INCUBATION HUMIDITY AS AN ENVIRONMENTAL STRESSOR ON THE OSMOREGULATORY DEVELOPMENTAL PROGRAM OF THE CHICKEN, *Gallus gallus domesticus*

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Fetal programming results from stressors during fetal development and may influence the occurrence of disease later in life. Maternal nutritional status and/or environment can affect renal development by inducing limited nephron endowment at birth, which results in diseases such as hypertension and coronary heart disease in mammals. Birds are likely to be effective models for this process because, like mammals, they have high pressure cardiovascular systems, mammalian-type nephrons and are homeothermic.

This project uses the chicken embryo to explore physiological responses of disrupted hydration state thereby providing insights into renal fetal programming. Under normal conditions the chorioallantoic membrane (CAM) and developing avian kidney work in unison to ensure a proper balance of ions and water within the egg. White leghorn chicken eggs were incubated at 37.5^{o}C±0.5^{o}C and either <35%, 55-60% (normal) or >85% relative humidity.

Amniotic fluid serves as the drinking source for the embryo late in development; its composition is important to salt and water homeostasis. High amniotic fluid osmolality increased the blood osmolality for embryos exposed to low humidity incubation thereby indirectly influencing the renal developmental program of the embryos from this group. Indeed estimated filtering capacity was doubled in the low humidity group (6.77 ± 0.43 mm^3) compared to normal (4.80 ± 0.33 mm^3) and high (3.97

± 0.30 mm^3) humidity groups. The increased filtering capacity seen for those embryos from low humidity may indicate the ability for more efficient recovery of water if similarly stressed as an adult bird.

All embryo populations maintained similar oxygen consumption (0.075 ml/min – 0.37 ml/min), hematocrit (15 % - 32 %) and hemoglobin values (4 g/dl – 9 g/dl), thus displaying control over these aspects of the internal environment despite the obvious environmental insult of extreme incubation humidity. These results signify the embryo's immature kidney, along with lower gastrointestinal tract, functions much like the adult form maintaining homeostasis, although the mechanisms may differ. The overall benefits of this research included better understanding of the role the kidney during embryonic development and determining whether environmental factors, such as humidity, leave an imprint on morphological and physiological aspects of the urinary system of the embryo and water compartments of the egg.

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CHAPTER 1

INTRODUCTION

Development and Environment

Embryological development in birds is influenced by a number of environmental factors, most notably temperature and humidity. Under normal conditions the chorioallantoic membrane (CAM) and developing avian kidney work in unison to ensure a proper balance of ions and water within the egg. The effects of relative humidity on these systems remain unclear. High (>85%) and low (<30%) humidity during incubation increases mortality rates in chicken embryos (Ar and Rahn, 1980), presumably due to osmotic stresses placed upon the embryo by inadequate water availability or absence of mechanisms that enable the removal of higher volumes of water. Due to the high mortality rates witnessed in extreme humidity conditions, a general hypothesis can be formed that extremes in humidity encountered during incubation result in morphological and physiological variations in developing chickens.

Critical periods, described as stages of development in which the embryo is most susceptible to environmental influences, can result in modification of the developmental program of organ systems (Spicer and Burggren, 2003). This change, which may be reflected morphologically or physiologically, can serve as a benefit to the embryo. Alternatively, when developmental trajectories are modified due to environmental stimuli, homeostatic set points can be affected thus initiating a series of events that can result in adult pathologies (Kuzawa, 2007).

Renal Fetal Programming

In most living organisms, water and sodium are major determinants of body fluid homeostasis. The body can compensate for deviations from the homeostatic state within limits via reflexes, behaviors, hormones, or a combination of these mechanisms (Guan *et al.*, 2008). An abundance of research has analyzed the systems involved in maintaining fluid and electrolyte homeostasis within adults (Fitzsimons, 1998), while much is to be uncovered with regards to how similar mechanisms operate during embryonic development. When faced with alterations in the maternal environment the fetus may adapt accordingly. Adaptation can cause an imprint in developing organ systems with long-term influence after birth.

Barker's hypothesis, also known as fetal origins hypothesis, refers to disturbances during critical periods of development which can serve as potential causes for pathologies in adults (Barker, 1995). This hypothesis is based on a relationship between birth weight and the lifetime risk for coronary heart disease (Barker, 1995). The emergence of this hypothesis started a landslide of questions regarding the impact of perturbations brought on by the maternal environment on the developmental trajectories of organ systems during the fetal period (Langley-Evans, 2008). The "Fetal Origins Hypothesis" proposes that coronary heart disease, and the diseases related to it, originate through responses to undernutrition during fetal life and infancy that permanently change the body's structure, physiology and metabolism (Barker, 1995).

An organism's development is not merely a gene-led process based on the switching on and off of genes in well-ordered sequences. Adaptive responses prompted by variations in the environment, as indicated through the maternal system,

can alter the profile of genes expressed at any given stage of development with permanent effects upon the morphology and physiology of the fetus (Langley-Evans, 2008).

Properly designed human group studies take several decades to determine whether a possible association between maternal nutrition, body composition or other factors are the reason for development of disease in adults (Ross and Desai, 2005; Langley-Evans, 2008). Mammalian models have shown that maternal nutritional status and/or environment can influence an embryo to experience hypertension, coronary heart disease, type II diabetes, obesity, asthma or osteoporosis (Ingelfinger, 2004; Mitchell *et al*, 2004; Ross and Desai, 2005). The mechanisms behind these pathologies in adults are not well understood.

A limited nephron population at birth has been implicated as the primary contributor to adult renal disease (Brenner *et al.*, 1988; Langley-Evans *et al.*, 1999; Hughson *et al.*, 2003). Since nephrogenesis is complete upon birth in most mammals, any reduction in functioning capacity of the kidney prior to birth results in increases in blood pressure to maintain glomerular filtration rate (GFR) and fluid homeostatic functions (Mackenzie and Brenner, 1995; Langley-Evans, 2008). Then a vicious cycle is established involving localized increased blood pressure as being the cause for further loss of nephrons due to tissue damage (Mackenzie and Brenner, 1995). Once this cycle is created, heart disease with subsequent renal failure results if proper lifestyle changes and medical measures are not taken.

An example of fetal programming of adult disease is seen in rats where high maternal salt intake during pregnancy causes rats to develop salt sensitive hypertension

resulting in tubular atrophy in subcortical regions and enlarged glomeruli (Rasch *et al.*, 2004). Fetal lambs experiencing maternal hypertonicity show signs of plasma hypertonicity, arterial hypertension, increase in hematocrit and total hemoglobin and hypernatremia (Desai *et al.*, 2003). Timing of the environmental insult is also critical to nephron endowment. When fetal sheep experience umbilico-placental embolization (UPE) close to the end of gestation, the results are intrauterine growth restriction (IUGR), decreases in kidney weights, and insignificant reductions in number of nephrons (Mitchell *et al.*, 2004). However, twinning in sheep causes IUGR with a significant reduction in number of nephrons with the resultant increase in glomerular volume to counterbalance lower nephron numbers. This study by Mitchell *et al.* (2004) signified that prolonged periods of growth restriction, as in the case of twins, has a more profound effect on kidney development compared to IUGR by UPE.

The renin-angiotensin system (RAS) and glucocorticoid exposure during critical periods are proposed mechanisms behind reduced nephron number resulting from fetal programming (Moritz *et al.*, 2003; Guan *et al.*, 2008). Intact RAS is necessary for maintenance and regulation of GFR *in utero* and angiotensin II (ANG II) is needed to sustain cellular differentiation and organ development (Lumbers, 1995; Guan *et al.*, 2008). Increased exposure to glucocorticoids during development augments angiotensin I and II receptors, resulting in apoptosis of metanephric mesenchymal cells and/or accelerated branching, which may then terminate prematurely before the regular number of nephrons have been established (Glassberg, 2002; Moritz *et al.*, 2003; Langley-Evans, 2008).

Use of mammalian models, like rat, sheep and pig, to study fetal programming has been useful due to their similarities with human development (Moritz *et al*, 2003; Mitchell, 2004). However, there are still limitations with using mammalian models, such as the difficulty in monitoring normal development within the mother during critical periods of maturation. The use of an alternative model, such as an avian model, may prove useful in answering questions common to fetal programming. There are many attributes that are shared between mammals and birds, such as kidney and endocrine development, presence of high pressure systems and endothermy (Braun, 1985). Due to these similarities, one could utilize an avian model to test the effects of environmental insults on normal developmental trajectories.

Testing the effects of osmotic stress imposed by extreme incubation humidity on embryonic chickens in this study will demonstrate how compensation during critical periods may alter the developmental programs of organ systems. This alteration during organ development, specifically kidney development, may cause the bird to become more susceptible to pathologies in the adult form similar to what is seen in mammals.

Chicken as an Animal Model

In comparative physiology, models are often chosen based on the August Krogh principle which states “For many problems there is an animal on which it can be most conveniently studied” (Krebs, 1975; Burggen, 1999). The chicken has long been shown to be a prominent paradigm for studying development since it exists in a self-contained environment that can be easily manipulated (Stern, 2005; Burt, 2007). Temperature and humidity-controlled incubators enable fertilized eggs to be easily maintained during the short incubation period of 21 days (Romanoff, 1960).

By placing embryos in extreme humidity conditions during incubation, we can observe not only the broad outcomes of the stressor on embryonic development, but we can also examine the mechanisms by which the embryo alters its development. This model lends to establishment of critical periods for organ growth and function. Understanding the critical periods of development for birds yields information about the development of other species that may be too difficult to study due to mechanical obstacles. For example, the avian kidney is an excellent model to study kidney development since onset of function is marked by expansion of the allantoic sac (Vleck *et al.*, 1980). Therefore, we can test the kidney's response to environmental stressors or teratogens.

In mammals, it is the maternal environment that imposes stress upon the embryo when nutrition is not adequate (Langley-Evans, 2008). Avian embryos may lead the way to understanding and possibly reversing or preventing health issues which affect the human population. Because we are able to examine the direct effects of environmental factors and manipulate avian embryos during critical time periods of development, birds may indeed become an excellent model for studying fetal programming.

The avian kidney is not the only example of modeling within the osmoregulatory system of the embryo; the allantois can also be a paradigm for mammalian distal nephron (Graves *et al.*, 1986). The chicken allantois shares many structural and functional similarities to the amphibian urinary bladder, a well-established model for mammalian distal nephron (Hoyt, 1979; Stewart and Terepka, 1969). In addition, both the chicken allantois and amphibian bladder can readily respond to hormonal

stimulation (aldosterone, arginine vasotocin and prolactin) to control salt and water balance (Murphy *et al.*, 1983; Doneen and Smith, 1982). Since the tadpole urinary bladder is small making developmental analysis difficult, the large, readily-accessible chicken allantois is a good model for studying epithelial ion transport (Graves *et al.*, 1986).

Avian Kidney: Adult Morphology

The avian kidneys are large, elongated, lobular organs that consist of cranial, medial and caudal divisions and reside deep within the synsacrum. Connective tissue and vasculature interweaving amongst the divisions of the kidneys makes it difficult to remove the organ *in toto*. All three divisions are drained by a single ureter; although, separate blood supply is sent to the cranial division via a branch of the descending aorta while the medial and caudal divisions share blood supply via renal artery branches from the ischiatic artery (Goldstein and Skadhauge, 2000).

The avian kidney consists of a series of lobules each containing a cortex and medulla that is shaped like a cone; hence the name medullary cones (Johnson 1968; Johnson and Mugaas, 1970a; Braun and Dantzler, 1972; Johnson 1974). Medullary cones are made up of loops of Henle, collecting ducts and vasa recta and function similar to mammalian renal medulla (Casotti *et al.*, 2000).

The unique characteristic about avian kidneys is the presence of both mammalian- (MT) and reptilian-type (RT) nephrons which intermingle with one another forming a disorganized arrangement when compared to highly regionalized mammalian kidneys (Wideman, 1989). Mammalian-type nephrons, also known as looped nephrons, have a loop of Henle and an ability to contribute to the osmotic gradient needed to create

hyperosmotic urine. Looped nephrons make up only 10-30% of the total nephron population while RT make up the vast majority, 70-90%, of the nephrons seen in birds (Casotti *et al.*, 2000; Goldstein and Skadhauge, 2000; Braun, 1985). The loops of Henle are seen projecting down into medullary cones at the center of each kidney division. Adult MT glomeruli diameters range from 81-110 µm while the RT glomeruli are much smaller, ranging from 49-75 µm in diameter (Wideman, 1988). The RT nephrons lie near the surface of the kidney divisions and do not possess a loop of Henle similar to nephrons in reptiles.

The organization of the avian nephron is similar to that seen in mammals. Each nephron contains a renal corpuscle consisting of a glomerulus (fenestrated capillaries), visceral and parietal layers of Bowman's capsule, Bowman's space, and a juxtaglomerular apparatus (JGA). The parietal layer of Bowman's capsule is made up of simple squamous epithelium while podocytes compose the visceral layer and contribute to the filtration pathway. The JGA located at the vascular pole of the corpuscle consists of a macula densa (cells lining a small region of the distal convoluting tubule that exhibit increased cell height and crowding) extraglomerular mesangium (mesangial cells), granular juxtaglomerular cells (located within in the wall of the afferent arteriole) and nerve fibers (Johnson and Mugaas, 1970b). The urinary pole is located opposite the vascular pole where the ultrafiltrate is drained via the proximal convoluted tubule (Wideman, 1988).

The proximal convoluted tubules are composed of cuboidal to columnar epithelial cells with a well-developed brush border. The avian thin descending limb of Henle's loop contains cuboidal cells while the thick ascending limbs have similar epithelial

structure and contain some apical microvilli and tightly stacked mitochondria. A region seen only in RT nephrons in place of the loop of Henle connecting the proximal and distal tubules is called the intermediate segment. This segment is analogous to the thick ascending limb of Henle's loop. The distal tubules begin near the vascular pole of the renal corpuscle and cells are cuboidal in shape and lack a brush border. These tubules empty into collecting tubules which have two main types of cells, intercalated cells similar in structure to cells seen in the distal tubules and mucus secreting cells filled with numerous granules of mucopolysaccharides. Finally, the collecting ducts show a decrease in number of intercalated cells and increase in tall, columnar mucus-secreting cell types (Wideman *et al.*, 1981).

Avian Kidney: Development

The avian kidney as a model to study kidney development permits the ability to combine morphological differentiation with function using urine formation, and thus formation of the allantoic sac, as markers for kidney function (Vleck *et al.*, 1980). There are three stages of avian kidney development: pronephros, mesonephros, and metanephros. These stages are similar to the development seen in both mammals and reptiles. The pronephros begins as a protrusion of cells from the intermediate mesoderm between the paraxial mesoderm and the lateral plate around day 1 of incubation (Hiruma & Nakamura, 2003). During this time, the pronephros resembles the kidney structure of both fish and amphibians. Initially, the pronephros was thought to serve to induce mesonephric development (Clark *et al.*, 1993). Recent findings by Hiruma and Nakamura, (2003) indicated the pronephros functions as early as incubation day 4. At this stage, the pronephros exhibits many of the characteristics of

the later mesonephros, such as nephric ducts and tubules. The main difference between the pronephros and mesonephros besides the onset of functioning during development is the presence of external glomeruli. The external glomeruli seen in the pronephros protrude into the body cavity and contain epithelial cells with foot processes, indicative of the visceral layer of Bowman' s capsule seen later, and both a basement membrane and fenestrated endothelium. Other distinguishable differences between the pronephros and the mesonephros include the narrower pronephric duct versus the wider mesonephric duct. Additionally, the pronephros exhibits shorter tubules. Around incubation day 6, degeneration of the pronephros ensues and the mesonephros becomes the functioning kidney of the embryo.

The mesonephros begins differentiation around day 2.5. The production of urine and thus functioning mesonephros begins around incubation day 3.5 (Narbaitz & Kapal, 1986). This is confirmed by the expansion of the allantoic sac and the presence of urine. Allantoic fluid may be called urine because of its ionic composition and presence of uric acid (Romanoff, 1967). Once this expansion has occurred, fusion of the allantoic sac and chorion follows forming the chorioallantoic membrane (CAM) which affects the interchange of gases with the environment through the porous shell. Narbaitz and Kapal (1986) stated that it appeared that the functioning kidney was associated to the presence of foot processes, which would later be referred to as pedicels of podocytes or the visceral layer of Bowman's capsule. Between days 5 and 7, the volume of urine in the allantoic sac increases volume by a factor of 6. Around incubation day 16, the mesonephros also begins degenerating much like that of the pronephros, but the

metanephros has already begun functioning by this point in development (Murphy *et al.*, 1991).

The metanephros begins functioning around incubation day 12 (Carretero *et al.*, 1997). It is around this day that the proximal convoluted tubules display their apical microvilli demonstrating their resorptive capabilities (Narbaitz and Kacew, 1978). All nephron types are seen and may be isolated by day 18 of incubation (Gambaryan, 1992). The metanephros does not degenerate and indeed continues to develop until approximately 30 days after hatching (Wideman, 1989). The metanephros remains the functioning form of the kidney throughout the chicken's life.

Avian Osmoregulation

The ability of birds to conserve water is vital to their survival. Birds are able to generate uric acid as their chief nitrogenous waste similar to reptiles, and produce hyperosmotic urine like fellow homeotherms to conserve body water while eliminating wastes (Braun, 1985).

As previously mentioned, birds have two types of nephrons, while the cumulative function of nephrons is to filter waste materials out of the blood and maintain fluid and salt balance; the MT nephrons function with a countercurrent multiplier system which creates a concentrated urine unlike the RT nephron which tends to undergo glomerular intermittency when water conservation is needed (Braun, 1985).

Filtration of the plasma through the glomerular capillary tuft is the first step in the formation of avian urine. The JGA functions in regulation of the single nephron glomerular filtration rate (SNGFR) by the action of the renin-angiotensin system (Skadhauge, 1981). If tubular fluid has not been adequately diluted or the flow rate is

too high, excess sodium chloride is delivered to the macula densa causing SNGFR to decrease by way of JG cells containing renin. The renin released from these cells cleaves angiotensinogen which is further reduced to angiotensin I forming angiotensin II by way of angiotensin converting enzyme (ACE). Angiotensin II will decrease SNGFR by increasing afferent arteriole resistance. The contractile elements of the mesangium may also aid in decreasing SNGFR by decreasing the surface area of glomerular capillaries. It will also increase the secretion of ADH (arginine vasotocin (AVT) or antidiuretic hormone) and aldosterone, and stimulate the hypothalamus to activate the thirst reflex, leading to increased blood pressure.

The proximal convoluted tubule (PCT) of the MT nephrons functions much like the mammalian PCT. The main function of this segment of the nephron is to recover the bulk of nutrients, fluid and electrolytes from the tubular lumen. Adult mammals exhibit reabsorption of 60-75% of total filtered volume upon reaching the end of the PCT, while micropuncture studies involving adult European starlings suggest reabsorption of an average of only 18-24% (Laverty & Dantzler, 1982). These results were limited since only small RT nephrons could be sampled via micropuncture and RT nephrons typically have a lower rate of fluid reabsorption compared to MT nephrons. Two functional secretory mechanisms exist in the avian PCT which become functional around embryonic day 9 (Rennick, 1969). Those mechanisms include organic cation and anion transport systems. The organic cation transport system secretes substances such as choline, acetylcholine, creatinine, epinephrine, histamine, serotonin, and amiloride into the tubular lumen (Wideman, 1988). This mechanism depends on oxidative metabolism and is coupled to both hydrogen ion counterport and sodium-

hydrogen ion antiport systems (Rennick, 1981). The organic anion transport system secretes uric acid. This action is seen exclusively in the RT nephron and not in MT nephron because it is speculated that uric acid precipitates may obstruct the loop of Henle (Dantzler and Braun, 1980).

The loop of Henle, which can only be seen in the MT nephrons, is a combination of two regions, the thin descending limb and thick ascending limb. The thin descending limb functions in active transport due to its large paracellular pathways and abundant mitochondria in the cuboidal cells lining this limb. This limb also demonstrates high permeability to sodium and chloride (Nishimura, 1985). The thick ascending limb is known as a diluting segment or energy source that is the driving force behind the operation of the medullary countercurrent multiplier system. This occurs by pumping sodium chloride into the medullary interstitium while the epithelium prevents osmotic equilibration due to its water impermeability (Miwa and Nishimura, 1986).

The intermediate segment is a region of the nephron only seen in RT nephrons extending from the final segment of the PCT to the beginning of the DCT at the vascular pole of the glomerulus. This segment is known as the primitive loop of Henle and it serves to dilute the tubular fluid further before reaching the macula densa (Wideman, 1988).

The distal convoluted tubule (DCT) is also a diluting segment with low water permeability (Stoner, 1985). Sodium/potassium ATPase is located at the basolateral membrane and it extrudes sodium from the cell and pumps potassium into the cell. Sodium and chloride enter the cell across the apical membrane (Wideman, 1988). The late distal tubules function in acidification of urine by secreting hydrogen ions into the

tubular lumen in exchange for potassium and reabsorption of additional quantities of sodium. Arginine vasotocin may modulate the water permeability of the late distal nephron (Stoner, 1985)

The collecting tubules (connecting tubule) function primarily in urinary acidification and water reabsorption. Cells in this region secrete mucus to both coat the tubules so that precipitated uric acid does not obstruct the collecting tubule lumen and also to convert uric acid to lyophilic colloids or layer spherules, trapping sodium and potassium between the spherule layers so that the stabilized uric acid may more readily be moved along the tubular lumen (Long and Skadhauge, 1983). The collecting ducts also secrete large quantities of mucus and AVT is thought to increase the water permeability in this segment (Wideman, 1988).

Renal-Gastrointestinal Coupling

The terminal portion of the gastrointestinal tract, specifically the urodeum of the cloaca, contributes significantly to the creation of hyperosmotic urine. Since birds do not possess a urinary bladder they use their lower intestines for urine storage and modification through reverse peristalsis. It is within the lower gastrointestinal tract that the urine is brought in contact with bacteria and mucosal epithelium, both of which participate in modifying the composition of the urine significantly (Braun, 1999a). This action enables the bird a final opportunity by which to recover water and vital proteins that bare significant energy costs due to uric acid excretion (Braun, 1999b). Little is known about renal-gastrointestinal coupling in embryos. When it begins to function or how the cloaca of the embryo will respond to extreme humidity is open for exploration.

Water Compartments in the Egg

There are two physiological processes that influence water amounts inside avian eggs during development. The first is a steady loss of water across the porous eggshell by diffusion, and the second is oxidation of yolk lipids producing metabolic water (Davis *et al.*, 1988). This section will review the development and functions of the water compartments and associated membranes in the chicken egg.

There are three main extra-embryonic fluid compartments in the egg: the sub-embryonic fluid (SEF), amniotic fluid and allantoic fluid. Through the formation and depletion of these fluid compartments, the embryo is able to remain hydrated through out development. The SEF compartment is the first new compartment which appears in the yolk sac beneath the embryo around 2-3 days of incubation. The water content in this compartment reaches a maximum on day 6 when it is composed of more than 95% water (Baggott, 2001). The mass of this compartment decreases after day 6. There is a critical period of growth for this fluid compartment between incubation days 3-7. Eggs that are not turned properly during this period have a decreased SEF volume and thus, the growth of all other extra-embryonic fluid compartments are retarded (Deeming, 1989).

The amniotic fluid compartment serves as mechanical protection to the embryo and achieves peak water content on day 13 (Baggott, 2001). This fluid compartment is unaffected by the hydration state of the egg (Ar, 1991). The amnion itself is formed from a layer of ectoderm and underlying vascular mesoderm immediately adjacent to the embryo. On day 4 of incubation, folds in the amnion fuse to form the amniotic sac over the head and tail of the embryo (Baggott, 2001). The sero-amniotic connection, a

duct connecting the amniotic sac and albumen sac, is formed on day 12 (Baggott, 2001). This connection allows the movement of albumen proteins and water into the amniotic fluid to be swallowed by the embryo.

The chorion develops in continuity with the amnion and away from the embryo. This membrane is comprised of an outside layer of ectoderm adjacent to the inner shell membrane and an inner avascular mesoderm which lines the extra-embryonic coelom (Baggott, 2001). The mesoderm of the chorion fuses with the vascular mesoderm of allantois to form the chorioallantoic membrane, or CAM around day 6. Ninety-eight percent of the eggshell membrane is covered by CAM by day 11, thereby acting as the primary respiratory surface for the embryo during the second half of incubation (Baggott, 2001). The water/gas interface is a diffusion limiting exchange system involving both the CAM and porous eggshell. Weight loss in chicken eggs is almost entirely due to water/gas interfaces diffusive properties (Romanoff, 1969; Ar *et al.*, 1974). By day 12 the CAM is completely formed and transport of sodium ions begins (Graves *et al.*, 1986).

