produces a markedly smaller and bradycardic embryo (Tate et al., 2012). A single study investigated the response to Ang II in embryonic alligators and concluded that while the embryonic cardiovascular system responds to Ang II, water-loss did not affect this action (Tate et al., 2012). While this initial study provided basic information regarding the Ang II response during embryonic alligator development additional questions remain. In adult vertebrates Ang II alters cardiovascular function via direct and indirect actions (Nishimura et al., 1994; Bernier and Perry, 1997). The direct cardiovascular actions of Ang II include receptor-mediated vasoconstriction of vascular smooth muscle, whereas indirect actions include stimulation of secondary release of catecholamines from the adrenal medulla (chromaffin tissue in fish), and sympathetic nerve terminals further accentuating direct effects of Ang II in adult chickens (Nishimura et al., 1994; Bernier and Perry, 1997). The extent to which these systems contribute to the Ang II response in embryonic animals including American alligators and its contribution to long-term cardiovascular homeostasis is unknown.

Cardiovascular homeostatic mechanisms that buffer rapid changes in blood pressure such as the baroreflex may also contribute to the embryonic response to dehydration. The baroreflex is a reciprocal relationship between arterial pressure and
the stimulatory tone on the heart and vasculature of the animal that is initiated by acute changes in blood pressure, detected by the stretch sensitive baroreceptors located throughout the vasculature in all vertebrates (Ito and Scher, 1978; Smith et al., 1981; Jones, 1973; Ishii and Ishii, 1986). Embryonic alligators possess a functional hypertensive baroreflex during incubation, however, lack the hypotensive reflex additionally the function of this reflex following dehydration has not been quantified (Crossley et al., 2003B). Sensitivity of baroreflex, the rate of change in heart rate following a change in arterial pressure, can be affected by humoral and central peptides and hyperosmolality (Bealer, 2003; Charkoudian et al., 2005; Mueller et al., 2013B). In embryonic chickens baroreflex sensitivity is decreased by Ang II and Ang II is critical component of the dehydration response (Mueller et al., 2013B). Dehydration during incubation could induce an increase in plasma osmolality, which may alter baroreflex sensitivity.

The objective of this study was to determine the impact of chronic dehydration of embryonic alligators on cholinergic and adrenergic tone on the heart rate and the vasculature, in addition to baroreflex function. Additionally the direct and indirect actions of Ang II on the system were investigated. It was hypothesized that the relative bradycardia following dehydration was due to β-adrenergic withdrawal on the heart. Additionally, the Ang II pressor response is mediated through an α-adrenergic receptor mechanism similar to that seen in adult birds. Finally baroreflex sensitivity was hypothesized to decrease following dehydration compared to control embryos.
3.2 Materials and Methods

3.2.1 Alligator Embryo Acquisition and Incubation

American alligator eggs (*A. mississippiensis*) were obtained from wild nests at the Rockefeller Wildlife Refuge in Grand Chenier, LA, USA by Dr. Ruth Elsey. Two eggs from each clutch were used for staging, according to Ferguson (1985), to accurately establish the initial percentage of incubation (total incubation period 72 days at 30 °C). Eggs were weighed, numbered, and transported to the Life Sciences complex at the University of North Texas, Denton, TX. Upon arrival, eggs were randomly distributed into Ziploc® boxes (1L, containing vermiculite mixed with water at a 1:1 ratio). Each box was placed inside a large Ziploc® bag (100 L) with fully saturated air passed through the bag at a rate of (1-2 L min⁻¹). Water content, determined by mass at the beginning of the study, was maintained by weighing the box twice weekly, with water added as needed. Eggs were maintained in an environmental chamber at 30 °C (Percival model #: I-66LLVL, Perry, IA), ensuring that all embryos were developing as females (Ferguson, 1985; Ferguson and Joanen, 1982).

3.2.2 Alligator Egg Dehydration Protocol

Eggs (N = 37) were randomly divided between dehydration (n = 18) and control groups (n = 19). The dehydration protocol consisted of three repeated dehydration events at 40%, 50%, and 60% of incubation based on initial staging. During each dehydration event egg mass was reduced by ≈ 5%, calculated prior to each water loss event based on each pre-drying event mass. For each dehydration bout, eggs were placed on plastic racks above Drierite (W.A. Hammond Drierite Co., Ltd., Xenia, OH,
USA) and weighed every 12 hours until 5% pre-dehydration egg mass was lost. Eggs lost approximately 11.1 ± 0.3 g equivalent to 15 ± 0.3 % of the eggs mass (Table 3.1). Following each dehydration event, eggs were returned to their respective box, containing vermiculite mixed with water at a 1:1 ratio.

3.2.3 Surgical Procedures

At 70% and 90% of incubation, control and dehydrated eggs were removed from incubation and candled to locate an accessible tertiary CAM artery. Eggs were placed in a temperature-controlled surgical chamber (30 °C), and a portion of the eggshell removed under a dissection microscope (Leica MZ6; Leica Microsystems, Waukegan, IL, USA). The CAM artery was isolated for arterial pressure monitoring and drug injection, as previously described. Briefly, the isolated artery was catheterized using heat-pulled, heparinized and saline-filled PE-50 tubing, and fixed to the eggshell with cyanoacrylic glue, as previously described (Crossley and Altimiras, 2005). Following catheterization, embryos were transferred to a four-chamber (730 ml chamber volume, with one embryo per chamber placed on cotton), water-jacketed, stainless steel experimental apparatus and allowed to recover for at least 1 hr. Temperature (30 °C) was maintained throughout the apparatus’ chambers by circulating water bath (VWR 1165; VWR International, LLC, West Chester, PA, USA). Each chamber in the apparatus had a stainless steel lid, with three small holes that allowed for the catheter line and airlines to enter the chamber. Room air warmed to 30 °C was pushed into each chamber from an aquarium pump (0.350 l min⁻¹).

The arterial cannula was attached to a pressure transducer (ADInstruments...
disposable transducer, Colorado Springs, CO, USA) connected to a bridge amplifier (4-Octal bridge, ADInstruments, Colorado Springs, CO, USA) and calibrated against a vertical saline column between 0 kPa, set at an even level with the transducer, and 1 kPa, set 10 cm above the transducer. Chart acquisition software (v 7.2.5 ADInstruments Colorado Springs, CO, USA) was used to record arterial pressure. Voltage output signals were converted with a data acquisition system (ML 785, Powerlab/8SP Colorado Springs, CO, USA) and stored in a computer for analyses.

Absolute blood pressure was corrected to account for the distance between the top of the egg from the top of the experimental chamber by adding the measured distance (cm) from the transducer to the top of the egg to record pressure (kPa). Pressure transducers were calibrated with a saline column prior to each measurement period, and heart rate ($f_H$) was calculated instantaneously from the arterial pressure trace using Chart acquisition software. For the purposes of drug treatments total injection volumes were normalized for each embryonic age to the estimated 5% of total blood volume, with drugs administered through a T connector in the arterial catheter line (~35 μl, 70%; ~50 μl, 90%), and each drug injection was flushed with saline (~35 μl, 70%; ~100 μl, 90%) to ensure the drug had entered the CAM artery (Crossley and Altimiras, 2005). Following recovery, embryos received a control injection of heparinized saline into the catheter, with the volume identical to each injection volume (drug plus saline flush).

3.2.4 Tonic Regulation and Ang II Injection Protocol

Following control saline (0.9% NaCl) injection control and dehydrated embryos
received the same series of injections; consisting of native alligator Ang II (2000 ng kg\(^{-1}\); Gift from J.M. Conlon), Atropine (3 mg kg\(^{-1}\), Sigma Aldrich, St. Louis, MO), Ang II (2000 ng kg\(^{-1}\)), propranolol (3 mg kg\(^{-1}\); Sigma Aldrich, St. Louis, MO), Ang II (2000 ng kg\(^{-1}\)), phentolamine (3 mg kg\(^{-1}\); Sigma Aldrich, St. Louis, MO). Following each injection of Ang II embryos were allowed to recover for 45–60 minutes until \(P_m\) and \(f_H\) values had reached stable values for at least 20 min. For cholinergic and adrenergic blockade (atropine, propranolol, and phentolamine) embryos were allowed to recover for 30–45 minutes prior to the subsequent injection.

3.2.5 Baroreflex Function

In a separate group of embryos at 90% of incubation baroreflex function was determined using the pharmacological manipulation of arterial pressure (Oxford method). Following control saline injection control and dehydrated embryos received the same series of injections; consisting of nitric oxide donor, sodium nitroprusside (SNP; Sigma Aldrich, St. Louis, MO) at increasing concentrations (25 \(\mu\)g kg\(^{-1}\), 50 \(\mu\)g kg\(^{-1}\), and 100 \(\mu\)g kg\(^{-1}\)). Following SNP, embryos received phenylephrine (PE; Sigma Aldrich, St. Louis, MO) at increasing concentrations (25 \(\mu\)g kg\(^{-1}\), 50 \(\mu\)g kg\(^{-1}\), and 100 \(\mu\)g kg\(^{-1}\)). Atropine (3 mg kg\(^{-1}\), Sigma Aldrich, St. Louis, MO) was delivered after the final dose of PE, followed by a final injection of PE (100 \(\mu\)g kg\(^{-1}\)). Following each injection embryos were allowed to recover for 45 – 60 minutes until \(P_m\) and \(f_H\) values had reached stable values for at least 20 min.

At the completion of each experiment the embryos were euthanized with an overdose of isoflorane and wet embryonic-yolk free mass, yolk, heart, lung, liver, kidney
wet masses were recorded and tissues were stored at -80 °C for future analyses. All experiments were carried out according to approved UNT Institutional Animal Care and Use Committee protocol number #11-007.

3.2.6 Data Analysis and Presentation

Control \( P_m \) and \( f_H \) were taken prior to any injection from five minutes prior to the first injection. The cardiovascular response to Ang II consisted of an acute hypertensive bradycardia, however the peak response in \( P_m \) and \( f_H \) were not concurrent, therefore \( P_m \) and \( f_H \) were taken individually.

\( P_m \) and \( f_H \) responses to atropine, propranolol, and phentolamine were collected from a 5 minute stable period approximately 25 min following. Within each developmental time point (70 or 90% of incubation) and condition (control and dehydrated), individual paired \( t \)-tests were used to determine if the individual \( P_m \) and \( f_H \) responses to atropine, propranolol and phentolamine were significant (Eme et al., 2011A). The proportional change was compared across incubation percentage and condition by arcsine square root transforming the proportional change of \( P_m \) and \( f_H \). Transformed \( P_m \) and \( f_H \) values were compared between and within incubation percentage and condition with a 2-way ANOVA, significant results were followed by a SNK post-hoc comparison to delineate values into distinct groups following significant results obtained from the ANOVA model. Separate repeated measures ANOVA were conducted for \( P_m \) and \( f_H \) response to Ang II within individual groups on untransformed values. For comparisons of the contribution of cholinergic and adrenergic receptors to the Ang II response for \( P_m \) or \( f_H \) response arcsine square root transformed values were
compared using a repeated measures ANOVA. In total Ang II was delivered four times; once before cholinergic and adrenergic blockade and three more times following removal of individual components of the cholinergic and adrenergic receptors. Significant effects in the ANOVA model were followed by a SNK post-hoc comparison to delineate values into distinct groups.

3.2.7 Calculation of Baroreflex Gain

The static method was employed to assess baroreflex function through collecting the longest duration of the maximal response for $P_m$ and $f_H$ following each injection to increasing doses of SNP and PE. $P_m$ (X) and $f_H$ (Y) responses were plotted for each embryo. A best-fit linear line was applied to each embryo only for responses that showed a baroreflex response. The absolute slope of the line represented the baroreflex gain ($G_{50}$). Gain was normalized ($G_{50N}$) for comparison between conditions according to the method suggested by Berger et al., $G_{50N} = G_{50} \times (P_m / f_H)$, with $P_m$ and $f_H$ values representing the baseline values prior to PE 25 μg kg⁻¹ (1980). Separate 1-way ANOVA’s were used to determine if dehydration during incubation significantly affected $G_{50}$ and $G_{50N}$.

Throughout the text, means are given ± standard error of the mean (± SEM). Statistical significance was determined based on $\alpha = 0.05$ (Statistica v12.0; StatSoft, Tulsa, OK, USA).
3.3 Results

3.3.1 Morphological Traits

All alligator eggs weighed significantly less when the final mass (70% or 90%) was compared to the initial (pre-water loss) mass (Table 3.1). Alligator egg mass was significantly affected by dehydration condition during incubation, alligator eggs exposed to dehydration lost approximately 15-16% of egg mass during incubation, whereas control embryos lost approximately 1-2% (Table 3.1). Embryonic wet mass was significantly affected by dehydration ($p < 0.0001$) and developmental age ($p < 0.0001$). Embryos exposed to dehydration during incubation were not significantly smaller than control embryos at 70% of incubation. At 90% of incubation embryos in both groups were larger than 70% embryos, however, dehydrated embryos were significantly smaller than control embryos at this stage of development, $\approx 25\%$ ($p < 0.001$). Yolk mass significantly decreased with incubation age ($p < 0.0001$), however, dehydration during incubation had no significant effect on residual yolk at the time of sampling (Table 3.2). Proportional organ mass (g organ g embryo$^{-1}$) for heart, and lung decreased with incubation age ($p < 0.001$), whereas relative kidney mass remained similar across incubation, dehydration had no effect on the growth of these organs. Dehydration significantly affected proportional liver mass ($p < 0.05$). Following SNK post-hoc comparison, the livers for 70% dehydrated embryos proportional mass was increased compared to control embryos at the same stage in incubation, and similar to control and dehydrated embryos at 90% of incubation (Table 3.2).
3.3.2 Baseline CAM Arterial Pressure and Heart Rate

$P_m$ increased with incubation age ($p < 0.05$), however, there was no significant difference between control and dehydrated embryos at either stage studied. Incubation age did not significantly affect $f_H$, whereas, dehydration during incubation significantly affected the baseline $f_H$ ($p < 0.05$). Dehydrated embryos at 70% of incubation were similar to control embryos, whereas embryos at 90% of incubation the baseline $f_H$ in dehydrated embryos was approximately 10% lower than control embryos (Table 3.3).

3.3.3 Cholinergic Receptor Blockade

Cholinergic receptor blockade with atropine (3 mg kg$^{-1}$) resulted in a significant hypotension at 90% of incubation control and dehydrated embryo ($p < 0.05$; Fig. 3.1A). Neither incubation age nor dehydration during incubation significantly affected the $P_m$ response cholinergic blockade. Dehydration during incubation significantly affected the $f_H$ response to cholinergic blockade ($p < 0.01$; Fig. 3.1B). Cholinergic blockade failed to increase $f_H$ in both control and dehydrated embryos at 70% of incubation, however, at 90% of incubation both control and dehydrated embryos significantly increased $f_H$ approximately, 3% and 9% respectively (Fig. 3.1B; $p < 0.05$). While dehydration significantly affected $f_H$ response to cholinergic blockade, the SNK post-hoc comparison did not reveal distinct groups.

