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Incubation relative humidity induces renal morphological and physiological remodeling in the embryo of the chicken (*Gallus gallus domesticus*)

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ABSTRACT

The metanephric kidneys of the chicken embryo, along with the chorioallantoic membrane, process water and ions to maintain osmoregulatory homeostasis. We hypothesized that changes in relative humidity (RH) and thus osmotic conditions during embryogenesis would alter the developmental trajectory of embryonic kidney function. White leghorn chicken eggs were incubated at one of 25–30% relative humidity, 55–60% relative humidity, and 85–90% relative humidity. Embryos were sampled at days 10, 12, 14, 16, and 18 to examine embryo and kidney mass, glomerular characteristics, body fluid osmolalities, hematological properties, and whole embryo oxygen consumption. Low and especially high RH elevated mortality, which was reflected in a 10–20% lower embryo mass on D18. Low RH altered several glomerular characteristics by day 18, including increased numbers of glomeruli per kidney, increased glomerular perfusion, and increased total glomerular volume, all indicating potentially increased functional kidney capacity. Hematological variables and plasma and amniotic fluid osmolalities remained within normal physiological values. However, the allantoic, amniotic and cloacal fluids had a significant increase in osmolality at most developmental points sampled. Embryonic oxygen consumption increased relative to control at both low and high relative humidities on Day 18, reflecting the increased metabolic costs of osmotic stress. Major differences in both renal structure and performance associated with changes in incubation humidity occurred after establishment of the metanephric kidney and persisted into late development, and likely into the postnatal period. These data indicate that the avian embryo deserves to be further investigated as a promising model for fetal programming of osmoregulatory function, and renal remodeling during osmotic stress.

1. Introduction

Avian developmental morphology and physiology has long been studied for the insights it gives into our understanding of the fundamentals of development in vertebrates. The large majority of these studies have been primarily directed at three targets: the cardiovascular system, acid-base regulation and overall metabolic rate (Mueller et al., 2015). Although the renal system is crucial to embryonic function, the ontogeny of the kidneys has been far less well studied. The gross morphology of the developing avian embryonic kidney has been well documented (Dressler, 2006; Mutnick et al., 1997), as are some aspects of the molecular signals leading to renal tissue differentiation during ontogeny (Grinstein et al., 2013; Schneider et al., 2015). However, far less is known of actual renal function during ontogeny. Certainly, it is understood that the mesonephric kidney begins to function by about day 5 through day 11 of incubation. The mesonephric kidney is then progressively replaced by the metanephric kidney at about day 11–12 (Alvine and Burggren, 2013; Bolin and Burggren, 2013; Evans et al., 2010; Robinson et al., 2010). This renal developmental morphological progression is reflected in the kidneys' increasing importance in water balance, ion regulation, and nitrogenous waste excretion as development proceeds and the osmoregulatory functions of the chorioallantoic membrane (CAM) are supplemented by renal processes (Clark et al., 1993; Gabrielli and Accili, 2010; Pacha et al., 1985).

Largely unexplored, however, is the phenotypic plasticity of the kidney – that is, to what extent the normal development of the renal system is shaped by internal or external stressors in either a natural or experimental context. Internal stressors potentially altering normal renal development in avian embryos could involve modified hormonal cues from the egg yolk (Deeming and Pike, 2013; Griffith et al., 2016; Ho and Burggren, 2010; Ho et al., 2011; Reed and Clark, 2011). However, a major stressor, which can arise through internal metabolic processes or external environmental conditions, involves variation in water content of the egg. As bird eggs develop and metabolize egg yolk and albumin, they produce metabolic water, which in most birds must be lost across the egg shell to avoid "drowning" the developing embryo. In the chicken (*Gallus gallus*), for example, eggs typically lose about 12% of

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their mass during normal development (Duchateau et al., 2015; Peebles et al., 2001; van der Pol et al., 2013), almost all as metabolically produced water. On its journey from metabolizing tissue through the egg shell to the external environment, this water potentially traverses the allantoic, amniotic, blood, and/or cloacal compartments (Graves et al., 1986; Hoyt, 1979; Mortola and Al Awam, 2010; Romanoff, 1960). The ultimate loss of water by water vapor diffusion is heavily influenced by relative humidity of the ambient air, and eggs losing too much or too little water exhibit a decrease in hatching success (Davis et al., 1988; van der Pol et al., 2013).

Excessive water loss or failure to eliminate sufficient water during incubation may very well be a key stressor altering normal renal development due to the modified osmoregulatory performance required by the developing kidneys to regulate water balance. As such, exposure to abnormal relative humidity during egg incubation is likely to alter avian embryonic renal morphology. Furthermore, since dynamic transport epithelium is known to remodel its morphological and cellular function in response to a change in demand, it is also likely that physiological changes will coincide with a change in kidney structure. Consequently, we hypothesized that the developing avian kidneys of the chicken (*Gallus gallus domesticus*) will show developmental phenotypic plasticity in morphology and physiology in response to high or low incubation humidity. Embryos were exposed to both high and low relative humidity throughout embryonic development and assessed for effects of these exposures on renal morphology and physiology as well as whole egg metabolism.

2. Materials and methods

2.1. Source and incubation of eggs

Fertilized but as yet un-incubated White Leghorn chicken eggs (*Gallus gallus domesticus*) were shipped from Texas A&M University (College Station, TX, USA) to the University of North Texas (Denton, TX, USA). All experimental procedures were approved by The University of North Texas' Institutional Animal Care and Use Committee (IACUC).

Upon receipt, eggs were immediately weighed and individually marked, and then randomly chosen for placement within three Hova-Bator incubators set to a temperature of 37.5 °C ± 0.5 °C at three relative humidities. Control eggs were incubated at 55–60% RH, which is the industry standard RH range for optimal development and hatch. The low and high RH treatment groups were incubated at 25–30% and 85–90% RH, respectively. Relative humidity was measured using wireless Baro-Thermo-Hydrometers (model BTHR968, Oregon Scientific). Sampling time points at day 10, 12, 14, 16, and 18 were chosen to include stages 36–44. These stages begin prior to the onset of metanephros function alongside the mesonephros (Stage 38) and continue through the time when the mesonephros has degenerated (Stage 42). All nephron types are seen (Stage 44) (Hamburger and Hamilton, 1951). Sample sizes varied in range from $N = 5$ in glomeruli assays to $N = 44$ in kidney/embryo mass assays, to up to $N = 128$ for % mortality, and are provided in figures for reference.

2.2. Mortality

Percent mortality was calculated for each humidity level over the course of 18 days of incubation. These values were gathered from a total of 128 eggs per incubation condition.

2.3. Measurement of egg, embryo and kidney masses

Egg mass was measured at Day 10, 12, 14, 16 and 18. Embryos were first euthanized by injection of 100 μl pentobarbital sodium (50 mg/ml) into a CAM vein exposed through small window in the eggshell. The embryo was then dissected from extra-embryonic membranes and yolk. After embryo wet mass was measured, the metanephric kidneys (hereafter termed simply "kidneys") were dissected and weighed to determine kidney wet mass. Tissues were then desiccated by heating at 70 °C for 24 h to determine kidney and embryo dry mass. Loss in mass during development was calculated as the percentage difference between the egg mass prior to incubation at D0, and the egg mass at each sampling day.

2.4. Glomerular characteristics and nephron perfusion

Aspects of renal morphology and physiology were assessed in embryos at each time point according to previously published methodology (Bolin and Burggren, 2013). Briefly, five kidneys (one per embryo) were dissected from terminally sampled embryos and placed in 50% ethanol at 4 °C overnight for determination of the number of glomeruli per kidney, glomerular volume and glomerular perfusion.

