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ORIGINAL PAPER

Renal, metabolic and hematological effects of trans-retinoic acid during critical developmental windows in the embryonic chicken

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Abstract All-trans-retinoic acid (tRA), an active metabolite of vitamin A, directly influences the developing kidney, and is a major regulatory signal during vertebrate organogenesis. The aim of the current study was to specifically target potential critical windows in renal development, and assess altered renal function through disruptions in embryonic fluid compartments. In addition, the effect of exogenous tRA administration on embryonic growth and metabolism was determined. Embryos were exposed to 0.1 or 0.3 mg tRA on embryonic day 8. Morphological and physiological measurements were made on days 12, 14, 16 and 18. Embryo wet mass on day 18 was reduced by 23 % (0.1 mg tRA) and 44 % (0.3 mg tRA). tRA exposure elevated mass-specific oxygen consumption in embryos exposed to 0.1 mg (21.2 ± 0.3 μL^{-1} g^{-1} min^{-1}) and 0.3 mg (23.4 ± 0.4 μL^{-1} g^{-1} min^{-1}) when compared to sham (18.9 ± 0.6 μL^{-1} g^{-1} min^{-1}) on day 14, but not subsequent incubation days. Osmolality of blood plasma was transiently lowered in embryos exposed to 0.3 mg tRA between days 14 and 16. Allantoic fluid osmolality was significantly elevated by tRA to $\sim$220 mmol L^{-1} from days 16 to 18 compared to controls. Blood plasma [Na^+] was reduced by $\sim$17 % over the same period, while allantoic fluid [Na^+] was elevated in tRA-treated embryos compared to control embryos. Collectively, our data indicates that exogenous administration of tRA produces significant alterations to the developmental trajectory of the developing embryonic chicken.

Keywords Chicken embryo · Blood osmolality · Retinoids · Developmental patterns

Introduction

Retinoids and their active metabolites, such as all-trans-retinoic acid (tRA), are major regulatory signals during vertebrate organogenesis (Means and Gudas 1995), acting via regulation of transcription factors. Retinoids are implicated in both cell differentiation and embryo homeostasis (De Luca 1991). In addition, the level of exogenous retinoids that the embryo is exposed to over development must be tightly regulated, because excessive or inadequate levels cause several developmental abnormalities (Yutzey et al. 1994; Osmond et al. 1991; Maden et al. 1998; Ross et al. 2000; Clagett-Dame and Deluca 2002). In the kidney, specifically, retinoic acid increases branching from the ureteric bud, leading to more sites for developing nephrons, essentially modulate the nephron number in a dose-dependent manner (Vilar et al. 1996). This dose-dependent stimulation of nephrogenesis induced by retinoic acid actually reverses at higher concentrations, where it inhibits nephron growth and differentiation (Vilar et al. 1996). While nephron number is indeed influenced by tRA, it remains unclear what effect, if any, tRA may have on the embryo's capability to maintain internal fluid homeostasis under situations where kidney structure may have been compromised.

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Relatively little is known of the early ontogeny of renal function in birds, and especially to what extent the renal system exhibits developmental plasticity in the face of changes in exogenous or endogenous regulators such as tRA. The developing embryonic kidney structure originates from the intermediate mesoderm, and development occurs through three relatively distinct stages: the pronephros, the mesonephros, and the metanephros (Romanoff 1960). During the final third of incubation, the developing kidney undergoes dramatic changes in metanephric morphology and physiology with changes in glomerular diameter, glomerular volume, and total and perfused nephron number (Bolin and Burggren 2013). Because of the distinct developmental stages of the kidney, and the dynamic period of renal growth and development over the last third of incubation, the avian embryo is a useful model to assess critical windows in which the whole embryo, or specific organ systems, may be sensitive to changes in the external or internal environment. In addition, it is possible to examine how perturbations during development may manifest themselves after hatch, as the metanephros continues to develop until 30 days after hatching (Wideman 1989).

The metanephros, the most highly developed and most complex kidney compared to the other two intermediate forms, begins functioning during the middle of incubation (Wideman 1989). In the current study, we attempted to target a potential critical window in metanephric nephrogenesis with exogenous tRA. We hypothesized that exposing the developing embryonic kidney to exogenous tRA would alter the developmental trajectory of the kidney, with physiological consequences being alterations in internal electrolyte and fluid osmolality homeostasis. In addition, we assessed whole embryo development, largely through monitoring organ mass and metabolic rate, to characterize how exogenous tRA may alter embryonic development. We tested this hypothesis by assessing the internal osmolality environment within tRA-treated eggs, as well as tracking the developmental changes that occur within embryonic and non-embryonic fluid compartments over the last third of normal embryonic development.