3.3.4 Adrenergic Receptor Blockade

$\beta$-adrenergic receptor blockade with propranolol (3 mg kg$^{-1}$) resulted in a significant increase in $P_m$ in all embryos studied, ranging from 11% to 32% ($p < 0.001$;
Fig. 3.2A) and reduced $f_H$ in all embryos ($p < 0.001$; Fig. 3.2B). Incubation age ($p < 0.001$) and dehydration during incubation ($p < 0.05$) significantly affected the $P_m$ response to β-adrenergic receptor blockade. However, SNK post-hoc comparison only revealed a significant difference between 70% control and 90% dehydrated embryos (Fig. 3.2A). β-adrenergic receptor blockade increased $P_m$ approximately 12% and 16% in control and dehydrated embryos at 70% of incubation respectively, whereas at 90% of incubation β-adrenergic receptor blockade increased $P_m$ approximately 25% and 32% in control and dehydrated embryos, respectively (Fig. 3.2A). β-adrenergic receptor blockade also resulted in a significant reduction in $f_H$ ranging from 33 to 58% (Fig. 3.2B). Neither incubation age nor dehydration during incubation significantly affected this response. α-adrenergic receptor blockade with phentolamine (3 mg kg⁻¹) significantly reduced $P_m$ in all embryos (Fig. 3.3A; $p < 0.001$). On average, the reduction in $P_m$ ranged from 15% to 20% in control and dehydrated embryos at 70% of incubation respectively. Whereas as incubation progressed so did the proportional change to α-adrenergic blockade, the decrease in $P_m$ was similar in control and dehydrated embryos, approximately 38% at 90% of incubation (Fig. 3.3A). $f_H$ was unaffected by α-adrenergic blockade in all groups with the exception of 70% control embryos, which significantly decreased $f_H$ 6 beats min⁻¹ (Fig. 3.3B).

3.3.5 Cholinergic and Adrenergic Receptor Contribution to the Ang II Cardiovascular Response

Ang II produced a hypertensive bradycardia in all embryos prior to receptor blockade (Fig. 3.4). The hypertension ranged from 46% to 53% at 70% and 90%, respectively (Fig. 3.4A). Neither incubation age nor dehydration affected the $P_m$
response to Ang II. However, the $P_m$ response was significantly affected by pharmacological blockade only after $\alpha$-adrenergic receptor blockade in both control and dehydrated embryos at 70% of incubation, and only dehydrated embryos at 90% of incubation. Pressor responses for control embryos at 90% of incubation, displayed a significant response to Ang II following blockade, and the response was similar to the Ang II response prior to cholinergic and adrenergic blockade, suggesting that late stage pressor responses are primarily due to direct actions of Ang II.

The Ang II induced bradycardia was significantly affected by incubation age ($p < 0.001$) dehydration during incubation did not significantly affect the response (Fig. 3.4B). At 70% of incubation Ang II caused a reduction in heart rate, 20% and 10% in control and dehydrated embryos, respectively. Whereas at 90% of incubation the Ang II induced bradycardia reduced $f_H$ 53% and 41% in control and dehydrated embryos, respectively. Following cholinergic blockade the reduction in $f_H$ was abolished in all embryos (Fig. 3.4B). Adrenergic receptor blockade did not contribute to the $f_H$ response throughout incubation and was not significantly affected by dehydration during incubation.

3.3.6 Cardiovascular Response to Sodium Nitroprusside and Phenylephrine

SNP caused a significant decrease in both $P_m$ in both control and dehydrated embryos at all concentrations that did not statistically differ in magnitude of change (Table 3.5). $f_H$ was unaffected following each injection. Delivery of PE at all doses caused a significant increase in $P_m$ ($p < 0.001$; Fig. 3.5A). The increase in $P_m$ was coupled with a significant depression of $f_H$ with the intermediate and highest dose of PE.
(p < 0.001), the lowest dose of PE (25 μg kg⁻¹) while eliciting a significant response for P_m was unable to induce a significant reduction in f_H (Fig. 3.5B).

3.3.7 Baroreflex Gain

SNP reduced P_m, however did not change f_H, confirming previous findings that embryonic alligators do not possess a hypotensive baroreflex (Crossley et al., 2003B). The increase in P_m following PE injections induced a transient reduction in f_H (Fig. 3.5). The mean baroreflex response to increasing concentration of SNP and PE is presented in Figure 3.6. The raw value comparison is for the heart rate and arterial pressure relationship is presented in Figure 3.7. G_50 was not significantly affected following dehydration during incubation (p = 0.07; Table 3.4). G_50N values were similar for control embryos as previously reported and dehydration during incubation did not return any significant difference compared to control embryos (p = 0.07; Table 3.5; Crossley et al., 2003B). Following cholinergic blockade with atropine (3 mg kg⁻¹) the increase in P_m from the highest concentration of PE the P_m response was similar in magnitude to the response generated prior to blockade (Fig. 3.8A). Cholinergic blockade abolished the bradycardia elicited by the increased P_m from the highest concentration of PE (Fig. 3.8B).

3.4 Discussion

Short term and long term cardiovascular regulatory systems were assessed following dehydration during incubation of the American alligator (A. mississippiensis) embryo. Dehydration impacted morphology of alligator embryos with marked effects on
whole embryo growth and increased relative liver mass at 70% of incubation. Dehydration resulted in a relative bradycardia compared to controls at 90% incubation. To determine the effects of dehydration on control $P_m$ and $f_H$, long and short-term effectors were assessed. Dehydration during incubation activated a cholinergic tonus on heart rate at 90% of incubation, whereas adrenergic control of $P_m$ and $f_H$ were unaffected. Ang II caused a hypertensive bradycardia in all embryos and an indirect vasoconstriction due to $\alpha$-adrenergic stimulation at most developmental points and conditions studied, with the exception of control embryos at 90% of incubation. The reduction in $f_H$ concurrent with the Ang II pressor response increased with incubation age and was not affected by dehydration during incubation. This response was abolished following cholinergic blockade, suggesting the Ang II $f_H$ response was the result of cholinergic stimulation. Assessment of baroreflex function failed to reveal any significant effect of dehydration during incubation.

Dehydrated alligator embryos were normotensive and bradycardic at 90% of incubation, which had previously been suggested to be a reduction of $\beta$-adrenergic tone due to plasticity in alligator embryos (Tate et al., 2012). Dehydration however did not result in decreased $\beta$-adrenergic tone but rather activated cholinergic tone decreasing heart rate slightly (Fig. 3.1B). Cholinergic activation has been reported in hypoxic incubated white leghorn embryonic chickens late in development, however this stress does not do the same in embryonic alligators (Eme et al., 2011A; Crossley and Altimiras, 2012). Activation of cholinergic tone in embryonic alligators has previously been reported in response to acute thermal stress suggesting the capacity for adjusting this regulator at 90% of incubation (Marks et al., 2013). Thus, while cholinergic function
is activated acutely by temperature, dehydrated embryos maintain cholinergic tone. The effects of dehydration on adrenergic control of $P_m$ in the embryos were less clear.

$\beta$-adrenergic tonic regulation of $f_H$ was not affected by dehydration; however, tonic $\beta$-adrenergic control of $P_m$ may be increased while $\alpha$-adrenergic tone did not differ. Dehydration increases relative blood volume in alligator embryos at 70% of incubation without altering $P_m$, given embryos trended to increase the response to the $\beta$-adrenergic, suggesting $\beta$-adrenergic receptor tone to baseline $P_m$ may be adjusted to compensate for the increased blood volume at 70% of incubation (Fig. 3.2A; Tate et al., 2012). Chronic hypoxia elicits $\beta$-adrenergic receptor mediated vasorelaxation of CAM arteries in embryonic chickens ($G.\ gallus$) and hypoxic alligator embryo increases $\beta$-adrenergic tone of heart rate (Eme et al., 2011A; Lindgren et al., 2011). Given the variable action of other stressors on $\beta$-adrenergic function in other studies and data presented here, it appears that dehydration stress during incubation responds in a similar manner. In addition to trends in $\beta$-adrenergic tonus, cholinergic tonus of heart rate appears to be affected by dehydration stress to lower heart rate.

3.4.1 Cholinergic and Adrenergic Contribution to the Cardiovascular Response to Ang II

The RAS is important component of humoral control of the cardiovascular system in adult vertebrates (Carroll and Opdyke, 1982). Multiple examples of embryonic birds and reptiles indicate that cardiovascular regulation by the central nervous system is not fully functional, and embryos may rely on humoral regulation during embryonic development (Crossley and Altimiras, 2000; Crossley and Altimiras, 2005; Eme et al., 2011A; Crossley and Altimiras, 2012; Eme et al., 2013B). The cardiovascular response
to Ang II increases with incubation age and contributes to tonic control of baseline arterial pressure as in chicken embryos; data presented here suggest that Ang II may serve a role in cardiovascular regulation in the alligator embryo as well (Crossley et al., 2010; Mueller, 2013).

Ang II produced a hypertensive bradycardia in alligator embryos, while the $P_m$ response remained constant across incubation age, the Ang II $f_H$ response intensified as incubation progressed (Fig. 3.4B). The pressure response to Ang II was constant over the final 30% of incubation and was not affected by dehydration (Fig. 3.4A). These findings differ from those previously reported for alligator embryos however the basis for this differences is unknown (Tate et al., 2012). In addition, the bradycardic response was significant in both control and dehydrated embryos at both stages studied, also different from results previously reported for the alligator embryo (Fig. 3.4B). Further investigations of possible secondary changes in paralleled regulatory mechanism are needed to understand these reported differences between studies in response to Ang II.

The cardiovascular response to Ang II in embryonic alligators consisted of both direct and indirect stimulation of the system. Blockade of the $\alpha$-adrenergic receptors eliminated the Ang II pressor response at 70% of incubation in control embryos whereas control embryos at 90% of incubation Ang II caused a significant pressor response (Fig. 3.4A). Following $\alpha$-adrenergic receptor blockade, the Ang II intensity fell from, 46% - 54% to 23% - 31% in all experimental groups excluding control 90% of incubation embryos (Fig. 3.4A). This suggest that Ang II elicits a secondary catecholamine release at least at 70% of incubation in both control and dehydrated embryos and at 90% incubation in dehydrated embryos only. Our results are consistent with adult
vertebrates, and it appears that Ang II elicits a pressor response through direct and indirect action, eliciting a secondary α-adrenergic stimulation (Zehr et al., 1981; Carroll and Opdyke, 1982; Nakamura et al., 1982; Silldorff and Stephens, 1991; Bernier and Perry, 1997; Slivkoff and Warburton, 2003). Thus catecholamines are a component of the Ang II response in most groups of embryonic alligators studies here.

The source of the catecholamine stimulation was not determined in the present study. In birds and mammals Ang II administered into systemic circulation elicits catecholamine release from the adrenal medulla, and sympathetic nerve terminals in multiple vertebrate examples (Zehr et al., 1981; Nishimura et al., 1994; Bernier and Perry, 1997). Ang II induced catecholamine release has been described in a limited number of ectothermic vertebrates. In fish (Oncorhynchus mykiss) catecholamines are suggested to originate entirely from chromaffin tissue, whereas the painted turtle (Psudemys scripta) sympathetic nerve terminal release of catecholamines has been suggested (Zehr et al., 1981; Bernier and Perry, 1997). While in adult vertebrates Ang II stimulates the catecholamine release from sympathetic nerve terminals, embryonic alligators maintain adrenergic tone exclusively via circulating catecholamines (Eme et al., 2011A). Given this, the Ang II induced catecholamine release in embryonic alligators is likely due to a non-neural mechanism, possibly from adrenal stimulation. However, the bradycardia originates from cholinergic stimulation, likely from vagal efferent signals stemming from a hypertensive baroreflex.

Ang II induced a hypertensive bradycardia was evident in the alligator embryos at both points studied (Fig. 3.4B). Similar observations have been described for in embryonic chickens (G. gallus) and a previous study in of embryonic alligator (Crossley
et al., 2010; Tate et al., 2012; Mueller et al., 2013A). We hypothesized the bradycardia induced by Ang II would be reduced in dehydrated embryos, and would be of cholinergic origin via the vagus. While dehydrated and control embryos did not differ, the bradycardia was due to cholinergic stimulation of the heart as previously suggested for embryonic chickens which may be a consequence of the immature autonomic regulation of the cardiovascular system in these species (Crossley, et al., 2010; Mueller et al., 2013A; Tate et al., 2012). Adult mammalian studies suggest that Ang II inhibits vagal efferent fibers evoked by baroreceptor stimulation (Lumbers et al., 1979; Potter, 1982; Garner et al., 1987; Reid and Chou, 1990; Reid, 1992). However, embryonic chickens and alligators do not possess tonic vagal control of heart rate during incubation possibly due to high plasma concentrations of Ang II which act on the CNS to inhibit vagal tone (Crossley et al., 2000; Crossley et al., 2005; Eme et al., 2011A; Mueller et al., 2013B). Given that vagal control of heart rate is absent in alligator embryos, further depression of vagal control is not possible. Therefore it is reasonable that stimulation of baroreceptors, activating a vagal mediated depression in heart rate is elicited by the increase in arterial pressure induced by Ang II rather than inhibited, possibly due to the lack of tonic vagal control of heart rate during embryonic development.

Baroreflex function, a metric of the capacity of the CNS to regulate cardiovascular function was unaffected by dehydration (Kirchheim, 1976). While sensitivity (G50) of the dehydrated embryos appeared higher than controls, no statistical difference existed between control and dehydrated embryos. G50N values for control embryos were similar to those previously reported (Crossley et al., 2003B). G50N for
dehydrated embryos resembled values previously reported for 80% of incubation alligator embryos (Crossley et al., 2003B). Dehydrated embryos did not develop a hypotensive baroreflex despite repeated dehydration events. Previous reports support findings presented here suggesting that the cardiac limb baroreflex response is not altered by dehydration in humans (Thompson et al., 1990; Ploutz et al., 1993). Heart rate at 30°C is largely controlled through β-adrenergic receptor stimulation with no input of cholinergic receptors, the early maturation of a hypotensive baroreflex would require the tonic vagal stimulation increasing heart rate during a decrease in arterial pressure (Marks et al., 2013). While a baseline bradycardia was present and tonic cholinergic tone was observed in dehydrated embryos, which suggest capability of a hypotensive baroreflex, however, dehydrated embryos did not develop a fully functional baroreflex, possibly due to the normotensive state.

In summary, dehydration during incubation significantly affects both morphological and baseline cardiovascular function in the embryonic alligator. The hypotheses were confirmed that dehydration would produce a smaller and bradycardic embryo. However, the bradycardia in dehydrated embryos was of cholinergic origin, not β-adrenergic as hypothesized. Tonic adrenergic regulation was unaffected by dehydration during incubation. The Ang II response was similar across the final 30% of incubation and similar to adult vertebrates, as Ang II induces secondary α-adrenergic receptor stimulation to augment the Ang II pressor response. Finally, baroreflex function was unaffected despite values in dehydration embryos that were similar to the maximal value reported for alligator embryos. Therefore American alligator (A. mississippiensis)
embryos may alter other mechanisms of cardiovascular regulation to compensate for dehydration, whereas whole embryo growth is marked.