Only a portion of nephrons have glomeruli that are perfused at any given time (Bolin and Burggren, 2013). To distinguish perfused from non-perfused glomeruli, a standard protocol using Alcian Blue glomerular staining was used (Bolin and Burggren, 2013; Wideman et al., 1987). This technique, similar to using entrapped microspheres to assess microcirculation in capillary beds, uses Alcian blue pigment that forms visible aggregates within the glomerular tuft of each perfused nephron. By enumerating the number of perfused versus non-perfused glomeruli in each sample, the proportion of perfused glomeruli can be calculated. Mannitol, a therapeutic diuretic, was injected intravenously to a major vein in the CAM to recruit the maximum possible number of functional nephrons into urine formation, following previously established protocols (Bolin and Burggren, 2013), After 10 min, a bolus of 0.2% Alcian Blue dye in saline equivalent to 3% blood volume was then injected and allowed to circulate for 30 min. Embryos were euthanized as above prior to kidney dissection. The kidneys were placed in 50% ethanol for 24 h at 4 °C, then placed into a bluing solution (equal parts of 50% ethanol and 1% ammonium hydroxide) for 90 min at 4 °C to enhance the blue color and harden the Alcian Blue micro-pellet forming within the glomerular tuft (Bolin and Burggren, 2013; Wideman et al., 1987). Kidneys were then partially digested with 20% hydrochloric acid at 37 °C for 2–3 h. The solution containing the kidney tissues was then gently stirred for 10 min with fresh deionized water to dissociate the tissue into individual nephrons, specifically allowing perfused glomeruli to be identified by the presence or lack of Alcian Blue micro-pellets and to create a homogenous mixture.

Aliquots (2 μl) of dissociated kidney tissue solution were placed on a microscope slide and examined under a Nikon Eclipse E200 microscope at 400× magnification. Total number of glomeruli in the 2 μl aliquots and perfused glomeruli was recorded using Image-Pro® Plus version 4.1 software (Media Cybernetics®). The number of perfused and non-perfused glomeruli were calculated and the glomerular circumference was measured using Image-Pro software as described in (Bolin and Burggren, 2013).

2.5. Body fluid collection, osmolality and uric acid concentration

To assess the ability of the embryo to osmoregulate during exposure to low and high RH, the osmolality of the blood was measured at each time point from day 10–18. The osmolality of the allantoic fluid compartment was also measured, as it is an indicator of dehydration/hydration during embryonic development, in addition to serving as a repository for waste. Amniotic fluid osmolality was also sampled at the same time points, as a potential source of water. Cloacal fluid osmolality was measured on day 16 and day 18 only, as there was little cloacal fluid available for analysis from earlier time points. Blood was drawn for analysis of whole blood osmolality. Separate 1 ml syringes were then used to remove ~100 μl of both allantoic and amniotic fluids from the allantoic and amniotic sacs. Embryos were then euthanized via exposure to isofluorane for 10 min followed by decapitation. Cloacal fluid was sampled by placing a capillary tube pulled to a tip diameter of 30 μm

(World Precision Instruments PUL48 1E pipette puller) into the cloaca. Osmolality of each fluid from a 10 μl sample was measured using a Wescor Vapro® vapor pressure osmometer (model 5520).

As a metric of water conservation efficiency, the concentration of uric acid in allantoic fluid, was determined colorimetrically using a QuantiChrom® uric acid assay kit (BioAssay Systems). As described in Bolin and Burggren (2013), 5 μl samples of allantoic fluid were assayed in duplicate for uric acid quantification and expressed as mmol/l uric acid according to standard curve of optical densities of provided standards.

2.6. Hematology

A separate subset of eggs was candled using a xenon light source to locate an artery for blood sampling in the CAM. To maintain temperature of the embryo while blood was being drawn, eggs were placed in a sand and water jacket with steady circulation of 37 °C water (Fisher Scientific Isotemp Refrigerated Circulator model 9100). A small portion of shell was removed to expose the CAM artery, then approximately 100–300 μl of blood was removed via a heparinized 1 ml syringe with 30 gauge needle. After a sample was taken, the embryos in the eggs were euthanized as described above.

For measurement of hematocrit (Hct, %), whole blood was injected from the sampling syringe into microhematocrit capillary tubes, which were then sealed with Critoseal (FISHERbrand®). The capillary tubes were then placed in a microhematocrit centrifuge for 3 min. Hematocrit was then determined by measuring the ratio of the length of packed red blood cells in the Hct tube to total length of blood in the tube.

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). Hematocrit and Hb were then determined using a Coulter counter (Coulter® AC _T Series Analyzer, Beckman Coulter, Inc) to verify Hct and [Hb] values obtained from the above and earlier experiments. No significant difference ($P > 0.05$, *t*-tests) was seen between the measurements obtained from each type of instrument.

2.7. Respirometry

Oxygen consumption ($\dot{V}_{O_2}$), expressed as ml O_2/egg/min was measured on embryonic days 10, 12, 14, 16 and 18 using conventional flow-through respirometry for avian embryos (Dzialowski et al., 2002; Mortola et al., 2013; Reyna, 2010). This system allowed for simultaneous recording of $\dot{V}_{O_2}$ from eight respirometers. Individual eggs from each of the three treatment groups were placed within a respirometer chamber (volume 300 ml). All respirometers were then placed within an incubator at 37 °C. Air at 37 °C flowed into each respirometer via a manifold system (Sable System MF-8 Airflow Manifold), first passing through a column of Drierite® and soda lime to scrub the air of water vapor and carbon dioxide, respectively. Inflowing air was adjusted by the manifold and distributed equally to the eight respirometers. Air flow into the respirometers was matched to embryonic age and embryonic metabolic rate, and so ranged from 25 ml air/min at day 10 (D10) to 75 ml air/min at day 18 (D18).

Gas exiting each respirometer passed again through soda lime then Drierite®, and then to a multiplexor (Sable Systems) that gated the flow from each respirometer to the oxygen sensor (Sable Systems FC-1B O2 Analyzer). The oxygen sensor was coupled to a hardware interface (Sable Systems Universal Interface II) and collected via a data acquisition system (DAS 2.0) connected to a desktop computer. Data was analyzed using Datacan V software (Sable Systems) to monitor and record the $\dot{V}_{O_2}$ of each egg during three successive 2 h readings. The three readings were then averaged to generate a single $\dot{V}_{O_2}$ value for each embryo at each measured developmental time.

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 relatively brief period of oxygen consumption measurement.

After measurement of $\dot{V}_{O_2}$, embryos from each of the three incubation groups were returned to their respective incubators for the remainder of the experiment.

2.8. Control vs experimental groups

As described above, both control and two experimental groups of eggs were incubated and analyzed in this study. Changes in metanephric kidney morphology (wet and dry masses, glomerular dimensions, nephron numbers and their perfusion) as well as body masses of the chicken embryo during normal development under control conditions have previously been described (Bolin and Burggren, 2013). Some of these previously published data comprise a portion of the "control' data for the current study, which are necessary to provide statistical and interpretive context for the actual experimental changes induced by relative humidity, and all control conditions were the same and physiological values did not differ between combined data sets. Importantly, the current study's description of the experimental results focuses exclusively on changes induced by incubation humidity levels over developmental time, rather than developmental effects per se presented in our earlier study.

2.9. Statistics

All data were tested for normality of distributions (Shapiro-Wilks test for normality) prior to specific statistical analyses. Changes in each parameter over developmental time and between incubation groups were assessed with two-way parametric ANOVA. Student-Newman Keuls (SNK) multiple range post hoc tests were run to differentiate distinct data 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 <0.05 were considered as statistically significant.

3. Results

3.1. Cumulative embryo mortality

Cumulative embryo mortality over the entire incubation period was 28 ± 3% in control groups (Fig. 1). However, incubation RH level significantly affected mortality ($p < 0.015$). Embryos exposed to low RH had the highest cumulative mortality of 45 ± 5% ($N = 128$), with the high RH group having a cumulative mortality of 37 ± 4% ($N = 128$).

