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# Metanephric kidney development in the chicken embryo: Glomerular numbers, characteristics and perfusion

Greta Bolin, Warren W. Burggren *

*Developmental Integrative Biology Research Cluster, Department of Biological Sciences, University of North Texas, 1155 Union Circle #305220, Denton, TX 76203-5017, USA*

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ABSTRACT

The developing metanephric kidneys and chorioallantoic membrane (CAM) work in unison to ensure ion and water homeostasis in the avian embryo within its egg. This study focused on how avian renal structure and glomerular perfusion change in concert during development, as well as on changes in body fluid compartment osmolalities. White leghorn chicken eggs were incubated at 37.5 °C and 55–60% relative humidity and were examined during days (D) 10–18 of development. Alcian blue, a stain that forms solid aggregations in actively perfused glomeruli of the metanephric kidney, was used to identify the proportion of glomeruli actually perfused. Total nephron number increased from 4705 ± 1599 nephrons/kidney on day 12 to 39,825 ± 3051 nephrons/kidney on day 18. Actively perfused nephrons increased ~23-fold from 761 ± 481 nephrons/kidney on day 12 (~16% of total nephrons) to 17,313 ± 2750 nephrons/kidney on 18 (~43% of total nephrons). Glomerular volume increased from days 12 to 14, remaining constant thereafter. Blood and cloacal fluid osmolality ranged from 270 to 280 mOsm/L. Amniotic fluid osmolality changed in a complex fashion during development but was comparable to blood on days 10 and 18. Allantoic fluid had the lowest osmolalities (175–215 mOsm/L) across development. Uric acid increased steadily within the allantoic fluid compartment, from 36 ± 1 mmol/L to 63 ± 4 mmol/L. The avian metanephric kidney thus shows a dramatic increase in both recruitment of nephrons and potential filtering capacity during the last half of incubation, in preparation for the degeneration of the allantoic membranes prior to internal piping and subsequent hatching.



## 1. Introduction

The regulation of water and ion balance in avian embryos involves a balance between water loss occurring through evaporation from the shell and water gain occurring from the accelerating rate of metabolism of yolk lipids as development progresses. The net effect of these two water fluxes in chicken embryos is in an overall loss in egg mass of approximately 12% during the incubation period (Lundy, 1969). Eggs losing too much or too little water exhibit lower hatchability (Davis et al., 1988). Early in development, the embryo depends on the chorioallantoic membrane (CAM) to regulate ion and water balance, but the mesonephric kidney, progressively replaced by the metanephric kidney, assumes increasingly important roles in water balance, ion regulation and nitrogenous waste excretion as development proceeds (for a review, see Gabrielli and Accili, 2010).

The CAM comprises amniotic and allantoic compartments. 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 on day 12 as a duct between the amniotic sac and albumen to allow the movement of proteins to the embryo (Baggott, 2001). The allantoic sac begins to expand on day 3.5, reflecting the early onset of mesonephric kidney function. This early filtrate only aids water regulation and the removal of waste and also contributes to the rapid expansion of the allantoic sac (Romanoff, 1967). Between days 5 and 7, the volume of the allantoic sac increases 6-fold, corresponding with both differentiation and growth in size of newly forming nephrons of the metanephros combined with retained mesonephros function (Friebova-Zemanova et al., 1982). Allantoic fluid, in addition to being a repository for waste materials, also serves the embryo as a water reservoir for embryonic hydration. Collectively, the allantoic and amniotic compartments of the CAM serve as sources/sinks for both fluid and electrolytes (Graves et al., 1986; Hoyt, 1979).

As the avian embryo continues to develop, the kidneys begin to develop in a process similar to that occurring in mammals and reptiles. The pronephros emerges first out of surrounding mesoderm but is then eclipsed during embryonic growth by the growth of mesonephros, which begins differentiating around embryonic day 3 (Romanoff, 1960). The mesonephros appears morphologically on days 3–4, functions from days 5 to 11 and degenerates about day 15 as it is replaced by the metanephros, or "definitive kidney," which further differentiates into the permanent functional kidney. Ultrastructural and biochemical observations of the metanephric kidney on embryonic day 12 reveal an active fluid resorptive process by

* Corresponding author. Tel.: +1 940 565 3952; fax: +1 940 565 4438.
*E-mail address:* burggren@unt.edu (W.W. Burggren).

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way of fully differentiated renal corpuscles and proximal convoluted tubules (Narbaitz and Kacew, 1978). The so-called "mammalian-type" and "reptilian-type" nephrons evident in adult birds are found in the embryonic metanephros. Interestingly, mammalian-type nephrons, characterized by more extended tubular loops and larger glomeruli, are the first to form (Narbaitz and Kacew, 1978; Wideman, 1989). These are followed by reptilian-type nephrons, characterized by typically shorter loops and smaller glomeruli, which do not replace but rather add to the adult configuration of nephron types evident at hatching.

In addition to a long-standing appreciation of the changing nephron types during avian development, the gross morphology of the developing avian embryonic kidney has been well documented (Klusonová and Zemanová, 2007). However, very limited information is available regarding developmental changes in actual nephron numbers in embryonic birds. Do all nephrons of each type occur concurrently and then grow by hypertrophy, or are nephrons added as development proceeds? Are all nephrons perfused soon after their first appearance, or might they be intermittently perfused (and thus intermittently functional) as in adults of other vertebrates (Yokota et al., 1985)? Uncertainty also surrounds how nephron numbers correlate with potential changes in embryonic regulation of water and salt balance and how changes in nephrons numbers might actually influence embryonic kidney performance. This study then explores metanephric kidney development during the second half of development of the chicken embryo and correlates morphological and physiological renal changes with osmotic characteristics of the embryo's main fluid compartments. We hypothesize that the nephron number will parallel metanephric kidney growth, and that all nephrons are potentially active in plasma filtration.