Materials and methods

Source and incubation of eggs

Fertilized white leghorn eggs (*Gallus gallus domesticus,* layer strain) were obtained from Texas A&M University (College Station, TX) and shipped to the Department of Biological Sciences at the University of North Texas (Denton, TX). On arrival, eggs were placed in incubation at 37.5 ± 0.5 °C and 55–60 % relative humidity, and were turned automatically every 3 h. Temperature and humidity in the incubator were monitored using wireless barothermo-hydrometers (model BTHR968, Oregon Scientific). On embryonic days 12–18 (stages 37–44; (Hamburger and Hamilton 1951) eggs were removed for experimental analyses.

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

Drug preparation

Concentrations of 0.1 mg and 0.3 mg all-trans-retinoic acid [Sigma-Aldrich (tRA)], containing 5 % dimethyl sulfoxide (Sigma-Aldrich) were prepared from a stock solution of tRA. In addition, a sham solution was prepared with chick ringer alone consisting of 5 % dimethyl sulfoxide. 1-ml aliquots were made from all solutions and stored at −20 °C until required. Solutions were then removed from cold storage, thawed, and briefly mixed with a Vortex mixer. Care was taken to prevent exposing the solutions with retinoic acid to long periods of UV light prior to use in the experiments, to prevent its degradation.

Injection protocol

Eggs were removed from the incubator at day 8 (stage 33) of embryonic development. Eggs were then candled to mark an injection site that would not interfere with any of the underlying vasculature of the CAM. Following candling, eggs were transferred to an incubator (HOVABATOR, model 1602 N) set at 37.5 ± 0.5 °C and 60 % relative humidity. A small hole was made in the eggshell, at the previously determined site, using an 18½ gauge needle. An injection of 100 µl of 1 mg^{-1} ml solution retinoic acid (total dose = 0.1 mg) or 3 mg^{-1} ml solution (total dose = 0.3 mg) or sham solution was then administered into the allantoic fluid via a 25 gauge needle attached to a 1-ml syringe. After the injection, the hole in the eggshell was sealed with a 2-cm piece of electrical tape followed by application of JB Quickweld® to bind the tape to the shell. The eggs were then returned to the original incubator for continued incubation.

Mortality assessment

Mortality of embryos receiving an injection of retinoic acid or sham solution was checked on embryonic day 10 by candling. Mortality percentage was calculated for 0.1 mg tRA, 0.3 mg tRA, and sham solution from the number of dead eggs divided by number of eggs injected × 100. Embryos from many different injection days were pooled together by treatment group.

Embryo, organ and yolk mass

Embryos were euthanized by 0.1-ml pentobarbital injection directly into the embryo on the selected study day, and then removed from the shell. Wet mass was determined (Denver Instrument Company XD-800) after removal of excess fluids with Kimwipes® (Kimtech Science). The embryo was then pinned to a surgical mat, where the abdominal cavity was opened, and with the assistance of a dissecting microscope, the heart, and each kidney was removed, lightly dried with Kimwipes® and wet mass was determined. The tissue was then placed in a tissue oven (Fisher Isotemp® 100 series model 106 G) for 2 days. Following the 2-day drying time, dry mass was determined for embryo, heart and kidneys (Denver Instrument Company XD-800). The remains of the embryo were disposed of according to IACUC protocol. Yolk wet mass was determined by first removing the yolk sac away from all other structures, briefly rinsing with water, blotting dry with a Kimwipe®, and then transferring to a tarred weight boat. Once wet mass was recorded, the yolk sac was placed in a tissue oven (Fisher Isotemp® 100 series model 106 G) for 2 days. Following the 2-day drying time, dry mass was determined for each yolk (Denver Instrument Company XD-800).