The allantois is merely a small bud of ectodermal cells on day 2 but by day 3.5 forms a sac growing out from the primitive hindgut. It is this sac that will supply blood to the CAM and amnion. The allantoic sac acts as a repository for kidney excretions first appearing by day 3.5 which enters via the allantoic duct from the cloacal region of the hindgut (Romanoff, 1967). The source of this fluid which reaches its maximum around day 13, is blood filtered by the embryonic kidney (Romanoff, 1967). Excretory medium for nitrogenous metabolites are emptied into the allantoic fluid as ammonia, urea and uric acid (urate) starting around day 5 (Bradfield and Baggott, 1993b). The uric acid

content in this fluid compartment increases throughout development with a large increase seen on day 10 (4.73 mg or 0.15 mg/100 µL) – day 18 (62.48 mg or 3.24 mg/100 µL) (Romanoff, 1967). Uric acid is essential for water balance of the embryo due to its low solubility which enables the embryo to reduce the amount of water required for storage. Also, oxidation of uric acid to protein produces more metabolic water then oxidation from fat. Additionally, uric acid precipitates with cations (sodium) when pH is above 6.2 (McNabb, 1986).

Between days 12 and 19 of incubation, allantoic epithelium transports sodium actively by generating a short circuit current (SCC) (Graves *et al.*, 1986). This SCC allows for reabsorption of hyperosmotic fluid into the embryo's circulation late in development. Movement of sodium out of the allantois may be affected by hormonal action, specifically arginine vasotocin (AVT). Arginine vasotocin mRNA can be detected as early as day 6 (Milewski, 1989) and is detectable in the blood by day 16 (Klempt *et al.*, 1992; Muhlbauer *et al.*, 1993). This hormone may act directly on the CAM to enhance sodium transport causing a passive movement of water out of the allantois. The result of this action would explain the drastic reduction of allantoic fluid volume after its peak on day 13. The kidney does not have the capability to respond to AVT on day 12, but the same may not be said for the allantois.

In high water loss conditions, more uric acid precipitate is exhibited in the allantoic fluid, which means that as uric acid increases, sodium will disappear from allantoic fluid at two times the rate of chloride (2mmol of sodium to 1 mmol of chloride) (Davis *et al.*, 1988). In experiments carried out by Davis and colleagues (1988), embryos of high water loss eggs showed signs of hematocrit (Hct) and plasma

osmolality stress. Significance was only seen on days 17-21 for Hct and plasma osmolality on day 17 and days 19-21 in which high water loss groups were significantly higher than control. Low water loss did not affect development, conversely, the embryos were simply larger and the excess water was left behind after hatch (Davis *et al.*, 1988).

Bradfield and Baggott, (1993a) also exposed eggs to three incubation conditions and measured the effects of water loss upon uric acid, urea and ammonia in the Japanese quail, *Coturnix coturnix japonica*. They found that uric acid increases as development proceeds—strongly dependent on the rate of water loss from the egg. The same result was not seen for urea or ammonia content of the egg. These high water loss eggs demonstrated lower tissue water content and a smaller allantoic fluid volume. As the uric acid increases in the allantoic fluid and more sodium ions are bound, the potential for water reabsorption into the blood across the allantoic membrane increases (Bradfield and Baggott, 1993a). Water inflow from the kidney and outflow, across the allantoic membrane, determines allantoic fluid volume.

Most research of the fluid and electrolyte balance in developing chicks has been done during the second half of incubation. Simkiss (1980b) concluded that when water was limited to the embryo, the size of the embryo was altered. It is important to note that this conclusion was drawn from studies starting day 13 of incubation, so these results may not reflect how development will be affected if eggs are exposed to dehydration earlier in development. I examined these effects with my research.

Relative humidity (RH) ranging from 40-70%RH produces the most satisfactory incubation conditions for incubation with optimal performance at 53%RH (Peebles *et al.*,

2001). Also, qualities like hatchability and chick size can be controlled by egg moisture loss dictated by incubation conditions (Davis *et al.*, 1988; Swann and Brake, 1990). Finite water concentration of the egg is mandatory for optimal hatching success and is seen to have the most influence when embryonic metabolism reaches a plateau (Swann and Brake, 1990). As detailed earlier, the variation in the rate of water loss during incubation alters volume and electrolyte composition of allantoic fluid (Davis *et al.*, 1988; Hoyt, 1979; Bradfield and Baggott, 1993b) as well as uric acid content.

Water loss influences the developing embryo most during the first half of incubation as shown by Snyder and Birchard (1982). They incubated eggs under both dry and humid conditions by rotating them from their initial incubator every other day. They found that percent hatch was directly dependent on the amount of water lost during the first half of incubation rather than total loss of water.

Research Objectives and Hypotheses

As evident in the literature review just presented, there is a wealth of information about the avian kidney and its development, but also an abundance of unanswered questions. This study concentrated on three sites of osmoregulation in the developing chicken, (1) kidney, (2) lower gastrointestinal tract and (3) chorioallantoic membrane (CAM). By rearing eggs under extreme humidity conditions, the consequences of high and low water loss on renal morphology and physiology of the embryo were examined.

In the first study (Chapter 2), morphological measurements, including embryo and kidney wet and dry masses, along with oxygen consumption rates were measured for embryos starting day 10 of incubation through day 18. I hypothesized that embryo

and kidney masses are different between humidity groups and that the observed differences consequently affect oxygen consumption rates.

In the second study (Chapter 3), physiological components of this research including measurements of hematocrit, total hemoglobin and blood osmolality were measured for embryos day 10-18. Analysis of fluid compartment osmolality and uric acid concentration will give an insight to whether the embryo can compensate for shifts in water and electrolyte balance within the egg. I hypothesized that those embryos exposed to low humidity incubation experience desiccation in the egg resulting in raised hematocrit, higher fluid osmolality and an increase in uric acid precipitate within the allantoic sac. Alternatively, those embryos in the high humidity group have opposing results demonstrating developing systems that do not have the capacity to remove excess amounts of water.

In the third study (Chapter 4), renal development was measured by determining glomerular number and size distributions for embryos day 10-18 after modifying an alcian blue technique used in adult birds. I hypothesized that there were differences in glomerular distributions between humidity groups resulting in alterations of the embryo's renal developmental program when exposed to extreme humidity conditions.

CHAPTER 2

INCUBATION HUMIDITY EFFECTS ON SURVIVAL, MORPHOLOGY, AND METABOLISM OF THE CHICKEN

Introduction

Water Balance within the Egg

Water loss is an important factor regulating both survival and hatchability of avian eggs during incubation (Ar and Rahn, 1980). In the natural environment, incubation time is inversely proportional to both water vapor conductance and overall water loss (Rahn and Ar, 1974). This relationship, accompanied by the observed narrow range of incubation temperature and continuous egg turning, promotes optimal conditions for development of the avian embryo (Drent, 1973; Baggott, 2001). During the course of natural incubation, avian eggs lose between 16-18% of their initial mass in the form of water (Rahn and Ar, 1974; Ar and Rahn 1980; Snyder and Birchard, 1982). More recent findings coupling hen age and incubation humidity suggested weight loss in the form of water should not exceed 12% for optimal hatchability for commercial hatcheries (Peebles *et al*., 2001). Although several authors have reported satisfactory results from incubation humidity ranging from 40%-70% relative humidity (RH) (Lundy, 1969; Simkiss 1980a), only gross morphological measurements were observed with no indication of how development of organs or organ systems, including cardiovascular, renal and endocrine, may have been altered.

The amount of water present at any time during bird development is attributed to two main processes (1) loss of water by diffusion through tube-like pores in the calcite shell (Board, 1980), and (2) gain of water by oxidation of yolk lipids into what is known

as "metabolic water" (Ar and Rahn, 1980). Romanoff (1960) demonstrated that the amount of water in fresh chicken eggs was approximately 74% of total egg mass with water in the hatchling equaling 73% of total hatchling mass. This indicates that water consumed is replaced by water produced by metabolism. So the embryo, with help from it's mother by way of albumen, presumably has no difficulty with water allocation during development. However, a specific water concentration within the egg is mandatory for optimal hatching success; therefore, it is necessary that the embryo lose water during incubation (Ar and Rahn, 1980). When eggshell conductance is decreased artificially, the embryo is left without a route for water loss (Swann and Brake 1990). Those embryos with reduced eggshell conductance end up with smaller air cells. As a result of water loss, the air cell enlarges, which increases the air reservoir. This mechanism ensures that the embryo has a reservoir of oxygen to support the conversion from chorioallantoic membrane (CAM) gas exchange to pulmonary gas exchange achieved via internal pipping into the air cell (Board, 1980; Ar and Rahn, 1980). Air cell enlargement and subsequent size has been used as an indicator of adequate water loss for successful hatching.

High Water Loss

Several experimental studies have demonstrated the importance of incubation humidity during avian development (Hoyt, 1979; Davis *et al.*, 1988; Tazawa and Whittow, 2000). High rates of water loss have a greater negative impact on hatching if experienced in the first half of incubation (Simkiss, 1980a; Snyder and Birchard, 1982). Simkiss (1980a) examined rates of water loss and hatchability of eggs after being exposed to humid (65% RH) conditions for the first half of incubation, then dry (20%RH)

conditions for the second half of incubation and vice versa. Davis and colleagues (1988) mechanically increased rates of water loss by drilling holes into the air cell and exposing the eggs to low humidity (0-10% RH) during the last week of incubation. Survival and wet mass of the body, muscle, head and skin were significantly affected by low humidity owing to an increase in water loss. They concluded that because embryos from the high water loss groups exhibited reduced water in their extra-embryonic compartments, the CAM must have collapsed around the embryo. The proximity of CAM to embryonic skin may have caused water movement from the skin into circulation resulting in the observed differences in wet masses.

Since water consumed is replaced by water produced by metabolism, what might happen if the principle water source to the embryo, the albumen, was altered early in development? Eighty-eight percent of albumen is water and at laying, 60% of the water in the egg is in the albumen (Romanoff, 1967). By embryonic day 10, 76% of albumen water has been utilized with only 2.2 g increase in water of the embryo (Baggott, 2001). Therefore water from the albumen does not move directly to the embryo, but is moved between developing fluid compartments of the egg. Water content of the embryo does not increase dramatically until the second half of incubation (Romanoff, 1967). So, unless the water source is altered at the beginning of incubation or metabolism is slowed, the avian embryo exposed to low incubation humidity encounters no obstacles regarding water allowances. In contrast, those embryos exposed to low rates of water loss, as seen in high humidity incubation, would demonstrate difficulties with water removal.

Low Water Loss

Davis and colleagues (1988) examined the implications of low water loss after exposing embryos to 85% RH over the course of development, and reported that wet masses were larger with no effect on dry mass. The hatching success was decreased presumably due to low volume of the air cell, hence a decrease in oxygen availability. This study demonstrated the optimal range for incubation humidity should be maintained between 55-60% RH.

Chapter Objectives

The ultimate goal of this research was to determine whether the regulatory mechanisms of the embryo were limited by the external variable of incubation humidity. This chapter intends to examine the morphological and metabolic outcomes of extreme incubation humidity. I propose to advance our understanding of renal development in vertebrates by: 1) increasing the magnitude of embryonic exposure to extreme incubation humidity, and 2) examining the entire time course of development as opposed to taking a "snapshot" in time. A novel contribution will be the comparison of the effects of humidity extremes on kidney masses. Since the kidney plays an important role in osmoregulation of the avian embryo, determining the effects that incubation humidity may have upon the development of this organ is imperative. Finally, to date there has been little investigation of how incubation humidity affects oxygen consumption of the avian embryo and these experiments will determine the metabolic effects of hydration and dehydration of the embryo.

Materials and Methods

Source and Incubation of Eggs

Fertilized White Leghorn eggs (*Gallus gallus domesticus*) were obtained from Texas A&M University (College Station, TX) and shipped to University of North Texas (Denton, TX), where they were held in Hova-Bator incubators. The University of North Texas' Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

Eggs were separated and randomly chosen for placement within three incubators at either <30%, 55-60% or >85% relative humidity (RH) and temperature of 37.5^{o}C ± 0.5 ^{o}C. Humidity was measured using wireless Baro-Thermo-Hydrometers (model BTHR968, Oregon Scientific). Eggs were weighed pre-incubation and on experimental days 10-18. Loss in mass during development was calculated as the difference between pre-incubation and experimental day whole egg mass. On embryonic days ranging from 10-18, (stages 36-44; (Hamilton and Hamburger, 1951) eggs were removed from each incubator for experimental analyses.

Surgical Protocol

Embryos were sacrificed by injection of 0.1 ml pentobarbital into a CAM vessel or exposure to isofluorane vapor for 10 min in a desiccation chamber followed by rapid decapitation. The embryo was removed from the shell and wet weight measured. Each kidney was removed under a dissecting scope (WILD M32 Heerbrugg, Switzerland) using fine-tipped forceps and surgical scissors. Embryo and kidney wet weight was measured after removal of excess moisture via Kimwipes® (Kimtech Science) followed by placement of embryo and kidneys in Fisher Isotemp® oven (100 series model 106G)

at 60 °C for three days to obtain dry weight. Kidneys used for light microscopy analyses were placed overnight into a vial of 10% neutral buffered formalin with a pH of 7.2 at 4 °C.

Mortality

Mortality percentage was calculated as number of unviable eggs divided by number of incubated eggs multiplied by 100 for each humidity level over the course of 18 days of incubation. These values were gathered over a period of 32 incubation runs consisting of 12 eggs per humidity level per run.

Respirometry

Oxygen consumption was measured on embryonic days 10 - 18 using conventional flow through respirometry for avian embryos (Ackerman and Rahn, 1981; Dzialowski *et al.*, 2002). Eggs were placed into individual respirometers and into an incubator at 37°C for a maximum of 2 h for each experimental trial. Compressed air flowed into each jar after first passing through a column of Drierite® and soda lime to rid the air of water and carbon dioxide, respectively (Figure 2.1). Once inside the incubator, tubing was connected to a manifold (Sable System MF-8 Airflow Manifold) where the inflowing air could be adjusted and distributed simultaneously to 8 respirometers. Gas flow into each respirometer ranged from 25ml/min (day 10) to 75ml/min (day 18). Gas from each respirometer passed through both Drierite® and soda lime and then to a multiplexor (Sable Systems) and finally to the oxygen sampler (Sable Systems FC-1B O_2 Analyzer). Oxygen signals were passed to a Sable Systems Universal Interface II connected to a computer. This system allowed for multi-channel recording of oxygen consumption. Data acquisition system (DAS) 2.0 was used to

collect data while Datacan V was used to analyze data. Eight respirometers were run simultaneously and each trial was run a total of 3 times. Chamber humidity remained close to 0% RH during each experiment. Despite exposure to a dry air stream, eggs showed no significant weight loss during the period of oxygen consumption measurement.

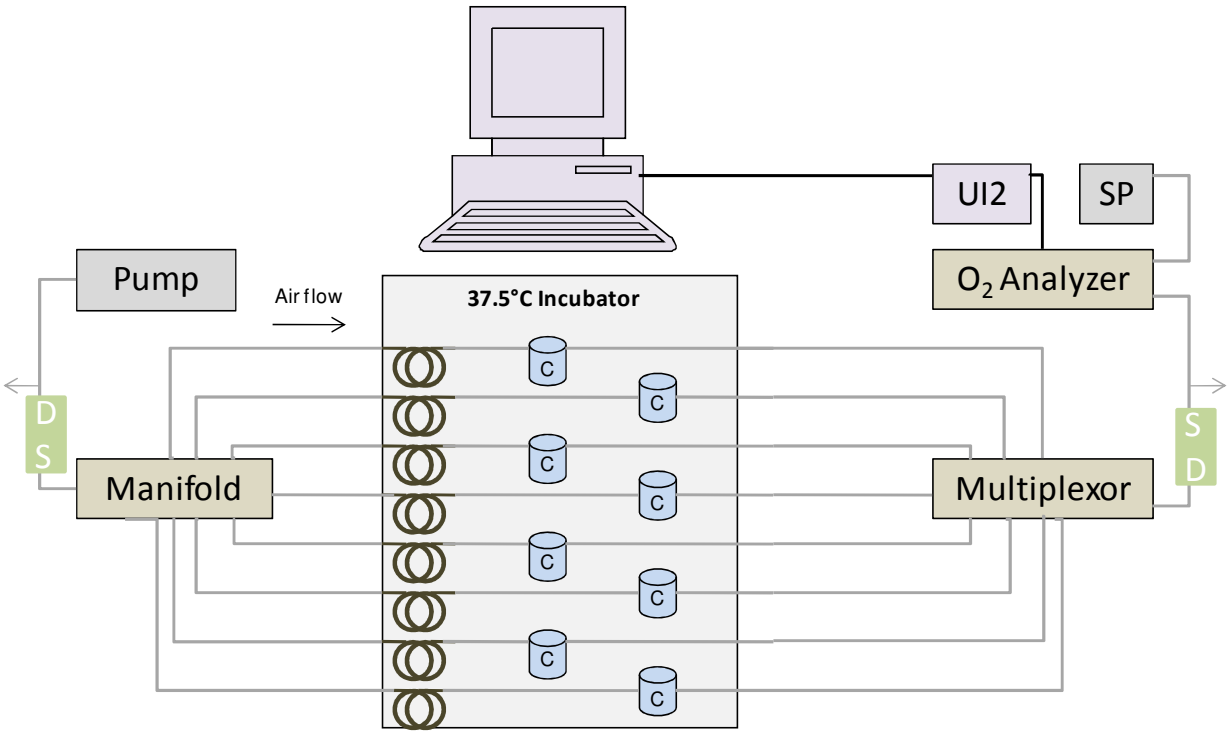

Figure 2.1: Respirometer used for measuring oxygen consumption in Day 10 and Day 18 chicken embryos (D: drierite; S: soda lime; Diagram by Kelly Reyna, 2009).

Statistics

All data were tested for normality of distributions (Shapiro-Wilks test for normality) before specific statistical analyses were performed. Two-way parametric ANOVA was used to test whether or not developmental day, humidity level or interaction between the two factors had an effect on the data. Student-Newman Keuls

(SNK) multiple range *post hoc* tests were run to separate means into distinct groups. Concatenation procedure was used when a significant interaction was seen between developmental day and humidity level. Linear regression analyses were conducted to determine whether significant relationships existed between parameters. All statistical analyses were conducted using SigmaStat 3.5, SigmaPlot 10.0, and SAS software. Decisions were made with a 0.05 level of significance.

Results

Mortality

Mortality percentage up to day 18 was calculated from 32 incubation trials in which 3 dozen eggs were incubated per treatment group per trial (Figure 2.2). A significant humidity effect (p=0.015) was seen in which cumulative mortality percentages were highest in low humidity (44.8 ± 4.5 %) and high humidity populations (37.2 ± 3.8 %) compared with the normal humidity population (27.5 ± 2.9 %).

Mass Analysis

A significant interaction was seen between humidity level and developmental day for whole egg mass loss (p<0.001, Figure 2.3). Student Newman-Keuls (SNK) *post hoc* test separated the data into three distinct groups in which low humidity exhibited twice the loss in mass to that of normal humidity and three times greater loss in mass compared to high humidity.

Analysis of embryo wet mass measured on days 10-18 demonstrated a significant interaction between development and humidity level (p<0.001, Figure 2.4A). On day 14, normal humidity embryos (11.9 ± 0.4 g) weighed significantly more than low humidity (10.0 ± 0.5 g) while high humidity embryo wet mass (10.7 ± 0.6 g) was

statistically similar to both normal and low groups. Normal humidity (17.8 ± 0.5 g) embryo mass on day 16 was different from high humidity (16.0 ± 0.6 g) but neither normal nor high humidity differed from low humidity embryo wet mass (17.2 ± 0.6 g). Finally, on day 18 of incubation, normal humidity wet mass (25.5 ± 0.5 g) was significantly larger than both high (22.6 ± 0.6 g) and low humidity (21.8 ± 0.9 g) groups.

As expected, embryo dry mass increased significantly as development progressed ($p<0.001$, Figure 2.4B). However, on day 18, a humidity effect was seen in which dry mass in the low humidity group was significantly larger at 5.26 ± 0.3g than both normal (4.1 ± 0.2 g) and high (3.94 ± 0.2 g) humidity incubation groups.

A significant relationship was observed between embryo and kidney wet masses for all humidity levels throughout development (Figure 2.5) ($p<0.001$). Over 90% of the variation in mean kidney wet mass was accounted for by embryo wet mass for all groups. There was a difference between slopes of normal and low humidity group ($0.02>p>0.01$) and normal and high humidity group ($0.005>p>0.002$), but there was no difference between low and high humidity groups.

Kidney wet and dry masses were measured on embryonic days 10-18 for all treatment groups (Figures 2.6A & 2.6B). Development had a significant affect on mean wet kidney mass ($p<0.001$). On day 16, kidney wet mass differed between normal (69.8 ± 3.6 mg) and high (54.9 ± 4.5 mg) incubation groups while the low humidity group (63.3 ± 3.6 mg) was not different from either group.

Both humidity and development had a significant impact on mean kidney dry mass ($p=0.004$; $p<0.001$, respectively, Figure 2.6B). Student-Newman Keuls *post hoc* analysis showed normal humidity to have a significantly higher mean kidney dry mass

of 5.1 ± 0.7 mg compared to both low (2.3 ± 0.5 mg) and high humidity (2.62 ± 0.8 mg) on day 14. Day 16 embryos were separated into two groups in which low humidity (9.3 ± 0.8 mg) was not different from either normal or high humidity; yet normal humidity (10.0 ± 0.7 mg) was significantly greater than high humidity group (8.0 ± 0.9 mg). Conversely, day 18 yielded significant differences between normal (13.9 ± 0.6 mg) and low humidity (11.6 ± 0.5 mg), while high humidity (12.6 ± 0.9 mg) remained statistically similar to both groups.

Oxygen Consumption

There was a significant interaction between humidity level and developmental day for oxygen consumption measured on embryonic days 10-18 (Figure 2.7) (p=0.03). Student-Newman Keuls *post hoc* test separated the populations into two distinct humidity groups. On day 18, normal humidity had a higher oxygen consumption of 0.37 ± 0.02 ml/min compared to low (0.28 ± 0.03 ml/min) and high (0.28 ± 0.03 ml/min) humidity groups which were not statistically different. The depressed values of low and high humidity groups indicated a failure to thrive compared to embryos incubated in the normal humidity group.

Discussion

Mortality

It is well known that any deviation from the incubation humidity used stringently by commercial breeders, tends to result in poor survival. Mortality rate was measured primarily to demonstrate that the extreme humidity groups did indeed impose stress on the developing embryo (Figure 2.2). Results of the present study were consistent with experiments by Davis and colleagues (1988) which showed mortality to be lower in

normal and high humidity conditions (10.8% and 29.4% respectively) compared with 75% for low humidity group. Snyder and Birchard (1982) demonstrated that survival was directly dependent on the amount of water lost during the first half of incubation rather than on the total loss of water.

Mass Analysis

Water loss from the egg was also used as a trial variable to demonstrate the differences between humidity levels. Under normal conditions, optimal mass loss should be about 12% of initial egg mass for 21 days of incubation (Lundy, 1969; Peebles *et al.*, 2001). The large differences seen in water loss between treatment groups in the present experiment confirmed prior research and were not surprising. Drastic increases of water loss between days 16 and 18 may have been due to the increased net water uptake in preparation for internal pipping and hatching.

Embryo wet masses measured across development showed that normal incubation conditions produced embryos with larger wet mass on days 14-18 (Figure 2.4A). These results indicated that embryos from normal conditions thrive in terms of whole body wet mass compared to the extreme humidity conditions.

Surprisingly, dry embryo masses were not affected in the same way as embryo wet masses (Figure 2.4B). The low humidity group exhibited a significantly larger whole embryo dry mass on day 18 of almost 2 grams more compared to both the normal and high humidity groups. Reasoning for this difference was not clear since normal conditions exhibited a larger embryo wet mass by almost 4 grams compared to low humidity group. Davis and colleagues (1988) showed similar results for embryo wet mass, but did not find dry masses to be significantly different, although they only

exposed embryos to high water loss starting on day 13. The increased dry mass of the low humidity group indicated that these embryos were actually larger and may have experienced an increased growth rate. Further experiments should be conducted to determine if the low humidity group experienced accelerated growth rate to compensate for the imposed stress.

Embryo wet mass was demonstrated to be a good predictor of kidney wet mass (Figure 2.5). This indicated that the kidney grows in proportion to embryo growth for all treatment groups. Kidney wet mass appeared to be relatively unaffected by alteration of humidity level, although differences were seen on day 16 between normal and high humidity groups (Figure 2.6A).