## 2. Materials and methods

### 2.1. Source and Incubation of Eggs

Fertilized white leghorn eggs (*Gallus gallus domesticus*) were obtained from Texas A&M University (College Station, TX, USA) and shipped to University of North Texas (Denton, TX, USA). Upon arrival eggs were weighed, individually marked and then randomly chosen for placement within one of three Hova–Bator incubators, which were ventilated with fresh air at 55–60% relative humidity (RH) and temperature of 37.5 °C ± 0.5 °C. Humidity was measured using wireless Baro-Thermo-Hydrometers (model BTHR968, Oregon Scientific).

Eggs were weighed on incubation days 10–18, corresponding to stages 36–44 (Hamburger and Hamilton, 1951). Loss in mass during development was calculated as the difference between pre-incubation and experimental day whole egg mass. On embryonic days 10, 12, 14, 16 and 18, eggs were removed from each incubator for experimental analyses (*n* values are provided in Results section).

All experimental procedures were approved by The University of North Texas' Institutional Animal Care and Use Committee (IACUC).

### 2.2. Kidney Harvesting

All embryos were sacrificed by injection of 100 μL pentobarbital sodium (50 mg/mL) into a CAM vein exposed through removal of a small portion of eggshell. The embryo was removed from the shell and separated from the extra-embryonic membranes, after which body wet weight was measured. The abdominal cavity was opened, and all organs on the ventral-side were removed first, revealing the mesonephros and metanephros, which were separately identified. Subsequently, the mesonephros with the gonads were removed. The extracted metanephric kidneys (hereafter termed simply "kidneys") were separated from the gonads, and their wet weight was measured after careful removal of surface fluid through blotting with Kimwipes® (Kimtech Science). Kidneys were then placed on aluminum foil squares and dried for 24 h at 70 °C prior to determining dry weight.

Kidneys to be used for light microscopy analysis were placed into 10% neutral buffered formalin with a pH of 7.2 at 4 °C for ~12 h. Kidneys to be used for glomerular distribution analysis were placed in 50% ethanol at 4 °C overnight.

### 2.3. Glomerular perfusion assessment

One objective of this study was to identify the maximum number of potentially functional glomeruli at any given point in chicken embryo development. While we did not assess actual glomerular filtration rate in the embryos in this study, glomeruli that were not perfused were *ipso facto* not creating urine, so the ratio of maximum possible perfused to total glomeruli was a key index of glomerular functionality.

#### 2.3.1. Dye injection and staining of perfused glomeruli

Eggs were candled to locate a CAM vein, the location of which was marked on the shell with a pencil. To maintain temperature of the embryo during the experiment, the pointed end of the egg was buried to a depth of ~3 cm into clean quartz sand maintained at 37 °C. A 1-cm square piece of egg shell was then removed over the previously located CAM vein, which was then cannulated with a bent 30 gauge needle connected to PE-50 tubing. The cannula was secured to the surface of the eggshell with putty, to eliminate vessel movement during the injections.

To stain individual glomeruli that were actually being perfused following mannitol stimulation, we employed the Alcian blue staining technique previously established as suitable for assessing glomerular perfusion in chickens (Wideman et al., 1987). Alcian blue is a large, positively conjugated dye molecule (Scott et al., 1964). Cationic isothiouronium groups in Alcian blue are thought to bind via electrostatic interactions to the anionic sulfate and carboxylate groups located within the carbohydrate moieties of mucin. Alcian differentially accumulates and stains the glomerular tuft, an aggregation of glomerular capillaries sitting within Bowman's capsule (Fig. 1A). Key to the experiments, such staining, will occur in perfused nephrons potentially capable of ultrafiltration, but not in non-perfused and therefore non-functional nephrons (Wideman et al., 1987). Importantly, Alcian blue not only visibly accumulates within the glomerular tuft of each perfused nephron, but given sufficient injected concentration and time for ultrafiltration, the aggregation actually forms a stable "micro-pellet" within the glomerular tuft of each nephron that is not disrupted by homogenation of the kidney tissue. The concentration of such micro-pellets can be used as a quantitative tissue perfusion marker, analogous to methodologies of using entrapped microspheres in the microcirculation (for a review, see Robertson and Hlastala, 2007). The advantage of this technique over the visual examination of individual histological slides is that with Alcian blue staining the average concentration of perfused glomeruli can be easily determined for the entire kidney from a single procedure. This is in contrast to histological examination, which would have required separate examination of large numbers of individual specific kidney regions within the field of view of a microscope, with the tenuous assumption that such spatial sampling represents the characteristics of the kidney overall.

Mannitol was first administered as an osmotic diuretic, acting indirectly to temporarily maximize nephron perfusion, after established protocols for avian kidney assessment (Bankir and Hollenberg, 1983; Unflat et al., 1985). An injection of 2.5% mannitol in saline comprising 3% of total blood volume (embryo wet weight × 0.15, after Rychter et al., 1955) was injected over a 1-min period via a glass Hamilton syringe through the cannula. Ten minutes after mannitol treatment, a bolus of 0.2% Alcian blue dye in saline (3% blood volume) was injected at the same rate as the mannitol injection. After 30 min of dye circulation, shown by pilot experiments to be sufficient for Alcian blue