3.2. Egg and embryo masses

Incubation humidity significantly ($P < 0.001$) affected cumulative mass loss of whole eggs when expressed as % mass loss (Fig. 2). Not surprisingly, as early as D10 low humidity eggs showed twice the % mass loss compared to controls, and three times greater % mass loss compared to high humidity (all group to group comparisons were significant at the level of $p < 0.001$)

Embryo wet mass measured over D10-D18 was significantly affected by incubation RH (Fig. 3A) ($p < 0.001$). On D14, low RH-exposed embryos were significantly lighter than controls (10.0 ± 0.5 g versus 11.9 ± 0.4 g, $p < 0.001$), while on D16 it was the high RH-exposed embryos that were significantly lighter than controls (16.0 ± 0.6 g versus 17.8 ± 0.5 g, $p < 0.001$). However, towards the final days of incubation (day 18), both low and high RH-exposed embryos were significantly

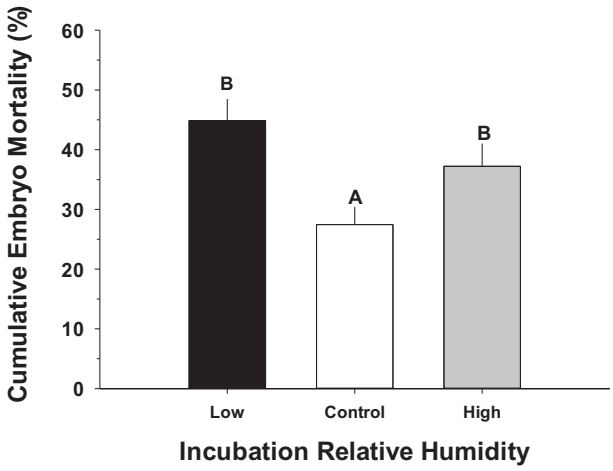

Fig. 1. Cumulative mortality percentage in chicken embryos produced by incubation in low (25–30%), control (55–60%) and high (85–90%) relative humidity levels. Means ± SE shown. Letters indicated statistical comparisons of groups. $N = 128$ eggs for each incubation condition.

lighter than the control embryos (21.8 ± 0.9 g and 22.6 ± 0.6 g, respectively, versus 25.5 ± 0.5 g, $p < 0.001$).

Only low RH altered embryo dry mass and only on day 18 (Fig. 3B) where dry mass was significantly ($p < 0.01$) higher (5.26 ± 0.3 g) compared to both control embryos (4.1 ± 0.2 g) and embryos exposed to high RH (3.94 ± 0.2 g). Finally, the ratio of wet mass to dry mass was ~5:1 at all measured incubation stages.

3.3. Body fluid osmolality

Osmolality of blood, allantoic fluid, amniotic fluid and cloacal fluid during development in the three incubation groups are presented in Fig. 4. Of the four fluid compartments, blood osmolality was the most constant, ranging from 260 to 300 mOsmol/l at all developmental stages and in all three RH groups (Fig. 4A). Blood osmolality in the low RH group was significantly different on day 12 ($p < 0.01$) compared to both control and high RH groups, and only slightly higher than the control group on day 16. Allantoic fluid osmolality, 175–250 mOsmol/l, was similarly quite stable across developmental time in each group, but there was a marked effect of incubation condition (Fig. 4B). Essentially, the low RH group had the highest allantoic fluid osmolality on all days (significantly so at D10, 12, 14 and 18), and the highest RH group has

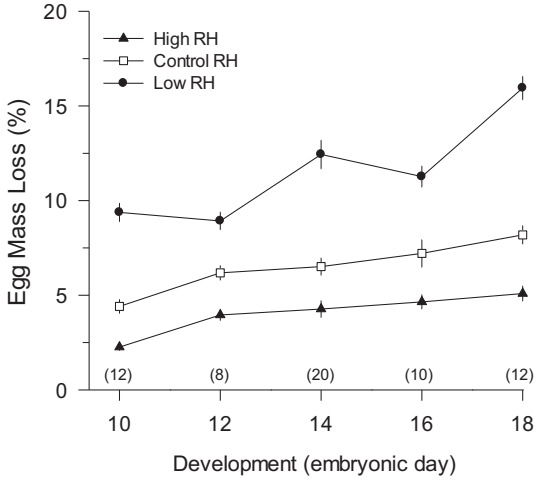

Fig. 2. Cumulative loss of whole egg mass (%) over development, as a function of incubation in low, control and high relative humidity. Mean ± SE are plotted. N values in parentheses.

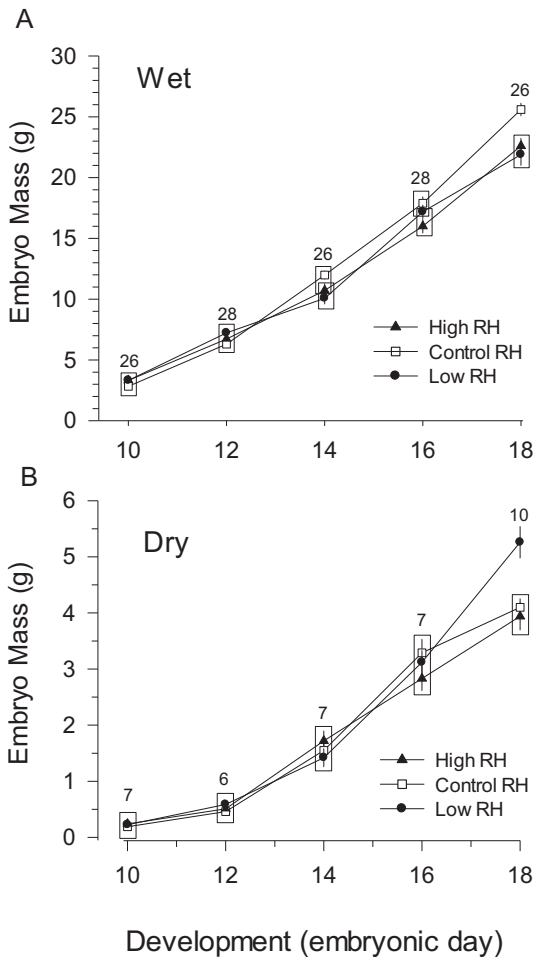

Fig. 3. Chicken embryo wet mass (A) and embryo dry mass (B) over development, as a function of incubation in low, control and high relative humidity. Means ± SE are presented. Statistically identical means are grouped within individual boxes. N values above each data point are identical for all three groups on any given day, unless otherwise indicated.

the lowest osmolality at D10, 12, and 14. However, with further development there was no significance difference ($p > 0.05$) between controls and the high RH group at D16 and D18. Amniotic fluid osmolality was 230–260 mOsmol/l at all developmental stages (Fig. 4C). The only effect of incubation humidity was at D14, when both the control and high RH embryos had a lower amniotic fluid osmolality.

Cloacal fluid could only be sampled beginning at D16. From D16 to D18 cloacal fluid osmolality was 275–310 mOsmol/l (Fig. 4D). Osmolality of cloacal fluid was significantly higher in the low RH group compared to either the control or the high RH group.

3.4. Allantoic uric acid concentration

Uric acid concentration steadily increased during development (Fig. 5). Uric acid concentration in allantoic fluid on D10 was 33.0 ± 4.2 mmol/l in control embryos, increasing to 62.5 ± 4.8 mmol/l on D18. All values for the low and high RH groups were statistically identical to those of the control embryos with the exception of D10, when the high RH group had a significantly ($p < 0.001$) depressed uric acid concentration of 14.0 ± 3.6 mmol/l compared to controls and low RH embryos.

3.5. Hematology

Hematocrit (Hct) was 15–20% on D10 in all three incubation groups, increasing significantly ($p < 0.001$) throughout development (Fig. 6A).