Table 3.1: Initial and final alligator egg mass loss across incubation in control (C) and dehydrated (D) eggs at 70% and 90% of incubation. Sample sizes are provided in parentheses. Asterisks indicate a significant difference between initial and final egg masses (p < 0.05). Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation %</th>
<th>Condition (n)</th>
<th>Initial mass (g)</th>
<th>Final mass (g)</th>
<th>Egg mass lost (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>C (6)</td>
<td>73.1 ± 2.3</td>
<td>72.3 ± 2.4</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>70</td>
<td>D (5)</td>
<td>74.5 ± 3.7</td>
<td>63.3 ± 3.4*</td>
<td>15.1 ± 0.5</td>
</tr>
<tr>
<td>90</td>
<td>C (13)</td>
<td>74.1 ± 1.8</td>
<td>72.2 ± 1.7</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>90</td>
<td>D (13)</td>
<td>74.7 ± 2.9</td>
<td>62.6 ± 1.2*</td>
<td>15.0 ± 0.4</td>
</tr>
</tbody>
</table>
Table 3.2: Embryonic wet mass of the American alligator (*Alligator mississippiensis*), including heart, liver, lung and kidney masses for control (C) and dehydrated (D) embryos at 70 and 90% of incubation. Sample sizes are presented in parentheses. Different Latin letters for embryo and yolk mass indicate statistically distinct groups following *post-hoc* comparison. For organ masses different Latin letters indicate distinct groups based on relative organ to embryos mass according to the *post-hoc* comparison. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation (%)</th>
<th>Condition (n)</th>
<th>Embryo (g)</th>
<th>Yolk (g)</th>
<th>Heart (g)</th>
<th>Liver (g)</th>
<th>Lung (g)</th>
<th>Kidney (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>C (6)</td>
<td>14.8 ± 1.2A</td>
<td>24.9 ± 1.2A</td>
<td>0.068 ± 0.003</td>
<td>0.159 ± 0.051A</td>
<td>0.188 ± 0.016A</td>
<td>0.134 ± 0.018</td>
</tr>
<tr>
<td>70</td>
<td>D (5)</td>
<td>11.4 ± 0.7A</td>
<td>24.4 ± 1.4A</td>
<td>0.056 ± 0.002</td>
<td>0.184 ± 0.025B</td>
<td>0.136 ± 0.008A</td>
<td>0.109 ± 0.046</td>
</tr>
<tr>
<td>90</td>
<td>C (13)</td>
<td>32.6 ± 0.9B</td>
<td>12.8 ± 1.1B</td>
<td>0.134 ± 0.005</td>
<td>0.572 ± 0.040B</td>
<td>0.328 ± 0.013B</td>
<td>0.266 ± 0.009</td>
</tr>
<tr>
<td>90</td>
<td>D (13)</td>
<td>24.4 ± 1.2C</td>
<td>15.1 ± 0.8B</td>
<td>0.107 ± 0.005</td>
<td>0.412 ± 0.034B</td>
<td>0.230 ± 0.013B</td>
<td>0.216 ± 0.030</td>
</tr>
</tbody>
</table>
Table 3.3: Baseline arterial pressure ($P_m$) and heart rate ($f_H$) in control (C) and dehydrated (D) alligator embryos. Sample sizes are provided in parentheses. Different Latin letters for $P_m$ and $f_H$ indicate statistically distinct groups following post-hoc comparison. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation (%)</th>
<th>Condition (n)</th>
<th>$P_m$ (kPa)</th>
<th>$f_H$ (Beats min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>C (6)</td>
<td>0.82 ± 0.05$^A$</td>
<td>77 ± 2$^{AB}$</td>
</tr>
<tr>
<td>70</td>
<td>D (5)</td>
<td>0.84 ± 0.03$^A$</td>
<td>76 ± 3$^{AB}$</td>
</tr>
<tr>
<td>90</td>
<td>C (13)</td>
<td>1.21 ± 0.06$^B$</td>
<td>82 ± 1$^A$</td>
</tr>
<tr>
<td>90</td>
<td>D (13)</td>
<td>1.39 ± 0.09$^B$</td>
<td>74 ± 2$^B$</td>
</tr>
</tbody>
</table>

Table 3.4: Arterial pressure ($P_m$) change in response to SNP injections at 25, 50, and 100 $\mu$g kg$^{-1}$ and sample sizes (n) in control (C) and dehydrated (D) alligator embryos at 90% of incubation. Different Latin letters for embryo and yolk mass indicate statistically distinct groups following post-hoc comparison. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>% Incubation</th>
<th>Condition (n)</th>
<th>SNP 25 $\mu$g kg$^{-1}$</th>
<th>SNP 50 $\mu$g kg$^{-1}$</th>
<th>SNP 100 $\mu$g kg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>C (6)</td>
<td>0.489 ± 0.057$^A$</td>
<td>0.232 ± 0.040$^{ns}$</td>
<td>0.278 ± 0.070$^A$</td>
</tr>
<tr>
<td>90</td>
<td>D (6)</td>
<td>0.541 ± 0.048$^A$</td>
<td>0.363 ± 0.061$^A$</td>
<td>0.342 ± 0.068$^A$</td>
</tr>
</tbody>
</table>

Table 3.5: Baroreflex assessment of gain (G50) and the normalized (G50N), mean arterial pressure ($P_m$) prior to phenylephrine injection, and sample sizes (n) for control (C) and dehydrated (D) alligator embryos at 90% of incubation. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>% Incubation</th>
<th>Condition (n)</th>
<th>G50 Beats min$^{-1}$ kPa$^{-1}$</th>
<th>G50N Unit less</th>
<th>$P_m$ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>C (6)</td>
<td>23.20 ± 4.58</td>
<td>0.32 ± 0.08</td>
<td>1.23 ± 0.13</td>
</tr>
<tr>
<td>90</td>
<td>D (5)</td>
<td>37.97 ± 6.20</td>
<td>0.72 ± 0.19</td>
<td>1.44 ± 0.12</td>
</tr>
</tbody>
</table>
Fig. 3.1: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B) at 70% and 90% of incubation in control (C; open bars) and dehydrated (D; closed bars) embryos following cholinergic blockade. An asterisk represents a significant response from the pre-injection value according to paired t-test ($p < 0.05$). Data are presented as the mean ± SEM.
Fig. 3.2: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B,) at 70% and 90% of incubation in control (C; open bars) and dehydrated embryos (D; closed bars) following β-adrenergic blockade. An asterisk represents a significant response from the pre-injection value according to paired t-test ($p < 0.05$). Similar Latin letters indicate a similar relative change according to SNK post-hoc comparison following the ANOVA model. Data are presented as the mean ± SEM.
Fig. 3.3: Mean change in arterial pressure (Pm; A), and heart rate (fH; B,) at 70% (70) and 90% (90) of incubation in control (C; open bars) and dehydrated (D; closed bars) embryos following α-adrenergic blockade. An asterisk represents a significant response from the pre-injection value (p < 0.05). Similar Latin letters indicate a similar relative change following post-hoc comparison. Data are presented as the mean ± SEM.
Fig. 3.4: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B) response to Angiotensin II before (open bars) and after cholinergic blockade (closed bars), after $\beta$-adrenergic blockade (shaded bars) and after $\alpha$-adrenergic blockade (dashed bars) at 70% and 90% of incubation in control (C) and dehydrated (D) embryos. An asterisk represents a significant response from the pre-injection value according to paired t-tests ($p < 0.05$). Similar Latin letters indicate a similar relative change following post-hoc comparison. Black lines below asterisks and Latin letters indicate similar responses within individual groups to Ang II injections. Data are presented as the mean ± SEM.
Fig. 3.5: Mean change in arterial pressure ($P_m$; A) and heart rate ($f_h$; B) to phenylephrine injection in control (open bars) and dehydrated embryos (closed bars). Asterisks indicate a significant change from baseline $P_m$ or $f_h$. Similar Latin letters indicate similar response generated within control or dehydrated embryos. Data are presented as the mean ± SEM.
Fig. 3.6: Mean baroreflex response to sodium nitroprusside and phenylephrine in 90% of incubation control (open diamonds) and dehydrated embryos (closed diamonds). Data are presented as mean ± SEM.

Fig. 3.7: Comparison of mean heart rate ($f_H$) response to increasing arterial pressure ($P_m$) relationship for 90% of incubation control (open diamond) and dehydrated (closed diamond) embryos. The diamond in each trace indicates the baseline $f_H$ and $P_m$ prior to phenylephrine delivery. Data are presented as mean ± SEM.
Fig. 3.8: Mean change in arterial pressure ($P_m$) and heart rate ($f_H$) to phenylephrine (PE; $100 \, \mu g \, kg^{-1}$) before (open bars) and after cholinergic blockade (closed bars) with atropine. Asterisks indicate a significant response to PE injection. Data are presented as mean ± SEM.
CHAPTER 4
HYPOXIC INCUBATION (15% O2) ALTERS THE CARDIOVASCULAR RESPONSE TO ANG I IN EMBRYONIC CHICKENS DURING PROLONGED HYPOXIA (*Gallus gallus*)

4.1 Introduction

Adult terrestrial vertebrates respond to hypoxia with multiple adjustments including, depressed cardiovascular function, reduced metabolic rate, increased ventilatory parameters, and numerous cellular responses serving to aid O2 delivery to metabolically active tissues (Frappell et al., 1992; Palmer et al., 1998; Powell et al., 1998). Investigations of a number of species spanning multiple taxa have been the primary model for investigation of the response to reduced oxygen; these studies suggest that during ontogeny these responses are underdeveloped (Crossley et al., 2005; Eme et al., 2011A; Lindgren et al., 2011; Crossley and Altimiras, 2012; Eme et al., 2013A,B; Iversen et al., 2014). Chicken embryos in particular, rely on adrenergic control of arterial pressure and heart rate. Studies demonstrate that tonic cholinergic tone is absent and sympathetic stimulation is limited, autonomic blockade reveals that adrenal chromaffin tissue may responsible for receptor stimulation (Crossley and Altimiras, 2000). Further, the cardiovascular response to acute hypoxia matures with incubation age, eliciting sympathetic nerve terminal release of catecholamines late in incubation, with a greater influence of humoral rather than neural catecholamine release (Crossley et al., 2003A). Lastly, humoral regulation of arterial blood pressure by Angiotensin II (Ang II), demonstrates increasing intensity of the pressor response as
embryos age and the response observed differs from that of adult chickens (Nakamura et al., 1982; Crossley et al., 2010).

Prior studies have reported a reduction in embryo mass, an increased relative heart mass, and a relative hypotension in chickens exposed to chronic hypoxia during incubation (Dzialowski et al., 2002; Lindgren and Altimiras, 2009; Lindgren et al., 2011; Crossley and Altimiras, 2012). These phenotypic differences are accompanied by the limited capacity for cardiovascular regulation throughout the majority of development in this species (Crossley et al., 2000; Crossley et al., 2003A; Crossley and Altimiras, 2012). The absence of functional autonomic tone, both parasympathetic and sympathetic has been previously suggested in embryonic chicken implying that humoral and local regulatory mechanisms such as circulating catecholamines are critical (Crossley et al., 2000; Crossley et al., 2003A). In the absence of functional autonomic regulation of the cardiovascular system, humoral control may have an increased role on maintaining cardiovascular homeostasis during development.

While plasma catecholamines have widely been recognized as a key mechanism to regulate cardiovascular function, studies have recently confirmed an active role of nitric oxide (NO), and Ang II in embryonic chickens in the maintenance of arterial pressure (Crossley et al, 2010; Iversen et al., 2014; Mueller et al., 2013A,B). Inhibition of NO synthase increases arterial pressure at 70% and 90% of incubation in embryos incubated in normoxic and chronic hypoxia (Iversen, et al., 2014). While NO tone remains relatively constant over the final 30% of incubation, the response to Ang II is age dependent, increasing the magnitude of the pressor response as incubation progresses suggesting an increased reliance (Crossley et al., 2010). These studies
provide critical information regarding cardiovascular regulation in the developing embryonic chicken in response to chronic hypoxic development however the components of the acute response to this stress have yet to be sufficiently elucidated.

Recent studies have focused on non-adrenergic mechanisms and humoral components that participate in the chronic and acute hypoxic cardiovascular response (Iversen, et al., 2014). While a hypertensive bradycardia was observed in a previous study, less severe bouts of acute hypoxia (10% O₂ for 5 minutes) produce a hypotensive bradycardia (Crossley et al, 2000; Crossley et al., 2003A; Mulder et al., 2001; Iversen et al, 2014). Based on these previous studies it appears that the cardiovascular response to acute hypoxia involves multiple mechanisms one such mechanism in addition to those already identified, is the renin angiotensin system (RAS).

The RAS is a cascade of cleavage events resulting in Ang II being produced from cleavage of Ang I through angiotensin converting enzyme (ACE) and had been primarily categorized as a response to reductions in blood volume. However the role of Ang II in the cardiovascular response to other stressors such as hypoxia is largely unknown. Evidence does suggest that renin production and circulating Ang II increase during acute hypoxic events in fetal sheep (Broughton Pipken et al., 1974; Giussani et al., 1996; Green et al., 1998). If embryonic chickens possess a similar mechanism, Ang II production could be elicited by reductions in oxygen, as well as blood volume and serve in the maintenance of cardiovascular function.

The cardiovascular response to Ang II has both direct and indirect components of the pressor response. In adult birds Ang II induces a secondary response mediated through α-adrenergic vasoconstriction, thus augmenting the direct vascular effects of
Ang II (Carrol and Opdyke, 1982; Nishimura, 1994). Elevated plasma catecholamine concentrations found in hypoxic embryos may dampen this contribution and the pressor response may consist of direct action of Ang II only (Carrol and Opdyke, 1982; Nishimura, 1994; Mulder et al., 2000; Lindgren et al., 2011). Further embryonic chickens maintain high circulating levels of Ang II during incubation (Crossley et al., 2010). Ang II Plasma concentration is higher in embryonic chickens for the duration of incubation than fetal sheep, concentrations are double plasma concentrations found in late gestation fetal sheep compared to 90% of incubation chicken embryos, whereas 90% of incubation is the lowest concentration of the days studied during embryonic incubation (Broughton Pipken et al., 1974; Green et al., 1998; Crossley et al., 2010). Elevated catecholamine and Ang II plasma concentrations may be accentuated to maintain resting arterial pressure in hypoxic embryos.

Elevated Ang II plasma concentrations throughout incubation in embryonic chickens, and the increase in circulating plasma Ang II during acute hypoxia in fetal sheep support further study for a role of the RAS during chronic hypoxic incubation in the embryonic chicken (G. gallus). We hypothesize that hypoxic embryos will: 1) attenuate the pressor response to exogenous Ang I and II, 2) reduce α-adrenergic contribution to the Ang II pressor response. 3) Ang II will significantly contribute to baseline arterial pressure in both embryos incubated in normoxia and hypoxia. 4) Inhibition of ACE will accentuate the hypotension during acute hypoxic exposure. The goal of this study was to determine if chronic hypoxic incubation alters the cardiovascular response to Ang I, Ang II, to determine the contribution of ACE on the
tonic control of arterial pressure, and lastly assess the RAS during acute and prolonged hypoxia.