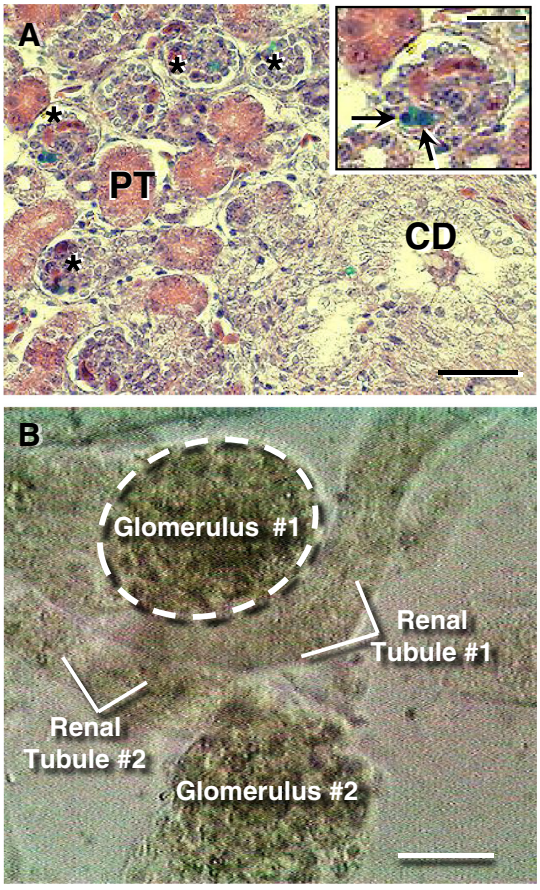


**Fig. 1.** (A) Light microscopy image of a histologically prepared metanephric kidney slice from a day 18 embryo. Aggregated Alcian blue dye pellets that accumulated in actively filtering glomeruli are indicated by an asterisk. PT, proximal tubule; CD, collecting duct. Scale bar = 25 μm. Insert shows a magnified view of a glomerulus with aggregated pellets of Alcian blue dye (arrows). Scale bar for insert = 10 μm. (B) Microscopic image of kidney homogenate derived from the macerated kidney of a day 18 embryo. Two glomeruli are pictured, each with an associated proximal renal tubule. Scale bar = 10 μm.

micro-pellet formation within the glomerulus, the embryo was sacrificed with an injection of 100 μL of pentobarbital introduced via the cannula. The kidneys were then dissected and weighed as described above.

Each Alcian blue-infused kidney was placed in a vial of 50% ethanol for 24 h at 4 °C to preserve the Alcian blue dye by partial dehydration of the kidney (Wideman et al., 1987). The kidneys were then placed into a "bluing solution" (equal parts of 50% ethanol and 1% ammonium hydroxide) for 90 min at 4 °C to increase the intensity of the Alcian blue stain and harden and solidify the Alcian blue micro-pellet within the glomerular tuft. Next, the kidney tissue was rinsed twice with cold deionized water to remove excess Bluing solution and placed in a vial with 20% hydrochloric acid (HCl) incubated at 37 °C for 2–3 h to begin the necessary tissue degradation. The acid was carefully decanted and the tissue was gently rinsed 3–4 times with cold deionized water. The tissue was left in the final water mix for 12–24 h at 4 °C. The kidney components were then dissociated by slow, gentle stirring for 10 min with fresh deionized water, bringing total volume of kidney and water to 6.5 mL. Previous assessment has demonstrated that there is no disassociation of the Alcian blue "micro-pellet" within the intact glomerulus by this procedure (Wideman et al., 1987).

*2.3.2. Determination of perfused/non-perfused glomeruli*

A 2-μL aliquot of homogenized solution containing dissociated kidney tissue components – but importantly, both intact glomeruli and Alcian blue pellets, prepared as described above – was removed with a Gilson micropipettor and deposited on a microscope slide for examination of the components of the dissociated kidney tissue. After allowing the tissues in the homogenate aliquot to settle on the slide for ~ 30 s, the homogenate was viewed with a Nikon Eclipse E200 binocular scope at 400× magnification. Fig. 1B shows two glomeruli, with attached proximal tubules, which are both clearly recognizable and distinguishable from each other.

The total number of glomeruli in the 2-μL homogenate aliquots of individual non-perfused, as well as the subset of perfused nephrons (identified by the presence within the glomerulus of an Alcian blue pellet) was measured using Image-Pro® Plus version 4.1 software (Media Cybernetics®). The number of both perfused and non-perfused glomeruli (per milligram kidney tissue) and the number of perfused and non-perfused glomeruli total per kidney were then calculated from the micro-pellet count/aliquot, the aliquot volume, the kidney tissue sample weight from which the homogenate was produced and the total kidney weight. The ratio of perfused to total glomeruli was then also determined. Five separately determined 2 μL aliquots from each embryo were counted, and the data averaged to provide the value used for each embryo in the calculation of mean values for all embryos.

Glomerular circumference (in μm) of the dissociated nephrons was determined from images of aliquots deposited on slides. Circumference was subsequently converted to glomerular diameter using the formula $d = C/\pi$, assuming the glomerulus to be circular. Glomerular volume was then calculated from the formula $V = 4/3\pi r^3$. Total glomerular volumes, and their distribution as a function of development, was then determined after the technique for assessing glomerular growth in birds established by Wideman (1989), which consisted of multiplying individual glomerular volume X estimated total number of glomeruli.

*2.4. Light microscopy for glomerular perfusion verification*

Unlike mammalian kidneys, embryonic avian kidneys do not hold their shape after extraction; therefore, the inability to consistently place and orient the kidneys in paraffin blocks obviated accurate quantification of kidney tissue morphometry from histological slides. Rather, light microscopy slides were simply used to verify that glomeruli were being stained with Alcian blue properly throughout the entire kidney—that is, that there were no regional staining variations.