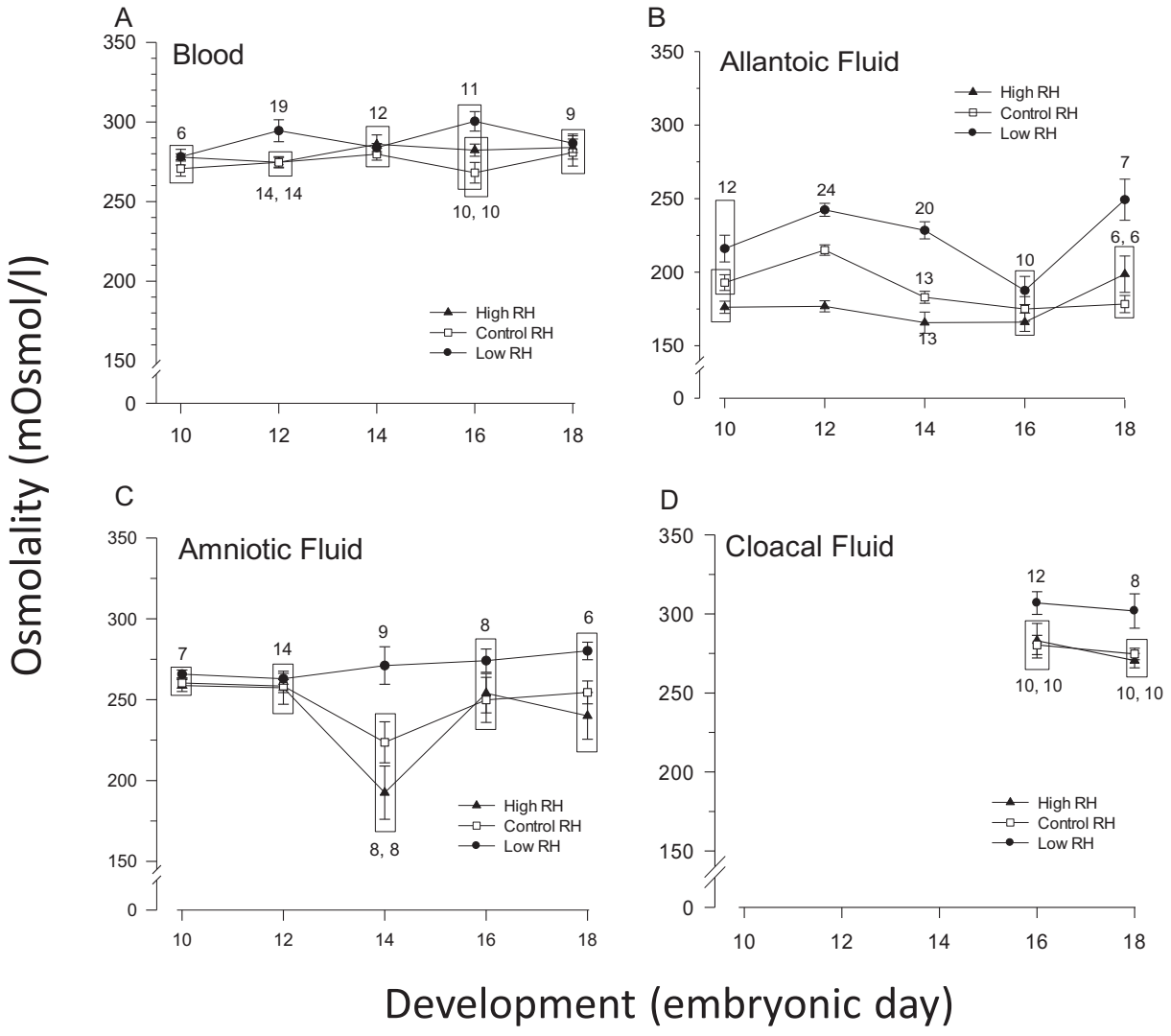

Fig. 4. Body fluid osmolalities as a function of incubation humidity across development of chicken embryos. A) Whole blood. B) Allantoic fluid. C) Amniotic fluid. D) Cloacal fluid. Means ± SE are presented. Statistically identical means are grouped within individual boxes. N values are for all three groups on any given day, unless otherwise indicated by an additional N value for a specific group or groups.

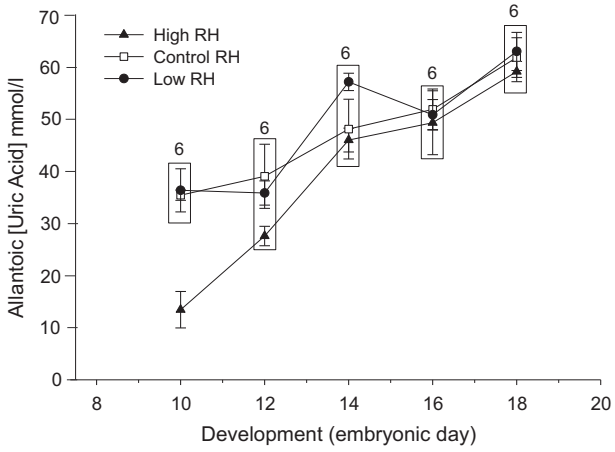

Fig. 5. Uric acid concentrations in allantoic fluid across development in chicken embryos. Means ± SE are presented. Statistically identical means are grouped within individual boxes. $N = 6$ for each data point for all three groups on any given day.

However, there were no significant differences between incubation groups at any day of development.

Blood hemoglobin concentration [Hb] increased significantly ($p < 0.05$) from 4 to 6 g Hb/dl to ~9 g Hb/dl at D18 (Fig. 6B). As for Hct, there were no significant differences between incubation humidity groups at any developmental stage.

3.6. Kidney characteristics

3.6.1. Kidney mass

Kidney wet and dry mass in the three incubation groups are indicated in Fig. 7. Kidney wet masses were essentially identical between groups on any given developmental day, with the exception of a small effect only on D16, with high RH-exposed embryos having significantly ($p < 0.05$) lower wet and dry mass than controls.

In aggregate, a close relationship ($r^2 > 0.90$) existed between kidney wet mass and embryo wet mass for all three groups over a mass range of <5 g (D10) to >25 g (D18) (Fig. 8). The wet kidney mass of embryos exposed to both low and high RH had statistically identical slopes that were both significantly ($p < 0.01$) higher than the control embryos, but not significantly different from each other (Fig. 8).

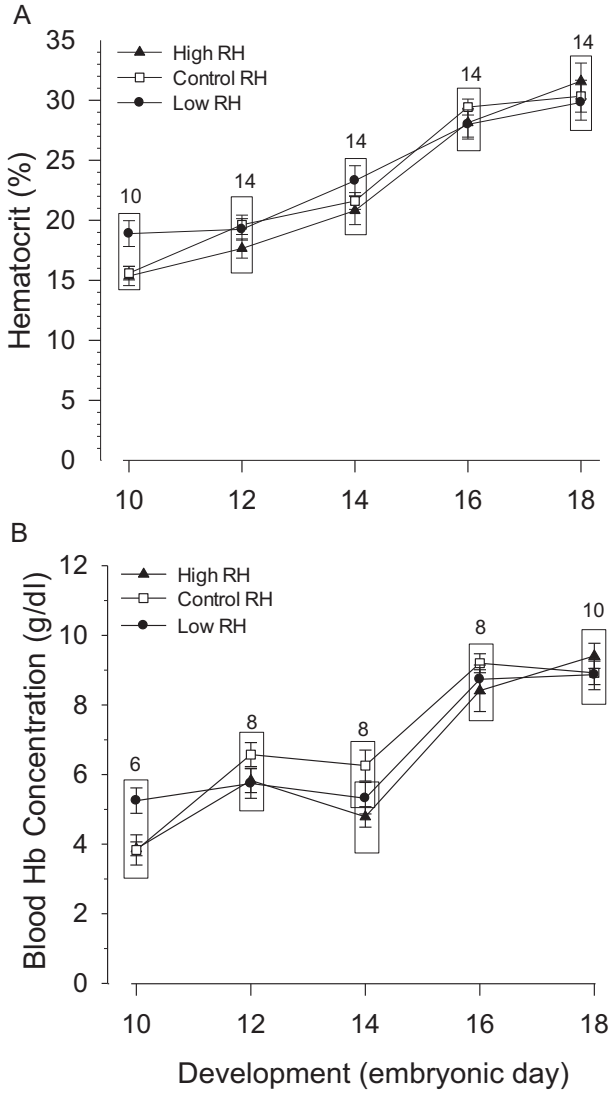

Fig. 6. Hematological properties as a function of incubation humidity level across development of chicken embryos. A) Hematocrit. B) Blood hemoglobin concentration. Means ± SE are presented. Statistically identical means are grouped within individual boxes. N values are for all three groups on any given day.

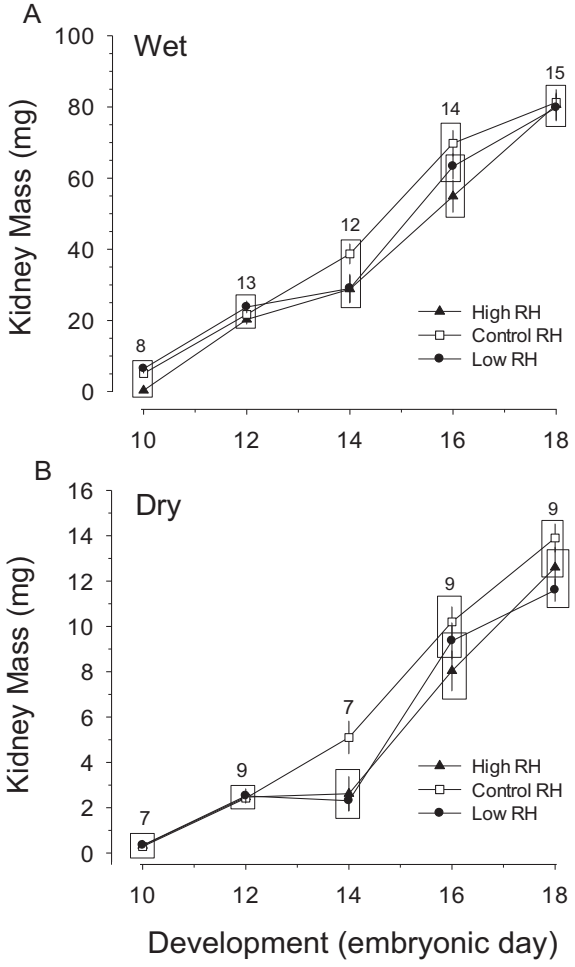

Fig. 7. Kidney wet mass (A) and kidney dry mass (B) as a function of incubation in low, control and high relative humidity. Means ± SE are presented. Statistically identical means are grouped within individual boxes. N values are for all three groups on any given incubation day.