4.2 Materials and Methods

4.2.1 Incubation Conditions

Fertilized white leghorn chicken (G. gallus) eggs were purchased from Texas A&M (College Station, TX, USA) and shipped to the University of North Texas (Denton, TX, USA). Eggs were weighed (± 0.1 g, Denver Instrument Company, Bohemia, NY, USA) and placed in one of two Grumbach incubator (Model # BSS 160; Grumbach Brutgeraete GmbH, Asslar, Germany) maintained at 38 ± 0.5 °C and 60% humidity and eggs were turned 60° every 3 h. Eggs were randomly selected for incubation condition, either under normoxia (21% O2; N) or hypoxia (15% O2; H). Embryos from both conditions were removed at day 15 of incubation (≈ 71% of total incubation) or day 19 (≈ 90% of total incubation) total 21-day incubation. Hypoxic incubation (15% O2) was maintained by mixing house air and nitrogen with rotameters (Model FC880; Brooks instrument, Hatfield, PA, USA) delivering the incubator with 15% O2. Gas composition was monitored throughout incubation with an Oxygen analyzer (S-3AI, Ametek Applied Electrochemistry, IL, USA). The normoxic incubator was left unaltered.

4.2.2 Vascular Catheterization and Experimental Setup

On days 15 (≈ 70% of incubation) and 19 (≈ 90% of incubation) eggs were removed from the incubator, weighed, and candled to locate an accessible tertiary artery in the chorioallantoic membrane (CAM). Eggs were then placed into a
thermostatically controlled surgical chamber (38 ± 0.5 °C) and small portion of (10 X 10 mm) the eggshell was removed with forceps and discarded from the site of the artery. An occlusive cannula using heat-pulled PE-50 tubing, was placed in the vessel, filled with heparinized (50 u ml⁻¹) 0.9% NaCl solution under a dissection microscope (Leica MZ6; Buffalo Grove, IL, USA) as previously described (Crossley and Altimiras, 2000). The catheter was secured to the shell with cyanoacrylic glue; the egg was then placed into a water-jacketed stainless steel experimental chamber. Each experimental chamber consisted of 6 individual chambers, each with a lid with two holes (2 X 6 mm diameter) for airlines (≈ 400 ml min⁻¹, pre-warmed to 38 ± 0.5°C) and externalization of the catheter. A water circulator (Jubalo model F32; Julabo USA Inc. Allentown, PA, USA) served to maintain 38 ± 0.5°C throughout the experimental apparatus.

Embryos were removed at 70% and 90% of incubation for all experiments with the exception of Series IV embryos (See below). The arterial catheter from each egg was attached to a pressure transducer (ADInstruments disposable transducer, Colorado Springs, CO, USA) connected to a bridge amplifier (ML228 octal bridge, ADInstruments, Colorado Springs, CO, USA) and the pressure signal was recorded using a PowerLab data acquisition system, 100 sample sec⁻¹ (ADInstruments, Colorado Springs, CO, USA) and LabChart software (v 7, ADInstruments, Colorado Springs, CO, USA). Heart rate (fH) was calculated in real time based on the arterial pressure (Pm) pulse. The pressure was calibrated using a vertical column of saline set at the top of the chamber. The distance between the top of the egg and top of the chamber was recorded (cm) and the pressure was corrected for this distance at the completion of each experiment.
4.2.3 Series I: Cardiovascular Response to Angiotensin Analogues and Selective Removal of ACE

Normoxic (70N, n = 13; 90N, n = 9) and hypoxic (70H, n = 8; 90H, n = 8) embryos were removed from respective incubation conditions. Following surgical instrumentation embryos were moved to an experimental chamber (as described above) and allowed to stabilize to establish baseline values prior to any manipulation. Once stable, the embryos received a control injection of saline (drug volume + saline flush) at 70% of incubation embryos received a 40ul drug injection and an 80ul saline flush, total 120ul and embryos sampled at 90% of incubation received a 50ul drug injection and a 100ul saline flush, total 150ul. Volumes were based on prior reported estimates of blood volume. Following control saline injections, Ang I (2000 ng kg\(^{-1}\)), Ang II (2000 ng kg\(^{-1}\)) were delivered. Embryos were allowed to recover for a minimum 1 hour following Ang I and Ang II injections. Following Ang I and Ang II embryos returned to baseline shortly after, < 30 min. Following the initial assessment of the cardiovascular response to Ang I and Ang II, ACE was inhibited with captopril (1.5 mg kg\(^{-1}\)), 30-60 minutes following inhibition of ACE, both Ang I (2000 ng kg\(^{-1}\)), Ang II (2000 ng kg\(^{-1}\)) were injected and the individual responses were recorded.

4.2.4 Series II: \(\alpha\) -Adrenergic Contribution to the Cardiovascular Response to Ang II

Following surgical manipulation normoxic (70N, n = 12; 90N, n = 9) and hypoxic (70H, n = 8; 90H, n = 8) embryos were allowed to stabilize prior to any pharmacological manipulation. Following control saline injection, Ang II (2000 ng kg\(^{-1}\)) was injected into the arterial catheter. Following Ang II, \(\alpha\)-adrenergic receptors were pharmacologically blocked using phentolamine (3mg kg\(^{-1}\); Sigma Aldrich, St. Louis, MO), 30-60 minutes
following α-adrenergic blockade, Ang II (2000 ng kg\(^{-1}\)) was again injected through the arterial catheter.

4.2.5 Series III: Acute Hypoxia and Angiotensin Converting Enzyme

Following surgical manipulation normoxic (70N, n = 8; 90N, n = 9) and hypoxic (70H, n = 9; 90H n = 10) embryos were allowed to recover and stabilize prior to any manipulation. Once \(P_m\) and \(f_H\) reached stable values, embryos were exposed to a five-minute acute exposure to 10% O\(_2\) through the use of a gas mixer (Sechrist; Anaheim, CA, USA). Pre-warmed Air (38 °C) was delivered into each individual chamber at a flow rate of 1.5 L min\(^{-1}\). Individual chamber O\(_2\) % was monitored with an oxygen analyzer (S-3AI, Ametek Applied Electrochemistry, IL, USA). Following the five-minute hypoxic period embryos were allowed to recover for approximately 1.5 - 2 hrs. \(P_m\) and \(f_H\) values stabilized quickly after, < 30 minutes, however, embryos were allowed additional time for recovery from lactate production from the hypoxic period. 1.5 - 2 hours after initial hypoxic period ACE was inhibited through captopril (1.5 mg kg\(^{-1}\)) as in Series I, this concentration was adequate to prevent conversion of exogenous Ang I into Ang II. Embryos were allowed to recover for 1 hour following ACE inhibition and exposed to another five-minute acute bout of hypoxia. \(P_m\) following ACE inhibition remained stable for the duration of the experimental protocol. The \(P_m\) and \(f_H\) response were collected from the final minute of hypoxic exposure for analysis for both hypoxic bouts.

4.2.6 Series IV Response to Angiotensin Analogues under Prolonged 15% O\(_2\)

Following surgical manipulation normoxic (90N, n = 9) and hypoxic (90H, n = 5)
embryos were allowed to recover for at least one hour at 21% O₂. Following the one-hour recovery at 21% O₂, oxygen percentage was reduced to 15% O₂ and embryos were exposed at this level for the duration of the experiment, allowing embryos to recover for 1.5 – 2 hours. Gas mixture was produced by mixing house air and house N₂ mixed with a gas mixer (Sechrist; Anaheim, CA, USA). Following the 1.5-2 hour stabilization period, embryos were delivered Ang I (2000 ng kg⁻¹), followed by Ang II (2000 ng kg⁻¹). Following each injection embryos were allowed to reach stable values prior to the subsequent injection.

Following all studies embryos were euthanized with an overdose of isoflorane and wet embryo, heart and yolk mass were recorded. All experiments were carried out according to approved UNT Institutional Animal Care and Use Committee protocol number #11-007.

4.2.7 Statistical Analyses for Mass Parameters

Embryonic and yolk wet mass was compared between incubation condition (e.g. hypoxia versus normoxia) and developmental age (e.g. 70% versus 90%) with a two-way analysis of variance (ANOVA). Proportional heart mass (mg organ mass g embryo mass⁻¹) was arcsine transformed and compared using a two-way ANOVA. Significant values from the ANOVA model were followed by a SNK post-hoc comparison to delineate statistically distinct groups.

4.2.8 Statistical Analyses for Cardiovascular Parameters

Baseline P_m and f_H were compared for each series (I, II, and III) using separate
two-way ANOVAs, with incubation condition and age as independent variables. A SNK post-hoc comparison was used to delineate distinct groups. Baseline Pm and fH were compared for series IV (90% of incubation only) using separate one-way ANOVAs between incubation conditions. Significant results from the ANOVA model were followed by a SNK post-hoc comparison to delineate distinct groups. Responses to Ang I and II within incubation and condition groups were assessed by using paired t-tests to determine if responses before and after ACE blockade were significant. For comparisons of the intensity of response, proportional changes to Ang I, and Ang II before and after ACE inhibition (Series I) or adrenergic blockade (Series II), were arcsine square root transformed and assessed using separate repeated measures two-way ANOVA for both Pm and fH (RM ANOVA) between incubation percentage and incubation condition. Significant results from the RM ANOVA model were followed by a SNK post-hoc comparison to delineate statistically distinct groups.

The Pm and fH following ACE inhibition (Series I,) or α-adrenergic (Series II) blockade, were assessed using separate paired t-tests for each incubation percentage and condition. For comparisons across incubation age and condition, proportional changes for Pm and fH were arcsine square root transformed and assessed using a two-way ANOVA. Significant results from the ANOVA model were followed by a SNK post-hoc comparison to delineate statistically distinct groups.

For series III, the longest uninterrupted section, for each individual embryo, available was collected from Chart© software within the final minute during acute hypoxia. Response in Pm and fH to acute hypoxia were assessed using separate paired t-tests for each incubation percentage and condition for each hypoxic event (before and
after ACE inhibition). For the first hypoxic event (before ACE inhibition) proportional changes were arcsine square root transformed and compared using a two-way ANOVA. Pm and fH response to ACE inhibition was treated the same as described above.

Throughout, data are presented as means ± standard error (± SEM). Statistical significance was determined based on α = 0.05 (Statistica v12.0; StatSoft, Tulsa, OK, USA).

4.3 Results

4.3.1 Morphology

Embryos incubated in 15% O2 were significantly smaller at 70% and 90% of incubation compared to control embryos incubated in 21% O2 across incubation (p < 0.05; Table 5.1). Relative heart mass (heart (mg) embryo (g)⁻¹) was significantly affected by hypoxic incubation in series I and II only (p < 0.05; Table 4.1).

4.3.2 Baseline CAM Arterial Pressure and Heart Rate

Resting Pm and fH for series I, II, and III are presented in table 4.2, values were similar to those previously reported for chronic hypoxic incubated embryonic chickens (Crossley and Altimiras, 2012; Iversen et al., 2014). In general as incubation progressed Pm significantly increased (p < 0.05) and fH remained similar in normoxic and hypoxic embryos at 70% and 90% of incubation (Table 4.2). Embryos incubated in hypoxia were significantly hypotensive compared to normoxic embryos at 90% of incubation (p < 0.05), with the exception of series II embryos (Table 4.2).
4.3.3 Series I Cardiovascular Response to Angiotensin I and II in Normoxic and Hypoxic Incubated Embryos

Injections of both Ang I and Ang II resulted in the typical embryonic chicken cardiovascular response to Ang II (Crossley et al. 2010; Mueller et al., 2013A). At both stages and conditions studied chicken embryos increased $P_m$ when given Ang I and II (Fig. 4.1A and 4.2A). The pressor response was coupled to a transient bradycardia followed by a tachycardia (Fig. 4.1B,C and 4.2B,C).

The Ang I pressor response significantly increased in magnitude as incubation progressed ($p < 0.001$; Fig. 4.1A). This increase in $P_m$ ranged from 8.6% to 38%, at 70% and 90% of incubation respectively. Following ACE inhibition, Ang I failed to generate a significant response in all cases (Fig. 4.1A). The Ang I induced bradycardia was similar between incubation age and condition (Fig. 4.1B). In addition a significant tachycardia followed the hypertensive bradycardia across incubation and conditions with the exception of normoxic embryos at 70% incubation (Fig. 4.1C).

Ang II produced a similar response as Ang I, significantly increasing $P_m$ that increased in magnitude as incubation progressed in both hypoxic and normoxic embryos ($p < 0.05$; Fig. 4.2A). The pressor response to Ang II significantly increased $P_m$ ranging from 24% to 62%, in hypoxic and normoxic embryos at 70% to 90% of incubation, respectively ($p < 0.05$; Fig. 4.2A). The cardiovascular response to ACE inhibition resulted in a significant sustained reduction in $P_m$ in all embryos ($p < 0.05$; Table 4.3) whereas $f_h$ was unaffected and was statistically similar across incubation in hypoxic and normoxic embryos.

Following ACE inhibition the magnitude of the pressor response to Ang II did not differ from the pre-inhibition change, with the exception of hypoxic embryos studied at
90% of incubation (Fig. 4.2A). Prior to ACE inhibition in hypoxic embryos, Ang II increased $P_m$ approximately 53%, following ACE inhibition the pressor response increased $P_m$ approximately 77% (Fig. 4.2A). The $f_H$ response to Ang II was unaffected by ACE inhibition (Fig. 4.2B,C).

4.3.4 Series II: Alpha Adrenergic Contribution to the Ang II Pressor Response

The pressor response generated by Ang II (2000 ng kg$^{-1}$) prior to $\alpha$-adrenergic blockade was similar to that observed in Series I in both hypoxic and normoxic embryos at 70% and 90% of incubation. Ang II elicited an increase in $P_m$ prior to $\alpha$-adrenergic blockade ranging from 25% to 62% at 70% and 90% of incubation respectively (Fig. 4.3A). $\alpha$-adrenergic blockade significantly reduced $P_m$ in all embryos, neither incubation age nor condition altered the relative change following $\alpha$-adrenergic blockade (Table 4.4). The response to Ang II following $\alpha$-adrenergic blockade significantly reduced the magnitude of the pressor response to Ang II in 70% hypoxic, 28% to 21% and 90% control embryos only, 64% to 41% (Fig. 4.3A). The bradycardic response present in series I was not significant in series III in 90% of incubation control embryos (Fig. 4.3B), however the secondary tachycardia persisted (Fig. 4.3C).

4.3.5 Series III: Acute Hypoxia and the RAS

Acute hypoxia (5 min, 10% $O_2$) caused a significant transient hypotensive bradycardia in both 70% and 90% of incubation in normoxic and hypoxic embryos, ranging from a 13% to 18% reduction in $P_m$ ($p < 0.05$; Fig. 4.4A). Neither incubation percentage, nor incubation condition significantly affected the acute $P_m$ or $f_H$ response.
to 10% O₂. Inhibition of ACE had similar effects on Pₘ as in series I, resulting in a sustained reduction in Pₘ without significantly affecting fₜ (p < 0.05; Table 4.3).

Inhibition of ACE abolished the hypotension with acute hypoxia at both 70% and 90% of incubation in both normoxic and hypoxic embryos (Fig. 4.4A). However, the fₜ response was unaffected by ACE inhibition, resulting in a similar change to the pre ACE inhibition response (Fig. 4.4B).