After Alcian blue staining and tissue fixation as described above, tissue was processed (formalin fixation, dehydration and paraffin infiltration) in a microwave rapid histoprocessor (Milestone RH1) and embedded in paraffin blocks (Shandon Histocentre 3 tissue embedder). Subsequently, 8-μM serial sections were cut with a microtome (Leica RM 2245). The microtome ribbons were mounted on labeled slides in a 55 °C tissue float bath (Fisher Scientific) and allowed to air dry for 24 h. After drying, 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 on top of the slides using Permount® SP15-100 (Fisher Scientific) after dehydrating with another graded ethanol series. The slides were then allowed to air dry for 24 h before viewing.

Gross morphological observations were made via Nikon Eclipse E200 binocular scope from 40× to 400× magnification, and images were digitized using Image-Pro® Plus version 4.1 software (Media Cybernetics®). Fig. 1B shows a typical light microscopy image of a kidney slice from a day-18 embryo, with Alcian blue staining of perfused glomeruli evident.

*2.5. Body fluid compartment osmolality*

A 25-gauge needle connected to a 1-mL syringe was used to remove ~100 μL allantoic and amniotic fluids from the allantoic and

amniotic compartments of embryos intact in their eggs at days 10, 12, 14, 16 and 18. Blood was obtained from an intravenous catheter as detailed in the previous section. Cloacal fluid was sampled from the cloaca of embryos that had been sacrificed via exposure to isoflurane for 10 min followed by rapid decapitation. Cloacal fluid was drawn by capillary action into a capillary tube pulled to a tip diameter of 10–30 μm (World Precision Instruments PUL-1E pipette puller). All fluid osmolalities were measured on 10 μL samples with a Wescor Vapro® vapor pressure osmometer (model 5520).

### 2.6. Uric acid analysis

Uric acid concentration from the allantoic fluid 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 appropriate reagents to measure optical density of the uric acid present in the mixture. Each sample was duplicated along with blanks and standards.

### 2.7. Statistics

All data were tested for normal distribution (Shapiro–Wilk test for normality) before specific statistical analyses were performed. One-way parametric ANOVA was used to test changes over time. Two-way parametric ANOVA was used to test the significance of developmental day, fluid compartment or interaction between the two factors on the data. Student–Newman–Keuls (SNK) multiple range *post hoc* tests were run to separate data into distinct groups. 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. For all of the statistical analyses performed, a $p$ value of less than 0.05 were considered as statistically significant.

## 3. Results

### 3.1. Metanephric kidney mass

Kidney wet mass increased linearly with embryo wet mass between embryonic days 10 to 18 (Fig. 2A) ($p < 0.001$, $r^2 = 0.88$). Both wet and dry kidney mass increased ~15-fold during this development period (Fig. 2B). Kidney wet mass remained ~0.3–0.4% of total wet body mass over the range 2–30 g between days 12 and 18. However, kidney dry mass, when expressed as a % of kidney wet mass, increased from approximately 11% on day 12 to 17% on day 18, suggesting an increase in overall water content of the kidney tissue as development progressed.

### 3.2. Nephron development

Nephron density (nephrons/mg kidney) increased steadily throughout development, increasing significantly ($p < 0.01$) from 196 ± 59 nephrons/mg kidney on day 12 to 477 ± 46 nephrons/mg kidney on day 18 (Fig. 3A). Total number of nephrons per kidney increased significantly ($p < 0.01$) by ~8-fold over the monitored 6-day period of development, from 4705 ± 1599 nephrons/kidney at day 12 to 39,825 ± 3051 nephrons/kidney at day 18 (Fig. 3B).

On days 12 and 14, there was no significant difference ($p > 0.05$) between total nephrons and those being actively perfused—i.e., all nephrons were perfused. However, there was a significant difference (two-way ANOVA, $p < 0.01$) in total vs. perfused nephrons on days 16 and 18, in which perfused nephrons fell to approximately 1/3 to 1/2 of total nephrons (Fig. 3B).

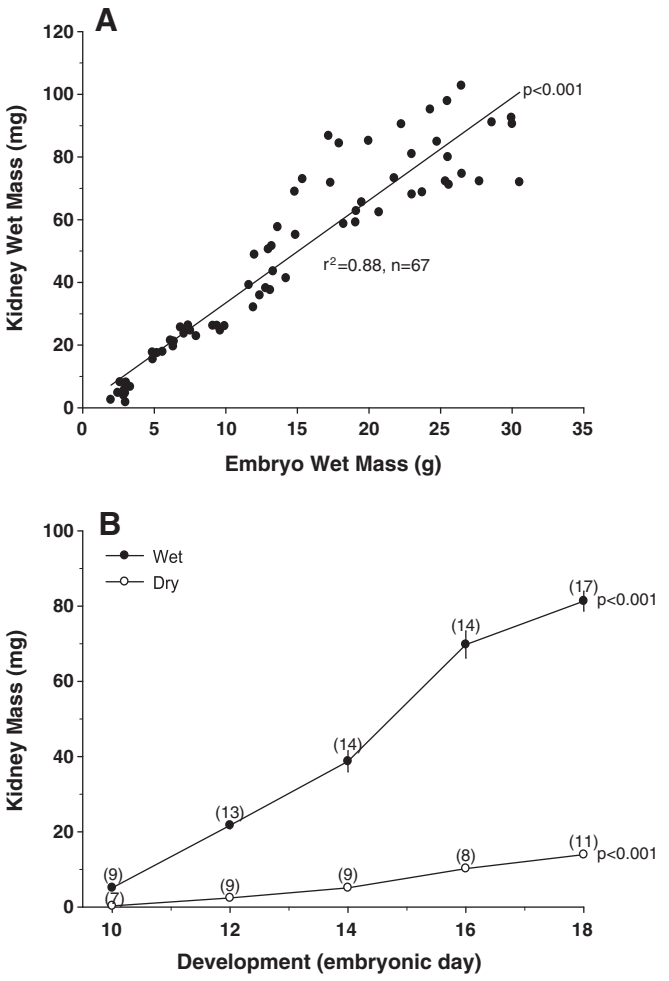


**Fig. 2.** Embryo and metanephric kidney mass. (A) Relationship between embryo wet mass and kidney wet mass. (B) Kidney wet and dry mass as a function of development. Mean values ± 1 SE and $p$ values via one-way ANOVA for kidney mass and development are provided. $n$ values in parentheses. Note that in some instances the standard errors are smaller than the size of the plotted symbols.