3.6.2. Glomerular density

Total number of glomeruli per kidney increased from ~5000/kidney to 35,000–50,000 glomeruli/kidney on D18. Glomerular numbers were statistically identical in all three groups at each day of incubation through day 16 (Fig. 9A). However, on D18 embryos from the low RH had a significantly higher number of glomeruli per kidney (50,500 ± 5200 glomeruli/kidney) compared to both control (39,800 ± 3100 glomeruli/kidney) and high (37,400 ± 3400 glomeruli/kidney) humidity groups ($p < 0.001$).

3.6.3. Glomerular volumes

Individual glomerular volumes in all groups declined significantly ($p < 0.01$) as a function of development (Fig. 9B). On D12 the average volume of the glomeruli in the low humidity incubation group was significantly larger ($p = 0.009$) (0.40 ± 0.55 nl) compared to both control (0.27 ± 0.04 nl) and high humidity incubation groups (0.22 ± 0.03 nl) ($p = 0.002$), although no other differences were found at any other point in development.

Total glomerular volume/kidney – the product of number of glomeruli/kidney (Fig. 9A) and their individual volume (Fig. 9B) – increased

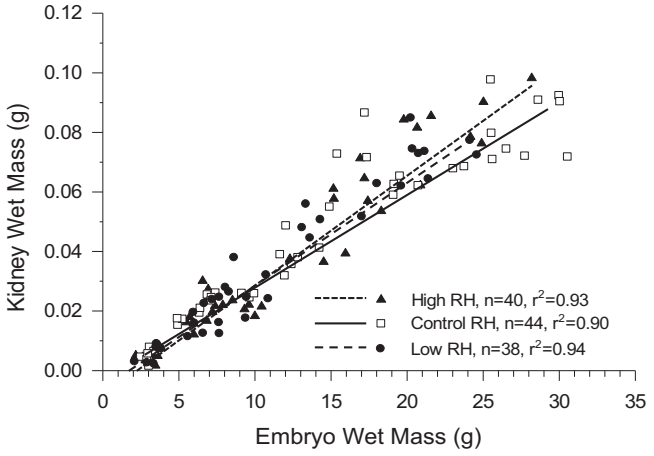

Fig. 8. Relationship between kidney wet mass and chicken embryo wet mass over the range of development from D10-D18. Regression coefficient and N values are shown for control RH, high RH and low RH groups. The slopes of all three lines were significantly different from 0 ($p < 0.01$), and the slopes of the lines for the low and high RH groups were identical but both significantly higher than the control group (see Results for further details).

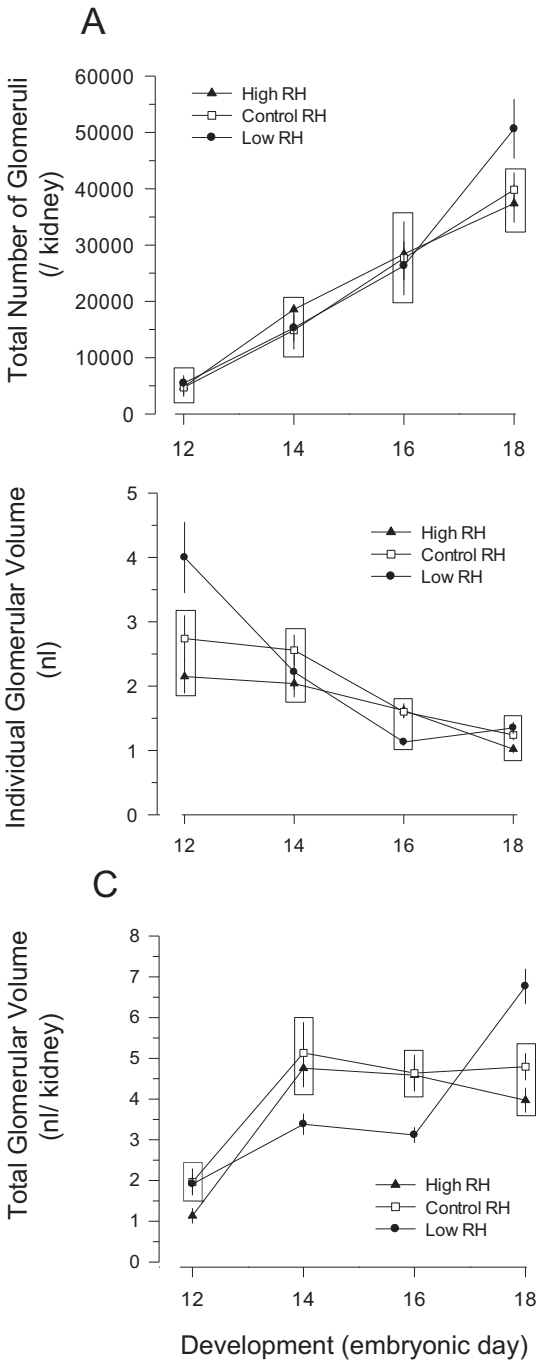

Fig. 9. Glomerular morphological characteristics as a function of incubation in low, control and high relative humidity. A) Total glomerular number per kidney. B) Volume per glomerulus. C) Total glomerular volume per kidney. Means ± SE are presented. Statistically identical means are grouped within individual boxes. $N = 5$ for each data point for all three groups on any given day.

significantly ($p < 0.001$) during development (Fig. 9C). This increase was due primarily to the sharp increase in glomerular numbers rather than individual glomerular size between D10 and D18, which declined with development. On D12 kidneys from the high RH-exposed embryos had a significantly ($p < 0.05$) lower total glomerular volume/kidney (1.15 ± 0.12 μl) than the normal (1.97 ± 0.25 μl) or low (1.94 ± 0.22 μl) RH-exposed (Fig. 9C). The low humidity group had a significantly lower total glomerular volume on days 14 and 16 (3.38 ± 0.26 μl; 3.12 ± 0.19 μl, respectively) compared to normal (5.13 ± 0.75 μl; 4.64 ± 0.44 μl) and high (4.76 ± 0.47 μl; 4.59 ± 0.32 μl) humidity groups, which were statistically identical (Fig. 9C). On day 18, the trend changed in which the low humidity group exhibited higher total glomerular volume/kidney (6.77 ± 0.43 μl compared to normal (4.80 ± 0.33 μl) and high (3.97 ± 0.30 μl) humidity groups which were not significantly different from each other.