4.3.6 Series IV: Prolonged 15% O₂ and the Cardiovascular Response to Ang I and Ang II

Prolonged hypoxic exposure (1.5 – 2 hours 15% O₂) significantly increased Pₘ approximately 10% in 90% control embryos (p < 0.05; Table 4.5). Hypoxic incubated embryos significantly reduced Pₘ, approximately 11% (p < 0.05; Table 4.5). fₜ significantly increased, approximately 5% in control embryos, whereas no significant change was observed in hypoxic incubated embryos (p < 0.05; Table 4.5). Injection of Ang I while exposed to 15% O₂ significantly increased Pₘ, 44 and 61% in control and hypoxic embryos, respectively (p < 0.05; Fig. 4.5A). Injection of Ang II resulted in a significant increase in Pₘ, approximately 52 and 67% in control and hypoxic embryos respectively (p < 0.05; Fig. 4.5B). Prolonged hypoxic exposure had a significant effect with incubation condition on the Pₘ response to Ang II, however SNK post-hoc comparison failed to separate distinct groups (p < 0.05; Fig. 4.5B).

4.4 Discussion

The RAS is essential in the maintenance of cardiovascular homeostasis in adult vertebrates (Carroll and Opdyke, 1982). Ang II specifically has been identified as an
important regulator of the cardiovascular system during embryonic life, maturing as incubation progresses in a species of bird and reptile (Crossley et al., 2010; Tate et al., 2012; Mueller et al., 2013A,B). The findings presented here provide more information regarding the function of the RAS demonstrating that the pressor response to Ang I and II increase with incubation percentage (Fig. 4.1A, 4.2A). Further, the RAS may be modified by hypoxic incubation in embryonic chickens. The data suggest that as incubation progresses Ang II receptor stimulation results in secondary release of catecholamines that contribute to the hypertensive response to Ang II as reported in adult chickens (Carroll and Opdyke, 1982; Nishimura, 1994). These findings do not support the initial hypothesis, that hypoxic incubation would attenuate the pressor response to Ang II embryos. While a tonic Ang II mediated constriction maintains $P_m$ in the embryonic chicken during the final 30% of incubation, hypoxic incubation has no impact on the Ang II vasculature tone. Further, the response to ACE inhibition did not result in an accentuated hypotension during acute hypoxia, but actually facilitated the hypotension. Finally, the response to Ang II is partially mediated by $\alpha$-adrenergic stimulation and is reduced in hypoxic embryos. These findings indicate that the Ang II response coupled with the RAS is developmentally plastic during chronic hypoxic incubation.

Incubation condition did not affect the $P_m$ or $f_H$ response to Ang I and II in embryos maintained at 21% $O_2$ during the experiment (Fig. 4.1, 4.2). However, following ACE inhibition the pressor response to Ang II increased in hypoxic embryos, suggesting that when production of circulating Ang II is prevented, the pressor response increases (Fig. 4.2A). While the basis for this response was not investigated in this study, prior
investigations suggest that hypoxic embryos may maintain higher angiotensin (AT) receptors (Marcus et al., 2010). In mammals, two AT receptors have been primarily studied, the AT1 and AT2 (Sasaki et al., 1991; Nakajima et al., 1995; Reviewed in Nishimura, 2001). The AT1 receptor stimulation primarily results in a vasopressor response while the AT2 receptor causes vasodilation (Tsutsumi et al., 1999). It is important to note that AT1 receptors persist into adulthood in rats while AT2 receptors are generally reserved to fetal life and specific adult tissues (Grady et al., 1991; Tsutsumi and Saavedra, 1991; Lenkei, et al., 1997; Munk et al., 2007). Thus hypoxic incubation may affect the cardiovascular response to Ang II when measured at 21% O2 however this difference is only revealed when ACE is inhibited possibly reflecting increased receptor density (Fig. 4.2A). However, further studies are needed to determine the contribution of Ang II to baseline $P_m$, considering the proportional change in $P_m$ did not differ following ACE inhibition between incubation conditions (Table 4.3).

The response to Ang I was dependent on the oxygen percentage at the time of study. In hypoxic embryos at 90% of incubation Ang I increased $P_m$ approximately 32% in room air, whereas in 15% O2, the pressor response of hypoxic embryos was approximately 61%, similar in magnitude to the Ang II pressor response prior to ACE inhibition at 21% O2, (Fig. 4.5A). Cultured porcine, bovine, and human pulmonary endothelial cells increase ACE protein expression when exposed to hypoxia, in addition this increase in ACE protein is regulated by HIF-1α (Krulewitz and Fanburg, 1984; King et al., 1989; Balyasnikova et al., 1998; Zhang et al., 2009). Thus, while additional studies are needed, embryonic chickens chronically exposed to hypoxic incubation (15% O2), may increase ACE protein expression or increase activity during this
developmental stress in turn generating a higher pressor response to Ang I during prolonged exposure to hypoxia.

In embryonic chickens circulating plasma Ang II is much higher than values reported for fetal sheep (Broughton Pipken et al., 1974; Green et al., 1998; Crossley et al., 2010). Further in fetal sheep hypoxic bouts increase circulating plasma renin and in turn plasma Ang II during gestation (Broughton Pipken et al., 1974; Green et al., 1998). Given this increased production of Ang II in embryonic chickens and the increase in activity in the RAS in fetal sheep, it is reasonable to expect an increase in circulating plasma Ang II in the hypoxic incubated chicken, thus attenuating the pressor response generated from exogenous Ang I and Ang II possibly through receptor saturation. In this study we observed no significant difference between Ang I, Ang II, nor ACE inhibition when measured at 21% O₂ between normoxic and hypoxic embryos (Fig. 4.1A, Fig. 4.2A; Table 4.3).

The Ang II response in normoxic incubated embryos was mediated in part through a secondary action of catecholamine release, hypoxic incubation eliminates this component of the Ang II pressor response (Fig 4.3A). Embryonic chickens have previously been reported to increase catecholamines in response to chronic hypoxic incubation in addition to acute bouts of low O₂ (Crossley et al., 2003A; Lindgren et al., 2011). This increase in circulating catecholamines in hypoxic embryos could saturate α-adrenergic receptors, thus eliminating any contribution in the Ang II pressor response.

4.4.1 Contribution of ACE to the Cardiovascular Response to Acute Hypoxia

Acute hypoxia in embryonic chickens decreases $P_m$ and $f_H$ while increasing
plasma catecholamine and lactate concentrations, similar responses were observed in the current study (Crossley et al., 2003A; Lindgren et al., 2011; Iversen et al., 2014). Blockade of Ang II production indirectly through ACE inhibition eliminated the hypotension observed with the cardiovascular response to acute hypoxia (Fig. 4.4A). NO and adrenergic mechanisms have been reported to be involved in the acute hypoxic response in fetal sheep and embryonic chickens (Green et al., 1996; Harris et al., 2001; Mulder et al., 2001; Crossley et al., 2003A; Iversen et al., 2014). Embryonic chickens recruit several regulatory components adrenergic, cholinergic, and NO in the cardiovascular response to acute hypoxia (Fig. 4.6; Mulder et al., 2000; Crossley et al., 2003A; Lindgren et al., 2011; Iversen et al., 2014). Inhibition of NO-synthase and β-adrenergic blockade abolish and significantly attenuate the hypotension during acute hypoxia, respectively (Crossley et al., 2003A; Iversen et al., 2014). Blockade of α-adrenergic and cholinergic receptors accentuate the hypoxic hypotension (Crossley et al., 2003A). Data presented here suggest that some components of the RAS is involved in the cardiovascular response to acute hypoxia, likely ACE or Ang II.

In adult chickens Ang II stimulates both NO and catecholamine release. Endothelial AT receptor stimulation, in reserpine treated adult chickens, induces a relaxation likely due to an endothelial-derived calcium entry NO mechanism this response is abolished if NO synthase is inhibited (Nishimura et al., 1994; Nishimura et al., 2003). Taken together, data presented in this study and previous studies suggest that the hypotension during acute 10% O₂ observed in chicken embryos involves a RAS
mediated mechanism, possibly Ang II induced NO release or Ang II induced release of catecholamine’s stimulating peripheral β-adrenergic receptors.

4.5 Summary

Collectively, components of RAS specifically Ang I conversion into Ang II, and the acute response to hypoxia suggest that the RAS is involved in both the chronic and acute hypoxic response in the embryonic white leghorn chicken (G. gallus). Ang I conversion to Ang II is increased in hypoxic conditions only in hypoxic incubated chicken embryos, implying increased ACE production or activity. This may suggest a role in cardiovascular regulation during prolonged hypoxic exposure, in hypoxic embryos only and also demonstrates plasticity of the RAS during prolonged hypoxia. Further, our data suggest that α-adrenergic receptors stimulation is involved in the Ang II pressor response in the chicken embryo but this is abolished by hypoxic incubation. Additionally, the hypotension during acute hypoxia appears to involve an Ang II mediated production of NO or adrenergic stimulation through secondary catecholamine release. While the role of Ang II during chronic and acute hypoxia is not fully elucidated, data presented here support further inquiry regarding the mechanism during acute hypoxia, as well as the role ACE serves during prolonged exposure to hypoxia warrants further investigation.
Table 4.1: Embryonic morphological characteristics and sample sizes (n) across experimental series (I, II, III, IV) in 70% and 90% of incubation chicken embryos incubated in 21% O₂ (N) and 15%O₂ (H). Similar superscript Latin letters denote a similar response for the relative change for respective masses within each experimental series according to the SNK post-hoc comparison. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation %</th>
<th>Condition</th>
<th>Series</th>
<th>Final egg mass (g)</th>
<th>Embryo mass (g)</th>
<th>Heart mass (mg)</th>
<th>Relative heart mass (mg g⁻¹)</th>
<th>Yolk mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 N (13)</td>
<td>I</td>
<td>55.4 ± 1.2</td>
<td>15.3 ± 0.6</td>
<td>114.3 ± 6.3</td>
<td>7.4 ± 0.2</td>
<td>11.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>70 H (9)</td>
<td>I</td>
<td>55.5 ± 1.7</td>
<td>11.6 ± 0.6</td>
<td>100.3 ± 5.5</td>
<td>8.7 ± 0.3</td>
<td>11.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>90 N (7)</td>
<td>I</td>
<td>54.8 ± 1.7</td>
<td>31.9 ± 1.0</td>
<td>156.3 ± 5.4</td>
<td>4.9 ± 0.0</td>
<td>9.2 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>90 H (5)</td>
<td>I</td>
<td>59.4 ± 0.8</td>
<td>19.9 ± 2.0</td>
<td>141.5 ± 6.9</td>
<td>7.7 ± 1.0</td>
<td>8.1 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>70 N (12)</td>
<td>II</td>
<td>57.9 ± 2.0</td>
<td>15.2 ± 0.5</td>
<td>119.6 ± 5.4</td>
<td>7.9 ± 0.3</td>
<td>12.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>70 H (9)</td>
<td>II</td>
<td>55.2 ± 1.3</td>
<td>11.8 ± 0.3</td>
<td>101.8 ± 3.0</td>
<td>8.7 ± 0.4</td>
<td>10.4 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>90 N (8)</td>
<td>II</td>
<td>55.0 ± 1.9</td>
<td>29.8 ± 2.0</td>
<td>149.2 ± 7.7</td>
<td>5.2 ± 0.6</td>
<td>10.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>90 H (8)</td>
<td>II</td>
<td>54.1 ± 1.6</td>
<td>21.6 ± 1.2</td>
<td>157.9 ± 12.1</td>
<td>7.4 ± 0.6</td>
<td>10.3 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>70 N (6)</td>
<td>III</td>
<td>54.2 ± 1.6</td>
<td>14.1 ± 0.4</td>
<td>111.6 ± 2.1</td>
<td>8.6 ± 0.3</td>
<td>11.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>70 H (7)</td>
<td>III</td>
<td>54.0 ± 1.9</td>
<td>12.0 ± 0.4</td>
<td>103.3 ± 6.8</td>
<td>8.6 ± 0.7</td>
<td>11.2 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>90 N (8)</td>
<td>III</td>
<td>54.7 ± 0.3</td>
<td>26.5 ± 0.3</td>
<td>181.1 ± 10.5</td>
<td>6.9 ± 0.5</td>
<td>11.1 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>90 H (10)</td>
<td>III</td>
<td>52.2 ± 1.0</td>
<td>21.9 ± 1.7</td>
<td>146.0 ± 7.5</td>
<td>7.0 ± 0.6</td>
<td>9.6 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>90 N (9)</td>
<td>IV</td>
<td>56.8 ± 2.3</td>
<td>25.6 ± 0.9</td>
<td>170.1 ± 7.2</td>
<td>5.9 ± 0.2</td>
<td>12.2 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>90 H (5)</td>
<td>IV</td>
<td>52.2 ± 1.9</td>
<td>21.6 ± 1.3</td>
<td>112.4 ± 5.7</td>
<td>5.2 ± 0.3</td>
<td>10.7 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2: Control arterial pressure ($P_m$), heart rate ($f_H$) and sample size (n) across experimental series (I,II,III) in chicken embryos incubated in 21% O$_2$ (N) and 15% O$_2$ (H) at 70% and 90% of incubation. Similar uppercase Latin letters denote a similar values for $P_m$ or $f_H$ within respective series according to the SNK post-hoc comparison. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation (%)</th>
<th>Condition</th>
<th>Series</th>
<th>$P_m$ (kPa)</th>
<th>$f_H$ (Beat min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>N (13)</td>
<td>I</td>
<td>1.38 ± 0.04$^A$</td>
<td>270 ± 5$^A$</td>
</tr>
<tr>
<td>70</td>
<td>H (9)</td>
<td>I</td>
<td>1.43 ± 0.07$^A$</td>
<td>265 ± 4$^A$</td>
</tr>
<tr>
<td>90</td>
<td>N (8)</td>
<td>I</td>
<td>2.21 ± 0.14$^B$</td>
<td>272 ± 6$^A$</td>
</tr>
<tr>
<td>90</td>
<td>H (8)</td>
<td>I</td>
<td>2.24 ± 0.10$^B$</td>
<td>250 ± 9$^B$</td>
</tr>
<tr>
<td>70</td>
<td>N (12)</td>
<td>II</td>
<td>1.50 ± 0.09$^A$</td>
<td>273 ± 3$^A$</td>
</tr>
<tr>
<td>70</td>
<td>H (9)</td>
<td>II</td>
<td>1.78 ± 0.08$^{A,B}$</td>
<td>275 ± 3$^A$</td>
</tr>
<tr>
<td>90</td>
<td>N (9)</td>
<td>II</td>
<td>2.54 ± 0.14$^C$</td>
<td>254 ± 6$^B$</td>
</tr>
<tr>
<td>90</td>
<td>H (8)</td>
<td>II</td>
<td>1.88 ± 0.07$^B$</td>
<td>260 ± 10$^{A,B}$</td>
</tr>
<tr>
<td>70</td>
<td>N (9)</td>
<td>III</td>
<td>1.52 ± 0.10$^A$</td>
<td>273 ± 4$^A$</td>
</tr>
<tr>
<td>70</td>
<td>H (9)</td>
<td>III</td>
<td>1.63 ± 0.09$^A$</td>
<td>270 ± 5$^A$</td>
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<tr>
<td>90</td>
<td>N (9)</td>
<td>III</td>
<td>2.36 ± 0.07$^B$</td>
<td>274 ± 4$^A$</td>
</tr>
<tr>
<td>90</td>
<td>H (10)</td>
<td>III</td>
<td>2.25 ± 0.19$^B$</td>
<td>266 ± 6$^A$</td>
</tr>
</tbody>
</table>