### 3.3. Glomerular morphometrics

Interactions between developmental changes in the distribution of glomerular diameters and nephron density are shown in Fig. 4. Evident at all developmental days was a broad distribution of glomerular diameter. Interestingly, nephron density was higher on days 16 and 18, but nephrons from kidneys on days 12 and 14 had significantly larger mean diameters. Thus, mean glomerular diameters actually decreased during kidney development as follows: day 12, 79 ± 3 μm; day 14, 76 ± 2 μm; day 16, 60 ± 1 μm; and day 18, 64 ± 1 μm ($n = 5$ for each day).

Total glomerular volume/kidney increased 2.5× from day 12 (1.97 ± 0.33 μL) to day 14 (5.13 ± 0.75 μL) but then leveled off after day 14 (Fig. 5). As expected from nephron perfusion data above, the glomerular volume of perfused nephrons was significantly lower than the volume of non-perfused nephrons on days 16 and 18.

### 3.4. Fluid compartment analyses

Developmental changes in osmolality of blood, amniotic fluid and allantoic fluid from days 10 to 18 of development are shown in Fig. 6A. Blood osmolality was approximately 270–280 mOsm/L during the entire second half of embryonic development. Amniotic fluid was statistically identical to blood on day 10 but was significantly lower than blood on days 12, 14 and 16, before rising back to statistically

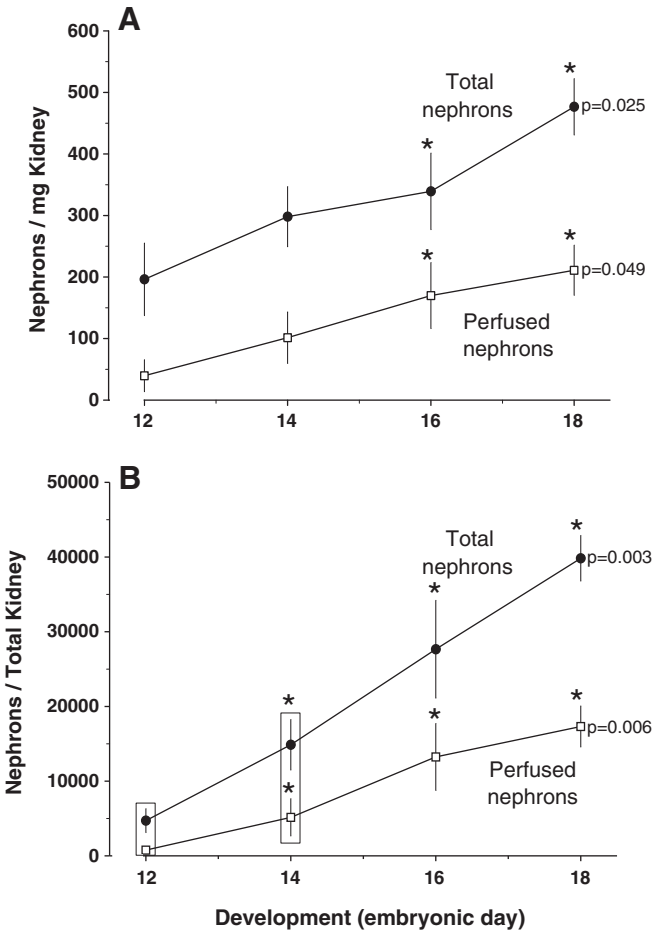


**Fig. 3.** Nephron characteristics in the developing metanephric kidney. Calculated number total and perfused nephrons as a function of development expressed as (A) per milligram kidney or (B) per total kidney. Mean values ± 1 SE and *p* values determined by one-way ANOVA for each variable across development are provided, and mean values that are significantly different from the mean at day 12 are indicated by an asterisk for both total and perfused nephrons. Mean values within a common outlined box are not significantly different from each other. $n = 5$ for each mean value at each day.