3.6.4. Size distribution of individual glomeruli

While Fig. 9B indicates mean individual glomerular volumes, there were interesting size variation patterns associated with these data. The glomerular size (volume) distributions of individual glomeruli, presented as % of total glomeruli of a given size, are presented in Fig. 10. In control embryos, the largest proportion (~40–60%) of individual glomeruli fell in the volume range of 1–2 nl D12-D16, with a shift towards smaller glomeruli in D18 (Fig. 10A). This developmental change in glomerular size distribution mirrors the fall in calculated mean individual glomerular volume with advancing development (Fig. 9B). Nonetheless,

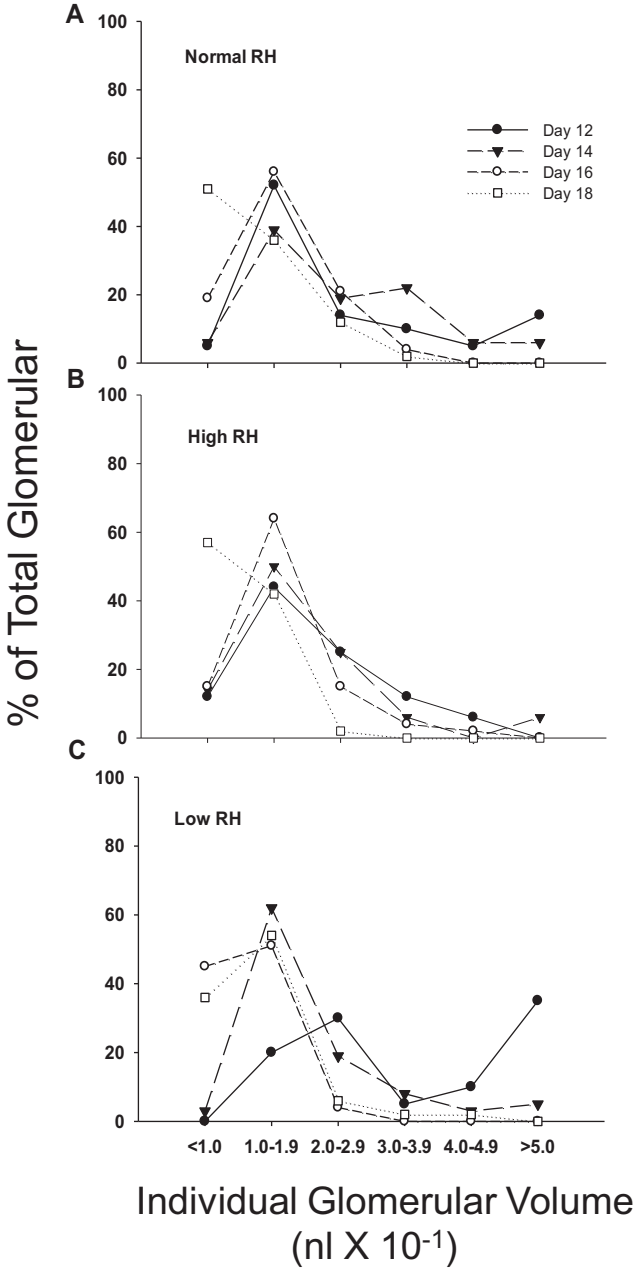

Fig. 10. Distribution of glomerular volume, expressed as percentage of glomeruli in each volume range, across development and under different incubation humidities.

small proportions (<5%) of larger glomeruli with volumes in the 2–5 nl range persisted at all stages. Embryos reared in high RH showed a pattern of developmental change in individual glomerular size distribution that closely resembled that of the control embryos. In contrast, embryos reared in low RH show a distinctly different pattern. In early D12 embryos, a general shift towards a higher proportion of larger glomeruli occurred, in accordance with the significantly higher calculated mean glomerular volume at this stage in the low RH embryos (Fig. 9B). In this different pattern of glomerular size distribution in the low RH group, early development (D12) was characterized by a broader distribution of individual glomerular volumes, including large percentages of glomerular volumes in the 2–3 and >5 nl range (Fig. 10C). Interestingly, this aberrant pattern was reversed, with later developmental stages in this low RH group showing a pattern of individual glomerular volume distribution similar to that seen in control and high humidity incubation groups.

3.6.5. Glomerular perfusion

The total number of perfused glomeruli/kidney (as distinct from total number of glomeruli) was <1000 on D12, but increased significantly ($p < 0.01$) throughout development in all RH groups through D16 (Fig. 11A). However, on day 18, the low RH group had a significantly ($p < 0.005$) higher number of perfused glomeruli ($3.58 \times 10^4 \pm 3.40 \times 10^3$ glomeruli/kidney) than either high RH-exposed embryos and controls ($2.21 \times 10^4 \pm 2.47 \times 10^3$ and $1.73 \times 10^4 \pm 2.75 \times 10^3$ glomeruli/kidney, respectively) (Fig. 11A).

The perfused glomerular volume per kidney followed the same developmental trend as for number of perfused glomeruli/kidney (Fig. 11B). No significant differences occurred from D12 through D16, but on D18 perfused glomerular volume per kidney was significantly ($p < 0.001$) higher in the low RH embryos (4.20 ± 0.23 μl) compared to either high RH or control embryos (1.89 ± 0.18 μl and 2.06 ± 0.21 μl, respectively) (Fig. 11B).

3.7. Embryonic oxygen consumption

Oxygen consumption ($\dot{V}_{O_2}$) in control embryos was ~0.08 ml O_2/min/egg at D10, increasing significantly ($p = 0.03$) to ~0.36 ml O_2/min/egg at D18 (Fig. 12). Oxygen consumption increased during incubation at high and low RH, though there was no significant effect upon oxygen consumption from D10 through D16. However, on D18 the $\dot{V}_{O_2}$ of both low and high RH groups was significantly ($p < 0.01$) depressed by about 25% (0.28 ± 0.03 ml O_2/min/egg in both groups) compared to controls (0.37 ± 0.02 ml O_2/min/egg.

4. Discussion

4.1. Incubation humidity and embryo mortality

Major deviation from a relative humidity of 55–60% results in poor embryo survival in chicken embryos (Ar and Rahn, 1980; Bruzual et al., 2000; Davis et al., 1988; Hamdy et al., 1991; van der Pol et al., 2013). In the present study, both the high (>85%) and low (<30%) RH groups experienced significantly depressed hatching success (Fig. 1), which is consistent with a previous study (Ar and Rahn, 1980). Interestingly, survival is directly dependent on the amount of water lost during the first half of incubation rather than on the total loss of water throughout incubation (Snyder and Birchard, 1982).

Increased mortality from abnormal incubation humidity is likely the result of osmotic stresses placed upon the embryo by either inadequate water availability during low humidity incubation or absence of mechanisms enabling the removal of higher volumes of water during high humidity incubation (Ar and Rahn, 1980). Within the developing chicken embryo, the chorioallantoic membrane and avian mesonephros and

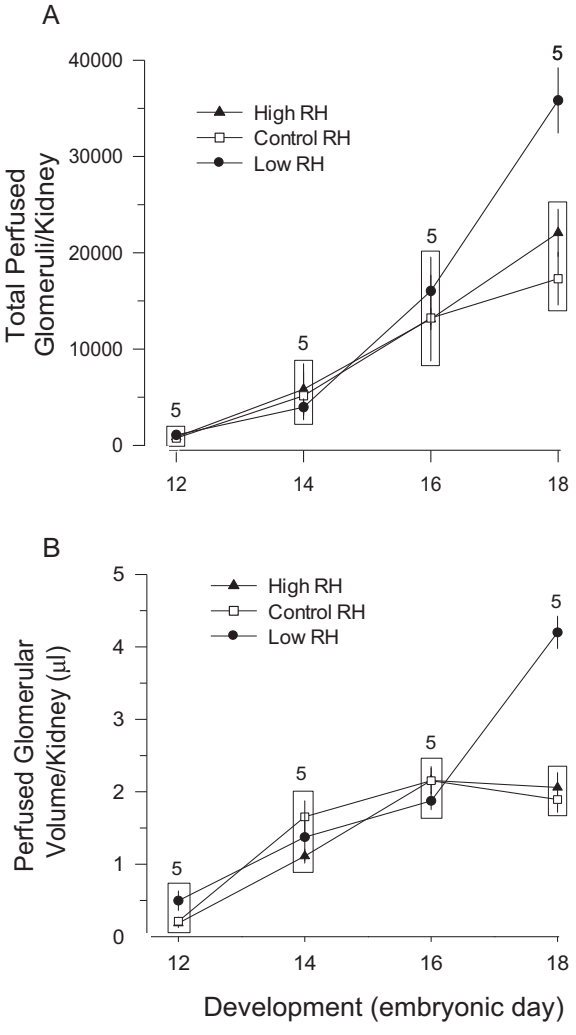

Fig. 11. Glomerular perfusion as a function of incubation humidity level across development in chicken embryos. A) Concentration of perfused glomeruli. B) Perfused glomeruli per kidney. Means ± SE are presented. Statistically identical means are grouped within individual boxes. $N = 5$ for each data point for all three groups on any given day.