The results for kidney dry mass differed from those seen in kidney wet mass since humidity was shown to significantly affect dry mass (Figure 2.6B). Normal incubation conditions yielded significantly larger kidney dry mass compared with high humidity conditions across developmental days measured. These results were different than originally predicted since it seemed logical that those kidneys in the low humidity group might have a drastic increase in kidney dry mass versus both the normal and high humidity groups. This assumption was based on what was found in adult birds. Adult coastal songbirds exhibited a redistribution of kidney tissue thus causing an increase in kidney mass attributed to increases in medullary tissue (Sabat *et al.*, 2004). Coastal songbirds exposed to salt water versus tap water showed no significant differences in kidney dry mass although kidneys exposed to salt water did have higher mean dry mass (Sabat *et al.*, 2004). The study on adult coastal songbirds demonstrated that changes in kidney dry mass occurred when water stress was imposed. Unfortunately, there are

little to no data to compare with the current results since the kidney has not been examined for embryos exposed to water stress via incubation conditions during development.

Oxygen Consumption

Embryos from normal conditions measured on day 18 showed oxygen consumption values significantly higher by 0.1 ml/min compared to both the high and low humidity groups (Figure 2.7). These data closely resembled what was found after analysis of embryo wet mass (Figure 2.4A). The higher oxygen consumption values seen under normal incubation conditions may have been attributed to larger embryo wet mass. Vleck *et al.* (1980) observed that dramatic increases in oxygen consumption during development parallel growth of the embryo. So the results from these analyses were similar.

Assumptions based on adult birds led me to expect low humidity groups to experience a reduction in oxygen consumption and high humidity groups to experience increased oxygen consumption. Yokota *et al.* (1985) commented that to rapidly remove water, high rates of water turnover must be induced by way of increasing glomerular filtration rate; thus oxygen consumption is increased. Additionally, one might consider how oxygen consumption is affected by hypoxia since a decrease in air cell volume, as seen in embryos under high humidity conditions, may lead to reduction in capacity to uptake oxygen during the pipping phase of breathing. Embryos exposed to 11% hypoxia experience a hypometabolic response (Mortola and Labbé, 2005). Growth rate was also seen to decline for embryos subjected to hypoxic conditions (Mortola and Cooney, 2008). Embryos exposed to decreases in oxygen availability by reduction in

shell area demonstrated low hatch rates presumably due to low energy needed for maneuvering in preparation to hatch (Tazawa *et al.*, 1971).

In contrast to the above mentioned observations, to conserve water, some birds are able to reduce glomerular filtration rate thereby indirectly reducing oxygen consumption. In the study by Tieleman *et al* (2003) adult larks exhibited a decrease in basal metabolic rate (BMR) and total evaporative water loss as aridity increased resulting in a reduced need for food and water with minimal heat production. Larks demonstrated "plastic" responses to changes in their environment when the need to conserve water was imperative for survival.

As is evident by the present data, embryonic exposure to changes in aridity of incubation may not be the same as what occurs in adult birds that are similarly stressed. Assumptions based on adult data may prove false and care must be taken when extrapolating performance of embryos to that of adults (Burggren, 2005).

Summary

In summary, morphology was affected by humidity level and recommended incubation humidity were the best for the embryo to thrive. Despite obvious changes in embryo and renal mass, embryos exposed to extreme humidity are able to maintain levels of oxygen consumption comparable to embryos exposed to recommended incubation conditions until day 18. Towards the end of development, both growth rate and oxygen consumption were depressed for embryos exposed to extreme humidity conditions. I hope to shed light on the mechanisms that led to the observations seen in this research in the chapters to follow.

Figures

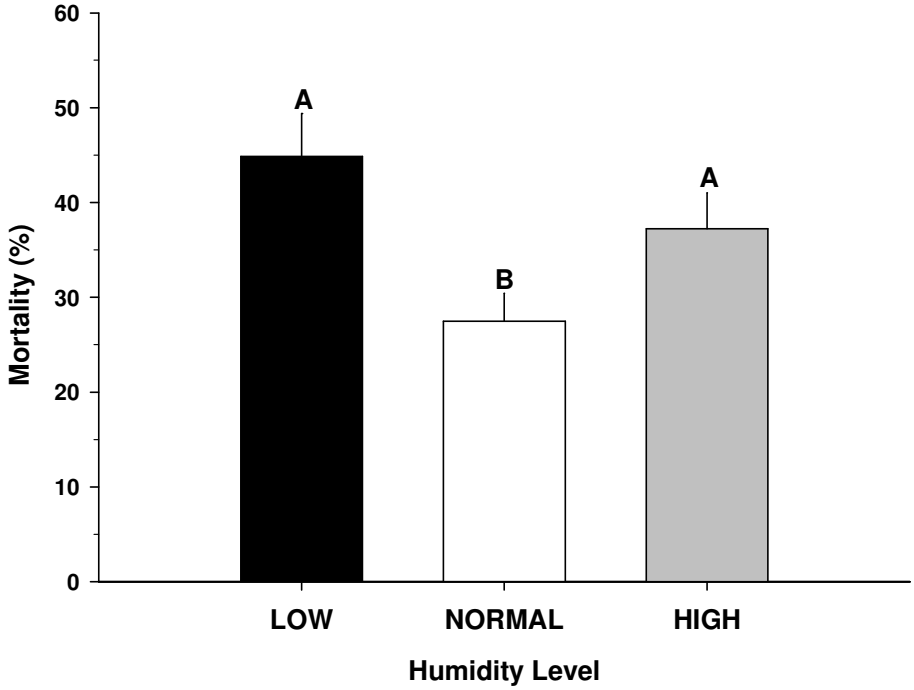

Figure 2.2: Cumulative mortality percentage produced by incubation in low, normal and high humidity levels (mean ± 1 SE shown, *n*=32 incubation trials). Letters indicate statistical grouping.

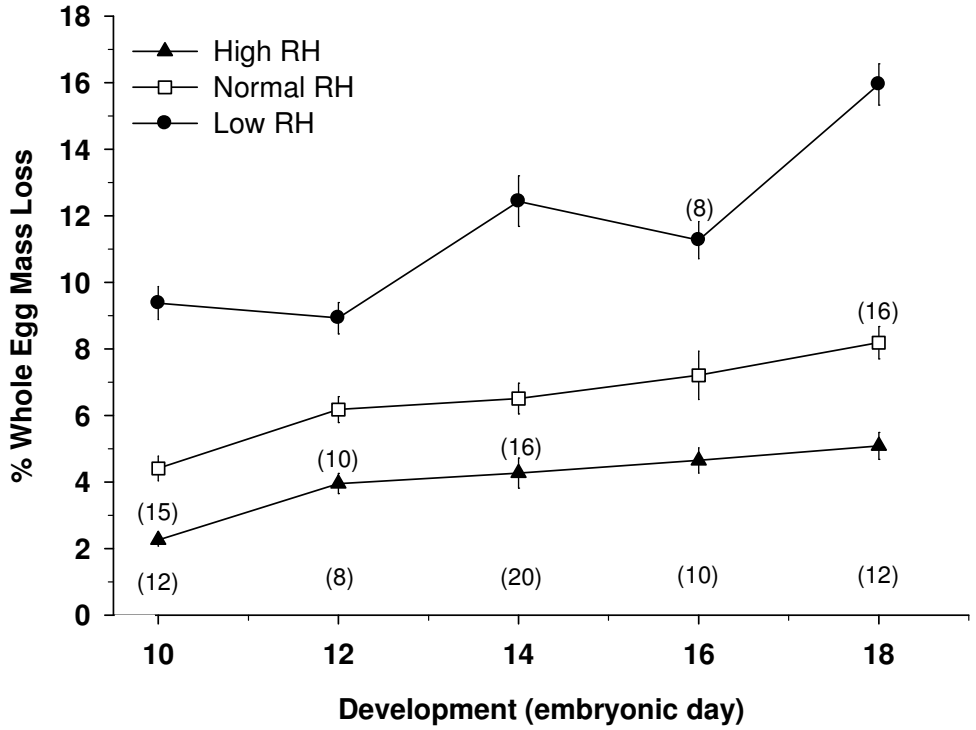

Figure 2.3: Whole egg mass loss (mean ± SE) as a function of development and humidity level. Sample sizes given in parentheses.

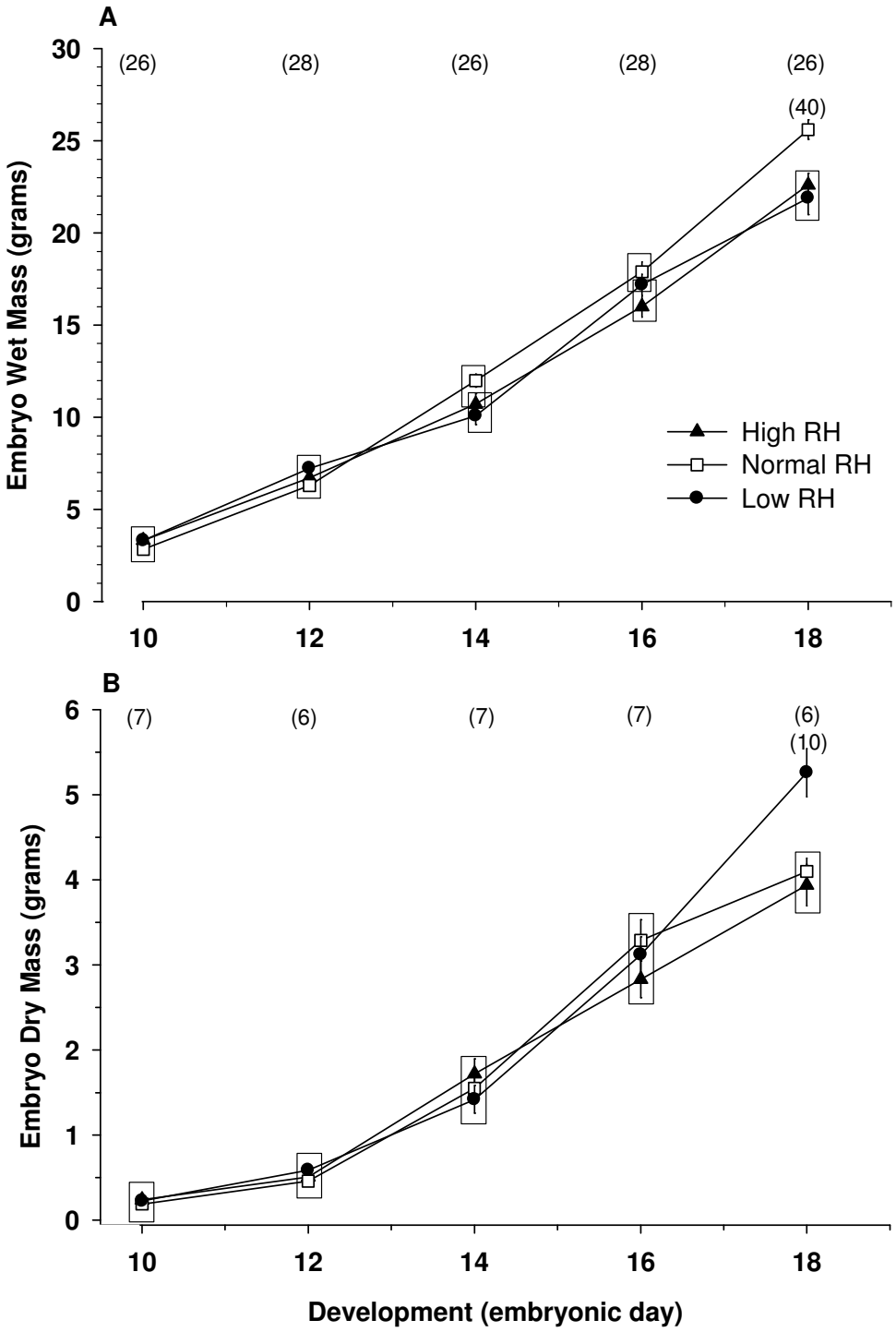

Figure 2.4: (A) Embryo wet mass; (B) Embryo dry mass (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

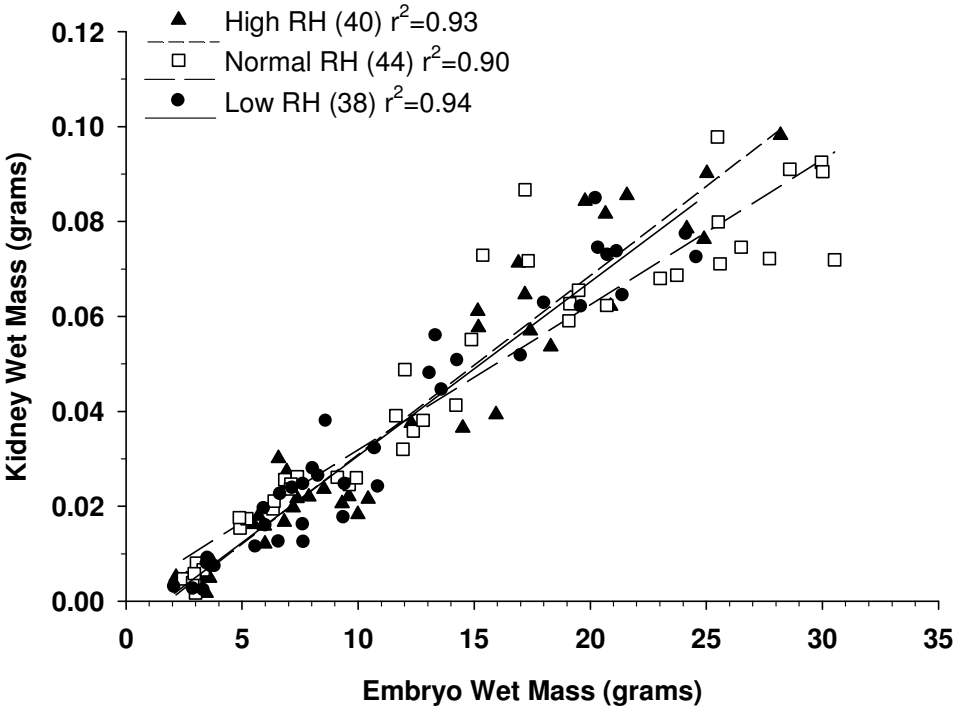

Figure 2.5: Relationship between kidney wet mass and embryo wet mass. Regression coefficient and *n* values are shown.

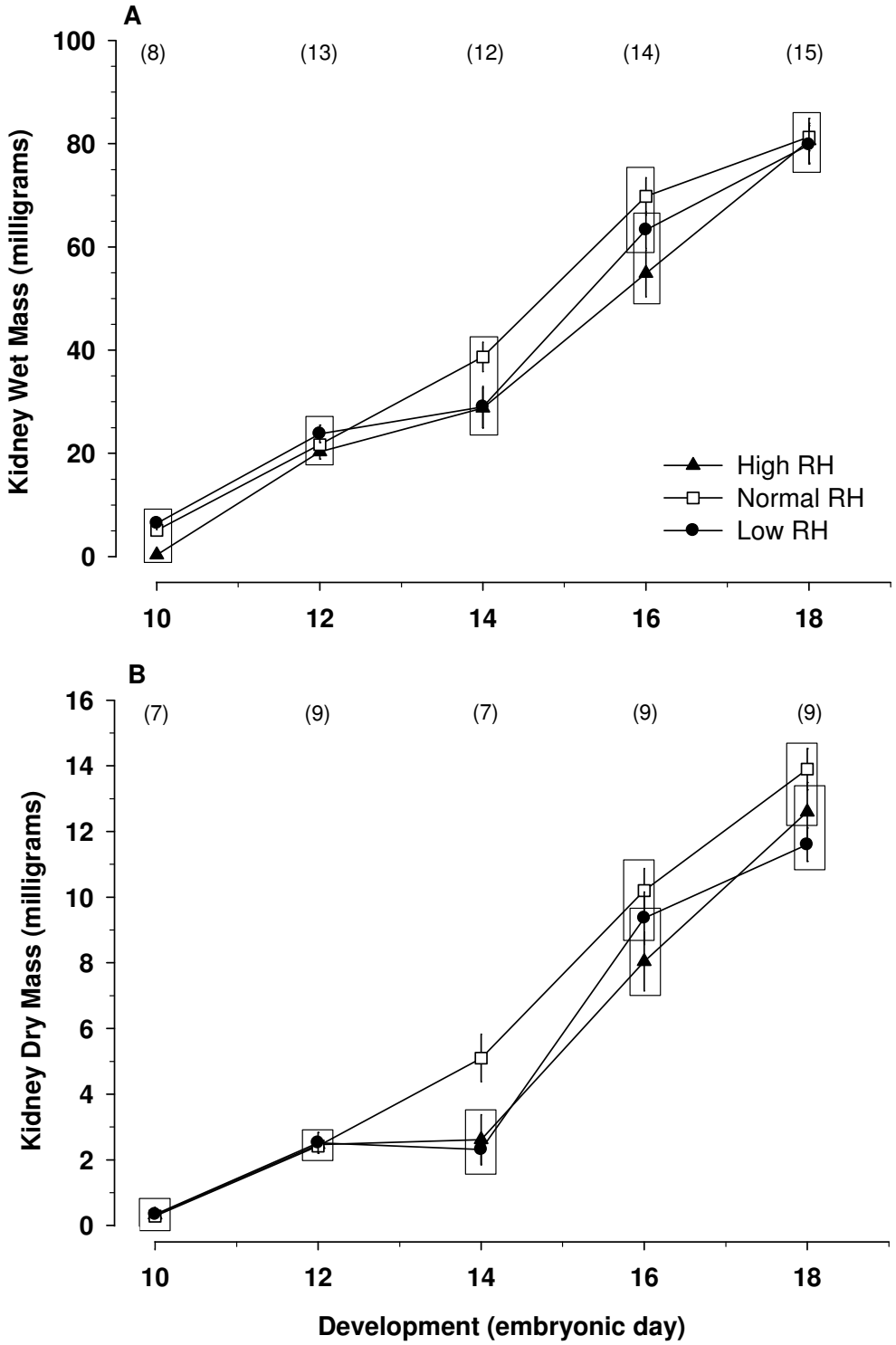

Figure 2.6: (A) Kidney wet mass; (B) Kidney dry mass (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

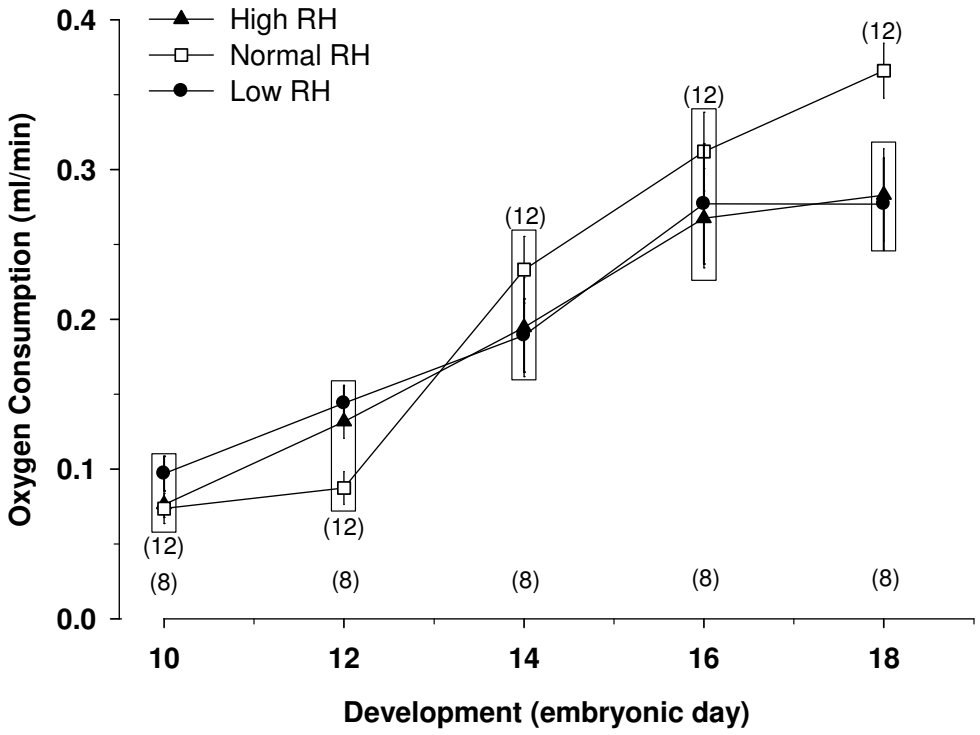

Figure 2.7: Oxygen consumption (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

CHAPTER 3

INCUBATION HUMIDITY AS AN ENVIRONMENTAL STRESSOR ON SALT AND WATER BALANCE IN THE DEVELOPING CHICKEN

Introduction

Renal Function in Adult Birds

When making assumptions on how the avian embryonic kidney may compensate for an osmotic challenge, one must understand renal function in the adult bird. The avian kidney, like the mammalian kidney, has the ability to create hyperosmotic urine. It is the mammalian-type (MT) nephrons together with vasa recta and collecting ducts that form medullary cones that make up the countercurrent multiplier system in birds (Johnson and Mugaas, 1970). Although birds possess similar morphological features that make up the countercurrent multiplier system compared to mammals, one major difference exists. While mammals must utilize both urea and NaCl to establish a concentration gradient, NaCl is the only solute that makes up the interstitial osmotic gradient in birds (Braun, 1985).

The capacity of the renal system has been tested in numerous adult bird species including chicken, desert quail, bobwhite quail and coastal songbirds. Goldstein and Braun (1989) looked at structure and concentrating ability of the avian kidney by dehydrating adults of several different species of birds. All birds in the study by Goldstein and Braun (1989) exhibited loss of body mass, increased hematocrit, blood and urine osmolality with the highest urine osmolality reported from the house sparrow at 1335 mOsmol/kg. Salt-loaded desert quail adults experienced a reduction in glomerular filtration rate (GFR) with a sharp increase in blood osmolality of 100 mOsmol

(Braun and Dantzler, 1972). This reduction in whole kidney GFR was presumably the result of glomerular intermittency where reptilian-type (RT) nephrons ceased functioning. The mechanism behind this action is thought to involve arginine vasotocin (AVT), the avian form of ADH, in which AVT causes the constriction of afferent arterioles to RT nephrons reducing the number of functioning glomeruli (Braun and Dantzler, 1972). These experiments demonstrated the ability of adult birds to conserve water in the face of extreme changes in hydration.

The lower gastrointestinal tract, specifically the cloaca, contributes significantly to the creation of hyperosmotic urine in adult birds. Since birds do not possess a urinary bladder, they utilize their lower intestines for urine storage and modification through reverse peristalsis. The avian ureters empty into the terminal portion of the gastrointestinal tract. Within the cloaca, the urodeum collects the ureteral urine. Braun (2003) reviewed the evolutionary rationales for reverse peristalsis activity of the lower gastrointestinal tract of the bird. First, the urinary bladder poses a significant weight thus potentially impeding flight. Also, the ureteral urine is only slightly hyperosmotic to blood (Braun, 1997). If the urine entering the lower intestines were extremely hyperosmotic, a net movement of water across intestinal mucosa with attendant water loss would result. Another example of the benefits for kidney-gastrointestinal coupling explained by Braun (2003) involves the formation of uric acid as the metabolic end product of protein metabolism. Uric acid is broken down in the lower gastrointestinal tract by uricolytic bacteria. This breakdown allows the liberation and recycling of nitrogen to the bird.

The kidney-gastrointestinal coupling also contributes to prevention of protein loss. Within the nephrons, uric acid crystals will coalesce if protein is not present. The protein, serum albumin in this case, keeps the uric acid spheres in a colloidal suspension (Braun, 2003). Once the urine enters the lower gastrointestinal tract, serum albumin is reabsorbed.

Production of uric acid benefits the avian embryo as well. Since uric acid is deposited into the allantoic sac as crystals, the limited water supply is not affected compared to the damage that would be caused by high volumes of ammonia or the accumulation of urea (Schmidt-Nielson, 1997). Uric acid accumulation has been measured in embryonic quails exposed to altered hydration state. Uric acid content increased steadily throughout development and was strongly dependent on the rate of water loss (Bradfield and Baggott, 1993a, 1993b). An increase in the uric acid precipitate in the allantois in response to high water loss was also seen in the chicken (Davis *et al.,* 1988).

Water Balance in Avian Embryos

Research on the responses of embryonic avian kidney to altered hydration has been minimal, although Murphy *et al.,* (1991) noted a spike in GFR between days 12 and 16 that corresponded to both an increase in mesonephric glomerular size and appearance of metanephric glomeruli.

A cleidoic egg (a term coined by Needham, 1942) is a semi-closed system, one in which only gases are exchanged with the environment while all excretory products remain within the egg. The egg, laid with a set amount of water, experiences water loss through evaporation and water gain from yolk lipid metabolism during the course of

development. The net effect of these two forces is in an overall loss in egg mass of 12% (Lundy, 1969). Eggs that lose too much or too little water exhibit lower hatchability (Davis *et al.*, 1988).

Throughout development the embryo depends on both the chorioallantoic membrane (CAM) and kidney to ensure proper water balance. Associated with the CAM are three main fluid compartments that contribute substantially to the embryo; the yolk, amniotic fluid and allantoic fluid. Usually, these compartments contain sufficient water, nutrients and energy for proper embryonic growth. The amniotic fluid is contained within the amnion, made up of ectoderm and avascular mesoderm lying adjacent to the embryo (Baggott, 2001). By embryonic day 5, the amniotic sac has formed with a contractile outer layer of mesoderm that fuses to the vascular mesoderm of the allantois. The sero-amniotic connection forms as a duct between the amniotic sac and albumen to allow the movement of proteins to the embryo (Baggott, 2001). This connection is established on day 12.