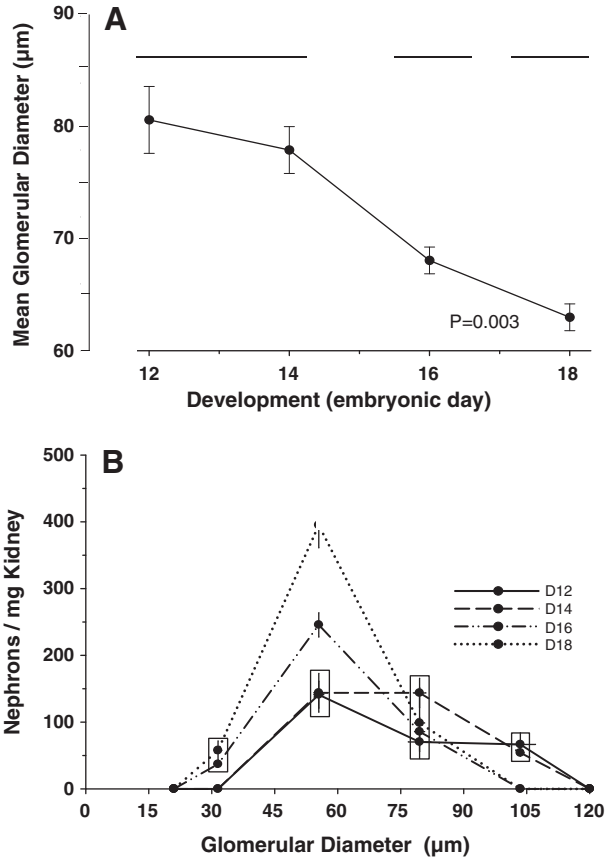


**Fig. 4.** Glomerular diameter and development. (A) Mean glomerular diameter changes during the last half of incubation. Mean values not significantly different from each other ($p < 0.05$) fall under the same horizontal line. (B) Changes in glomerular diameter distribution during the last half of development. Mean values within a common outlined box are not significantly different from each other. Mean values ± 1 SE are provided. $n = 5$ for each day in both panels.

identical values to blood on day 18. Allantoic fluid had a significantly lower osmolality than all of the sampled body compartments across the measured developmental range, varying between 180 and 220 mOsm/L. Finally, cloacal fluid on days 16 and 18 (the only days that could be measured) was statistically identical to that of blood, at ~280 mOsm/L.

Uric acid, a nitrogenous waste product of metabolism, was sampled in allantoic fluid, where its concentration increased significantly ($p = 0.03$) over development (day 10 = day 12 < day 18) from 36 ± 1 mmol/L to 63 ± 4 mmol/L (Fig. 7).

## 4. Discussion

Much recent studies of avian developmental morphology and physiology have focused on characteristics of the respiratory or cardiovascular system, with relatively little attention paid in comparison to the renal system and osmoregulatory capabilities of the avian embryo. Certainly, a basic understanding exists of the osmoregulatory functions of the developing renal tissues in the avian embryo. Essentially, the pronephros begins to function as early as day 4 of chicken development (Hiruma and Nakamura, 2003) but then begins to degenerate by day 6, with renal osmoregulatory functions transitioned to the mesonephros around this point in development. Indeed, the mesonephros is making large contributions to urine formation by days 5–7 (Narbaitz and Kapal, 1986). However, the mesonephros itself begins to degenerate around day 16, with renal osmoregulatory function fully assumed by the metanephros at this point. The metanephros (the renal structure examined in this study) begins functioning around day 12 (Romanoff, 1960), progressively assuming the renal osmoregulatory functions as the degeneration of the mesonephros accelerates (Carretero et al., 1997), and

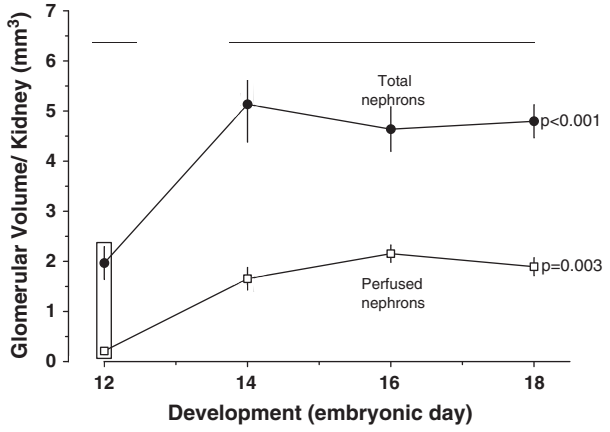


**Fig. 5.** Estimated total and perfused glomerular volume as a function of development. Mean values ± 1 SE and *p* values determined by one-way ANOVA for each volume across development are provided. Mean values not significantly different from each other ($p < 0.05$) fall under the same horizontal line. $n = 5$ for condition on each day. Mean values within a common outlined box are not significantly different from each other.

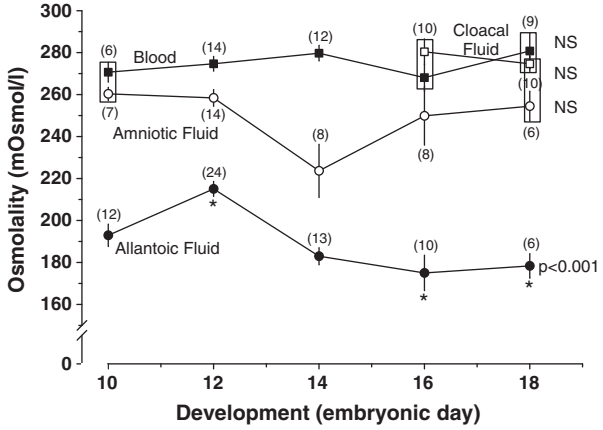


**Fig. 6.** Developmental changes in osmolality of blood and fluids in the amniotic, allantoic and cloacal body compartments. Note: cloacal fluid could only be sampled from days 16 to 18. Mean values ± 1 SE and *p* values determined by one-way ANOVA for fluids across development are provided, and mean values that are significantly different from the mean at day 12 are indicated by an asterisk for both total and perfused nephrons. *n* values are in parentheses. Mean values within a common outlined box are not significantly different from each other. NS = not significant.

continues to develop for ~30 days after hatching (Wideman, 1989). Although these basic transitions in renal osmoregulatory function during development have been documented in the chicken embryo, prior to our study, there has been no information available regarding metanephric nephron characteristics (e.g., glomerular size and number) and only very limited data on metanephric kidney growth across the second half of normal avian kidney development.