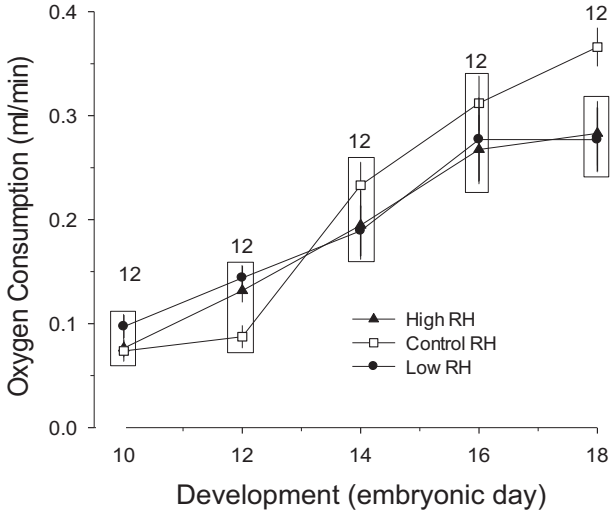

Fig. 12. Oxygen consumption as a function of incubation humidity across development of chicken embryos. Means ± SE are presented. Statistically identical means are grouped within individual boxes. $N = 12$ for each data point for all three groups on any given day.

then, with further development, the metanephric kidneys work collectively to regulate osmotic and ionic homeostasis. The present study has demonstrated significant humidity-induced modification of kidney structure, associated with major disturbances of body fluid osmolalities. This suggests that osmotic stress brought about by renal insufficiency combined with decreased osmoregulatory efficiency of the extraembryonic membranes is likely involved in the increase in mortality in embryos exposed to low and high RH during embryogenesis.

4.2. Incubation humidity and body mass

Based on chicken embryo masses, chicken embryos thrive in terms of body growth at a RH of 55–60%, and fail to thrive as RH moves away from optimal in either direction, especially late in development. Importantly, the pattern of dry versus wet embryo mass change was the same in the three treatment groups until a distinct departure on D18, when embryos were nearing hatching (Fig. 3A–B). On this final measurement day, embryos in non-ideal incubation humidity showed lower wet mass, suggesting both a failure to thrive in late incubation and the importance of the ability to regulate water balance in the ultimate weight of the embryo at hatching. Interestingly, embryos exposed to low humidity had a significantly larger dry mass compared to both the control and high humidity groups, despite the low humidity group having a lower wet mass (Fig. 3A–B). This indicates that the low humidity embryos had lower water content compared to controls and high RH-exposed embryos, and underscores the presumption that an osmoregulatory deficiency contributes to the increased mortality seen in low RH-exposed animals. Similar results for chicken embryo wet mass of birds incubated in low humidity have been reported (Davis et al., 1988), but that study found no effect of incubation humidity on dry masses. This discrepancy between studies may arise from methodological differences – the study by Davis et al. (1988) only exposed embryos to low relative humidity starting on D13. A critical window for affecting embryonic growth and body composition may thus occur earlier rather than later in development, even though the mass differences found here manifest themselves closer to hatching. Conversely, the change on D18, despite a close relationship from day 10–16, may indicate a developmental effect associated with metanephric kidney function, rather than a progressive exertion of effects on the system (see below.)

4.3. Fluid osmolalities

4.3.1. Blood

Plasma in chicken embryos is typically hypertonic to both amniotic and allantoic fluid, by up to 80 mOsmol/l (Davis et al., 1988; Gabrielli and Accili, 2010; Simkiss, 1980) 2010), reflecting the significant transport of ions and other solutes to the embryonic and extraembryonic membrane fluid compartments. All blood osmolality values in the present study were within the normal range (Fig. 4a) (Bolin and Burggren, 2013; Braun, 1999; Davis et al., 1988; Gabrielli and Accili, 2010; Simkiss, 1980). Although statistically significant differences in plasma osmolality occurred between incubation groups on D12 (7% rise) and D16 (12% rise), the physiological significance of these small deviations from control are unclear. In the ovine fetus, dipsogenic stimulation occurs when blood osmolality increases by only ~10%, evoking the thirst response (Xu et al., 2001). Avian embryos also swallow amniotic fluid during incubation as part of emerging thirst and hunger mechanisms (Karcher et al., 2005). Whether the chicken embryo increases swallowing behavior to prevent increases in plasma osmolality is unknown.

4.3.2. Allantoic fluid

Allantoic fluid, formed as a result of kidney filtration of blood, typically remains hypo-osmotic to avian embryonic plasma and all other measured fluid compartments throughout the entire incubation period (Murphy et al., 1991). This is thought to be the result of active ion transport with subsequent water movement from allantois to blood (Hoyt, 1979; Simkiss, 1980; McNabb and McNabb, 1975; Davis et al., 1988; Bolin and Burggren, 2013). Allantoic fluid showed larger effects of incubation humidity than did plasma osmolality, with significantly elevated values in the low RH group and a significantly depressed value in the high RH group at D12, D14 and D18. The decline in allantoic fluid osmolality between D12 and D16 in low RH and control groups has previously been reported (Romanoff, 1967). This influence on allantoic fluid is not surprising, as the allantoic fluid compartment, underlying the egg shell, is the most proximate buffer between the interior of the egg and the environment – whether characterized by high or low relative humidity.

4.3.3. Amniotic fluid

Previous studies have shown that amniotic fluid is unaffected by the egg's hydration state prior to ~day 17, the point in development at which the allantoic fluid department has been emptied and the CAM has begun to deteriorate (Davis et al., 1988; Hoyt, 1979). The present study varies slightly in that the sharp transient decrease in amniotic fluid in both control and high RH-exposed embryos on D14 did not occur in the low RH-exposed embryos, whose amniotic fluid remained relatively steady throughout the experiment (Fig. 4C). Avian embryos begin to swallow amniotic fluid coincident with the formation of the sero-amniotic connection on day 12 (Lopez de Torre et al., 1993). Amniotic fluid is likely diluted in control and high humidity groups by albumen entering the amniotic fluid compartment via the sero-amniotic connection, which could account for the decrease in fluid osmolality in normal and high humidity groups on D14 (Bolin and Burggren, 2013). The amniotic fluid osmolality of the low humidity group does not drop significantly on D14 presumably due to the albumen becoming depleted of water before the sero-amniotic connection has formed. Ingestion of this high osmolality amniotic fluid may account for the slight increase of blood osmolality occurring on D16 in the low humidity group (Fig. 4A).

4.3.4. Cloacal fluid

In adult birds the combined actions of the kidney and gastrointestinal tract produce hyperosmotic urine (Braun, 2003). For all treatment groups cloacal fluid was approximately isosmotic with blood, and hypertonic to allantoic and amniotic fluid (Fig. 4). On D16 and D18, cloacal fluid osmolality was elevated in embryos exposed to low RH. However, little is known about the relative contributions of the gastrointestinal tract and the kidneys to cloacal fluid during development. Further investigation is warranted to understand the roles of the gastrointestinal tract and the kidney in the onset of cloacal fluid modification during development, and their respective contributions to osmoregulation.

4.4. Uric acid concentration

In avian embryos, nitrogenous metabolites are emptied into the allantoic fluid as ammonia, urea and uric acid (urate), starting around day 5 of incubation (Bradfield and Baggott, 1993). Uric acid is an essential part of water balance in developing avian embryos (Bradfield and Baggott, 1993). Oxidation of protein to urate produces more metabolic water than oxidation of fat. Uric acid storage also requires less water than other metabolic end-products, increasing nitrogenous waste excretion efficiency (Laverty and Skadhauge, 2008; McNabb and McNabb, 1980; Singer, 2003). Additionally, uric acid precipitates with cations (sodium) when pH is above 6.2 (McNabb, 1986), which can result in reduced fluid osmolality.

Uric acid concentration in allantoic fluid under control incubation conditions, increased nearly two-fold during chicken embryo development in the present study (Fig. 5), confirming earlier studies (Bradfield and Baggott, 1993; Romanoff, 1967). In Japanese quail embryos incubated under dehydrating conditions, allantoic fluid showed reduced osmolality because of an increase in concentration of uric

acid, which presumably sequestered sodium ions in the allantoic fluid (Bradfield and Baggott, 1993). The results of the current study differ for those of quail, with no appreciable effects of dehydrating conditions brought about by low relative humidity incubation at any measured point in development. Noteworthy, however, was the profound effect of high incubation humidity only on D10, where allantoic uric acid concentration was much lower than either control or low humidity groups. This could represent a major change in nitrogenous metabolism on D10 but, if so, such a change is not reflected in oxygen consumption. Alternatively, or in addition, these data could reflect a lingering influence of the mesonephros. Clearly warranted for avian embryos is a future comprehensive, multi-compartment analysis of nitrogenous end-products and their physiological consequences.