The chorion obtains a vascular supply upon fusion with the allantois to form the CAM. The allantois begins as an out pocketing of the hindgut on day 3.5 and becomes the waste compartment for the embryo for the duration of development. The allantoic fluid is maintained hypoosmotic to the blood (Hoyt, 1979).

In addition to being a repository for waste materials, the allantoic fluid also serves the embryo as a source of hydration. The allantoic fluid compartment, along with amniotic fluid, reaches maximum volume on day 13 (Romanoff, 1967). During the last half of development, the CAM begins to resemble an amphibian urinary bladder resulting in extensive reabsorption of both fluid and electrolytes as needed by the

embryo (Graves *et al.*, 1986; Hoyt, 1979). This membrane is sensitive to exogenous antidiuretic hormone (ADH) causing an enhanced fluid reabsorption (Doneen and Smith, 1982). When increased desiccation is induced experimentally, the allantoic fluid compartment becomes greatly reduced and is the first fluid compartment to experience a decline in volume (Davis *et al.*, 1988; Hoyt, 1979).

The kidney plays a role in water and ion balance in the developing embryo in conjunction with the CAM. The avian kidney develops in a sequence of pronephros, mesonephros and metanephros similar to growth seen in both mammals and reptiles. The pronephros appears to function similarly to the mesonephros until day 6 (Hiruma and Nakamura, 2003). The allantoic sac begins to expand on day 3.5 as evidence of the functioning mesonephros (Hamburger and Hamilton, 1951). The mesonephric filtrate contributes not only to the removal of waste and water regulation, but also to the rapid expansion of the allantoic sac which increases respiratory surface area of the embryo (Freeman and Vince, 1974). Between days 5 and 7 the volume of the allantoic sac increases by a factor of 6 (Romanoff, 1967). This increase corresponds with both differentiation and growth in size of newly formed nephrons (Friebová-Zemanová, 1982). The metanephros, or definitive kidney, appears on day 4 and begins functioning on day 12 (Romanoff, 1960).

Osmoregulation of the avian embryo differs from that in adults, and is an involved process that includes many organs working in unison to maintain fluid and electrolyte homeostasis. This section of my research examines the effects of extreme incubation humidity on the water balance of the egg, including blood parameters and external fluid compartments. Blood parameters, e.g. hematocrit and blood osmolality, are important

to consider when investigating the kidney since the kidney's functions are intertwined closely with the cardiovascular system. Several studies have looked at the effects of high and low water loss on embryos, but none have examined these effects during the time in which the mesonephros is the only functioning kidney (day 10). In addition, the lower gastrointestinal tract significantly affects the urine of the adult, so I hypothesize that low incubation humidity will cause an increase in osmolality of the cloacal fluid in the embryo.

Materials and Methods

Source and Incubation of Eggs

Fertilized White Leghorn eggs (*Gallus gallus domesticus*) were obtained from Texas A&M University (College Station, TX) and shipped to University of North Texas (Denton, TX), where they were held in Hova-Bator incubators. The University of North Texas' Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

Eggs were separated and were randomly chosen for placement within three incubators at either <30%, 55-60% or >85% relative humidity (RH) and temperature of 37.5^{o}C ± 0.5 ^{o}C. Humidity was measured using wireless Baro-Thermo-Hydrometers (model BTHR968, Oregon Scientific). Eggs were weighed pre-incubation and on the designated experimental day. Weight loss during development was calculated as the difference between pre-incubation and experimental day whole egg mass. On embryonic days ranging from 10-18, (stages 36-44; (Hamilton and Hamburger, 1951) eggs were removed from each incubator for experimental analyses.

Hematology

Eggs were candled to locate an artery in the chorioallantoic membrane (CAM) from which to take a blood sample. Eggs were placed in a sand and water jacket with steady circulation of 37°C water to maintain temperature of the embryo while blood was being drawn (Fisher Scientific Isotemp Refrigerated Circulator model 9100). Once the CAM artery was exposed approximately 100-300µL of blood was removed via a heparinized 1 mL syringe with 30 gauge needle. The egg was then placed in a desiccator with a lethal amount of isofluorane.

For measurement of hematocrit (Hct, %), blood samples were injected into FISHERbrand® microhematocrit capillary tubes and sealed with cryostat sealant. Immediately following blood collection, the capillary tubes were placed in a microhematocrit centrifuge for 3 minutes and Hct was determined as volume of packed red blood cells to total volume of blood.

Total hemoglobin (tHb, g/dl) was measured by injecting 35 µL of blood into Safecrit® heparinized plastic microhematocrit tubes (Statspin®Inc) and aspirating the blood into an OSM™3 Hemoximeter™ (Radiometer Copenhagen) for analysis. A Coulter counter (Coulter® A^{C}●T Series Analyzer, Beckman Coulter, Inc) was also used to verify Hct and tHb values obtained from earlier experiments. No significant difference was seen between the measurements from each type of instrument.

Fluid Collection and Osmolality

A 25 gauge needle and 1 ml syringe was used to remove ~100 ml allantoic and amniotic fluids from the allantoic and amniotic sacs. Cloacal fluid was sampled by placing capillary tube pulled to a tip diameter of 10-30µm (World Precision Instruments

PUL-1E pipette puller) into the cloaca of embryos that had been sacrificed via exposure to isofluorane for 10 min followed by rapid decapitation. Blood was obtained via methods detailed in the previous section. Samples of 10 µL were injected into Wescor Vapro® vapor pressure osmometer (model 5520) to determine osmolality of all fluids.

Uric Acid Analysis

A sample of allantoic fluid was removed as described in the previous section. Uric acid concentration was determined by quantitative colorimetric uric acid determination (QuantiChrom® uric acid assay kit, Scion analysis system). A 5 µL sample of allantoic fluid was placed in a 96-well microtiter plate along with a mixture of various reagents to measure optical density of the uric acid present in the mixture. Each sample was duplicated along with blanks and standards. Calculation of uric acid in sample was carried out as follows:

$$= \frac{OD_{SAMPLE} - OD_{BLANK}}{OD_{STANDARD} - OD_{BLANK}} \quad X\ 10\ (mg)/dL)$$

Statistics

All data were tested for normality of distributions (Shapiro-Wilks test for normality) before specific statistical analysis was performed. Two-way parametric ANOVA was used to test whether or not developmental day, humidity level or interaction between the two factors had an effect on the data. Student-Newman Keuls (SNK) multiple range *post hoc* tests were run to separate data into distinct groups. Concatenation procedure was used when a significant interaction was seen between developmental day and humidity level. All statistical analyses were conducted using

SigmaStat 3.5, SigmaPlot 10.0, and SAS software. Decisions were made with a 0.05 level of significance.

Results

Hematology

Total hemoglobin content increased significantly ($p<0.001$) between 10 and 18 days in all treatment groups (Figure 3.1). On day 14, the normal humidity group had a significantly higher hemoglobin content of 6.26 ± 0.44 g/dl compared to the high humidity group (4.78 ± 0.29 g/dl) while the hemoglobin content of the low humidity group (5.32 ± 0.45 g/dl) was not statistically different from either normal or high groups.

Mean hematocrit increased significantly ($p<0.001$) from 15% on day 10 to 30% on day 18 for all treatment groups (Figure 3.2). Humidity did not affect mean hematocrit.

Body Compartment Fluid Osmolality

A significant interaction ($p<0.001$) was seen between fluid compartment osmolality and developmental day for all humidity levels (Figure 3.3, Table 3.1). The SNK *post hoc* analysis revealed that blood and cloacal fluid osmolality was not statistically different for all humidity levels. Amniotic fluid osmolality was statistically identical to blood and cloacal fluid osmolality for low humidity embryos on day 18 and high humidity embryos on both days 16 and 18.

Amniotic fluid osmolality demonstrated a significant interaction between humidity level and development for all treatment groups (Figure 3.4) ($p=0.008$). SNK *post hoc* analysis revealed significant differences on day 14 and 18. Low (228 ± 9 mOsmol), normal (180 ± 4 mOsmol) and high (154 ± 7 mOsmol) humidity groups were significantly

different from one another on day 14. The same relationship was seen for day 18 in which low humidity (280 ± 5 mOsmol) had a higher osmolality compared to normal (254 ± 7 mOsmol) and high (240 ± 14 mOsmol) humidity groups.

Blood osmolality in the control population ranged between 265 and 280 mOsmol (Figure 3.5). Blood osmolality in the low humidity group was significantly higher than both normal humidity and high humidity groups across development (p=0.004). Embryonic days 12 and 16 showed the largest significant difference between low (294 ± 7 mOsmol; 300 ± 6 mOsmol, respectively) and normal humidity groups (274 ± 4 mOsmol; 268 ± 6 mOsmol, respectively). The high humidity values for day 12 (274 ± 3 mOsmol) and 16 (283 ± 4 mOsmol) were not different from the normal humidity group but were significantly less than low humidity on day 12.

Cloacal fluid was measured only on embryonic days 16 and 18 since fluid could not be collected in adequate quantities for analysis on days 10-14. Humidity level significantly affected both developmental days measured (Figure 3.6) (p=0.001). The SNK *post hoc* analysis separated the embryos into distinct groups. The low humidity group on days 16 and 18 (307 ± 7 mOsmol, 302 ± 11 mOsmol, respectively) was significantly greater than both normal humidity (280 ± 6 mOsmol, 275 ± 4 mOsmol) and high humidity (283 ± 11 mOsmol, 270 ± 4 mOsmol) groups which were not different from one another.

There was a significant interaction (p<0.001) between humidity level and development for mean allantoic fluid osmolality (Figure 3.7). The SNK *post hoc* test separated all experimental days into distinct groups except for day 16. On day 10, the low humidity osmolality of 216 ± 9 mOsmol was significantly higher than the high

humidity group (176 ± 4 mOsmol), but the normal humidity group (193 ± 5 mOsmol) was statistically similar to both low and high humidity groups. All groups were distinctly different on days 12 and 14: 12 low (242 ± 4 mOsmol) > normal (215 ± 4 mOsmol) > high (177 ± 4 mOsmol); 14 L (228 ± 6 mOsmol) > N (183 ± 4 mOsmol) > H (166 ± 7 mOsmol). On day 18, the low humidity group (249 ± 14 mOsmol) had a significantly elevated osmolality compared to high (199 ± 12 mOsmol) and normal (178 ± 6 mOsmol) humidity groups which were statistically similar. These results follow closely to previous reports on allantoic fluid osmolality.

Uric Acid

Uric acid concentration increased significantly between days 10-18 and was affected by both development and humidity (Figure 3.8) ($p<0.001$). The SNK *post hoc* analysis separated humidity level into two groups, low (85 ± 6 mg/dL) and normal (82 ± 6 mg/dL) humidity groups had a higher uric acid concentration compared to high (68 ± 5 mg/dL) humidity group. On day 10, low (62 ± 7 mg/dL) and normal (61 ± 2 mg/dL) humidity groups were not statistically different from each other but were higher than high (23 ± 6 mg/dL) humidity group. Uric acid concentration increased with development as expected.

Discussion

Osmoregulation of the Avian Embryo – Revisited

Within the embryonic environment, water fluxes exist between extra- embryonic compartments over the entire course of development. Altered incubation conditions affect water and ion balance of the embryo. Hoyt (1979) was the first to alter humidity during incubation. His results were similar to the results seen in this experiment,

although he focused on day 17 which merely provided a snapshot of the effects of altered incubation humidity. A closer examination of these effects on earlier days lends to discovery of critical periods during embryonic development. Davis *et al.,* (1988) also demonstrated effects of both high and low water loss on avian embryos, although the experimental design differed from the current research. For instance, high water loss was induced on day 13 of incubation by drilling multiple holes in to the egg. The information gained from this experiment was informative, but by day 13 significant changes in ion and water distribution have already occurred. Albumen progressively loses water up to day 10, while both the amniotic and allantoic fluid compartments reach their maximum by day 13 (Baggott, 2001). Crucial changes in the distribution of resources had already taken place by the time water stress was brought upon the embryo in the previous experiment by Davis *et al.,* (1988).

Hematology

Mean hematocrit was altered by dry conditions in avian embryos, beginning on day 17 (Davis *et al*., 1988; Hoyt, 1979); mean hematocrit for each of the experiments referenced using domestic chickens ranged from 29% (Hoyt, 1979) to 38% (Davis *et al*., 1988). Surprisingly, the same results were not seen in this study, although my values were similar to those demonstrated by Hoyt (1979). In the present experiment, the low humidity group had higher hematocrit values on days 10-14, but no significant difference was seen compared to the normal or high humidity groups, indicating that blood remained unaffected by incubation humidity (Figure 3.2).

Total hemoglobin concentration demonstrated a developmental effect as expected with a separation between normal and high humidity groups on day 16 (Figure

3.1). Reasons for this significant difference were not clear but may indicate a reduction in concentration of the hemoglobin in high humidity owing to a possible increase in blood volume. However, blood volume was not measured for embryos in the present study.

Amniotic Fluid

Amniotic fluid serves as a mechanical protection for the embryo during development. Osmolality of this fluid compartment peaks on day 13 followed by a slow decline until hatching (Baggott, 2001). The current opinion is that amniotic fluid is unaffected by hydration state of the egg until around day 17 when the allantoic fluid department has been exhausted and the CAM begins to deteriorate (Baggott, 2001; Davis *et al*., 1988; Hoyt, 1979). In contrast, my research showed that mean amniotic fluid osmolality exhibited a significant interaction between humidity level and developmental day (Figure 3.4). The low humidity group showed a significantly greater mean amniotic fluid osmolality compared to normal and high humidity groups on day 14, before the deterioration of the CAM begins. This is an important observation since the embryo begins to consume amniotic fluid once the sero-amniotic connection is formed on day 12 (Baggott, 2001). The influx of albumen via the sero-amniotic connection may dilute the amniotic fluid thus explaining the drop in fluid osmolality in normal and high humidity groups on day 14. The low humidity amniotic fluid osmolality does not drop significantly on day 14 presumably due to the albumen becoming depleted of water before the sero-amniotic connection has formed. If the embryo ingested this high osmolality amniotic fluid, the effects may be reflected in an increased blood osmolality as was observed in this study. Another effect of this action might result in altered

kidney morphology as seen in mammals. The potential for embryonic programming of kidney structure and function is great and will be examined in Chapter 4.

On day 18, the low humidity group had significantly greater osmolality compared to normal and high humidity groups. This difference may be reflected by lack of effective osmoregulatory control since the CAM has atrophied dramatically (Stewart and Terepka, 1969).

Blood Osmolality

Blood osmolality in the low humidity group was significantly higher than both normal and high humidity groups, with particular emphasis on days 12 and 16 (Figure 3.5). The physiological underpinnings for the peaks observed in mean blood osmolality on days 12 and 16 were not clear, although an increase in osmolality may be due to a reduction in blood volume caused by the osmotic stress of high water loss or increase in sodium in the blood.

Due to the peaks in blood osmolality on days 12 and 16, it can be assumed that the embryo increases its swallowing of the amniotic fluid as a thirst response to compensate. In ovine fetus, dipsogenic stimulation occurs when blood osmolality increases by 10% (Xu *et al.*, 2001). Low humidity incubation caused mean blood osmolality to increase by 7% on day 12 (when amniotic swallowing begins) and 12% on day 16. Whether the embryo increases its swallowing to compensate for increased blood osmolality is not known, although if this mechanism is utilized, the low humidity embryo is not able to quench its thirst due to the high osmolality of the amniotic fluid for embryos from this group.

Cloacal Fluid

To date, cloacal fluid has not been evaluated for water-stressed embryos despite the known coupling between the kidney and gastrointestinal tract. The kidney and lower gastrointestinal tract have coupled functions to produce hyperosmotic urine in adults, but little is known about the action of this coupling in the embryo. The urodeum of the cloaca receives the ureteral urine. It is this region of the cloaca in which the allantoic stalk is connected. Cloacal fluid osmolality was measured on day 16 and 18 only (Figure 3.6). There was a humidity effect seen for these data. Mean cloacal fluid was separated into two distinct groups in which low humidity measurements were significantly higher than normal and high humidity groups.

When comparing fluid compartment osmolality, the cloacal fluid was not different from the blood for all treatment groups (Table 3.1). The similarity between osmolality in this case indicates that the kidney is indeed creating concentrated urine. The cloacal fluid in embryos from the low humidity group was hyperosmotic to the blood.

Allantoic Fluid

The allantois accumulates fluid primarily as a result of kidney filtration of plasma; the filtrate enters the allantois by the allantoic stalk causing its initial expansion (Murphy, 1991). This fluid becomes increasingly hypoosmotic to embryonic plasma during incubation due to the movement of sodium and chloride across the allantois to the blood. Several studies have documented the drastic decrease in volume beginning after day 13 (Romanoff, 1967; Baggott, 2001) and how this loss in volume increases when eggs are placed in dehydrating environments. In the present experiment, allantoic fluid was affected by the hydration state of the embryo. As predicted, low

humidity incubation caused the allantoic fluid osmolality to increase compared to the normal humidity group. The opposite trend was seen for the high humidity group in which the lowest osmolality values across development were seen. There was no difference in allantoic fluid when a drop was observed on day 16. This decline may have been due in part to removal of ions by CAM transport or uric acid sequestering of sodium ions (McNabb and McNabb, 1975). On day 18, an increase in allantoic fluid osmolality was presumably the result of the reduction in allantoic fluid volume. Both Hoyt (1979) and Davis *et al.,* (1988) demonstrated similar results, but neither showed significant differences before day 14.

Uric Acid Concentration

Uric acid concentration increases approximately two-fold during development (Romanoff, 1967; Figure 3.8). High rates of water loss tend to affect the amount of uric acid seen in the allantois. Bradfield and Baggott (1993a) proposed that uric acid is essential for the water balance of the embryo since oxidation of protein to urate produces more metabolic water than oxidation of fat. Also, it requires less water to store uric acid compared to other metabolic end-products (McNabb and McNabb, 1980). In Japanese quail, the allantoic fluid exhibited a reduction in osmolality because of the increase of uric acid sequestering sodium ions (Bradfield and Baggott, 1993b). Beyond a steady increase in uric acid concentration, results for the current experiment differed from what has been seen in quail and chicken since low humidity incubation did not cause increased uric acid concentration. The differences between the data may have been the result of not taking the volume of allantoic fluid into consideration.

Altering the hydration state of the embryo had a significant affect on water balance during development. However, since no other study has found an increase in amniotic fluid osmolality, I propose that this result of low humidity incubation may result in the programming of renal tissues within the developing embryo, namely the kidney. The following chapter considers renal morphology during development to test this hypothesis.

Figures

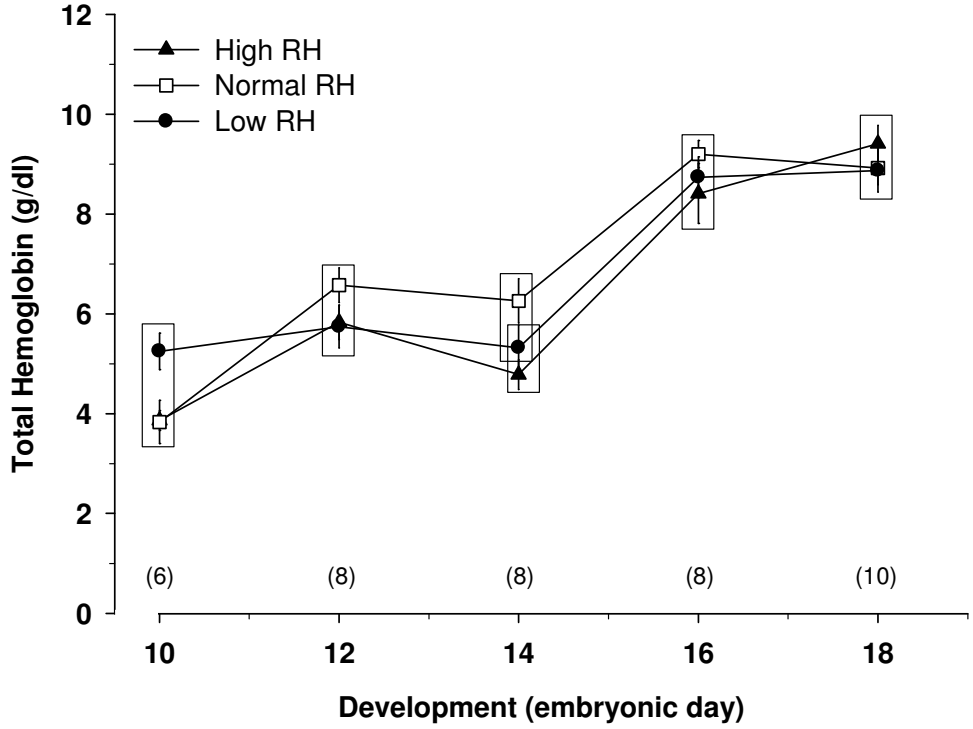

Figure 3.1: Total hemoglobin concentration (mean ± SE) compared across development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

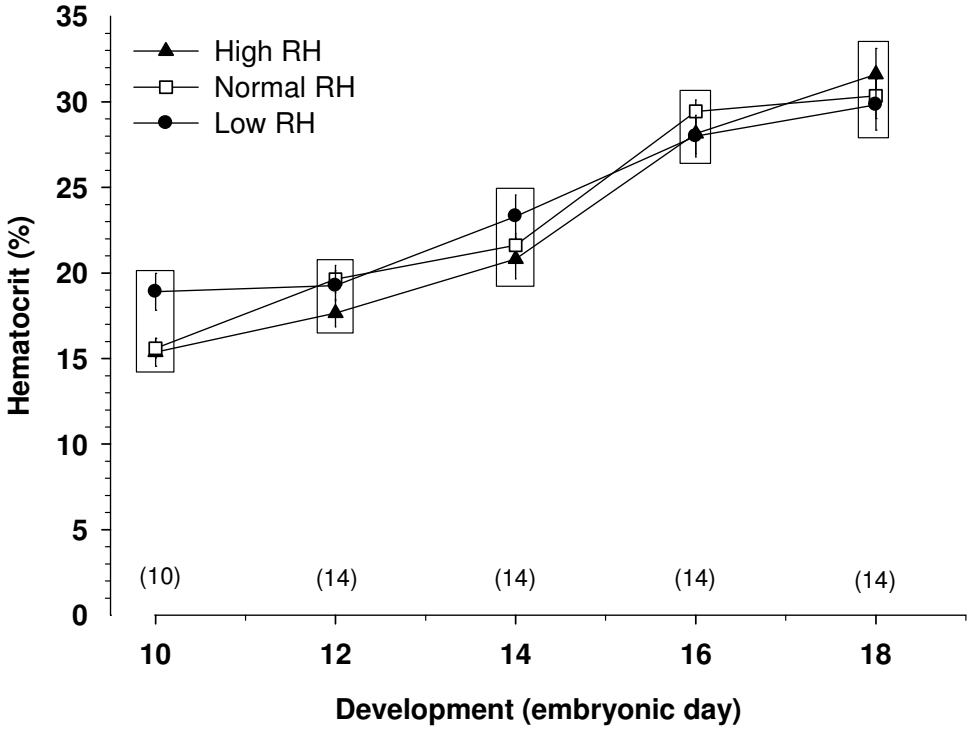

Figure 3.2: Hematocrit (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

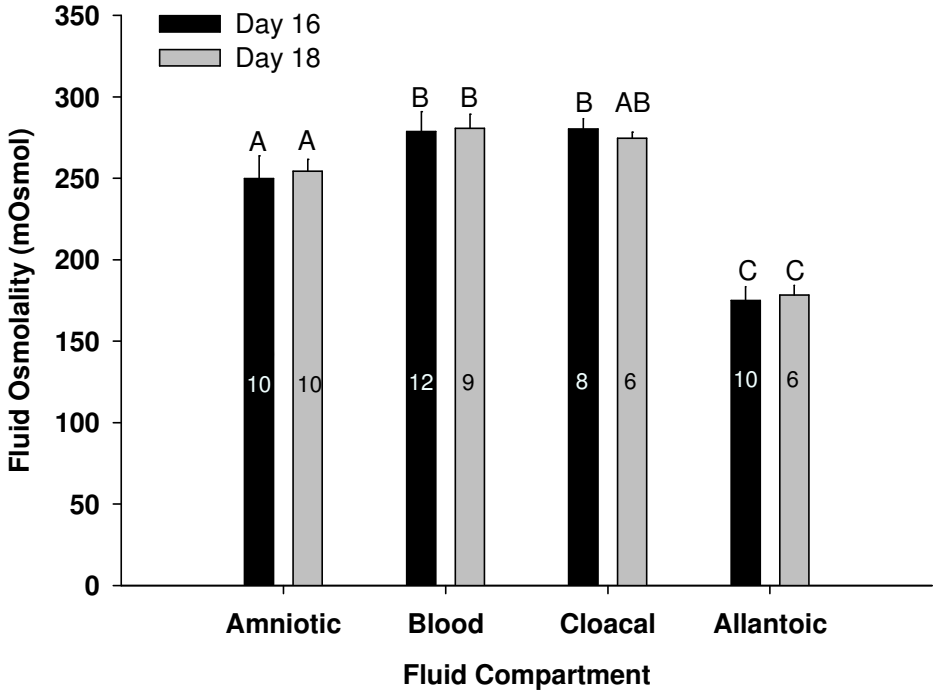

Figure 3.3: Fluid osmolality values seen under normal conditions for days 16 and 18 of development. Mean ± SE are plotted. *n* values for each mean, at each day, are shown. Letters indicate statistically identical groupings.