### 4.1. Metanephric kidney mass changes during development

Embryo wet mass accurately predicted kidney wet mass, with kidney growth occurring in direct proportion to embryo growth during development (Fig. 2A). Kidney wet and dry mass increased approximately in proportion during the last half of development (Fig. 2B). This suggests that not just the overall water content of renal tissue grew in proportion as the kidneys developed but also the volume of the kidneys' internal fluid compartments such as Bowman's capsule of the glomerulus, renal tubules and vascular spaces.

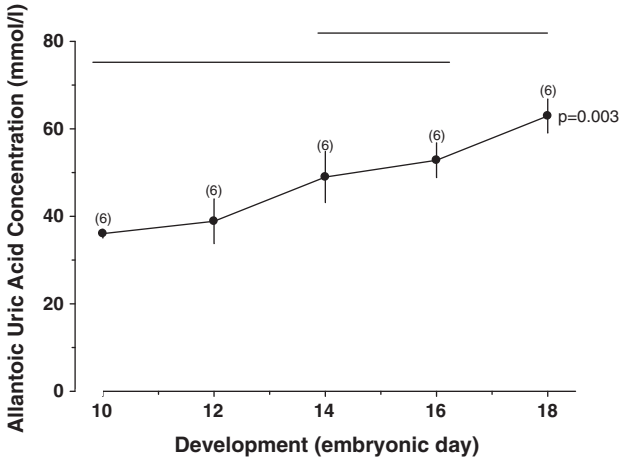


**Fig. 7.** Uric acid concentration as a function of development. Mean values ± 1 SE are provided. *n* values are in parentheses. Mean values not significantly different from each other ($p < 0.05$) fall under the same horizontal line. *p* values determined by one-way ANOVA for uric acid concentration across development are provided.

### 4.2. Glomerular characteristics during development

The so-called "mammalian-type" nephrons, which have large glomeruli, appear first in the development of the chicken embryo, followed later by reptilian-type nephrons with relatively smaller glomeruli. In fact, reptilian-type nephrons continue to appear not only throughout embryonic development but even far into the post-hatch period for up to 30 weeks (Wideman, 1989). This so-called "inside-out" or mammalian-type to reptilian-type nephron sequence of development results in adult bird kidney structure that is 10–30% mammalian-type and 70–90% reptilian-type (Casotti et al., 2000; Goldstein and Skadhauge, 2000; Wideman, 1989).

Alcian blue staining of glomeruli, a previously validated technique for assessing functionality of the developing avian kidney (Wideman, 1989), combined with conventional light microscopy, allowed us to estimate the total nephron numbers and, as a subset, the number of perfused nephrons as well as glomerular diameters and volumes in the embryonic chicken kidney. In the present study, glomerular diameters changed dramatically during development from days 12 to 18 (Fig. 4), likely reflecting this changing distribution of reptilian- and mammalian-type nephrons with their considerably different glomerular sizes. The largest mean glomerular diameter of 79 ± 3 μm occurred on the first measurement day (day 12), likely reflecting the higher proportion of mammalian nephrons. More and more reptilian nephrons develop from days 12 to 18, skewing the mean glomerular diameter towards lower values (Fig. 4). The glomerular diameter data from the present study on developing chicken embryos are an interesting contrast to the post-hatch pattern of change in chicken hatchlings and juveniles described by Wideman (1988). In that study, the mean glomerular diameter ranged from ~22 to 134 μm (approximately the same range as in our study on embryos) but increased beginning at week 1 and continued to week 30.

Total glomerular volume/kidney increased 2.5-fold in the 48 h between days 12 and 14, thereafter stabilizing (Fig. 5). This volume increase occurred through an increase in total number of nephrons balanced against decreasing glomerular diameter associated with the development of reptilian-type nephrons during this period of renal development (Wideman, 1989). Generally, glomerular volume is only a poor indicator of filtering capacity across species due to differences in, for example, the elongation of the capillary network surrounding the tubules (Dantzler, 1980). However, glomerular volume may be a useful index of changes in filtration capacity over development within a species. Indeed, glomerular volume rose sharply between days 12 and 14, leveling off for the remainder of development (Fig. 5), likely due to continuing growth in numbers of reptilian-type nephrons (Wideman, 1989). Interestingly, the timing of these morphological changes coincides with a fall in both amniotic and allantoic fluid osmolality (but not blood osmolality) (Fig. 6).

The present study revealed a steady increase in the total number of nephrons, reaching ~40,000 nephrons/kidney on day 18, at ~30 g embryo body mass. These data compare favorably with the reported average glomerular number of 105,000 nephrons per 100 g body mass in 23 species of adult birds (Yokota et al., 1985).

A steady increase occurred during development from days 12 to 18 not only in total number of nephrons but also in a smaller subset of nephrons that were actually perfused (Fig. 3). That both perfused and non-perfused nephrons co-exist during development raises two key questions. First, are nephrons that are perfused actually fully functional, or does their perfusion mask immaturity on other aspects of nephron structure and function? It is possible, for example, that nephron perfusion develops prior to that nephron's ability to actually fully process urine, perhaps because the tubules are yet not fully populated with appropriate ion transporters or the endocrine regulation of ion transport is immature (Doneen and Smith, 1982a,b). A second unanswered question is whether the subpopulation of non-perfused nephrons actually lack mature vasculature, or are simply not perfused at the time of

fixation. In the kidneys of adult birds and mammals "glomerular intermittency" occurs, wherein fully functional but non-perfused nephrons are capable of being dynamically "recruited" to increase the overall filtering capacity when changes in urinary output are required (Braun, 1976; Dantzler, 1980). Of course, the embryos of most birds incubated within their eggs will not experience fluctuating hydric or other conditions that might demand the need for major recruitment or de-recruitment of nephrons, although there are interesting exceptions in the form of bird eggs that are left unattended for hours on end in extremely hot, arid environments (e.g., the gray gull *Larus modestus* from Chile). Thus, while the potential exists for acute recruitment of formerly non-perfused nephrons during the second half of development, testing of this hypothesis awaits further research. Noteworthy is that in our experiments we attempted to induce maximum diuresis through pretreatment with mannitol, which would be anticipated to maximize glomerular perfusion so that we could determine the upward limit on perfused nephron numbers. Unknown is whether the base condition of the developing kidney involves even less nephron perfusion than we observed under our experimental conditions.