Table 4.3: Mean arterial pressure ($P_m$) response and sample size (n) to ACE inhibition in experimental series I and III, in chicken embryos incubated in 21% O$_2$ (N) and 15% O$_2$ (H) at 70% and 90% of incubation. Asterisks indicate a significant $P_m$ following ACE inhibition within groups. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation (%)</th>
<th>Condition</th>
<th>Series</th>
<th>$\Delta P_m$ (kPa)</th>
<th>Relative $\Delta P_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>N (13)</td>
<td>I</td>
<td>-0.21 ± 0.03$^*$</td>
<td>-0.15 ± 0.02</td>
</tr>
<tr>
<td>70</td>
<td>H (9)</td>
<td>I</td>
<td>-0.08 ± 0.03$^*$</td>
<td>-0.06 ± 0.02</td>
</tr>
<tr>
<td>90</td>
<td>N (8)</td>
<td>I</td>
<td>-0.18 ± 0.04$^*$</td>
<td>-0.08 ± 0.04</td>
</tr>
<tr>
<td>90</td>
<td>H (8)</td>
<td>I</td>
<td>-0.30 ± 0.04$^*$</td>
<td>-0.14 ± 0.02</td>
</tr>
<tr>
<td>70</td>
<td>N (8)</td>
<td>III</td>
<td>-0.29 ± 0.06$^*$</td>
<td>-0.18 ± 0.03</td>
</tr>
<tr>
<td>70</td>
<td>H (9)</td>
<td>III</td>
<td>-0.31 ± 0.05$^*$</td>
<td>-0.19 ± 0.03</td>
</tr>
<tr>
<td>90</td>
<td>N (9)</td>
<td>III</td>
<td>-0.49 ± 0.10$^*$</td>
<td>-0.20 ± 0.04</td>
</tr>
<tr>
<td>90</td>
<td>H (10)</td>
<td>III</td>
<td>-0.22 ± 0.04$^*$</td>
<td>-0.11 ± 0.02</td>
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</table>
Table 4.4: Mean arterial pressure ($P_m$) response and sample size (n) to $\alpha$-adrenergic blockade in chicken embryos incubated in 21% $O_2$ (N) and 15% $O_2$ (H) at 70% and 90% of incubation from experimental series II. Asterisks indicate a significant reduction in $P_m$ within N and H following $\alpha$-adrenergic blockade ($p < 0.05$). Similar superscript Latin letters indicate a similar relative change in $P_m$ across incubation percentage and condition according to the SNK post-hoc comparison. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation %</th>
<th>Condition (n)</th>
<th>Series</th>
<th>Pre $P_m$ (kPa)</th>
<th>Post $P_m$ (kPa)</th>
<th>Relative $\Delta P_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>N (12)</td>
<td>II</td>
<td>1.81 ± 0.08</td>
<td>1.43 ± 0.09$^*$</td>
<td>-0.23 ± 0.02$^A$</td>
</tr>
<tr>
<td>70</td>
<td>H (9)</td>
<td>II</td>
<td>1.66 ± 0.07</td>
<td>1.25 ± 0.08$^*$</td>
<td>-0.25 ± 0.03$^A$</td>
</tr>
<tr>
<td>90</td>
<td>N (8)</td>
<td>II</td>
<td>2.54 ± 0.16</td>
<td>2.22 ± 0.15$^*$</td>
<td>-0.13 ± 0.03$^B$</td>
</tr>
<tr>
<td>90</td>
<td>H (8)</td>
<td>II</td>
<td>1.82 ± 0.09</td>
<td>1.38 ± 0.12$^*$</td>
<td>-0.25 ± 0.04$^A$</td>
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</tbody>
</table>
Table 4.5: Mean arterial pressure ($P_m$), heart rate ($f_H$) and sample size (n) for the response to chronic 15% O$_2$ in normoxic (N) and hypoxic (H) chicken embryos sampled at 90% of incubation for series IV. Asterisks indicate a significant change in $P_m$ within N and H incubated embryo responses during prolonged 15% O$_2$ ($p < 0.05$). Similar superscript Latin letters indicate a similar $P_m$ across incubation percentage and condition in 15% O$_2$ according to the SNK post-hoc comparison following the initial ANOVA model. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation %</th>
<th>Condition (n)</th>
<th>Series</th>
<th>21% O$_2$ $P_m$ (kPa)</th>
<th>15% O$_2$ $P_m$ (kPa)</th>
<th>Relative Δ $P_m$ (kPa)</th>
<th>21% O$_2$ $f_H$ (Beats min$^{-1}$)</th>
<th>15% O$_2$ $f_H$ (Beats min$^{-1}$)</th>
<th>Relative Δ $f_H$ (Beats min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>N (9)</td>
<td>IV</td>
<td>2.05 ± 0.08</td>
<td>2.24 ± 0.09$^A$</td>
<td>0.10 ± 0.04$^*$</td>
<td>248 ± 6</td>
<td>260 ± 5</td>
<td>0.05 ± 0.02$^*$</td>
</tr>
<tr>
<td>90</td>
<td>H (5)</td>
<td>IV</td>
<td>2.19 ± 0.07</td>
<td>1.93 ± 0.09$^A$</td>
<td>-0.12 ± 0.04$^*$</td>
<td>254 ± 15</td>
<td>265 ± 13</td>
<td>0.05 ± 0.04</td>
</tr>
</tbody>
</table>
Fig. 4.1: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B, C) to angiotensin I before (open bars) and after angiotensin converting enzyme (ACE) inhibition (closed bars) at 70% (70) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response from the pre-injection value according to paired t-tests ($p < 0.05$). Similar letters indicate a similar relative change according to SNK post-hoc comparison. Data are presented as the mean ± SEM.
Fig. 4.2: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B, C) to angiotensin II before (open bars) and after angiotensin converting enzyme inhibition (closed bars) at 70% (70) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response from the pre-injection value according to paired t-tests ($p < 0.05$). A black bar over the 70H response represents similar pre and post angiotensin converting enzyme inhibition response to Angiotensin II. Similar letters indicate a similar relative change according to SNK post-hoc comparison. Data are presented as the mean ± SEM.
Fig. 4.3: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B, C) to Angiotensin II before (open bars) and after β-adrenergic blockade (closed bars) at 70% (70) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response from the pre-injection value according to paired t-tests ($p < 0.05$). Similar letters indicate a similar relative change according to SNK post-hoc comparison. Data are presented as the mean ± SEM.
Fig. 4.4: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B, C) to a five minute exposure to 10% $O_2$ before (open bars) and after angiotensin converting enzyme inhibition (closed bars) at 70% (70) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response from the pre-inhibition value according to paired t-tests ($p < 0.05$). A bar with an asterisk directly above indicates a significant response in $f_H$ in all groups. Data are presented as the mean ± SEM.
Fig. 4.5: Mean change in arterial pressure ($P_m$), to angiotensin I (A) and angiotensin II (B) at 21% $O_2$ (open bars) and 15% $O_2$ (closed bars) at 70% (70) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response from the pre-injection value according to paired t-test ($p < 0.05$). Similar letters indicate a similar relative change according to SNK post-hoc comparison. Data are presented as the mean ± SEM.
Fig. 4.6: Representative trace of the change in arterial pressure to acute hypoxia in a 90% normoxic embryo. Within boxes are adrenergic, cholinergic, nitric oxide (NO), angiotensin converting enzyme (ACE), the central nervous system (CNS) and the contribution to the acute hypoxic hypotensive response. Arrows indicate the effect that respective regulatory systems have on the arterial pressure ($P_m$) response during acute hypoxia, as indicated by receptor blockade or enzyme inhibition. Subscript numbers correspond to the literature cited. 1: Crossley et al., 2000, 2: Crossley et al., 2003A, 3: Iversen et al., 2014. ACE contribution is presented in the current study.
5.1 Introduction

In response to chronic hypoxia, adult vertebrates modify the central nervous system (CNS), endocrine and local regulation of the cardiovascular system in an attempt to maintain oxygen transport. Under the stress of chronically reduced PO2, terrestrial vertebrates decrease parasympathetic tone while increasing sympathetic tone on the cardiovascular system (Butler, 1967; Hammill et al., 1979; Mazzeo et al., 1991; reviewed in Favret and Richalet, 2007). While the CNS responds to chronic hypoxia with changes in autonomic nervous system function, humoral mechanisms increase in function and consist of several components, including natriuretic peptides, arginine vasopressin (arginine vasotocin for non-mammalian vertebrates), endothelin, and adenosine (Rose et al., 1984; Saito et al., 1998; Horio et al., 1991; Jackson et al., 1985).

Adenosine, a purinergic nucleoside, is produced from degradation of AMP, cyclic AMP, and ATP, through ecto-nucleotidases located on the surface membrane of a variety of cells (Mian and Marshal, 1991; Eltzschig et al., 2003). Adenosine in adult vertebrates is a potent vasodilator in the heart and brain, and ischemic peripheral tissues (Katori and Berne, 1966; Winn et al., 1980; Bush et al., 1989; Koos and Doany, 1991; Yoneyama and Power, 1992). Adenosine acts as a ligand to a number of receptor subtypes. These include adenosine type 1 receptors (A1) that are localized on the
sinoatrial (SA) and atrioventricular (AV) node, activating adenosine $K^+$ channels that hyperpolarize cells (West and Belardinelli, 1985; Belardinelli et al., 1988; Wang et al., 199). Adenosine also inhibits catecholamine sensitive adenylate cyclase through direct and indirect effects (Belardinelli et al., 1988; LaMonica et al., 1985).

The cardiovascular response to adenosine has been well documented in adult vertebrates; however, during ontogeny when CNS control of cardiovascular function is immature, humoral regulators such as adenosine may play a greater role in maintaining cardiovascular homeostasis (Crossley et al., 2000). While this is a logical speculation, outside of a few model mammalian species the role of humoral regulators including adenosine in cardiovascular regulation during ontogeny is largely unknown. In mice, adenosine receptors are present early during ontogeny and tonic adenosine stimulation on the cardiovascular system is present prior to cholinergic and adrenergic receptor maturation (Hofman et al., 1997; Porter and Rivkees, 2001; Momoi et al., 2008). Adenosine has also been reported to increase cerebral flow during hypoxia in fetal sheep and is a potent contributor to the overall cardiovascular response to this stress (Mori et al., 1987; Giussani et al., 2001; Blood et al., 2002). In addition to these representative mammals, adenosine may also be important in embryonic birds. An original study by Adair demonstrated that adenosine might be important for vascular development in embryonic chickens, however, *in vivo* measurements documenting its function were not reported (1989). Therefore, additional studies in the embryonic chicken are required to address the deficiency in our current understanding.

Like fetal mammals, cardiovascular regulation in the embryonic chicken is immature, with a predominance of non-neural mediated adrenergic tone, nitric oxide
tone and tonic angiotensin II stimulation (Mulder et al., 2001; Crossley and Altimiras, 2000; Crossley et al., 2000; Chiba et al., 2004; Crossley and Altimiras, 2012; Mueller et al., 2013). Previously, adenosine has been reported to induce angiogenesis during hypoxic exposures and to reduce structural resistance in chicken embryos (Dusseau et al., 1986; Dusseau and Hutchins, 1988; Adair et al., 1989). Additionally, adenosine induces vascular endothelial growth factor expression in multiple sites including vascular smooth muscle (Grant et al., 1999; Gu et al., 1999). Further, isolated cardiac strip preparations suggest stimulation of adenosine receptors causes an A₁ receptor mediated negative inotropic response (Shryock et al., 1989). These studies suggest that adenosine may play a role in regulation of both the vasculature and heart of embryonic chickens.

This investigation was formulated to understand the functional role of adenosine in cardiovascular regulation of embryonic chickens. Based on reported data adenosine action on isolated atria from embryonic chickens, the hypoxic induced release of adenosine in fetal sheep, and angiogenic properties of adenosine in the embryonic chicken it is likely adenosine elicits actions on blood pressure and heart rate in the chicken embryo. Thus, it was hypothesized that exogenous adenosine will reduce heart rate and arterial pressure, and that hypoxic incubation will reduce these responses. Additionally, non-selective blockade with theophylline will reveal a tonic contribution of adenosine to the baseline heart rate in normoxic embryos, and will be reduced in hypoxic embryos.
5.2 Materials and Methods

5.2.1 Egg Source and Incubation

Fertilized white leghorn chicken (*Gallus gallus*) eggs were purchased from a commercial supplier (Sunnyside Hatchery; Beaver Dam, WI). Eggs were weighed (± 0.1 g, Denver Instrument Company, USA) and placed in a Grumbach incubator (model # BSS 160; Grumbach Brutgeraete GmbH, Asslar, Germany) maintained at 38 ± 0.5 °C and 60% humidity, and eggs were turned 60° every 3 h. Eggs were randomly selected for incubation condition, either under normoxia (n = 23; 21% O2; N) or hypoxia (n = 24; 15% O2; H).

Hypoxic incubation was achieved by mixing house air and compressed nitrogen with a gas mixer (Sechrist; Anaheim, CA, USA) delivering the incubator with 15% O2. Gas composition was monitored with an Oxygen analyzer (S-3AI, Ametek Applied Electrochemistry, IL, USA). The normoxic incubator was left unaltered. Embryos from both conditions were removed at days 13, 15, 17, and 19 (≈ 60%, ≈ 71%, ≈ 80%, ≈ 90% of total incubation respectively) out of a 21-day total incubation length.

5.2.2 Vascular Catheterization and Experimental Setup

On days 13 (≈ 60% of incubation), 15 (≈ 70% of incubation), 17 (≈ 80% of incubation), and 19 (≈ 90% of incubation) eggs were removed from respective incubation conditions, weighed, and candled to locate an accessible tertiary artery in the chorioallantoic membrane (CAM). Eggs were then placed into a thermostatically controlled surgical chamber (38 ± 0.5 °C) and small portion of (10 X 10 mm) the eggshell was removed with forceps exposing the artery. In the exposed artery an
occlusive catheter (heat-pulled PE-50 tubing; Braintree Scientific, Braintree, MA), filled with heparinized (50 u ml\(^{-1}\)) 0.9% saline was inserted under a dissection microscope (Leica MZ6; Buffalo Grove, IL, USA; Crossley and Altimiras, 2000). The catheter was secured to the shell with cyanoacrylic glue, and the egg was then placed into a water-jacketed stainless steel experimental apparatus. Each experimental apparatus consisted of 6 individual chambers, each with a lid with two holes (2 X 6 mm diameter) for airlines (≈ 400 ml min\(^{-1}\), pre-warmed to 38 ± 0.5 °C through 1 m coiled tubing at the base of each experimental chamber) and externalization of the catheter. A water circulator (Julabo model F32; Julabo USA Inc. Allentown, PA, USA) served to maintain 38 ± 0.5 °C throughout the experimental apparatus.