Oxygen consumption

Oxygen consumption was recorded via flow-through respirometry on incubation days 14, 16, and 18. Each egg was placed in its own metabolic chamber, which was then placed into a modified incubator set at 37.5 ± 0.5 °C. A separate metabolic chamber was designated as a "blank" or control chamber and remained empty throughout the experiment. The eggs were allowed to acclimate to the chamber for 30 min before the experiment began. Compressed normoxic air was pumped into a Sable System MF-8 airflow manifold (Sable Systems International). Airflow rates were adjusted to 100 ± 1 ml min^{-1} (Liu et al. 2008; Reyna 2010). Before the compressed air entered each individual metabolic chamber, it was passed through copper coils to warm incubator to temperature. From the metabolic chambers, exiting gas flowed to a Sable Systems multiplexor and through a column of drierite and soda lime, to remove excess water and carbon dioxide. Each chamber was sampled at 5 s consecutive intervals for 15 min by pulling a gas sample through an oxygen sensor (Sable Systems FC-1B O_2 Analyzer). Oxygen measurements from the analyzer were sent to a computer and processed by the Data acquisition system 2.0 and Datacan V data analyzer. Oxygen consumption was measured as the difference in oxygen concentration between the normoxic air flowing out of the "blank" chamber and the gas flowing out of each metabolic experimental chamber.

Fluid osmolality

On the designated sampling day (12, 14, 16, 18 days of embryonic development), ~100 µl of blood and allantoic and amniotic fluids were separately removed from the embryo with 25 gauge needles attached to 1-ml syringes. Approximately 100 µl whole blood samples were centrifuged (Fisher micro-centrifuge model 235A) for 5 min at 5,000*g* and the plasma was removed for osmolality analysis. Osmolality measurements for all fluids were obtained by injecting 10 µl of sample into a vapor pressure osmometer (Wescor® VAPRO, 5520).

Sodium and potassium fluid concentrations

Sodium (Na^+) and potassium (K^+) ion concentrations were measured in both plasma and allantoic fluid from a separate series of embryos comprising control embryos, sham-treated embryos, embryos exposed to 0.1 mg tRA, and embryos exposed to 0.3 mg tRA on embryonic day 8. Approximately 300 µl of whole blood was removed from the chorioallantoic vein for these experiments with a 25 gauge needle attached to a heparinized 1-ml syringe. The blood was immediately centrifuged (Fisher micro-centrifuge model 235A) for 5 min at 5,000*g* and the plasma removed, placed in liquid nitrogen, and stored at −80 °C. A new syringe needle combination was used to sample 300 µl of allantoic fluid from the same embryo sampled for blood. Allantoic fluid was quickly placed in liquid nitrogen and stored at −80 °C.

Blood and allantoic fluid samples were removed from storage and briefly mixed in a Vortex mixer. 50 µl samples of both plasma and allantoic fluid were diluted 400 times in ultrapure water to a final volume of 20 ml. After dilution, each plasma and allantoic fluid sample was separated into two different 5-ml aliquots, since only one ion can be measured concurrently. Standard concentrations for both Na^+ and K^+ were serially diluted from a pre-made 20 ppm standard (SCP Science) with the same ultrapure water, used to dilute the blood and plasma samples. Assessments of [Na^+] and [K^+] ion plasma and allantoic fluid concentrations were made using flame photometer (Perkin Elmer Instruments AAnalyst 330).

Hematology and blood pH and bicarbonate

A set of eggs were removed from incubation on days 12, 14, 16, and 18 and ~300 µl of blood was sampled as described above. For measurement of hematocrit, red blood cell count, and total hemoglobin, 35 µl of blood was injected into a Coulter counter (COULTER® Ac.T) for hematologic analysis.

Blood pH and bicarbonate ion concentration [HCO_3^-] were measured on 125 µl of freshly drawn blood using a Radiometer (Copenhagen Radiometer ABL 5).

Statistical analysis

All data were first tested for normality of distributions (Shapiro-Wilks test for normality) before additional statistical tests were performed. Two-way parametric ANOVA was used to test the effects of developmental day, treatment and the interaction between the two. Post hoc Student-Newman Keuls (SNK) multiple range tests were run if no significant interaction occurred to separate data into distinct groups. If a significant interaction was seen between development and treatment, concatenation was performed to separate statistically distinct groups within each developmental stage. All statistical analyses were performed using SAS 9.2 software. Statistical decisions were made at an alpha level of 0.05. Data are presented as mean ± SE.

Results

Mortality

Mortality from the sham-treated embryos was not different from control embryos or embryos injected with 0.1 mg trans-retinoic acid (tRA) when assessed on embryonic day 10 (Fig. 1, $p > 0.05$). However, a significant mortality increase occurred when embryos were exposed to a single injection of 0.3 mg tRA (33 ± 8 %) compared to control (8 ± 2 %), sham (10 ± 2 %), or 0.1 mg tRA treatments (19 ± 12 %) (Fig. 1, $p < 0.05$).