FLUID COMPARTMENT	LOW		NORMAL		HIGH	
	16	18	16	18	16	18
Blood vs Allantoic	S	S	S	S	S	S
Blood vs Amniotic	S	NS	S	S	NS	NS
Blood vs Cloacal	NS	NS	NS	NS	NS	NS
Cloacal vs Allantoic	S	S	S	S	S	S
Cloacal vs Amniotic	S	NS	S	NS	NS	NS
Allantoic vs Amniotic	S	S	S	S	S	S

Table 3.1: Fluid compartment comparisons between humidity levels on days 16 and 18 of development; significant difference (S); not significantly different (NS) via two-way ANOVA.

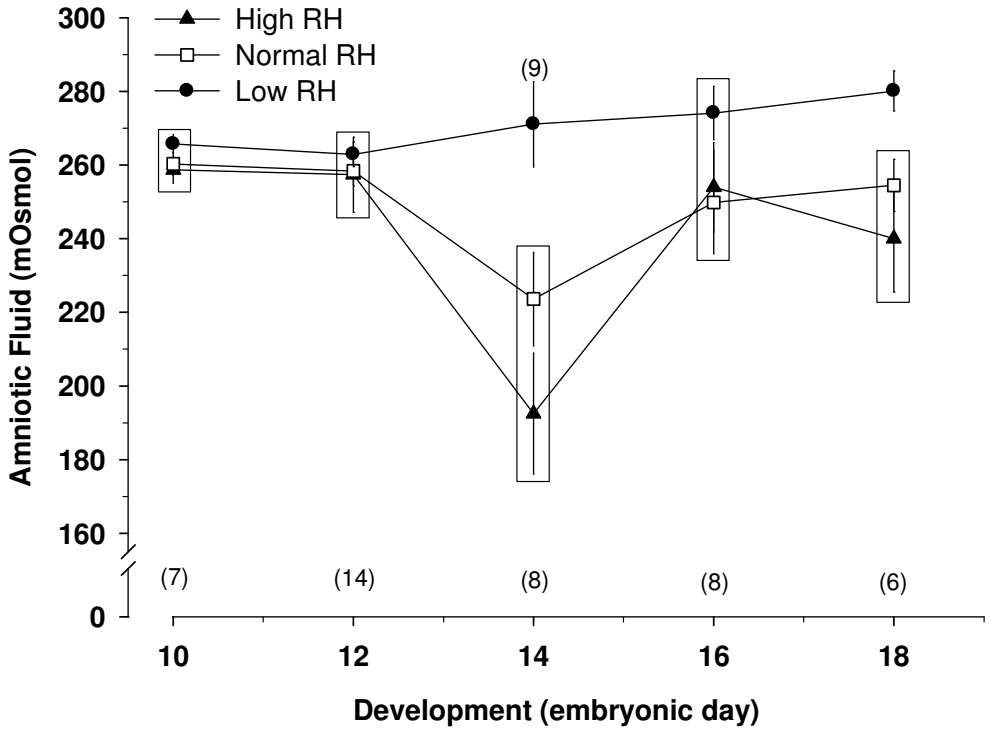

Figure 3.4: Amniotic fluid osmolality (mean ± SE) compared as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses

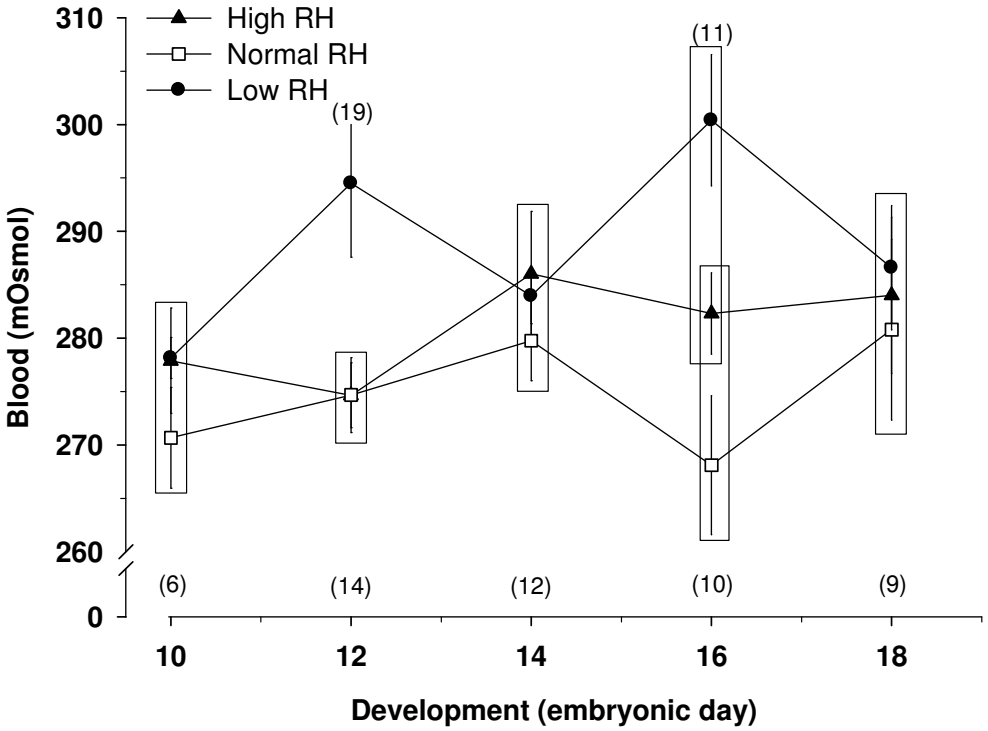

Figure 3.5: Blood osmolality (mean ± SE) compared as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

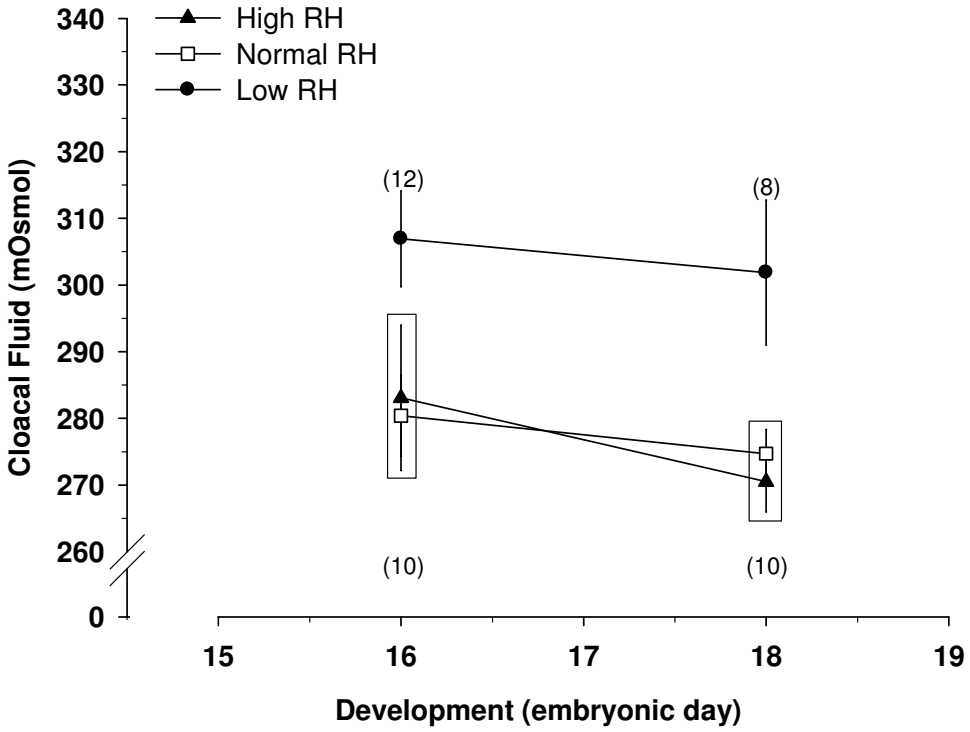

Figure 3.6: Cloacal fluid osmolality (mean ± SE) compared as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

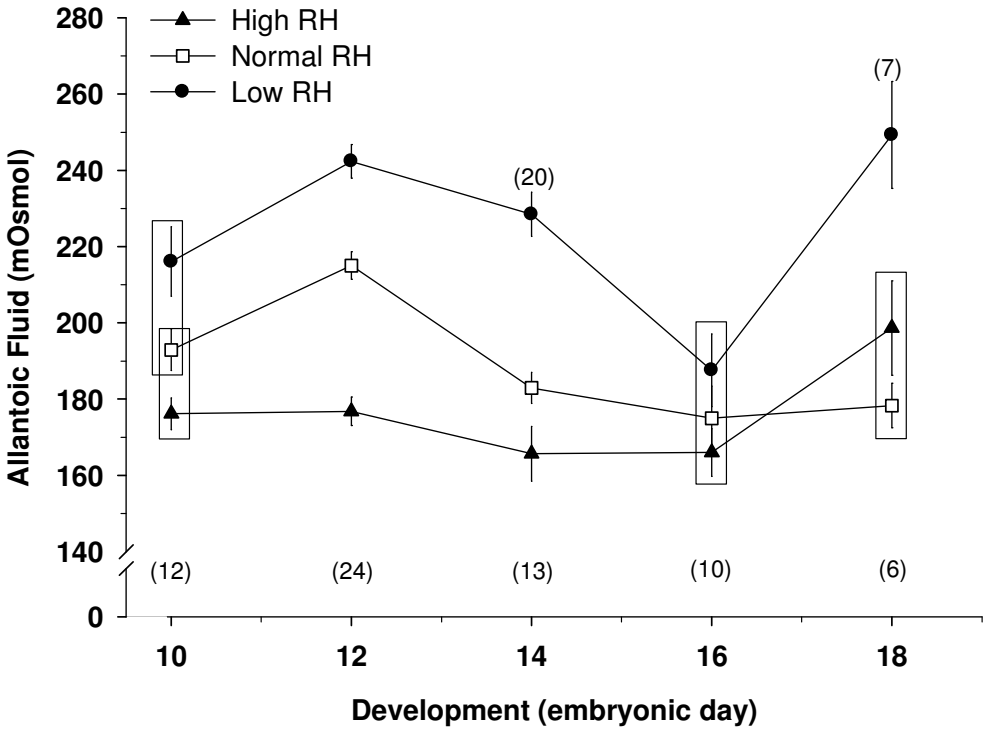

Figure 3.7: Allantoic fluid osmolality (mean ± SE) compared as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

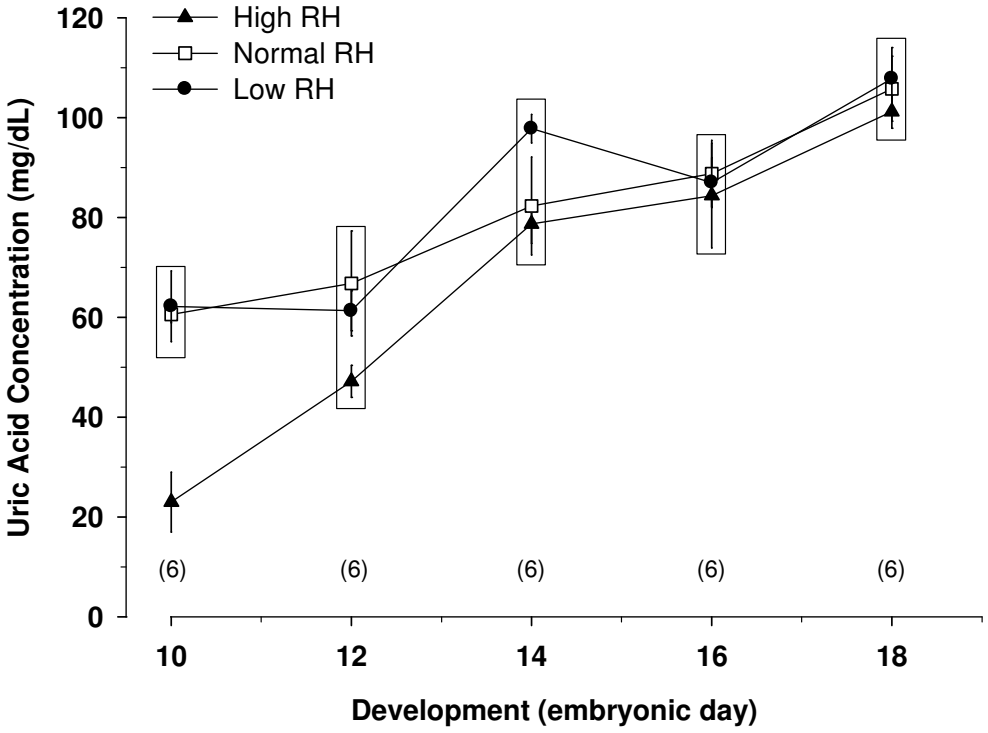

Figure 3.8: Uric acid determination (mean ± SE) compared as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

CHAPTER 4

RENAL DEVELOPMENT IN THE CHICKEN: NORMAL PATTERNS AND INFLUENCE OF INCUBATION HUMIDITY

Introduction

Fetal Programming – An Overview

In most living organisms, water and sodium are major determinants of body fluid homeostasis. The body can compensate for deviations from the homeostatic state within limits via reflex, behavior, hormones, or combination of these mechanisms (Guan, *et al.*, 2008). There is an abundance of research that has analyzed the systems involved to maintain fluid and electrolyte homeostasis within adults forms, while much is to be uncovered in regards to how similar mechanisms operate during embryonic development. When faced with alterations in the maternal environment the fetus may adapt accordingly. This adaptation can cause an imprint in developing organ systems with long-term influence after birth.

Recently a hypothesis has emerged that points to disturbances during critical periods of development as potential causes for pathologies later in life. These "disturbances" occur due to perturbations of the maternal environment. The effect of altered embryonic environment or under nutrition during fetal life and infancy permanently changes the body's structure, physiology and metabolism (Barker, 1995).

Mammalian models have shown that maternal nutritional status and/or environment can influence an embryo to experience adult pathologies like hypertension, type II diabetes, and obesity (Ingelfinger, 2004; Mitchell *et al.*, 2004; Ross and Desai, 2005). The mechanisms behind these diseases in adult life are not well understood.

Limited nephron endowment at birth has been implicated as the primary means by which development of certain adult diseases, like hypertension, may stem. Any reduction in functioning capacity of the kidney prior to birth in humans results in increases of blood pressure to maintain glomerular filtration rate (GFR) and fluid homeostatic functions (Langley-Evans, 2008). A cycle is then established where localized increases in blood pressure cause further loss of nephrons due to tissue damage. Heart disease with subsequent renal failure is the result if medical measures are not taken.

What is the mechanism behind reduced nephron number? The renin-angiotensin system (RAS) and glucocorticoid exposure during critical periods may be the means by which limited nephron endowment occurs (Moritz *et al.*, 2003). Intact RAS is necessary for maintenance and regulation of GFR *in utero* and angiotensin II (ANG II) is needed to sustain cellular differentiation and organ development (Lumber, 1995; Guan *et al.*, 2008). Increased exposure to glucocorticoids during development act to augment angiotensin I and II receptors resulting in apoptosis of metanephric mesenchymal cells and/or accelerated branching before the regular number of nephrons have been established (Glassberg, 2002; Moritz *et al.*, 2003).

During pregnancy, high maternal salt intake causes rats to develop salt-sensitive hypertension resulting in tubular atrophy in subcortical regions and enlarged glomeruli (Rasch *et al.*, 2004). Similar results are seen in fetal lambs exposed to maternal hypertonicity where there were increases in plasma hypertonicity, arterial hypertension, increases in hematocrit and total hemoglobin and hypernatremia (Desai *et al.,* 2003).

Timing of the environmental insult is also critical to nephron endowment. When fetal sheep experience umbilico-placental embolization (UPE) close to the end of gestation, the results are intrauterine growth restriction (IUGR), decreases in kidney weights, and reductions in number of nephrons (Mitchell *et al.*, 2003). However, twinning in sheep causes IUGR with a significant reduction in number of nephrons with the resultant increase in glomerular volume to counterbalance lower nephron numbers. This study by Mitchell *et al.,* (2003) signified that prolonged periods of growth restriction, as in the case of twins, has a more profound effect on kidney development compared to IUGR by UPE.

There are noticeable obstacles in the use of mammalian models for studying fetal programming, such as difficulty in monitoring normal development with the mother during critical periods of maturation. The use of an alternative model, like an avian model, may prove valuable in answering universal questions concerning fetal programming.

Mammals and birds share many commonalities including kidney and endocrine development, presence of high pressure systems and homeothermy (Braun, 1985). Due to these similarities, the avian model can be used to test the effect of environmental insults on normal developmental trajectories.

Avian Kidney and its Development

The avian embryonic kidney, in conjunction with the chorioallantoic membrane (CAM) maintains fluid and electrolyte balance during development. In birds, the paired kidneys are multi-lobed and divided into three, cranial, medial and caudal divisions. Three types of nephrons exist within avian kidneys. The first two types, mammalian-

type (MT) nephrons with long and short loops of Henle, resemble mammalian juxtamedullary and cortical nephrons. The third type of nephron is the reptilian-type (RT) which is similar to superficial nephrons of mammals and yet no loop of Henle is present (Gambaryan, 1992). Glomerular size and volume distributions are not different between left and right kidneys or among cranial, medial, and caudal divisions (Wideman *et al.*, 1992). Depending on the species, 10-30% of nephrons are MT while 70-90% of nephrons exist as RT nephrons in adult birds (Wideman, 1988).

In the chicken, average glomerular circumference of RT nephrons range between 0.15mm to 0.24mm while the larger MT nephrons contain glomeruli ranging from 0.25mm to 0.34mm (Wideman, 1988). Although a bimodal distribution between these two main types of nephrons seems likely, a unimodal relationship is observed. Glomerular sizes exist as a continuum arising from the intralobular artery to the periphery of the kidney (Wideman, 1988; Wideman, 1989). Nephron populations vary between species of birds from 48,000 glomeruli/kidney seen in desert quail (Braun and Dantzler, 1972) to 600,000 glomeruli/kidney observed in broiler hens (Wideman, 1988).

Avian kidneys, like those of mammals and reptiles, show a common developmental pattern in which the pronephros arises first and is then eclipsed by the growth of mesonephros and finally the maturation of the adult kidney form or metanephros. The pronephros appears first out of surrounding mesoderm and was thought to be non-functional in bird embryos (Romanoff, 1960). Hiruma and Nakamura (2003) demonstrated via histological techniques that the pronephros functions similarly to the mesonephros until day 6. There are no clear distinctions between the pronephros

and mesonephros once the mesonephros begins to develop (Hiruma and Nakamura, 2003).

The mesonephros begins differentiating around day 3 with the appearance of podocytes containing pedicels, foot processes that increase podocyte surface area, by day 3.5 (Romanoff, 1960; Narbaitz and Kapal, 1986). The mesonephric filtrate contributes not only to the removal of waste and water regulation, but also to the rapid expansion of the allantoic sac increasing respiratory surface area of the embryo (Freeman and Vince, 1974; Hamburger and Hamilton, 1951). The volume of the allantoic sac increases by a factor of 6 between embryonic days 5 and 7 (Romanoff, 1967). This increase corresponds with both differentiation and growth in size of newly formed nephrons (Friebová-Zemanová, 1982).

Vascular degeneration of both the pronephros and mesonephros begins with collapse of vessel walls, consequently obliterating the lumen. The degeneration of the endothelium and adventitia follow the initial collapse (Carretero *et al.*, 1997).

The metanephros, or definitive kidney, appears on day 4 and begins functioning on day 12 in chicken embryos (Romanoff, 1960). Vasculogenesis of the metanephros is the result of migration of extra-renal blood vessels (Wallner *et al.*, 1997). It is in this newly formed kidney that we can differentiate between the three nephron types. The MT nephrons are the first to form in the embryonic kidney (Narbaitz and Kacew, 1978); therefore, the kidney grows in an inside-out fashion resulting in the growth of RT nephrons last. This fact led Wideman (1989) to make a statement about recapitulation, “ontogeny does not recapitulate phylogeny in the instance of avian kidney development, as MT nephrons development precedes RT nephron development”. Indeed a

continuum exists within the kidney from the base to the periphery with respect to decreasing glomerular size, decreasing length of tubular elements and decreasing single nephron glomerular filtration rate necessary to produce glomerular ultrafiltrate (Braun and Dantzler, 1972; Wideman, 1988). Upon hatching, the avian kidney continues to grow in number, length of nephrons and size of glomeruli up to 30 weeks posthatch. The inner most MT nephrons are the largest and longest of the three types of nephrons (Wideman, 1989).

Avian Osmoregulation

The avian kidney, like the mammalian kidney, has the ability to create hyperosmotic urine. Medullary cones formed from MT nephrons together with vasa recta and collecting ducts make up the countercurrent multiplier system in birds (Johnson and Mugaas, 1970). The capacity of this system has been tested in numerous adult bird species including chicken, desert quail, bobwhite quail and coastal songbirds. Adult birds exposed to dehydration experience loss of body mass, increased hematocrit, blood and urine osmolality (Braun, 1985; Goldstein and Braun, 1989).

Osmotic stress also affects glomerular filtration rate (GFR). When birds are salt-loaded, GFR becomes reduced presumably due to a mechanism known as glomerular intermittency (Dantzler, 1966; Braun and Dantzler, 1972; Yokota, 1985). Glomerular intermittency results from the cessation of blood flow to the RT nephrons due to constriction of the afferent arterioles. Arginine vasotocin (AVT), the avian form of anti-diuretic hormone (ADH), causes the constriction of afferent arterioles to RT nephrons which reduces the number of functioning glomeruli and leads to the redistribution of renal blood flow (Braun and Dantzler, 1972).

Wideman *et al* (1987) demonstrated a case in which reduced GFR did not result from cessation of blood flow to RT nephrons. In their experiment, two populations were raised on either tap water or 0.6% NaCl (saline) drinking water. The saline-adapted birds exhibited significantly lower GFR although number of functioning nephrons did not change. The assumption for why this occurred was that glomerular intermittency may take place in a pulsating fashion different from the infusion rate of alcian blue used in this experiment to indicate number of functioning nephrons (Wideman *et al*., 1987).

The previous examples have shown how avian kidneys adapt functionally to changes incurred by osmotic stress. The kidney is also capable of altering its structure when faced with an osmotic insult.

Coastal songbirds are exposed to varying salinities due to food availability during their lifetime. Their kidneys undergo phenotypic plasticity where kidney renal medullary mass is altered (Sabat *et al*., 2004). It is the need to eliminate greater amounts of salt and nitrogen that yield hypertrophy of medullary tissue. The glomeruli of chickens also become hypertrophied when exposed to high salt intake (Wideman *et al*., 1987; Greg and Wideman, 1990). When severe dehydration or salt load is imposed, adult birds must slow the rise in blood osmolality by a combination of extrarenal excretion of ions, concentrating urine, and decreasing GFR.

These experiments demonstrate the ability of adult birds to conserve water in the face of extreme changes in hydration. Whether or not this phenomenon is demonstrated in the avian embryo will shed light on the maturity of kidney function during development.

How is the avian embryonic kidney affected by osmotic stress? Research on the responses of embryonic avian kidney to altered hydration has been minimal. Murphy *et al* (1991) noted a spike in GFR between days 12 and 16 that corresponded to both an increase in mesonephric glomerular size and appearance of metanephric glomeruli. Additionally, GFR and urine flow rate decrease with development presumably as a mechanism to control water as allantoic sac ceases to function (Clark *et al.*, 1993).

Chapter Objectives

There is limited information regarding developing nephron populations in embryonic birds. This research is intended (1) to give insight into normal kidney development, (2) to show how developmental trajectories of the kidney might be altered due to exposure to extreme humidity and (3) modify an alcian blue technique for estimation of glomerular number and functionality for avian embryos. Because number of filtering RT nephrons decreases in response to the need to conserve water for adult birds (glomerular intermittency, Braun, 1985), it will be interesting to see whether or not the same phenomenon is seen during development under osmotic stress. Therefore, I hypothesize that imposing osmotic stress by extreme incubation humidity on bird embryos will demonstrate how compensation during critical periods alter the developmental program of the kidney. If data from this chapter supports this hypothesis, the avian embryo may prove a good model for answering current questions in mammalian fetal programming involving limited nephron endowment.