The arterial catheter from each egg was attached to a pressure transducer (ADInstruments disposable transducer, Colorado Springs, CO, USA) connected to a bridge amplifier (ML228 octal bridge, ADInstruments, Colorado Springs, CO, USA) and the pressure signal was recorded using a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO, USA) and LabChart software (version 7, ADInstruments, Colorado Springs, CO, USA). Heart rate (\(f_H\)) was monitored throughout the experiment through a tachometer based on the arterial pressure (\(P_m\)) pulse. The pressure was calibrated using a vertical column of saline with the 0 set even with the top of the egg and top of the chamber was recorded (cm) and the pressure was corrected for this distance at the completion of each experiment.
5.2.3 Adenosine Dose Response Curve

Embryos were removed at desired incubation points from normoxic (60% n = 5, 70% n = 6, 80% n = 6, 90% n = 6) and hypoxic (60% n = 6, 70% n = 5, 80% n = 6, 90% n = 7) incubation conditions. Following surgical procedures cardiovascular parameters were allowed to stabilize prior to any manipulation, at least one-hour. After the embryos had maintained relatively constant $P_m$ and $f_H$ a control injection of saline that was equal to the drug injection volume + saline flush volume was injected as previously reported (Crossley and Altimiras, 2000; Crossley et al., 2003A). For 60% embryos this equated to 30 μl drug injection and 60 μl saline flush, 70% embryos 40 μl drug injection and 80 μl saline flush, 80% embryos 50 μl drug injection and 100 μl saline flush, and 90% embryos 50 μl drug injection and a 100 μl saline flush. Following saline, the $P_m$ and $f_H$ response to increasing concentrations of adenosine were recorded (0.267 mg kg$^{-1}$, 2.67 mg kg$^{-1}$, and 26.7 mg kg$^{-1}$). Following each injection parameters were allowed to reach stable values prior to subsequent injection (approximately 30 minutes). Following the initial three injections of adenosine, theophylline a non-selective competitive adenosine receptor antagonist (4 mg kg$^{-1}$) was delivered. Following the blockade, 10-30 minutes, adenosine (2.67 mg kg$^{-1}$) was injected and responses were recorded. At the completion of the study embryos were euthanized with an overdose of isoflorane, and the wet embryo and heart mass was recorded.

5.2.4 Statistical Analysis for Mass Parameters

Embryonic wet mass was compared between incubation conditions (e.g. hypoxia versus normoxia) and developmental age (60%, 70%, 80% and 90% of incubation) with
a two-way analysis of variance (ANOVA). Proportional heart mass (mg heart mass g embryo mass$^{-1}$) was arcsine transformed and compared using a two-way ANOVA. Significant values from the ANOVA model were followed by a Student Newman Keuls (SNK) *post-hoc* comparison to delineate statistically distinct groups.

5.2.5 Data and Statistical Analyses for Cardiovascular Parameters

Baseline $P_m$ and $f_H$ measurements were collected prior to the lowest injection of adenosine. $P_m$ and $f_H$ responses to increasing concentrations of adenosine were collected from the peak response, generally within the first 10 seconds following adenosine delivery. $P_m$ and $f_H$ response to theophylline were collected 5 minutes following delivery. Baseline $P_m$ and $f_H$ were compared using a two-way ANOVA. Separate one-way repeated measures ANOVA were used for comparisons within incubation age and between conditions for $P_m$ and $f_H$. For comparisons of the overall response relative to baseline cardiovascular values across incubation and condition, the proportional changes to adenosine were arcsine square root transformed and assessed using separate repeated measures two-way ANOVA for $P_m$ and $f_H$. For $P_m$ and $f_H$ response to theophylline separate paired t-test, within individual groups were used to determine if $P_m$ and $f_H$ were significantly affected. For the change in $P_m$ relative to baseline for the response to theophylline, proportional changes were treated as described previously. Significant returns in the ANOVA models were followed by a SNK *post-hoc* comparison to delineate distinct groups. Throughout the text, responses are presented as means ± standard error (± SEM). Statistical significance was determined based on $\alpha = 0.05$ (Statistica v12.0; StatSoft, Tulsa, OK, USA).
5.3 Results

5.3.1 Morphological Response to Chronic Hypoxic Incubation

Embryo mass significantly increased with incubation age (p < 0.001; Table 5.1). Embryos incubated in 15% O₂ were significantly smaller at 90% of incubation (p < 0.05). Relative heart mass (mg heart g embryo⁻¹) was significantly affected by both hypoxic incubation (p < 0.05) and incubation age (p < 0.05), however, SNK post-hoc analysis did not reveal distinct groups although there was a trend for a decrease in relative mass as incubation progressed in both normoxic and hypoxic embryos (Table 5.1).

5.3.2 Baseline Parameters

Baseline $P_m$ and $f_H$ for all embryos (Table 5.2) were similar to those previously reported for chronic hypoxic and normoxic incubated embryonic chickens (Crossley and Altimiras, 2000; Crossley et al., 2003A; Crossley and Altimiras, 2012; Iversen et al., 2014). In general, as incubation progressed $P_m$ significantly increased (p < 0.05) and $f_H$ remained similar in normoxic and hypoxic embryos across incubation (Table 5.2). Hypoxic embryos were significantly hypotensive compared to normoxic embryos at 90% of incubation (p < 0.05; Table 5.2).

5.3.3 Adenosine Dose Response Curve

Adenosine injection caused significant age and dose dependent reduction in $P_m$ and $f_H$ across all incubation ages and conditions sampled (Fig. 5.1, Fig.5.2A,B). The lowest concentration (0.267 mg kg⁻¹) of adenosine failed to elicit a significant change in $P_m$ in all cases. The intermediate concentration of adenosine (2.67 mg kg⁻¹) significantly
reduced $P_m$ only at 80% and 90% of incubation in normoxic embryos ($80\% \approx -23\%; 90\% \approx 34\%; p < 0.001$) and hypoxic embryos ($80\% \approx -15\%, 90\% \approx 20\%; p < 0.01$). The highest concentration (26.7 mg kg$^{-1}$) of adenosine resulted in the maximal response elicited in all embryos and intensified with age ($p < 0.001$), ranging from 32% at 60% of incubation to 53% at 90% of incubation (Fig. 5.2A). Hypoxic embryos responded in a similar manner as normoxic embryos to the highest concentration.

A clear bradycardic response to adenosine in all age groups was only observed following the maximal dose of adenosine and intensified with age increasing from 31% to 79% at 60% and 90% of incubation, respectively (Fig. 5.2B). The intermediate dose of adenosine only induced a significant reduction in $f_H$ in 80% normoxic as well as both 90% normoxic and hypoxic embryos (Fig. 5.2B) The intermediate concentration significantly lowered $f_H$ in normoxic embryos on average 26% at 80% of incubation ($p < 0.001$; Fig. 5.2B). The intensity of the $f_H$ response to adenosine at 90% of incubation did not differ between incubation conditions.

5.3.4 The Cardiovascular Response to Non-Selective Adenosine Receptor Blockade

Non-selective competitive adenosine receptor antagonist, theophylline (4 mg kg$^{-1}$), did not significantly alter $f_H$ at any incubation percentage or condition (Fig. 5.3B). However, blockade significantly increased $P_m$ at 80% and 90% of incubation in normoxic embryos only (Fig. 5.3A; $p < 0.05$). Hypoxic embryos significantly increased $P_m$ following adenosine receptor blockade only at 90% of incubation (Fig. 5.3A; $p < 0.05$). Theophylline failed to block the $P_m$ response to adenosine in normoxic embryos at 80% of incubation, whereas at 90% of incubation the $P_m$ response was abolished in
both normoxic and hypoxic embryos (Fig. 5.4A). Adenosine receptor blockade effectively blocked the \( f_H \) response in all groups (Fig. 5.4B).

5.4 Discussion

The first in vivo measurement of the embryonic cardiovascular response to exogenous adenosine in chickens is presented here. The \( P_m \) and \( f_H \) response to adenosine is age dependent and a tonic adenosine mediated vasodilation on arterial pressure is present at 80% of incubation in normoxic embryos and hypoxic incubation delays this until 90%. Hypoxic incubation was hypothesized to blunt the \( f_H \) response to adenosine. However, hypoxic embryos did not respond to adenosine at the intermediate concentration until 90% of incubation, whereas normoxic embryos responded at 80% of incubation. Based on data presented here hypoxic incubation affects actions of adenosine primarily on \( P_m \) and less so \( f_H \). In addition to differences following injections of adenosine blockade of receptors further supports an effect of hypoxic incubation on the cardiovascular response to hypoxia.

The reduced cardiovascular response to exogenous adenosine was only apparent for the intermediate concentration of adenosine in hypoxic embryos for \( P_m \) only. Bolus injections of the highest concentration adenosine were unaffected by hypoxic incubation. The primary findings were that, a clear maturation in the reduction in \( P_m \) and \( f_H \) response was apparent as incubation progressed (Fig. 5.2A,B). This type of maturation has been reported for \( A_1 \) receptors at earlier days in incubation, however, these studies focused on the chronotropic and inotropic response of isolated cardiac tissue (Blair et al., 1989; Shryock et al., 1989). Adenosine increases in effectiveness as
incubation progresses similar to reports for other humoral systems (Blair et al., 1989; Liang, 1989; Crossley et al., 2010). Angiotensin II (Ang II) causes an age dependent increase pressor response to injections of Ang II, however, baseline tone, remains similar across incubation (Crossley et al., 2010; Chapter 4). It is apparent that multiple humoral mechanisms mature as incubation progresses, but the tonic role to the regulation of $P_m$ and $f_H$ do not appear to be affected following hypoxic incubation but may serve a role in the maintenance of arterial pressure.

The $f_H$ response to adenosine is present during the final 40% of incubation in chicken embryos, this is not surprising considering adenosine receptors are present as early as 15% of incubation, however, unresponsive until 30% of incubation (Blair et al., 1989; Hatae et al., 1989). Adenosine directly slows $f_H$ in mammals through $A_1$ receptors on the sinoatrial and atrioventricular nodes, $A_1$ receptor binding of adenosine induces in inward current of $K^+$, hyperpolarizing the cardiac cells slowing $f_H$, and antagonizing action of catecholaminergic stimulation on the SA node, and reduces calcium entry into the cell following isoproterenol, a $\beta$-adrenergic agonist (Belardinelli et al., 1988). $f_H$ reductions due to adenosine were likely due to similar mechanisms as evident by the responses generated by the highest concentrations of adenosine which, elicited decreases in $f_H$ up to 79% in 90% of incubation embryos. This decrease in $f_H$ could have secondary effects on arterial pressure.

The reduction in $P_m$ to adenosine could originate from three possible mechanisms: AV block, negative inotropy, or vasculature vasodilation, or a combination of these effects. The AV interval increases following administration of adenosine agonists in multiple mammalian examples (Dimarco et al., 1983; Belardinelli, et al.,
In single atrial cells of the guinea pig, adenosine induces a K⁺ channel current (Belardinelli and Isenberg, 1983; Kurachi et al., 1986). Adenosine decreases contractility (negative inotropy) of both atrial and ventricular tissue in chicken embryos and is negatively coupled with adenylate cyclase (Liang, 1989; Shryock et al., 1989). Depressor responses to adenosine are largely suggested to involve the A₂ receptor in mammalian coronary arteries (Hein et al., 2001; Sato et al., 2005). Additionally, hypotensive actions of adenosine are mediated through A₁ and A₂A receptors. A₁ receptors specifically have been suggested to be involved in the maintenance of arterial pressure in mice, as suggested by increased baseline arterial pressure when A₁ receptors or knocked out (Brown et al., 2006; Andersen et al., 2011). Furthermore, peripheral responses to adenosine in the rabbit and guinea-pig support direct action of adenosine induced vasodilation (Sakai et al., 1998; Brodmann et al., 2003). Therefore, the decrease in Pₘ could be explained through any one of these actions of adenosine on the cardiovascular system, or a combination of the adenosine mechanisms. Additionally, hypoxic embryos did not respond to the intermediate concentration of adenosine at 80% of incubation, whereas normoxic embryos decreased Pₘ and fₜ at this age. Overstimulation of adenosine receptors down regulates and desensitizes receptors in chicken embryos (Shryock et al., 1989). Additionally, adenosine deaminase, breaks down extracellular adenosine, and increases during hypoxic exposure to cultured human endothelial cells, and has been identified in the adult chicken (Iwaki-Egawa and Wantanabe, 2002; Eltzschig, 2006). While this is possible, this could also be the result of delayed maturation in adenosine receptor activity.
Embryonic chicken baseline $P_m$ is regulated through multiple regulatory units including $\alpha$ and $\beta$-adrenergic receptors, Ang II, and NO. Adenosine receptor blockade increased $P_m$ in normoxic embryo at 80% and 90% of incubation and hypoxic embryos at 90% of incubation. Thus adenosine could serve a role in maintenance of $P_m$. However, this response could be the result of secondary actions that could achieve a similar response rather than receptor antagonism. Methyxanthines, caffeine and theophylline increase plasma catecholamines, which could account for the hypertensive response. However, additional studies are necessary to determine if blockade of adenosine receptors or a secondary response is responsible for the hypertension (Robertson et al., 1978; Higbee et al., 1982).