While the present study adds to the continuum of understanding of glomerular development, a longitudinal study of glomerular structure over the entire range of development from renal organogenesis through senescence in older birds is warranted.

### 4.3. Fluid analyses and embryonic osmoregulation

The mesonephric kidney begins functioning on the fifth day of development, resulting in expansion of the allantoic sac (Baggott, 2001; Simkiss, 1980). By day 12, blood osmolality is ~270–280 mOsm/L, remaining at this level over embryonic until day 18. Blood over this period is hypertonic to amniotic and especially to allantoic fluid (Fig. 4). Blood osmolality in other studies of avian embryos has been reported to range from 270 to 330 mOsm/L (Braun, 1999; Davis et al., 1988; Hoyt, 1979) and, as in the present study, tends to be constant over the full range of development. Despite water fluxes between extra-embryonic compartments of the bird egg over most of embryonic development (Baggott, 2001; Simkiss, 1980), osmotic gradients of up to 80 mOsm/L are maintained between compartments, especially between blood and allantoic fluid. These gradients reaffirm that significant transport of ions and other solutes must be occurring across these membranes (Davis et al., 1988; Gabrielli and Accili, 2010; Simkiss, 1980).

Amniotic fluid serves as a mechanical protection for the embryo during development, cushioning the movements of the embryo within the egg or of the egg itself. Amniotic osmolality peaks on day 13 followed by a slow decline until hatching (Baggott, 2001). Amniotic fluid is unaffected by hydration state of the egg until around day 17, at which time the fluid in the allantoic compartment has been drawn down and the CAM begins to deteriorate (Baggott, 2001; Davis et al., 1988; Hoyt, 1979). An influx of albumen via the seroamniotic connection may ultimately dilute the amniotic fluid (Murphy et al., 1991), which could explain the decrease in fluid osmolality on day 14 (Fig. 6).

The allantoic compartment accumulates fluid primarily as a result of kidney filtration of plasma; the filtrate enters the allantois by the allantoic stalk causing the initial expansion of the allantoic compartment (Murphy et al., 1991). This fluid remains hypo-osmotic to embryonic plasma throughout incubation by way of fluid and ion movement via active transport across the allantoic membrane to the blood (Davis et al., 1988; Simkiss, 1980). In the current study, allantoic fluid had significantly lower osmolality at all developmental stages compared to all other fluid compartments. We observed significant decrease in the osmolality after day 12 (Fig. 6), which coincides with several studies that have documented the drastic decrease in allantoic volume beginning around day 13 (Baggott, 2001; Romanoff, 1967).

The cloacal urodeum of adult birds functions as urine storage organ and actually modifies urine by reverse peristalsis (Braun, 2003). The combined actions of the kidney and gastrointestinal tract produces urine that is hyperosmotic in adult birds (Braun, 2003), but little is known of this coupling during development. In embryos, the urodeum of the cloaca receives the ureteral urine. It is this region of the cloaca in which the allantoic stalk is connected. Prior to our study, the osmolality of cloacal fluid had not been evaluated, although we were only able to sample sufficient cloacal fluid on day 16 and day 18 only (Fig. 6). In fact, cloacal fluid osmolality was not different from blood osmolality on days 16–18. Perhaps the kidney alone may have created hyperosmotic urine or the urine may have been further modified by gastrointestinal tract through reverse peristalsis (Braun, 1999). Further investigation is warranted to understand the onset of cloacal fluid modification during development and the role that it plays in the late embryo.

Uric acid concentration increases approximately 2× during development (Fig. 7). Uric acid is essential for avian embryonic water balance since oxidation of protein to urate produces more metabolic water than oxidation of fat (Bradfield and Baggott, 1993). In adult chickens, a concentration of 40–100 mmol/L in urine occurs as uric acid plus urate precipitates. This accounts for 60–90% of total uric acid plus urates excreted (Long and Skadhauge, 1983). Maximum solubility of uric acid is below 30 mM (McNabb and McNabb, 1980), with higher values resulting in precipitate formation, which accounts for the cloudy appearance of allantoic fluid (Lundy, 1969). Our observed increases in uric acid concentration during development are likely indicative of the volume decreases in allantoic fluid towards the end of development (Romanoff, 1967).

## 5. Conclusions

This study evaluated normal nephron development in the chicken embryo between days 12 and 18. Additionally, fluid osmolalities and uric acid concentrations were assessed during the last half of incubation. Most striking is the significant number of non-perfused nephrons at all measured stages of development. Whether these nephrons can be dynamically recruited to participate into the overall process of urine formation, or whether they require further development to become functional, remains to be determined. Nephron endowment (nephron numbers at birth) is a predictor for pathologies that can develop in adult mammals (Langley-Evans, 2009). Renal function in mammals and birds share many similarities, most importantly in the development and maturation of the kidney. Although the avian kidney continues to accumulate reptilian-type nephrons after hatching, it may prove to be a key model to use for investigation of the effects under nutrition or environmental insults have on developmental trajectories of the kidney.

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