Materials and Methods

Source and Incubation of Eggs

Fertilized White Leghorn eggs (*Gallus gallus domesticus*) were obtained from Texas A&M University (College Station, TX) and shipped to University of North Texas (Denton, TX), where they were held in Hova-Bator incubators. The University of North Texas' Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

Eggs were separated and randomly chosen for placement within placed three incubators at either <30%, 55-60% or >85% relative humidity (RH) and temperature of 37.5^{o}C ± 0.5 ^{o}C. Humidity was measured using wireless Baro-Thermo-Hydrometers (model BTHR968, Oregon Scientific). Eggs were weighed pre-incubation and on experimental days. On embryonic days ranging from 10-18, (stages 36-44; (Hamilton and Hamburger, 1951) eggs were removed from each incubator for experimental analyses.

Surgical Protocol

Embryos were sacrificed by 0.1ml pentobarbital injection into a CAM vessel. The embryo was removed from the shell and wet weight was taken. After being pinned to a surgical mat, the abdominal cavity was opened using surgical scissors and all organs were removed superior to the kidneys. Each kidney was removed using fine-tipped forceps, surgical scissors, and dissecting scope (WILD M32 Heerbrugg, Switzerland). Kidney wet weight was measured after removal of excess moisture via Kimwipes® (Kimtech Science). Kidneys used for light microscopy analysis were placed into a vial of 10% neutral buffered formalin with a pH of 7.2 at 4 ^{o}C overnight. Kidneys used for

glomerular distribution analysis were placed in 50% ethanol at 4 °C overnight. The remains of the embryo were then bagged and disposed of according to IACUC standards.

Glomerular Distribution Analysis

The following protocol was adapted from Wideman *et al.,* (1992) and Unflat *et al.,* (1985). Eggs were candled to locate a vein in the chorioallantoic membrane (CAM). To maintain temperature of the embryo during the experiment, eggs were placed in sand in a glass container with steady circulation of 37°C water (Fisher Scientific Isotemp Refrigerated Circulator model 9100). The CAM vessel was cannulated by PE-50 tubing attached to a bent 30 gauge needle by J●B® Weld. The cannula was fixed to both the surface of the eggshell and sand bath to ensure minimal movement during the injections. A 2.5% Mannitol injection, which induced diuresis, was administered via glass Hamilton syringe through the cannula in a bolus slowly over the course of 1 min. The amount injected into the embryo was 3% of the total blood volume, determined by estimating blood volume (embryo wet weight X 15%) (Rychter *et al*., 1955). After a period of 10 min, a bolus of 0.2% Alcian Blue was injected in the same manner. This injection was left to circulate for a total of 30 minutes. A Reptilian incubator (model TXR by LYON) was placed over the egg after each injection to help further maintain temperature. An injection of 0.1ml pentobarbital was introduced via cannula after the 30 min circulation time concluded. The kidneys were then dissected and weighed as described in the surgical protocol above.

Each kidney was placed in a vial containing 50% EtOH for 24 h at 4 °C. The kidneys were then placed into a Bluing mix (50:50 mixes of 50% EtOH and 1%

ammonium hydroxide (HNH_4OH)) for 90 min at 4 ^oC. Next the kidney was rinsed twice with cold deionized water and placed in a vial with 20% hydrochloric acid (HCl) and incubated at 37ºC for 2-3 h. The acid was decanted and the tissue was rinsed 3-4 times with cold deionized water. The tissue was left in the final water mix for 12-24 h at 4ºC. A stirring bar was added along with fresh deionized water to bring the total volume of kidney and water to 6 mL. Stir speed was set to medium for 10 min. A volume of 2 µl was removed via Gilson micropippetor and placed on a microscope slide. After allowing the aliquot to settle (approximately 30 seconds) tissue was viewed via Nikon Eclipse E200 binocular scope at 40X, 100X and 400X magnifications. Glomeruli were counted at 400X magnification and glomerular number per milligram and per kidney were estimated based on kidney wet weight. Glomerular circumferences were measured using Image-Pro® Plus version 4.1 (Media Cybernetics®). Functioning glomeruli were those which possessed alcian blue within the glomerular tuft. The filtration surface area of a three-dimensional capillary tuft is proportional to available volume within the glomerulus; by multiplying the glomerular volume by estimated number of glomeruli seen, glomerular volume distribution profiles will be seen (Wideman, 1989).

Light Microscopy

After proper fixation of the tissue by 10% neutral-buffered formalin, pH of 7.2, tissue was processed (formalin fixed, dehydrated, and paraffin) in a microwave rapid histoprocessor (Milestone RH1), embedded in paraffin blocks (Shandon Histocentre 3 tissue embedder) and 8 µM serial sections were made with a microtome (Leica RM 2245). The microtome ribbons were mounted on labeled slides in a 55ºC tissue float bath (Fisher Scientific) and allowed to air dry for 24 hours.

Once dry, the slides were rehydrated and stained using hematoxylin with a counter stain of eosin for viewing of both nuclear and cytoplasmic organelles. Coverslips were placed atop slides using Permount® SP15-100 (Fisher Scientific) after dehydrating with another series of graded ethanol. The slides were then allowed to air dry for 24 h before viewing.

Gross micro-morphological observations were made via Nikon Eclipse E200 binocular scope from 40X to 400X magnification and ImagePro® Plus version 4.1 imaging system (Media Cybernetics®). Light microscopy slides were used to verify that glomeruli were being stained properly through out the entire kidney.

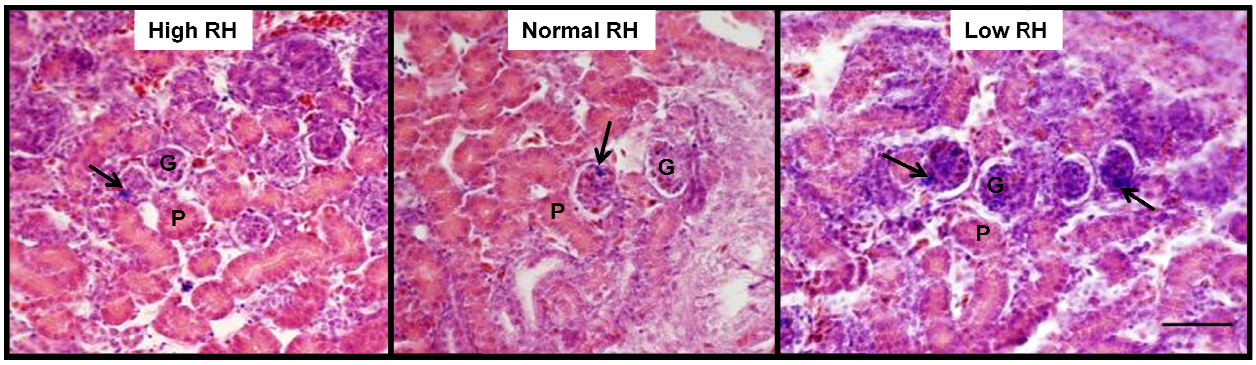

Figure 4.1: Light microscopy images of 8 µm kidney slices from high, normal and low humidity embryos, (G: glomerulus; P: proximal convoluted tubule; arrow: alcian blue dye; 50 µm scale).

Statistics

All data were tested for normality of distributions (Shapiro-Wilks test for normality) before specific statistical analysis was performed. Two-way parametric ANOVA was used to test whether or not developmental day, humidity level or interaction between the two factors had an effect on the data. Student-Newman Keuls (SNK) multiple range *post hoc* tests were run to separate data into distinct groups. Concatenation procedure was used when a significant interaction was seen between

developmental day and humidity level. Linear regression was run to identify whether relationships existed between development, glomerular estimates and circumferences as well as kidney wet weights, glomerular estimates and circumferences. All statistical analyses were conducted using SigmaStat 3.5, SigmaPlot 10.0 and SAS software. Decisions were made with a 0.05 level of significance.

Results

Kidney Wet Mass

Development had a significant affect on mean wet kidney mass ($p<0.001$) in all three populations, as anticipated (Figure 4.2). Incubation humidity had little effect on kidney wet mass, with a significant but small decrease in mass for control values evident in the high and low populations only on day 16.

Analysis of Total Glomerular Size and Distribution

Raw glomerular counts increased with kidney wet weight (Figure 4.3). A significant relationship existed between glomerular counts and kidney wet weight for all humidity groups (Low: $r^2=0.79$; Normal: $r^2=0.80$; High: $r^2=0.72$; $p<0.001$ for all groups). This relationship enabled the subsequent use of kidney wet weight in calculations for estimated glomerular number within the kidney.

Estimated total glomeruli increased significantly during development (Figure 4.4) ($p<0.001$). However, differences between incubation populations were only apparent on day 18, when the low humidity group had a significantly higher estimated glomerular number of $5.05 \times 10^4 \pm 5.24 \times 10^3$ glomeruli/kidney compared to both normal ($3.98 \times 10^4 \pm 3.05 \times 10^3$ glomeruli/kidney) and high ($3.74 \times 10^4 \pm 3.36 \times 10^3$ glomeruli/kidney) humidity groups.

There was a significant interaction between development and humidity level for mean glomerular volume (Figure 4.5) (p<0.001). On day 12, the volume of the glomeruli in the low humidity group was larger (4.00 ± 0.55 X 10^{-4} mm^3) compared to both normal (2.74 ± 0.36 X 10^{-4} mm^3) and high (2.15 ± 0.26 X 10^{-4} mm^3) humidity groups.

Glomerular volumes were grouped into bins to demonstrate the shift in size distributions during development (Figure 4.6). The number of glomeruli in each bin was estimated as a percentage (number of glomeruli observed for humidity level and developmental day divided by total glomeruli seen for the same humidity and developmental day).

Total glomerular volume per kidney indicated the kidney's capacity for filtration (Wideman, 1992). There was a significant interaction between humidity level and development for mean total glomerular volume/kidney (p<0.001) (Figure 4.7). The low humidity group had a significantly lower total glomerular volume on days 14 and 16 (3.38 ± 0.26 mm^3; 3.12 ± 0.19 mm^3, respectively) compared to normal (5.13 ± 0.75 mm^3; 4.64 ± 0.44 mm^3) and high (4.76 ± 0.47 mm^3; 4.59 ± 0.32 mm^3) humidity groups which were statistically the same. On day 18, the trend changed in which the low humidity group exhibited higher total glomerular volume/kidney (6.77 ± 0.43 mm^3 compared to normal (4.80 ± 0.33 mm^3) and high (3.97 ± 0.30 mm^3) humidity groups which were not different.

Analysis of Functioning Glomerular Size and Distribution

A significant interaction occurred between developmental day and humidity level for mean functioning glomeruli (Figure 4.8) (p=0.004). On day 18, low humidity group

(3.58 X10^4 ± 3.40 X 10^3 glomeruli/kidney) had a higher number of functioning glomeruli compared to high (2.21 X 10^4 ± 2.47 X 10^3 glomeruli/kidney) and normal (1.73 X 10^4 ± 2.75 X 10^3 glomeruli/kidney) humidity groups.

A significant interaction was seen between humidity level and development for functioning glomerular volume (p<0.001) (Figure 4.9). On day 12, low humidity group had a mean glomerular volume of 4.55 ± 1.25 X 10^{-4} mm^3 which was statistically larger than both normal (1.88 ± 0.339 X 10^{-4} mm^3) and high (1.59 ± 0.54 X 10^{-4} mm^3) humidity groups. On day 14, low (3.47 ± 0.84 X 10^{-4} mm^3) and normal (3.16 ± 0.41 X 10^{-4} mm^3) groups were larger than high humidity group (1.84 ± 0.16 X 10^{-4} mm^3).

There was a significant interaction between humidity level and development for functioning glomerular volume/ kidney (p<0.001) (Figure 4.10). On day 18, low humidity group had a considerably higher mean functioning glomerular volume / kidney (4.20 ± 0.23 mm^3) compared to normal (1.89 ± 0.18 mm^3) and high (2.06 ± 0.21 mm^3) humidity groups which were not statistically different.

Discussion

Avian Kidney Development Revisited

The kidney begins functioning on day 4 of development, as evident from the expansion of the allantoic sac. This information indicates that the immature kidney has the capacity to contribute to osmoregulation and may be affected by the hydration state of the embryo. To date, there has been no information regarding the influence of extreme humidity on kidney development. Additionally, there is a lack of data regarding total and functioning glomerular size and number or how these parameters are affected when the embryo is exposed to alterations in hydration state via incubation humidity.

Since nephron endowment is a predictor for pathologies that can develop in adult mammals, it was important to see if the same developmental principles of fetal programming apply to birds (Langley-Evans, 2008). Mammals and birds share many similarities, such as the development and maturation of the kidney. Although the avian kidney continues to accumulate reptilian-type (RT) nephrons after hatching, it may prove to be a key model to use for investigation of the affects under nutrition or environmental insults have on developmental trajectories of the kidney.

Analysis of Total Glomerular Size and Distribution

The estimated total glomeruli remained relatively unaffected by hydration state until day 18 of development where the low humidity group exhibited a marked increase in glomerular number. Since kidney wet weight was a part of the calculation for total glomeruli, it was important to demonstrate that there were no significant differences on day 18 that might lead to inflation of the total number of glomeruli estimated.

When exposed to high salt or low protein during development, fetal mammals have a reduced number of glomeruli presumably due to low RAS or over expression of arginine vasopressin (Bouby and Fernandez, 2003; Rasch *et al.*, 2004). In this study, the avian kidney did not behave in the same manner when exposed to dehydration from the embryonic environment. Instead, the low humidity kidney increased the number of total glomeruli thus increasing the filtering capacity of their kidney. My prediction is that had the low humidity birds hatched, they would have been more tolerant to dehydrating conditions due to the increased number of glomeruli present within the kidney.

Since the volume of glomeruli of the low humidity group exceeds that of the normal and high humidity groups on day 12, it can be assumed that there are more

mammalian-type (MT) nephrons. The more MT nephrons present, the greater the benefit for those embryos exposed to low humidity incubation to conserve water. There were no noticeable differences between glomerular volumes towards the end of development indicating that the immature kidney does not experience hypertrophy of MT nephrons when exposed to dehydrating conditions like kidneys of adult birds (Wideman *et al*., 1987; Greg and Wideman, 1990).

When comparing the glomerular volumes found in the present study to those found in previous studies, glomerular size differed dramatically (Gambaryan, 1992). This difference was due to the methods employed to analyze glomerular volume between the previous study and the present research. When kidneys are prepared for light microscopy observation, severe dehydration of the tissue is inevitably the result. The alcian blue method used in this study caused swelling of the glomeruli since kidneys were held overnight in water.

Mammalian-type nephrons represent a small portion (10-30%) of the total nephron population in avian kidneys (Wideman, 1988). So, the shift seen in size distributions from Figure 5 are to be expected since there is an increase in both number and growth of smaller nephrons as development progresses.

Kidneys from the low humidity group exhibited a sharp, significant increase in total available filtering capacity compared to the normal and high humidity groups. This steep increase supports the suggestion that the embryo was adapting its kidney to dry conditions via increased nephron development in preparation for hatching.

Analysis of Functioning Glomerular Size and Distribution

When plasma osmolality increases in adult birds, arginine vasotocin (AVT) constricts the afferent arterioles thus resulting in reduced blood flow and eliminating filtration of the RT nephrons (Wideman, 1989). Since the embryonic kidney does not have a substantial number of RT nephrons, the capability of the kidney to exhibit the adaptive response of glomerular intermittency may be absent. A smaller number of functioning glomeruli was expected for those embryos exposed to low humidity if the developing kidney mimicked the adult kidney. My results concluded that this was not the case. Indeed, there were more functioning glomeruli in the low humidity group compared to the normal and high humidity groups on day 18 of development. This indicated that the immature kidney does not respond to dehydrating circumstances like adult birds. As was postulated for the total number of glomeruli/kidney, low humidity embryos presumably altered their kidneys in preparation for hatching

Functioning glomerular volumes measured on day 12 of development were larger in the low humidity group compared to the normal and high humidity groups. The differences seen could have been the result of more MT nephrons present in the low humidity kidneys indicating that more MT glomeruli were sampled compared to smaller more peripherally distributed types of glomeruli.

The filtering capacity of functional glomeruli of the low humidity group far exceeded that of the other humidity groups in the present study. Although the glomerular circumferences on day 18 were not different between treatment groups, the combination of size and number demonstrated that those embryos raised under low humidity have kidneys more adapted for dry environments.

Speculations on Early Renal Development

Kidneys from day 10 embryos were also examined via the alcian blue method described above. There were no visible glomeruli stained or otherwise within the water suspensions used to count glomeruli. This information indicated that either the negatively charged glycoproteins along the glomerular filtration barrier did not exist at this stage of development, or the number of glomeruli present was too small to count via these methods. If the former was the case, plasma proteins may have been able to pass freely into Bowman's space which would require further recovery of these proteins once they entered the allantoic sac.

Summary

Kidneys from embryos raised under low humidity conditions were significantly altered via marked increases in glomerular number and volume. This statement refutes the idea that the immature avian kidney mimics the adult form when dehydrating circumstances are imposed. Additionally, the avian embryonic kidney does behave similarly to mammals exposed to high salt intake by reducing the number of nephrons. Instead, the kidney undergoes increased organogenesis in preparation for hatching under dry conditions. A survival benefit was observed for those embryos that possessed the ability to adapt their kidneys in light of an environmental insult such as incubation humidity.

The chicken is a good model for observing how an environmental insult, such as extreme incubation humidity, can modify the developmental program of the kidney. Homeostatic set points altered due to changes in the hydration state of the embryo may

prove similar to what is seen in mammals exposed to maternal hypertonicity (Desai *et al.*, 2003); however, a closer look at the development of both RAS and AVT is needed.

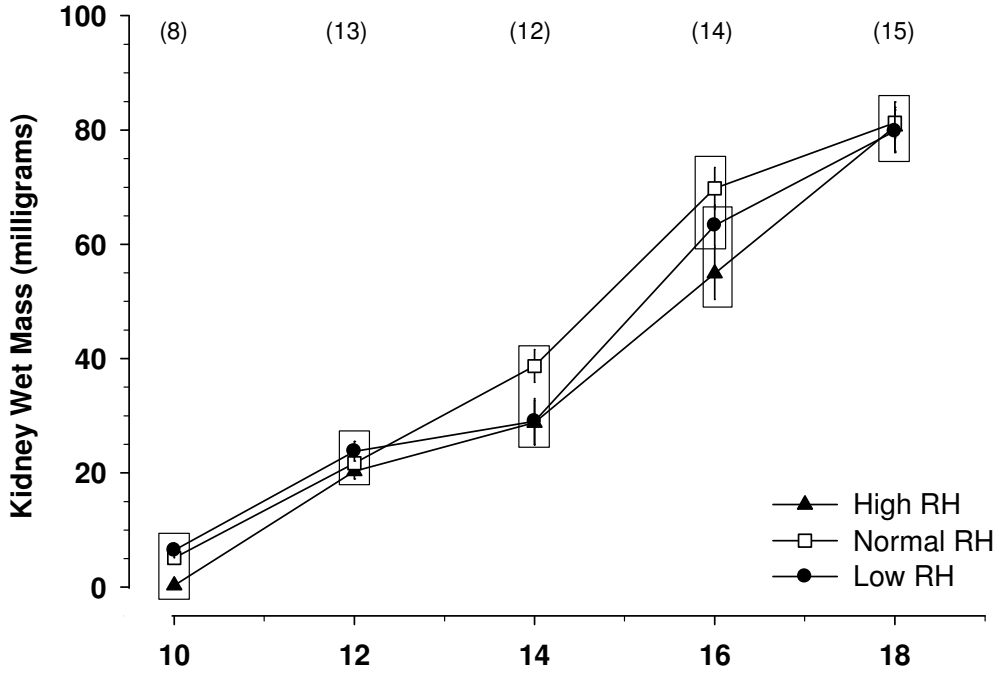

Figure 4.2: Kidney wet mass (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

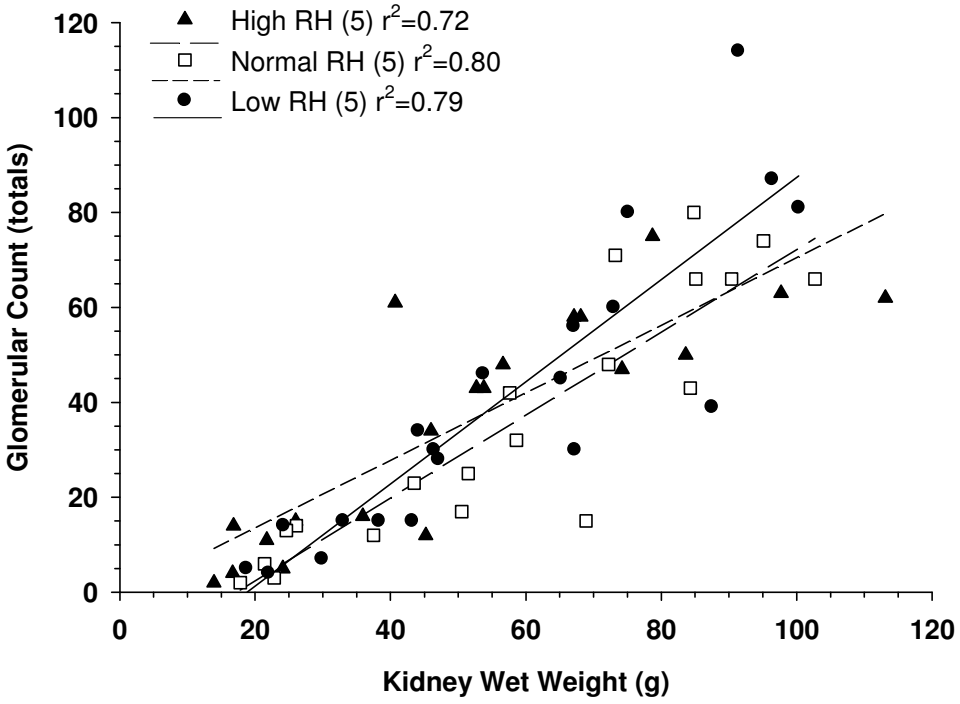

Figure 4.3: Raw glomerular count as a function of kidney wet weight for all humidity groups during development. Regression coefficients and *n* values are shown.