4.5. Hematology

Hematocrit and [Hb] values at any given stage in avian embryonic development vary considerably in the literature – for recent discussions of avian embryonic hematology see (Andrewartha et al., 2011; Mueller et al., 2015; Tazawa et al., 2012). These differences may arise from different experimental treatments, methods and even season and flock. Despite inherent variation, the hematological values for control embryos in the present study show similar absolute values and rates of change between D10 and D18 as previously reported.

We had predicted that exposure to low and high RH during early development would result in altered Hct and [Hb] compared to controls and low and high RH-exposed embryos, reflecting changes in embryonic hydration state. Yet, we found no significant differences in these hematological variables at any point during embryonic development as a function of experimental treatment (Fig. 6), which correlates well with stable blood osmolalities.

4.6. Renal characteristics and remodeling

Kidneys of adult birds show varying degrees of phenotypic plasticity. For example, in response to altered salt levels in their diet, adult coastal songbirds experience an increase in kidney mass, and a tissue redistribution favoring additional medullary tissue (Sabat et al., 2004). In chicken embryos older than day 14, the metanephric kidney is developed and functional (Bolin and Burggren, 2013; Hiruma and Nakamura, 2003; Schneider et al., 2015; Schneider, 2016), so the embryo essentially has the adult renal architecture at this time. It follows then, that later stage chicken embryos may, like in adult birds, be similarly capable of renal remodeling.

One of the most basic possible modifications is change in kidney mass, though how and why the kidneys might respond is variable. For example, it could be argued that the kidneys of the low incubation humidity group would have larger kidneys in an effort to recapture filtered water. Alternatively, they might have produced smaller kidneys to avoid water loss through urine in the first place. Finally, in the simple scenario of overall embryonic failure to thrive, the kidneys might be smaller, but not out of proportion to the reduced body mass. Similarly, for the high humidity incubation group it might be posited that there would be smaller kidneys because less plasma filtration and urine formation would be required given the pathological abundance of water. On the other hand, it could be argued that larger kidneys are needed to handle the increased water elimination burden. Despite these various plausible scenarios, the present study showed that neither kidney dry or wet mass was greatly affected by incubation humidity across the range of embryo body masses from D10-D18 (Fig. 7).

Despite lack of change in kidney mass as a result of incubation RH, kidney morphology (glomerular numbers, volumes and size distributions) underwent significant tissue-level changes at the nephron level (Figs. 9 and 10). When mammalian fetuses are exposed to high salt loads, their kidneys show reduced numbers of glomeruli (Bouby and Fernandes, 2003; Koleganova et al., 2011; Moritz et al., 2011; Rasch et al., 2004), presumably due to an underdeveloped renin-angiotensin system or over expression of arginine vasopressin. It is not clear how the observed increases in glomerular number (and individual glomerular volume in early development) in the chicken embryo exposed to low incubation relative humidity translate into actual changes in renal function, although it is clear that the capacity for kidney function is considerably increased (depending on circulatory capacity), as glomerular numbers increase.

Physiological remodeling is evident in the fact that after D14 the low RH group perfused a higher proportion of its existing glomeruli compared with the control group (Fig. 11). At any given time, typically only a subset of all glomeruli in vertebrate kidneys are perfused, generating a reserve filtration capacity that can be tapped by recruiting non-perfused nephrons into service (Aukland, 2001; Brown and Rankin, 1999; Dantzler, 1980). This phenomenon, termed "glomerular intermittency", also applies to the chicken embryo, where on D18 controls, for example, only about 50% of the ~40,000 nephrons per kidney remain perfused under normal incubation conditions (Bolin and Burggren, 2013).

In the present study, exposure to low RH resulted in nearly a doubling of the number of perfused nephrons and total perfused glomerular volume (Fig. 11). This could have resulted from remodeling to produce more nephrons and increased glomerular capacity in late development. However, if this remodeling produced a simple increase in nephron number (Fig. 9A) with no increase in individual glomerular volume (Fig. 9B), then it is likely that the changes in total perfused glomerular volume occurred when the embryos exposed to low RH adjusted their unperfused glomerular reserve to reduce functional nephron numbers on D14 and D16 and elevated functional nephron numbers on Day 18 to increase total glomerular volume (Fig. 9A, C). Important to note is that, given the lack of significant change in osmolality or hematology, that there was no dehydration per se. However, low humidity and the stress of potential dehydration caused by incubation in low RH may have stimulated subtle hormonal or other signals affecting kidney development, even in the absence of an increase in blood osmolality, for example.

Collectively, these data suggest at least a partial renal morphological and physiological remodeling to compensate for altered incubation hydration state. Interpretation of what an increase in total glomerular perfusion is achieving awaits further investigation. However, the lack of major changes in plasma osmolality apparently reflects the ability of the remodeled kidney to osmoregulate effectively.

4.7. Incubation humidity and oxygen consumption

Incubation humidity had little effect on embryonic $\dot{V}_{O_2}$ until late in embryonic development where both low and high RH-exposed embryos had significant depression of $\dot{V}_{O_2}$ compared to the controls (Fig. 12). This mirrors the pattern of change over development seen with embryo wet mass (Fig. 3), indicating that these departures from the control may correlate with osmoregulatory demands and the functional ability of the metanephric kidney at this developmental stage, rather than being indicative of a progressive effect of poor incubation conditions. Still, the dramatic increases in $\dot{V}_{O_2}$ during avian development parallel growth of the embryo (Dzialowski et al., 2002; Szdzuy et al., 2008; Vleck and Vleck, 1987). Thus, the depressed $\dot{V}_{O_2}$ values seen in the current study under low and high humidity incubation are most likely the direct result of smaller embryo wet mass on D18 in the experimental groups.

Additional underlying factors at play, however, might explain some of these changes in $\dot{V}_{O_2}$. For example, the volume of the air cell is directly proportional to water loss over the incubation period, so the high and low humidity groups might be expected to have smaller and larger air cells, respectively. This may have implications for gas exchange in late incubation, especially during internal pipping when respiration begins to shift from the CAM to the lungs (Mueller et al., 2015). At this stage,

a distinctly smaller air cell volume might lead to embryonic hypoxia, which in chicken embryos leads to reduced $\dot{V}_{O_2}$ and growth rate (Dzialowski et al., 2002; Mortola and Cooney, 2008; Mortola and Labbe, 2005).

Humidity conditions could also affect metabolic rate because of the metabolic costs of osmoregulation. Chronically high rates of urine formation in adult birds would require an increase in glomerular filtration rate (Yokota et al., 1985), as for mammals. The associated increase in renal perfusion would likely result in an increase in$\dot{V}_{O_2}$. Interpolating to embryonic development, the elevated routine $\dot{V}_{O_2}$ on D18 of the embryos exposed to high or low RH in the present study may have resulted from shifts in renal performance. However, $\dot{V}_{O_2}$ data are reported on a per embryo (whole egg) basis. Using mean embryo masses and $\dot{V}_{O_2}$ values from Fig. 3, $\dot{V}_{O_2}$ on a per gram basis were 0.0124 ml O_2/g/min and 0.00122 ml O_2/g/min for the high RH-exposed group compared to 0.0134 ml O_2/g/min for the control group. These calculations suggest that in fact, mass-specific routine metabolic rate is *not* greatly elevated by either high or low RH incubation conditions. Clearly, additional experiments, ideally combining glomerular filtration rate, water fluxes within and through the egg, and $\dot{V}_{O_2}$.will be required to sort out the complex interplay between these factors, and how incubation humidity affects them.

5. Conclusions

This study has established that incubation relative humidity influences the developmental morphology and physiology of the major osmoregulatory organs and tissues, as well as overall growth and metabolism, of the developing chicken embryo. Generally speaking, low humidity resulted in more profound changes in physiological function, and these changes occurred mostly after the onset of metanephric kidney function. This change in physiology due to exposure to low humidity was linked to a remodeling of the metanephric kidney architecture and functional properties, indicating that renal plasticity increases throughout ontogeny. The relative roles of other tissues involved in osmoregulation, and the timing of the onset of hormonal communication between the renal, cardiac and digestive systems remain unclear. Major differences in kidney morphology suggest that functional changes may persist at least into the perinatal period, indicating that the avian embryo may serve as a promising model for further exploration of renal fetal programming.

Acknowledgments

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