A possible role for adenosine in the cardiovascular response to acute hypoxia may be present given exposure to acute hypoxia decreases in $P_m$ and $f_H$ in embryonic chickens (Crossley et al., 2003A). $P_m$ responses to acute hypoxia suggest that the hypotension involves NO, $\beta$-adrenergic receptors, and may involve the renin angiotensin system (Crossley et al., 2003A; Lindgren et al., 2011; Iversen et al., 2014; Chapter 4). However, the reduction in $f_H$ during acute hypoxia in chicken embryos is not due to cholinergic stimulation, and is currently suggested to be the result of direct action of low oxygen on the heart (Crossley et al., 2003A). Adenosine released during hypoxia could mediate this depression in $f_H$ as reported in rats and should be considered as a possible mechanism in the embryonic chicken (Kaplan et al., 2003). Given the activity of adenosine, and that hypoxic events initiate release, further consideration should be given to the involvement of adenosine on the $f_H$ response to acute hypoxia.
5.5 Summary

Collectively, when measured at normoxic conditions embryos incubated in chronic hypoxia do not demonstrate a decrease in intensity of the $f_H$ response. Adenosine did not contribute to the maintenance of $f_H$ but a possible role in the maintenance of baseline $P_m$. The acute pressure responses to adenosine could have appeared due to several mechanisms, not investigated here, including secondary effects due to AV block, contractility of cardiac tissue, or direct vasodilatory properties of adenosine. Finally, adenosine receptor blockade significantly increased arterial pressure in normoxic embryos starting as early as 80% of incubation; hypoxic incubation delayed the contribution until 90% of incubation, although this could be due to secondary effects of theophylline. Thus it appears that adenosine elicits substantial responses in $P_m$ and $f_H$ and hypoxic incubation appears to delay maturation or desensitize adenosine receptors.
Table 5.1: Morphological characteristics for chicken embryos incubate in 21% O2 or 15% O2 at 60%, 70%, 80%, and 90% of incubation. Presented are the sample size (n), wet embryo, heart mass, and the relative heart mass. Different uppercase Latin letters indicate statistically distinct values for embryonic wet mass. Data are presented as the mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation %</th>
<th>Condition</th>
<th>Embryo mass (g)</th>
<th>Heart mass (mg)</th>
<th>Relative heart mass (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>N (5)</td>
<td>7.52 ± 0.19⁰</td>
<td>55.2 ± 2.0</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>60</td>
<td>H (6)</td>
<td>6.19 ± 0.33⁰</td>
<td>51.2 ± 3.6</td>
<td>8.4 ± 0.6</td>
</tr>
<tr>
<td>70</td>
<td>N (6)</td>
<td>13.04 ± 0.18⁰</td>
<td>61.0 ± 13.2</td>
<td>4.9 ± 0.2</td>
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<tr>
<td>70</td>
<td>H (5)</td>
<td>11.62 ± 0.31⁰</td>
<td>77.8 ± 3.1</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>80</td>
<td>N (6)</td>
<td>18.21 ± 0.53⁰</td>
<td>114.6 ± 7.5</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>80</td>
<td>H (6)</td>
<td>15.36 ± 1.46⁰</td>
<td>103.3 ± 4.2</td>
<td>7.2 ± 0.1</td>
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<tr>
<td>90</td>
<td>N (6)</td>
<td>23.98 ± 1.55⁰</td>
<td>134.3 ± 4.8</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>90</td>
<td>H (7)</td>
<td>28.88 ± 1.29⁰</td>
<td>136.2 ± 6.6</td>
<td>5.7 ± 0.1</td>
</tr>
</tbody>
</table>

Table 5.2: Baseline arterial pressure (Pₘ) and heart rate (f_H) and sample size (n) for normoxic (N) and hypoxic (H) incubated embryos at 60%, 70%, 80%, and 90% of incubation. Similar letters indicate similar values for Pₘ or f_H according to the SNK post-hoc comparison following the initial ANOVA model. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Incubation %</th>
<th>Condition</th>
<th>Pₘ (kPa)</th>
<th>f_H (Beats min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>N (5)</td>
<td>0.94 ± 0.07⁰</td>
<td>237 ± 4⁰</td>
</tr>
<tr>
<td>60</td>
<td>H (6)</td>
<td>1.04 ± 0.07⁰</td>
<td>240 ± 12⁰</td>
</tr>
<tr>
<td>70</td>
<td>N (6)</td>
<td>1.46 ± 0.08⁰</td>
<td>260 ± 7⁰</td>
</tr>
<tr>
<td>70</td>
<td>H (5)</td>
<td>1.44 ± 0.09⁰</td>
<td>260 ± 11⁰</td>
</tr>
<tr>
<td>80</td>
<td>N (6)</td>
<td>1.91 ± 0.05⁰</td>
<td>245 ± 8⁰</td>
</tr>
<tr>
<td>80</td>
<td>H (6)</td>
<td>1.93 ± 0.19⁰</td>
<td>264 ± 5⁰</td>
</tr>
<tr>
<td>90</td>
<td>N (8)</td>
<td>2.42 ± 0.17⁰</td>
<td>239 ± 6⁰</td>
</tr>
<tr>
<td>90</td>
<td>H (7)</td>
<td>2.09 ± 0.14⁰</td>
<td>252 ± 6⁰</td>
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</table>
Fig. 5.1: Representative arterial pressure response to 26.7mg kg⁻¹ adenosine in a 70% (A) and 90% (B) normoxic incubated chicken embryo. The solid bar indicates 10 seconds, whereas the arrow indicates the injection of adenosine into the CAM arterial catheter.
Fig. 5.2: Mean change in arterial pressure ($P_m$; A), and heart rate ($f_H$; B) response to increasing concentrations of adenosine; 0.267 mg kg$^{-1}$ (open bars), 2.67 mg kg$^{-1}$ (shaded bars), 26.7 mg kg$^{-1}$ (closed bars) measured at 60% (60), 70% (70), 80% (80) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response. Similar uppercase Latin letters indicate a similar relative change according to SNK post-hoc comparison. Data are presented as the mean ± SEM.
Fig. 5.3: Mean arterial pressure ($P_m$; A), and heart rate ($f_H$; B) before (open bars) and after (closed bars) adenosine receptor blockade with theophylline 4 mg kg$^{-1}$ at 60% (60), 70% (70), 80% (80) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response from the pre-injection. Similar letters indicate a similar relative change according to SNK post-hoc comparison. Data are presented as the mean ± SEM.
Fig. 5.4: Mean change in arterial pressure (P_m; A), and heart rate (f_H; B) response to adenosine (2.67 mg kg⁻¹) before (open bars) and after (closed bars) adenosine receptor blockade at 60% (60), 70% (70), 80% (80) and 90% (90) of incubation in normoxic (N) and hypoxic embryos (H). An asterisk represents a significant response from the pre-injection value (p < 0.05). Similar letters indicate a similar relative change according to SNK post-hoc comparison. Data are presented as the mean ± SEM.
Collectively, the findings presented in these studies have characterized the effects of developmental stress on the cardiovascular system in two embryonic archosaurs. The cardiovascular system begins function very near to the onset of its organogenesis both inherited and environmental factors affect the gene expression and the subsequent phenotype. Under these conditions the organ system must develop and function, however, the function may alter gene expression that then in turn alters the organ system. Chronic stress alters the regulatory control of the cardiovascular system, resulting in marked effects on both cardiovascular regulation and morphological phenotype.

Dehydration during incubation produced smaller and bradycardic embryos. However cardiovascular response to Ang II was not affected by dehydration. The Ang II response was similar but distinctly different from adult vertebrates. Ang II caused a secondary $\alpha$-adrenergic mediated vasoconstriction and decreased heart rate through cholinergic receptors. Dehydration induced a cholinergic tonus on heart rate in dehydrated alligators suggesting cholinergic control of heart rate is plastic. While studies in fetal sheep support the importance of Ang II during acute perturbations during development, the cardiovascular response to Ang II did not appear to be plastic following dehydration.

Chronic hypoxic incubation affected the cardiovascular response to Ang I when measured at 15% $O_2$, this could suggest that the RAS may be plastic in chronic incubation in hypoxia, and that chicken embryos incubated in hypoxia may alter
angiotensin converting enzyme function to support cardiovascular function. The vasodepressor response to acute hypoxia was abolished following angiotensin converting enzyme inhibition, suggesting the renin angiotensin system is involved in the acute hypoxic response. Given Ang II function was altered by hypoxic incubation, other humoral regulators of cardiovascular function may be altered as well. Therefore the effect of hypoxic incubation on the cardiovascular response to adenosine was investigated. The maintenance of blood flow in the fetal sheep to essential organs includes adenosine to reduce vascular resistance and promote blood flow. The cardiovascular response to adenosine in the embryonic chicken included a substantial bradycardia and a depression in arterial pressure. Data here support adenosinergic regulatory capacity in the chick embryo and that hypoxic incubation alters the cardiovascular response. Following these studies a clear role for humoral control of the cardiovascular system during incubation was evident.

Within the studies presented here, key features have been identified during ontogeny to complement the existing understanding of cardiovascular regulation during incubation stress in archosaurs. A collective assessment with data presented here and previous embryonic studies suggest that multiple regulatory mechanisms serve a role in the maintenance of blood pressure and heart rate in birds and crocodilians. Stress during ontogeny can induce presence or alter intensity of features investigated here and previous studies. Presented in Table 6.1 is a summary of regulatory systems that have been identified thus far among archosaurs studied to date and the effect of stress on cardiovascular function during embryonic development. Several regulatory features that are involved in cardiovascular maintenance during embryonic development have been
identified in multiple archosaur species; however, the effect of stress on cardiovascular regulation has only been assessed in two species, the American alligator (*A. mississippiensis*) and domestic chicken (*G. gallus*).

Clear similarities and differences in cardiovascular regulatory patterns exist within the archosaur lineage. Cholinergic control of heart rate is variable among archosaurs studied, whereas adrenergic control is apparent (Crossley et al., 2000; Eme et al., 2011A; Tate et al., 2012; Swart et al., 2013). Humoral control through multiple systems suggests an active role in maintenance cardiovascular function to supplement cholinergic and adrenergic regulation (Crossley et al., 2010; Iversen et al., 2014). The impact of stress during ontogeny has only been investigated in the chicken and alligator embryo. These studies have revealed plasticity of cardiovascular regulation to maintain cardiovascular function.

Stress during ontogeny can induce the presence of features that are not present under control conditions responsible for cardiovascular maintenance; such examples have been presented in the studies presented here in addition to previous work (Iversen et al., 2014; Chapter 3; Chapter 4; Chapter 5). Cholinergic tone during control incubation conditions is induced during ontogeny if stress is present in the alligator and chicken embryo. However, the stressor that induces cholinergic control of heart rate differs between alligator and chicken embryos. Dehydration stimulates cholinergic tone in alligators, whereas in chicken strains that do not normally possess tone, cholinergic tone on heart rate is induced by hypoxic incubation (Crossley and Altimiras, 2012; Tate et al., 2012). While cholinergic tone is likely mediated through neural mechanisms, adrenergic tone primarily relies on circulating catecholamines from non-neural sources,
suggesting that alterations in adrenergic tone may be a reflection of receptor density or sensitivity (Lindgren and Altimiras, 2009; Lindgren et al., 2011; Eme et al., 2011A).

Cardiovascular regulation through humoral components suggests an active role in the maintenance of cardiovascular function. In the embryonic alligator acute responses to Ang II suggest that dehydration does not alter the response, whereas hypoxic incubated chicken embryos appear to alter response to components of the renin angiotensin system, suggesting plasticity during hypoxic incubation conditions (Tate et al., 2012; Chapter 4). The acute response to multiple humoral components is affected by developmental stress, however, tonic contribution to baseline values of arterial pressure and heart rate do not differ from control embryos suggesting that humoral systems studied thus far may be plastic in the acute response but not long-term maintenance (Iversen et al., 2014; Chapter 4; Chapter 5).

Collectively, studies presented in this document further support the importance of the developmental environment on embryonic growth and maturation of the cardiovascular system. Further, mechanisms involved in regulation of cardiovascular function during normal and sub-optimal embryonic development were elucidated and demonstrated a clear effect of stress on regulatory patterns. In conclusion, regulatory control of cardiovascular function can be modified by stress during incubation; tonic regulatory mechanisms suggest that parasympathetic control is plastic, whereas, humoral control of cardiovascular function is less plastic in regards to tonic control.

6.1 Future Studies

Studies presented in chapters 2-5 have provided an assessment of the effects of
developmental stress on morphological and cardiovascular regulation in two archosaurs. Future studies should address any deficiencies in the cardiovascular physiology during stress during ontogeny.

6.1.1 Experiment 1

Measurements of the cardiovascular response to acute dehydration may better elucidate the embryo response to dehydration in ovo. Determining blood volume measurements after each dehydration event will quantify if water is preferentially removed from circulation other water compartments within the alligator egg. The current studies have provided evidence that alligator embryos defend blood volume following dehydration, acute measurement may reveal different trends and support the hypothesis that alligator embryos exposed to dehydration events will reduce blood volume. Measurement of embryonic blood pressure and heart rate at this time may reveal the acute mechanisms that govern cardiovascular function during embryonic development.

6.1.2 Experiment 2

Direct vagal recordings will provide assessment of the vagal capacity to function during development of alligator embryos. Studies presented here, suggest that cholinergic stimulation is responsible for the bradycardia in dehydrated alligator embryos. Vagal and sympathetic recordings in dehydrated alligator embryos will provide important information on the operation of the parasympathetic and sympathetic
regulation of heart rate and arterial pressure following dehydration stress during development.

6.1.3 Experiment 3

Angiotensin converting enzyme inhibition abolished the hypotension during acute hypoxia in chicken embryos. This suggests that angiotensin II may be involved in the acute response to hypoxia. Further studies are necessary to determine the mechanisms through which this occurs. Investigation into the effect of nitric oxide on the angiotensin II may reveal mechanisms of angiotensin II action similar to adult chickens where binding of angiotensin II stimulate nitric oxide release.

6.1.4 Experiment 4

Experiments presented here suggest that angiotensin converting enzyme is altered following incubation in hypoxia while measured in prolonged hypoxia. Determination of plasma concentrations and activity of angiotensin converting enzyme will address this gap in the current knowledge and provide a potential role of the renin angiotensin system during hypoxic events.

6.1.5 Experiment 5

Further investigations should distinguish the role that specific adenosine receptors contribute to the cardiovascular response to individual heart rate and blood pressure responses. Given non-selective adenosine receptor blockade increased arterial pressure, specific adenosine receptor antagonists should be utilized to
determine the individual receptor contribution. Also of consideration, the role of adenosine should be investigated for the acute hypoxic cardiovascular response.

6.1.6 Closing

Studies presented here provide important information regarding the response to stress during development in two species of Archosaurs. Further, information has been presented emphasizing the role of the developmental environment on embryonic growth. Additionally, variation in the incubation environment can leave the developing embryo with a modified cardiovascular phenotype. Cardiovascular regulation by both central and humoral components was assessed and data presented suggests that the plasticity of regulatory systems varies by species and the imposed developmental stress. Expansion of studies into other taxa both within and outside Archosaurs and alternative humoral regulatory systems may better define the effects of stress during incubation and the patterns of cardiovascular regulation present.
Table 6.1: Regulatory patterns and the cardiovascular response in archosaurs during control incubation (C) and the effect on function following dehydration or chronic hypoxic stress during incubation. No change, increase and decrease indicate response of control unit, if the system alters the response compared to control incubated embryos. For cardiovascular control by cholinergic receptors only yes is provided due to variable presence across studied species. A dash represents systems that have not been assessed during the specific stress listed at the top of the column. Sub-script numbers correspond to the literature cited: 1 Lindgren et al., 2011; 2 Crossley and Altimiras, 2012; 3 Eme et al., 2011A; 4 Tate et al., 2012; 5 Iversen et al., 2014. Items without a citation are provide are provided within the text of this document.

<table>
<thead>
<tr>
<th>Control unit</th>
<th>Present (C)</th>
<th>Dehydration</th>
<th>Chronic Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-adrenergic</td>
<td>Yes</td>
<td>No change</td>
<td>No change¹</td>
</tr>
<tr>
<td>β-adrenergic</td>
<td>Yes</td>
<td>No change</td>
<td>Increase¹,²,³</td>
</tr>
<tr>
<td>Cholinergic</td>
<td>Some</td>
<td>Yes</td>
<td>Yes² (Chickens)</td>
</tr>
<tr>
<td>(Alligators)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baroreflex</td>
<td>Late</td>
<td>No change</td>
<td>-</td>
</tr>
<tr>
<td>NO</td>
<td>Yes</td>
<td>-</td>
<td>Decrease⁵</td>
</tr>
<tr>
<td>RAS</td>
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<td>No change</td>
<td>Possible changes</td>
</tr>
<tr>
<td>Adenosine</td>
<td>Yes</td>
<td>-</td>
<td>Decrease</td>
</tr>
</tbody>
</table>
REFERENCES


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