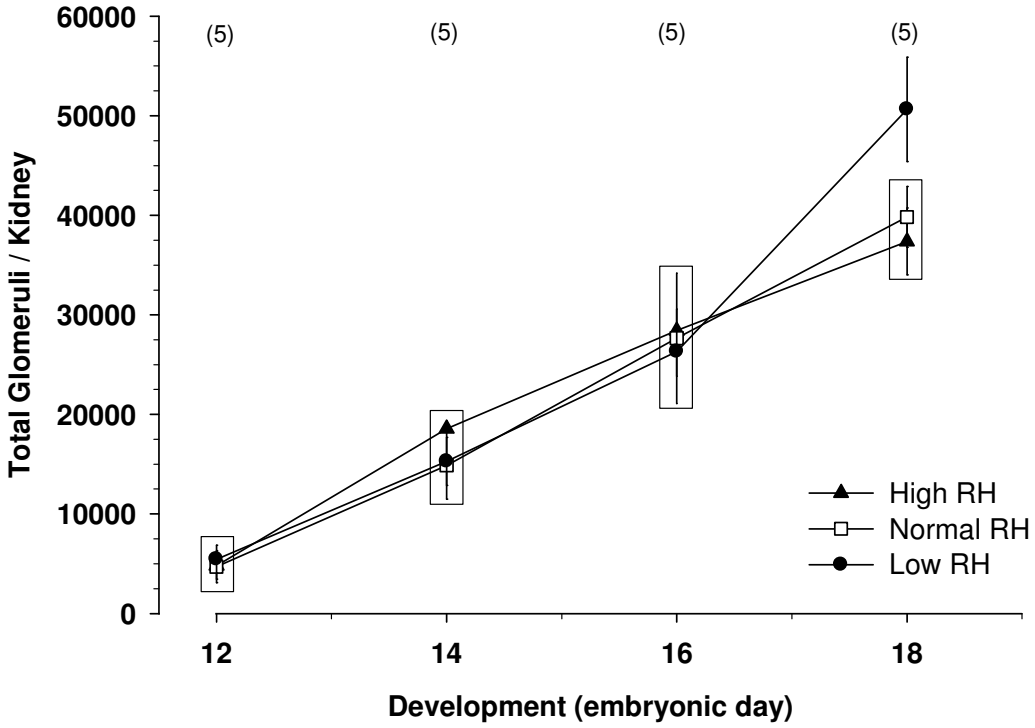

Figure 4.4: Estimated total glomeruli per kidney (mean ± SE) as a function of development and humidity levels. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

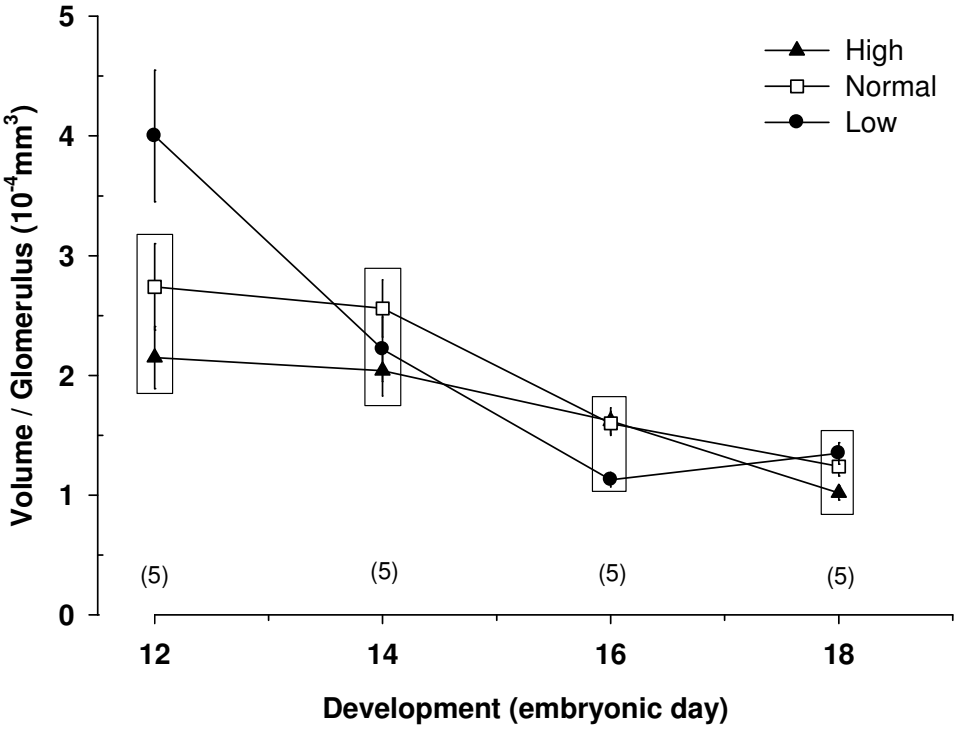

Figure 4.5: Volume per glomerulus (mean ± SE) as a function of development and humidity levels. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

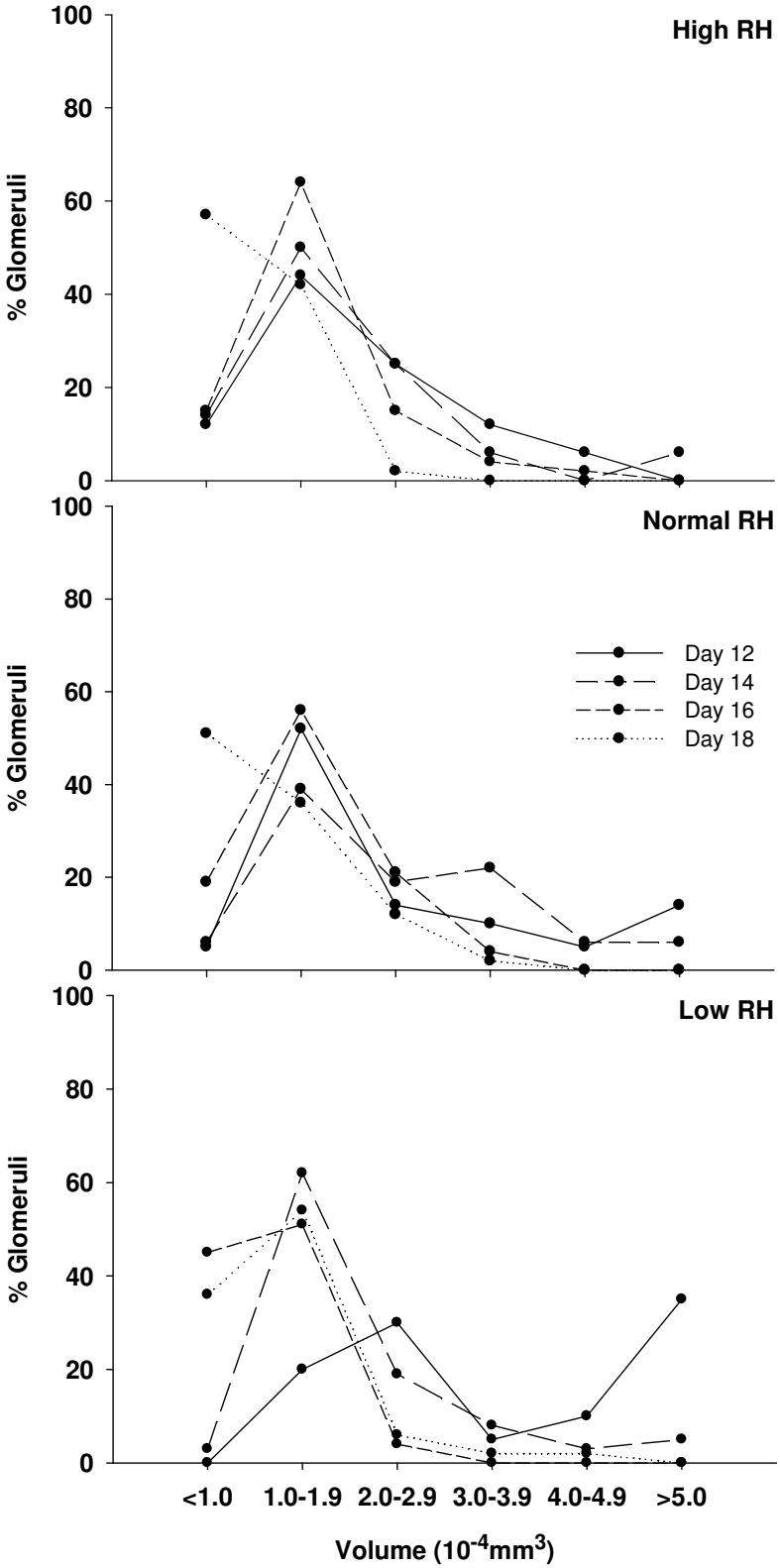

Figure 4.6: The percent of glomeruli in each volume bin for humidity level across development.

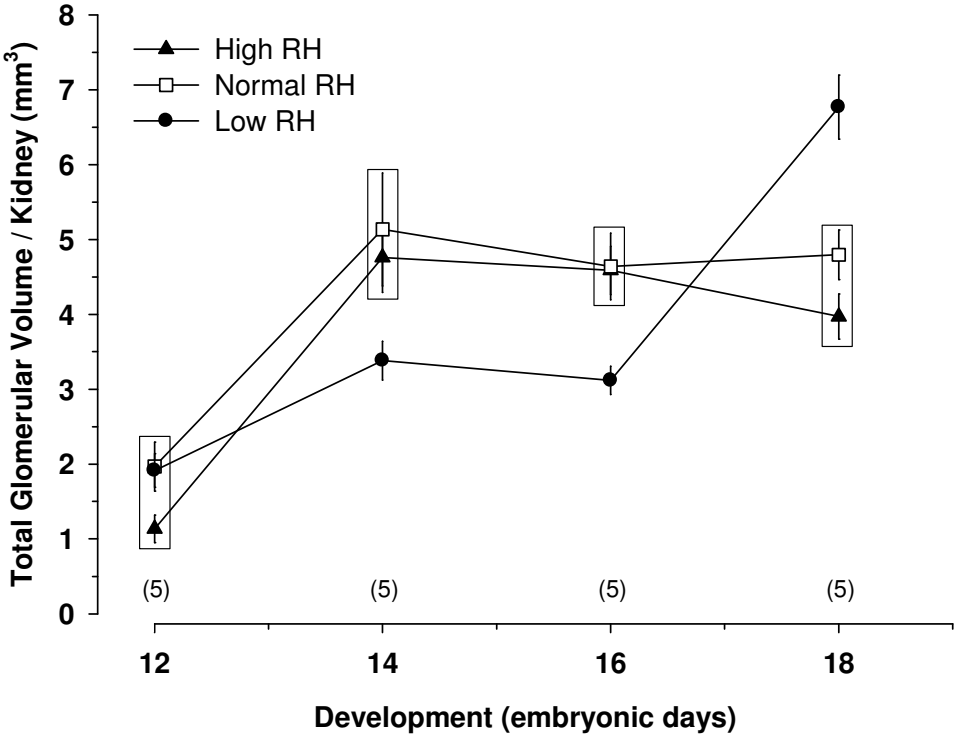

Figure 4.7: Total filtering capacity of the kidney (mean ± SE) as a function of humidity level and developmental day. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

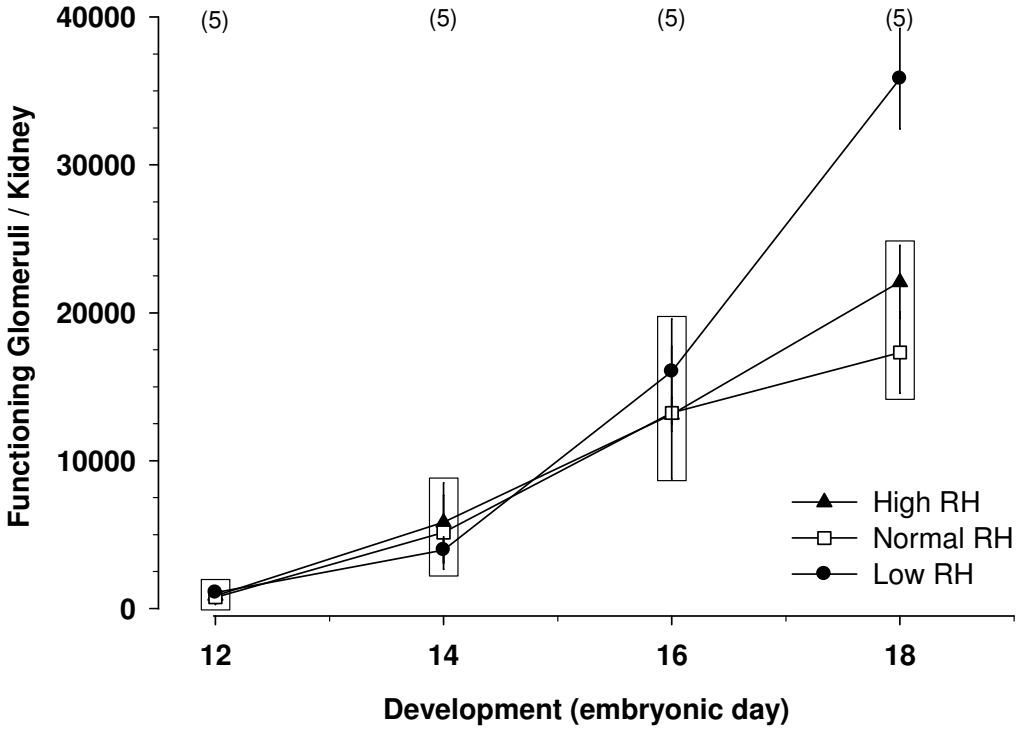

Figure 4.8: Functioning glomeruli per kidney (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

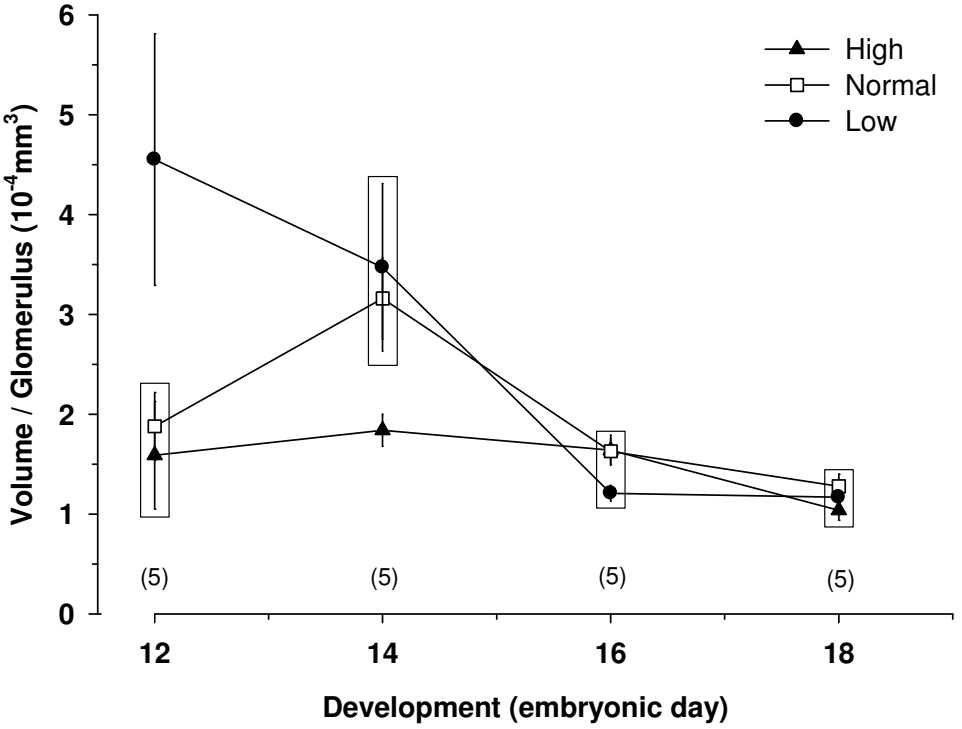

Figure 4.9: Functioning of glomerular volume (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

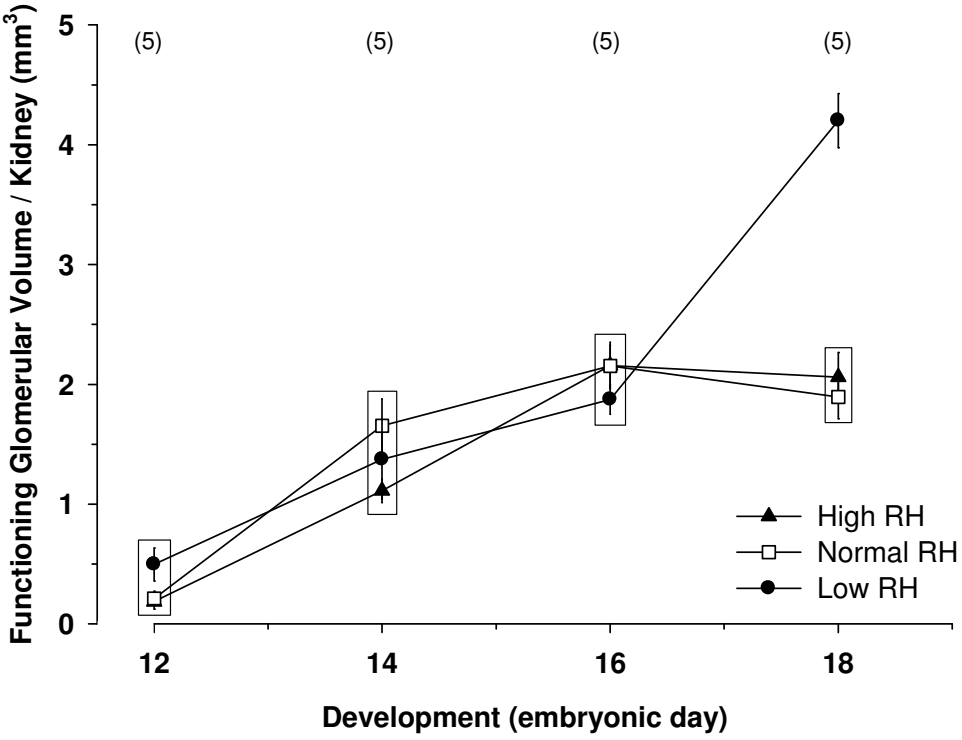

Figure 4.10: Functioning filtering capacity of the kidney (mean ± SE) as a function of development and humidity level. Statistically identical means are grouped within boxes. Sample sizes given in parentheses.

CHAPTER 5

CONCLUSION

Summary of Findings

Research Objectives Revisited

The chicken develops within a closed environment that can be subjected to outside manipulation to answer pertinent questions about development. In this study, my ultimate goal was to determine how the chicken embryo would compensate its morphological and physiological renal development in light of the environmental insult of extreme incubation humidity.

In addition to the broad goal of this research, I hoped to accomplish several specific objectives which included (1) increasing in the magnitude of embryonic exposure to extreme incubation humidity, (2) measuring affects of extreme humidity during the second half of development prior to internal pipping, and (3) examining kidney morphology as altered by extreme humidity. Also, a technique used to assess filtering capacity of adult birds was modified for embryos to evaluate normal kidney development and estimate functioning nephrons relative stress imposed by extreme incubation humidity.

Chapter 2 Conclusions

To accomplish my first and second objectives, embryos were incubated within treatment groups (low: <30% relative humidity (RH); normal: 55-60% RH; high: >85% RH) for the entire course of development and humidity affects were assessed on days 10 through 18. It was important for that the embryos were exposed to the treatment conditions for the entire incubation since loss of water during the first half of incubation

results in a greater impact on development (Snyder and Birchard, 1982). In addition, 76% of albumen water disappears by day 10, so any study that altered water after this time may not show a significant impact on the embryo (Baggott, 2001; Romanoff, 1960).

Embryonic days 10-18 were chosen for analysis since this time represents when the mesonephros functions alone (day 10-12; Murphy *et al*., 1991), then the metanephros starts functioning with the mesonephros (day 12-16; Carretero *et al*., 1997; Murphy *et al*., 1991) and finally the metanephros functions alone and all nephron types can be seen (day 18; Gambaryan, 1992). Also, hormones necessary for water conservation begin to function during this time including arginine vasotocin (AVT; Klempt *et al*., 1992) on day 15 and aldosterone on day 17 (Doneen and Smith, 1982). Outside the developing embryo, important developmental events also occur between days 10-18. The sero-amniotic connection is formed around the same time the chorioallantoic membrane (CAM) is completed and allantoic and amniotic fluid compartments peak (day 12-13; Baggott, 2001; Hoyt; 1979; Romanoff, 1960). Additionally, it is around day 17 of incubation that there is an increase in net water uptake in preparation for hatching (Davis *et al*., 1988; Romanoff, 1960).

Now that the first two objectives have been outlined and the value of the objectives has been detailed, I can move forward to analyze how the extreme humidity conditions influenced the embryo and the internal environment surrounding the embryo. There were no differences between treatment groups for embryo wet mass and oxygen consumption until day 18 of incubation. Despite the obvious environmental insult, as indicated by mortality rates examined in chapter 2, embryos from the extreme humidity groups were able to grow and consume oxygen at a steady pace with the normal

humidity group until just before pipping. On day 18, embryos exposed to extreme humidity had smaller embryo wet masses with resultant lower oxygen consumption thus demonstrating a failure to thrive compared to the optimal or normal humidity incubation.

This study was the first to examine how humidity stresses the developing avian kidney. The chicken kidney grows in proportion to the embryo in all incubation conditions. Exposure to extreme humidity does not especially alter this trajectory. Therefore, extreme humidity had no affect on the developing avian kidney in terms of gross morphology.

Chapter 3 Conclusions

Hematocrit, total hemoglobin and uric acid concentration followed expected and well-documented values (Davis *et al.*, 1988; Hoyt, 1979; Romanoff, 1960). Although deviations in hematocrit and uric acid precipitate were anticipated due to the literature reviewed, no differences between treatment groups show the ability of the embryo to maintain homeostasis.

Of all the physiological parameters examined, fluid compartment osmolality showed the most profound results. Humidity incubation coupled with development had a significant affect on fluid compartment osmolality, and treatment groups were separated into two to three distinct clusters with embryos from the low humidity group showing the biggest deviations from normal humidity incubation for all fluids analyzed.

Amniotic fluid serves as a mechanical protection during development and drinking source for the embryo once the sero-amniotic connection is formed on day 12 (Baggott, 2001). Its composition is important to salt and water homeostasis. Amniotic fluid osmolality, previously thought to remain stable, showed significant alteration. The

low humidity group had a higher osmolality compared to the other treatment groups across development. Blood osmolality was also affected by low humidity incubation presumably due to ingestion of the high osmolality amniotic fluid. Since blood osmolality was affected by low humidity incubation, the kidney's natural response was to conserve water. The developing kidney of embryos exposed to low humidity would then need to increase absorption of water to compensate for higher blood osmolality.

The avian kidney is coupled to the lower gastrointestinal tract, specifically the urodeum of the cloaca. The urine passes from the kidney through the ureters to the cloaca and is moved by reverse peristalsis into the lower gastrointestinal tract for further water and protein reabsorption. Embryos exposed to low humidity incubation exhibited higher cloacal fluid osmolality compared to both normal and high humidity groups. The cloacal fluid osmolality was not significantly higher than the blood for days 16 and 18 for all treatment groups demonstrating the maturity of the renal-gastrointestinal coupling prior to hatch.

The allantoic fluid is a constant source of water and electrolytes needed to maintain homeostasis of the embryo throughout development. The volume of this fluid compartment peaks around day 12 of incubation then steadily declines until day 16 when osmolality declines due to movement of sodium across CAM and sodium sequestering by uric acid (Davis *et al.*, 1988; Baggott, 2001; Romanoff, 1967). Increases in allantoic osmolality on day 18 demonstrated a decline in fluid volume and decreased function of CAM due to atrophy (Davis *et al.*, 1988). Embryos exposed to varying rates of desiccation appear to osmoregulate by altering the rate at which they absorb water from the allantoic fluid (Hoyt, 1979). The present study agrees with

previous research on the affects of changes in incubation humidity on allantoic fluid since the low humidity group had distinctly higher osmolality compared to the normal and high humidity groups.

Chapter 4 Conclusions

The final objective of this research was to quantify the changes in renal morphology exposed to extreme humidity. To accomplish this goal, I had to modify a proven method used in adult birds to estimate renal filtering capacity in embryos (Unflat *et al.*, 1985; Wideman *et al.*, 1987; Wideman, 1989; Wideman *et al.*, 1992). Once alcian blue concentrations and injection timing was adjusted for embryos, filtering capacity of each kidney could be estimated for days 12-18 of development. Glomerular size appeared to decrease with age; however, this phenomenon was quickly explained once nephron development was considered. Mammalian-type (MT) nephrons are the first to develop and are larger than reptilian-type nephrons (RT) which appear towards the end of development. So, glomerular volumes from day 12 were significantly larger than the ones on days 16 and 18 due to established renal maturation sequences. The estimated total number of glomeruli per kidney increased with age as expected with no differences seen between humidity groups until day 18. The low humidity group had significantly greater estimated total glomeruli and functioning glomeruli towards the end of development. Since the low humidity group demonstrated higher filtering capacity, it was assumed that these embryos would have been able to more efficiently conserve water when faced with a dry environment. These are profound results in light of the subtle differences between humidity groups found in chapters 2 and 3.

Finally, I hypothesize that in extremely dry incubation conditions, amniotic fluid osmolality increases affecting blood osmolality which indirectly causes increased renal organogenesis in response to the stressful environment. The increased filtering capacity of low humidity embryos was demonstrated upon examination of cloacal fluid. On days 16 and 18, cloacal fluid osmolality was higher in the low humidity group exhibiting their increased filtering efficiency. This adaptation in renal morphology prepares the embryos from the low humidity group for dry conditions upon hatching. I can conclude that the renal developmental program can be changed when exposed to extreme humidity incubation.

Future Directions

Further Kidney Analysis

Since it has been established that renal morphology can be changed by exposure to extremely dry incubations during development, many more questions resonate. For example, is glomerular filtration rate altered due to increased filtering capacity in low humidity embryos? Also, if hatchlings from all treatment groups are exposed to dehydrating conditions, do those from the low humidity group thrive? Additionally, what triggers the onset of glomerular intermittency? And finally, since the avian kidney continues growth up to 30 weeks post-hatch (Wideman, 1989) does kidney growth continue to be accelerated in the low humidity group? There are many unanswered questions with regards to renal functioning for altered humidity during development. Of all the possible directions my research could move, I am most interested in analysis of hormonal control of the kidney during development.

Arginine Vasotocin

Hormones play an integral role in salt and water balance in adult birds. Arginine vasotocin (AVT), which is structurally and functionally similar to mammalian anti-diuretic hormone (ADH), aids in water conservation in birds. When adult birds are exposed to dehydration or salt-loading, AVT is released from neurosecretory cells from the posterior pituitary. Glomerular filtration rate (GFR) is then reduced to allow for increased tubular reabsorption of water.

Experimental data suggests that AVT begins to perform its osmoregulatory action as early as day 12 in response to injection of AVT (Kisliuk and Grossman, 1994). Arginine vasotocin mRNA has been seen as early as embryonic day 6 by *in situ* hybridization (Milewski *et al.*, 1989), while immunocytochemical methods give positive results for AVT from day 8 (Tennyson *et al.*, 1986). Radioimmunoassay of the blood has shown detectable levels of AVT on day 14 of 6.9 ± 0.3 pg/ml (Muhlbauer *et al.,* 1993) while a separate study demonstrated that embryos exposed to osmotic challenge experienced an increase of AVT concentration in blood starting around day 15 (Klempt *et al.*, 1992). Would embryos exposed to extremely low humidity show evidence of AVT mRNA prior to day 6? If this is the case, will AVT appear in the blood sooner than day 15?

The primary target for AVT in adult mammals is the renal collecting duct, while both the vasculature and organs for osmoregulation (kidneys, bladder and skin) are targets in amphibians (Uchiyama, 1994). It is likely since the avian kidney structure is an intermediate between both mammals and amphibians that the target of AVT may also combine these elements (Goldstein, 2006). The role of AVT in kidney tubules may

be restricted to collecting ducts, and although vasotocin stimulates the production of cAMP in avian medullary tissue, an avian V2 homolog has not been sequenced or identified. Thus both vascular and tubular receptors for AVT remain undefined (Jenkins and Porter, 2004). A few landmark studies demonstrated that vasotocin exerts its antidiuretic effect by constricting afferent arterioles and thereby lowering GFR in select nephron populations, namely RT nephrons when birds were salt-loaded or AVT was administered (Braun and Dantzler, 1972; Braun, 1976); although this has yet to be rigorously tested, especially in the avian embryo.

Since AVT receptors have neither been localized or identified in the chicken, many efforts have been put forth to evaluate whether AVT has a direct effect on renal tubules. First, Goldstein and Bradshaw (1998) found that birds under normal conditions experience fractional tubular water reabsorption of 95% and yet those under extreme hydrated conditions demonstrated as low as 70% tubular water reabsorption. Another study which used inulin as a filtration marker, showed that the urine-to-plasma ratio can increase up to 10-fold from diuretic to anti-diuretic states in the absence of any change to GFR (Stallone and Braun, 1985). These results plainly suggest tubular water permeability may have been affected since GFR remained unchanged.

Isolated, perfused tubule experiments carried out by Nishimura and colleagues showed that once collecting ducts were stimulated by a high concentration of AVT, water permeability at this location was increased (Nishimura *et al.*, 1996). There has even been a mathematical model of the avian kidney suggesting that urine concentration can be influenced by AVT-induced changes in collecting duct water permeability (Layton *et al.*, 2000).

Because the renal tubular (v2) receptors for vasopressin in mammals are coupled to adenylyl cyclase leading to production of cyclic adenosine monophosphate (cAMP) as a second messenger system, several researchers are under the assumption that AVT would similarly be coupled to cAMP (Goldstein, 2006). A study which examined cAMP accumulation in individual renal tubules of house sparrows (*Passer domesticus*) revealed a dose dependent AVT-stimulated adenylyl cyclase activity in both thick ascending limbs and collecting ducts (Goldstein *et al.*, 1999).

Aquaporins

In mammals, aquaporins are the cellular link between cAMP production and enhanced tubular water reabsorption. Aquaporins (AQP) are molecular water channels that aid in rapid membrane water transport. There are 12 different aquaporins (0-11) that have been identified and sequenced in mammals. They are characterized as either orthodox, water-selective (0, 1, 2, 4, 5, 6, 10), or multifunctional, also known as aquaglyceroporins which transport water, glycerol and other solutes (3, 7, 8, 9) (Casotti *et al*, 2007). To date, 3 aquaporins have been identified in birds, AQP 1, 2, and 4.

Aquaporin 1 has been identified in the vascular smooth muscle cells (VSMCs), ectodermal and endodermal epithelia, and vascular endothelium located in kidney, lower intestinal tract and CAM of birds (Nielson and Agre, 1995; Casotti *et al.*, 2007). A study looking at developmental expression of AQP 1 in chick CAM using western blot and immunohistochemical techniques under normal conditions found strong expression on days 6, 10 and 14 (Ribatti *et al.*, 2002). One of the CAM's major roles is to preserve osmotic conditions in the blood and fluid around the embryo to promote healthy growth by regulating water and electrolyte transport from its lumen to embryonic circulation

(Simkiss, 1980; Davis *et al.*, 1988). Aquaporin 1 is thought to be the source of high rates of water movement. Further studies resulting in the alteration of normal environmental conditions may lead to a better understanding of this water channel when stress is placed upon the embryo.

Aquaporin 2 (AQP 2) has been identified as early as day 10 in the collecting ducts of the developing deep medullary regions of quail kidney. In a recent study, quail embryos were subjected to 5-10% egg white withdrawal (EwW) or 48 hours post-hatch food deprivation (FD) to examine nephron growth and quail AQP 2 (qAQP 2) mRNA expression, and effects of EwW and FD on qAQP 2 mRNA responses to 72 hours water deprivation posthatch (Nishimura *et al.*, 2007). This examination concluded that qAQP 2 mRNA signals increased in intensity in developing metanephros from day 10 through to the postnatal period. Those EwW and FD birds demonstrated delayed nephrogenesis and exhibited smaller numbers of glomeruli (Nishimura *et al.*, 2007). Most importantly, it appears that due to the impairment of the kidney, water and electrolyte homeostasis may have been altered, possibly indefinitely, in the adult bird similar to what is seen in perinatal programming of adult hypertension and kidney disease in mammals (Marchand and Langley-Evans, 2001).

The final AQP that has been identified in chickens is AQP 4 which has been associated with central osmoreception. The brain has been implicated as the primary expression site for AQP 4 via northern blotting analysis. Chickens exposed to water deprivation demonstrated increased AQP 4 mRNA in the hypothalamus, along with increased plasma osmolality, and hypothalamic mRNA levels of AVT (Saito *et al.*, 2005). Since hypothalamic AVT mRNA levels increased due to hyperosmotic

conditions, it might seem logical to speculate that increased AQP 4 mRNA may be the result of the increase in AVT. Further studies of these mechanisms must be tested before any true conclusions may be drawn.

Because of the work that has been carried out previously, one might think it valuable to know how extreme environmental conditions may affect AVT and AQP function and concentration. By looking at the presence and action of aquaporins in chicken development, one could determine whether the environment from which they are raised has a lasting and significant effect on the chicken's ability to conserve water.

As evident from these areas for future experimentation, the field of avian renal development is and will continue to be a rich one for scientific investigation.

APPENDIX A

CHICKEN OSMOREGULATORY DEVELOPMENTAL TIMELINE

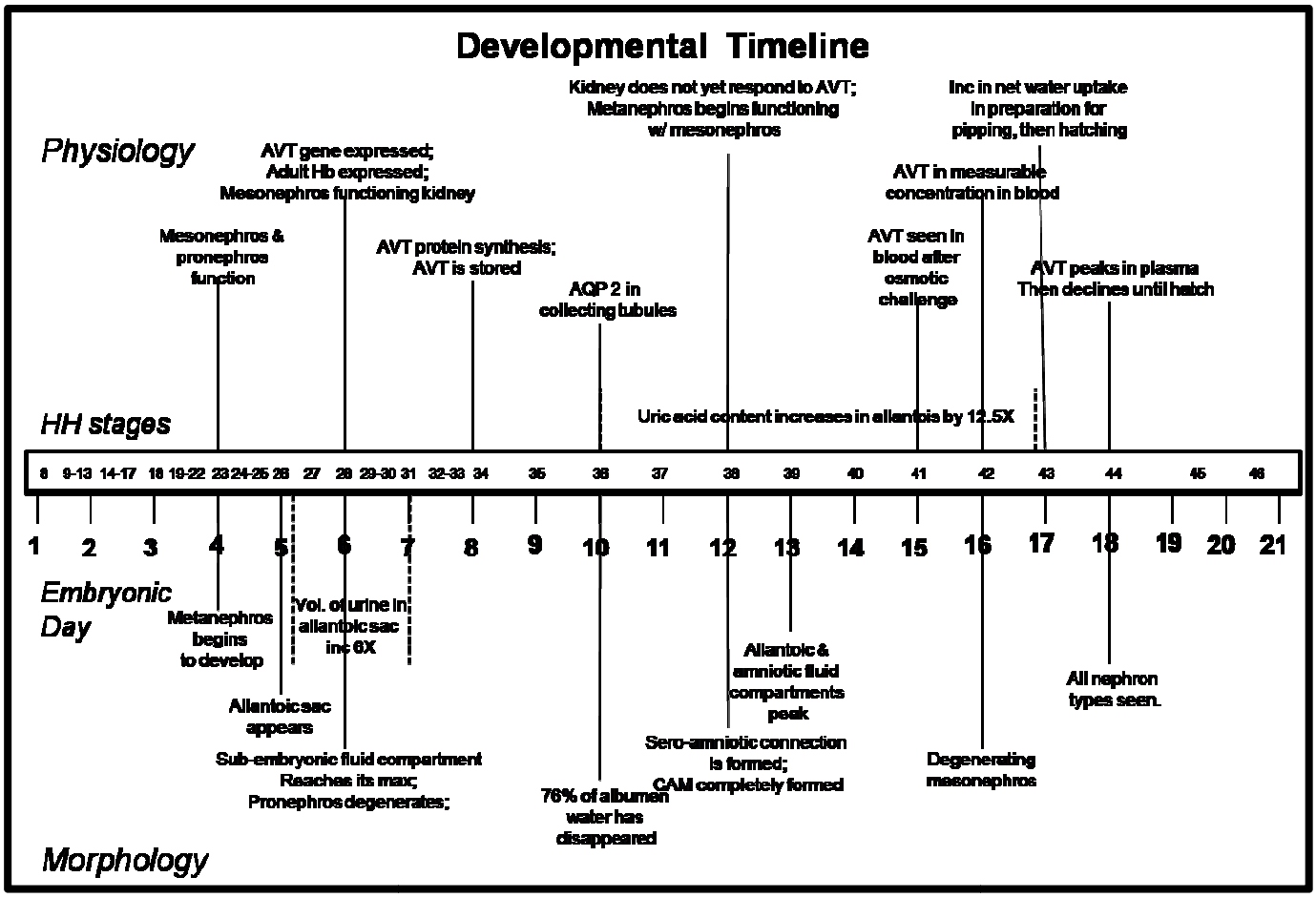

Figure A.1: Avian osmoregulatory developmental timeline. (Hamilton-Hamburger (HH); arginine vasotocin (AVT); aquaporin (AQP); hemoglobin (Hb); chorioallantoic membrane (CAM)).

APPENDIX B

ADDITIONAL FIGURES

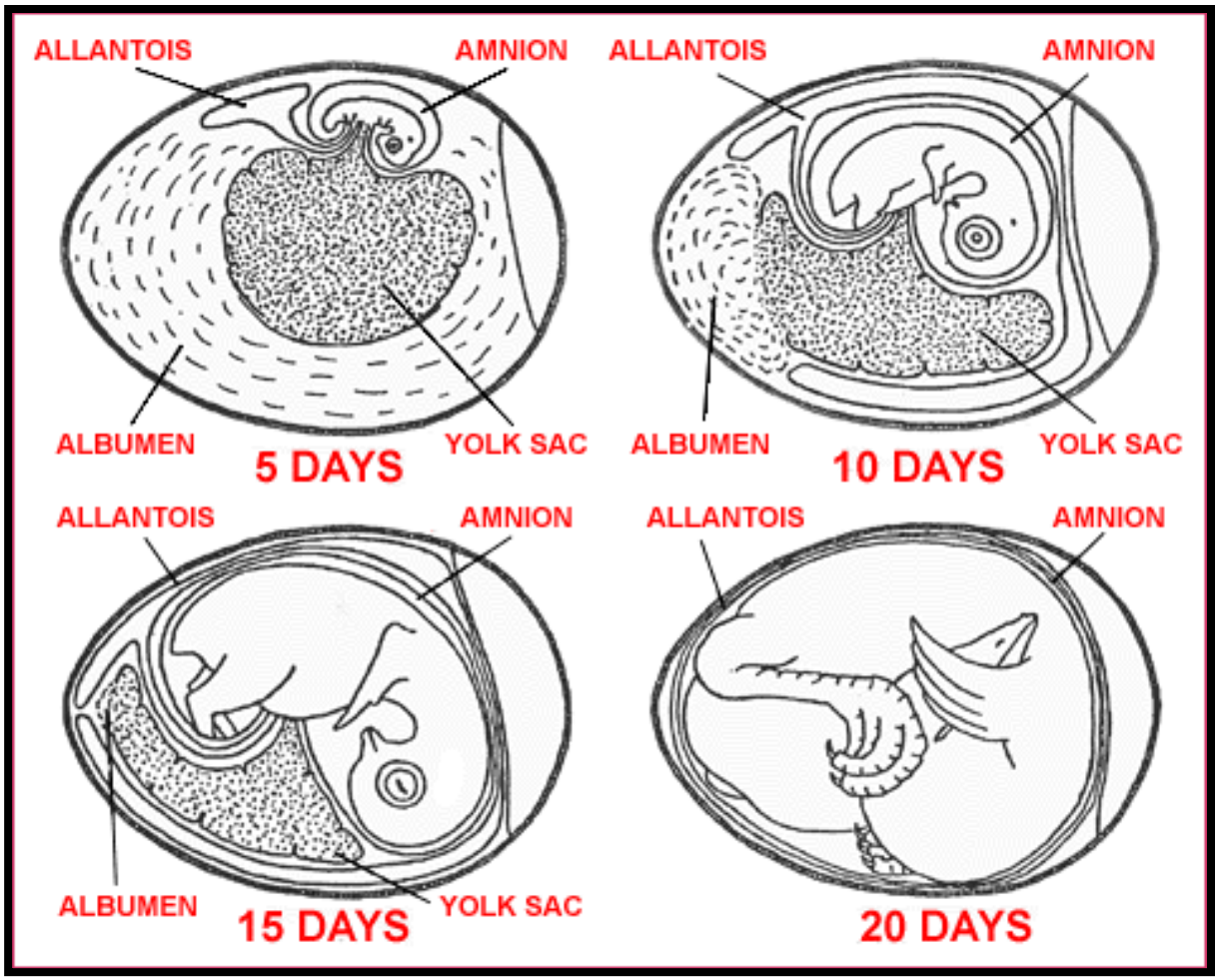

Figure B.1: Chicken development (Romanoff, 1960).

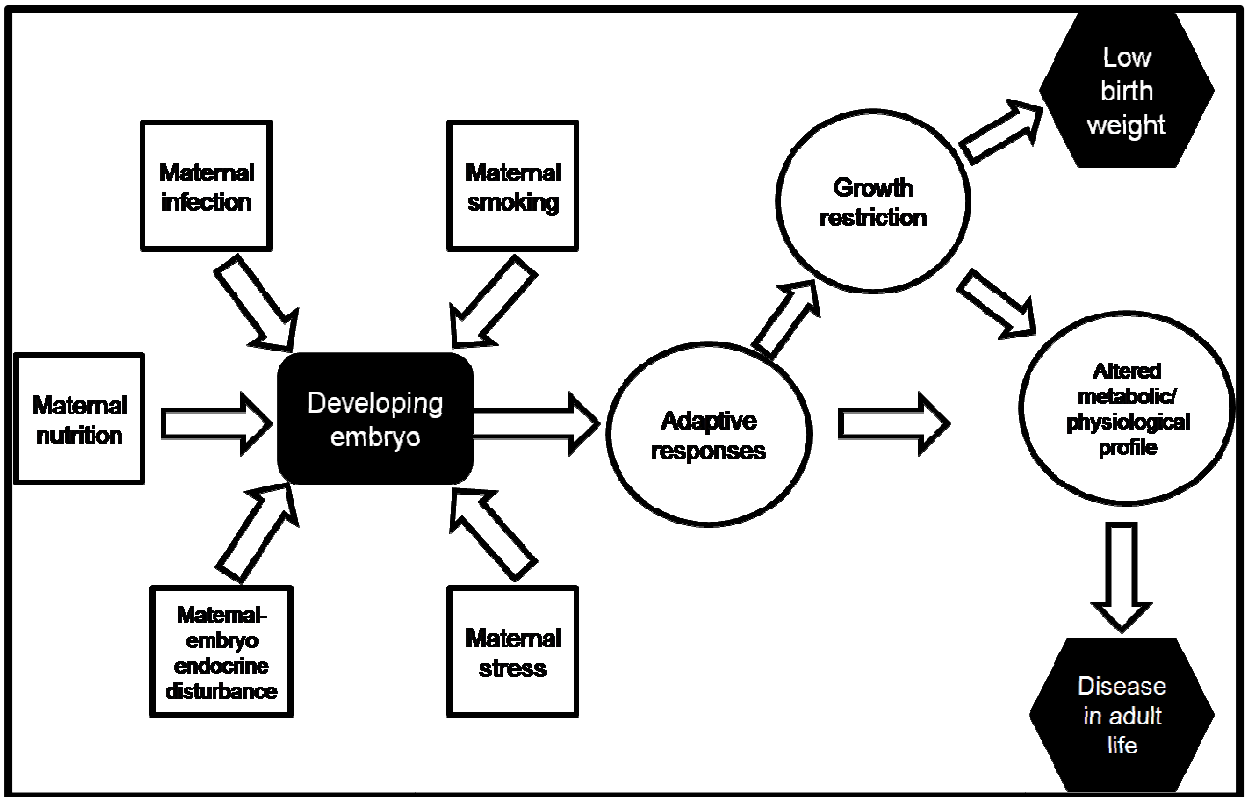

Figure B.2: General pattern seen in fetal programming of adult disease (Adapted from Langely-Evans, 2008).

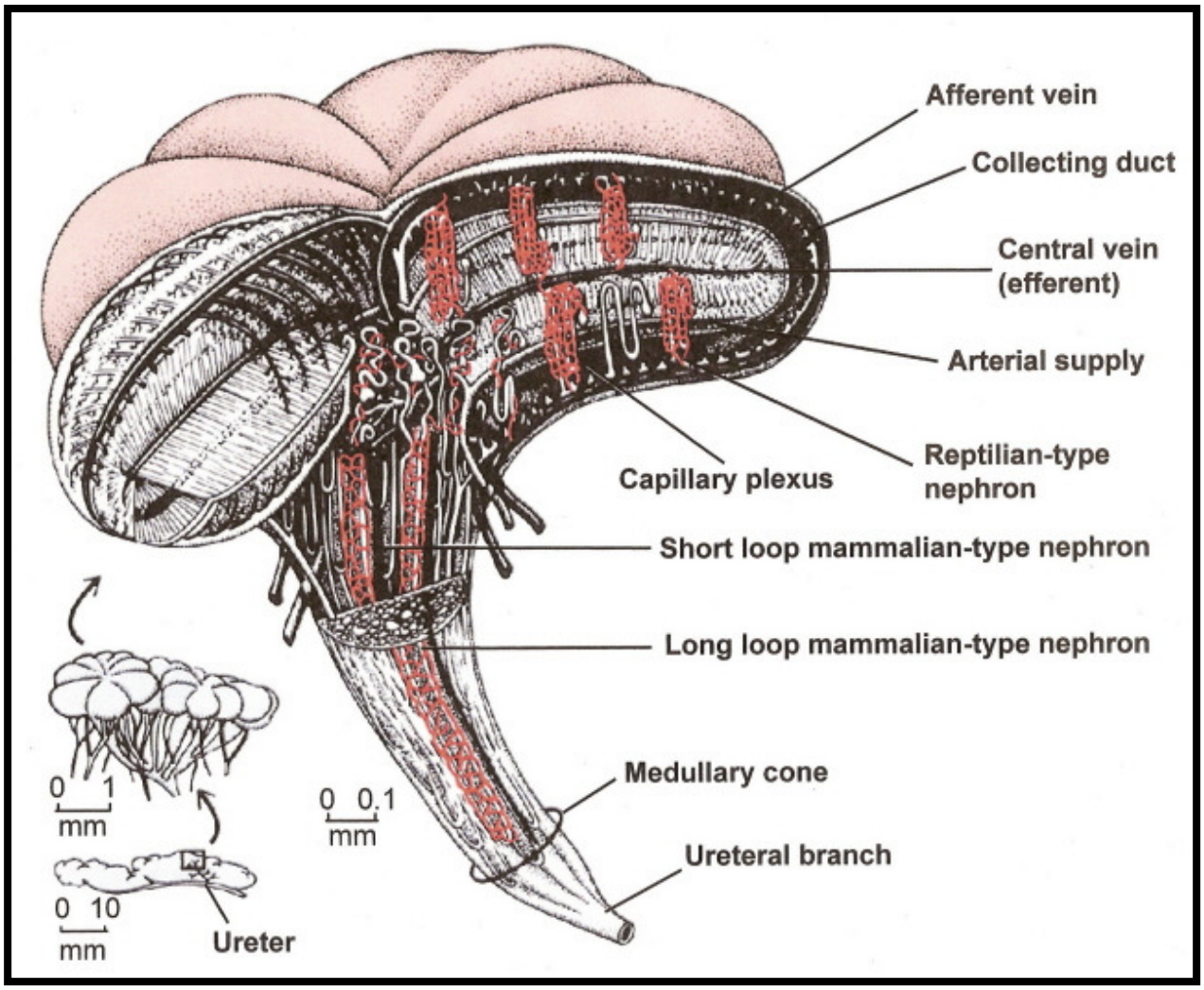

Figure B.3: Three-dimensional drawing of avian kidney showing nephron types and their relative position and relationship to other renal structures (Braun and Dantzler, 1972).

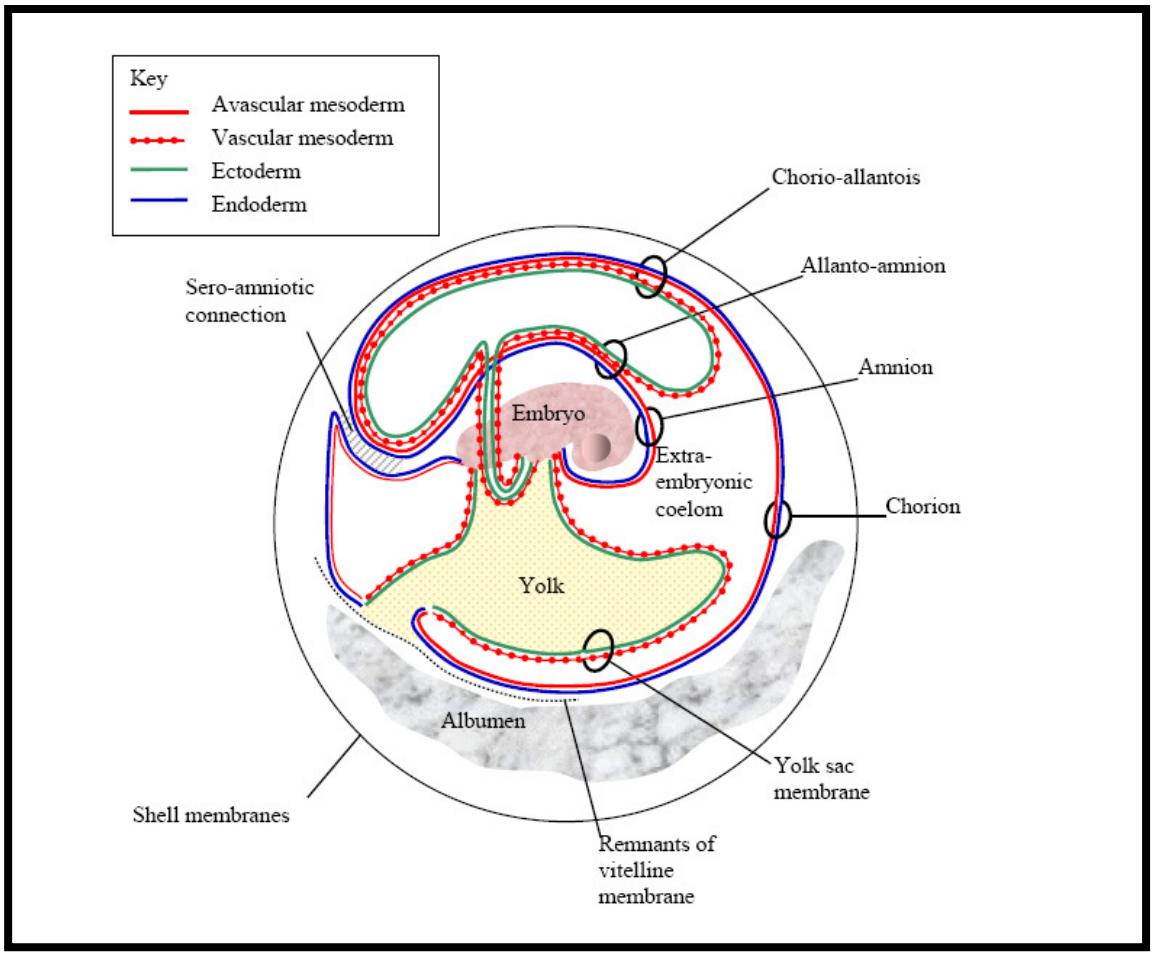

Figure B.4: A diagram of the extra-embryonic membranes and fluid compartments for the chicken embryo around a third of the way through incubation (Baggott, 2001).

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