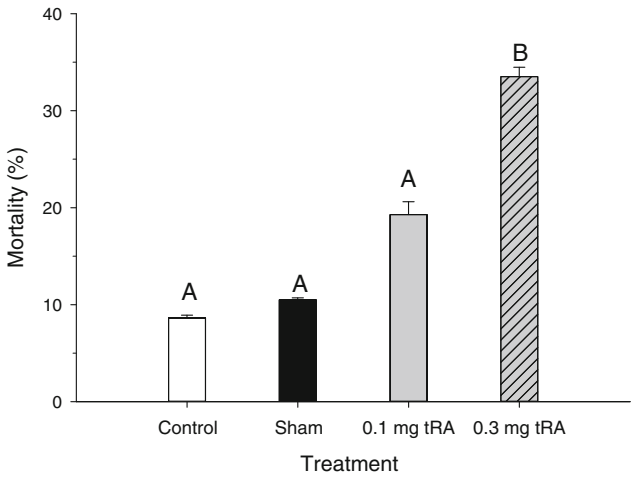

Fig. 1 Percent mortality assessed on embryonic day 10 in chicken embryos exposed on embryonic day 8 to a single injected dose of either sham or trans-retinoic acid (tRA). *Different letters* indicate statistically distinct groups. $N = 80$ for all groups. Data are presented as mean ± SE

Embryo mass

In control embryos, embryo wet and dry mass increased significantly ($p < 0.05$) from embryonic days 12 to 18 (Figs. 2a, 3a, respectively). Heart wet mass of control embryos increased throughout development ($p < 0.001$) with its largest mass increase (11 X), from day 12 (45 ± 3 mg) to day 14 (95 ± 3 mg) (Fig. 2b). In addition, heart dry mass increased significantly over development ($p < 0.001$) roughly doubling in size from day 12 (5 ± 0.2 mg) to day 14 (12 ± 0.4 mg) (Fig. 3b). Kidney wet mass of control embryos showed the largest proportional increase, roughly doubling in mass, from day 12 (52 ± 3 mg) to day 14 (109 ± 6 mg) (Fig. 2c). Similarly, kidney dry mass showed the largest increase, almost tripling in mass from day 12 (7 ± 0.3 mg) to day 14 (17 ± 1 mg) (Fig. 3c).

A significant interaction occurred between treatment and development for embryo wet and dry mass (Figs. 2a, 3a, respectively, $p < 0.0001$). No significant differences were seen between control and treatment embryo wet or dry mass on days 12 and 14. However, on day 16, control embryo wet mass (18 ± 0.3 g) was significantly greater compared to both 0.1 mg tRA (15 ± 0.3 g) and 0.3 mg tRA (14 ± 0.5 g) (Fig. 2a, $p < 0.05$). Embryo dry mass was not significantly different among control, sham, and 0.3 mg tRA, but a significant decrease was seen on day 16 in embryos treated with 0.1 mg tRA (Fig. 3a, $p < 0.05$). On day 18, tRA-treated embryos showed a dose-dependent decrease of 31 and 79 % from control and sham embryos with treatment of 0.1 mg tRA and 0.3 mg tRA, respectively (Fig. 2a, $p < 0.05$). While tRA decreased embryo dry mass on embryonic day 18, no difference between tRA concentrations was observed (Fig. 3a, $p > 0.05$). No differences occurred over any point studied between control- and sham-treated embryos.

Heart mass

A significant interaction occurred between treatment and development for both wet and dry heart mass (Figs. 2b, 3b, respectively, $p < 0.0001$). Neither heart wet mass nor dry mass was affected by either concentration of tRA or sham from day 12 to 14. Treatment with 0.3 mg tRA produced a significant decrease in heart wet mass (138 ± 6 mg) compared to control (172 ± 5 mg) and sham treatment (163 ± 6 mg) on day 16 (Fig. 2b, $p < 0.05$), with no differences between tRA treatments (Fig. 2b, $p > 0.05$). Heart dry mass was reduced by treatment of 0.1 mg tRA (148 ± 6 mg) compared to other treatments on day 16 (Fig. 3b, $p < 0.05$). On day 18, both 0.1 mg tRA (162 ± 7 mg) and 0.3 mg tRA (149 ± 5 mg) treatment, although not different from each other, produced a

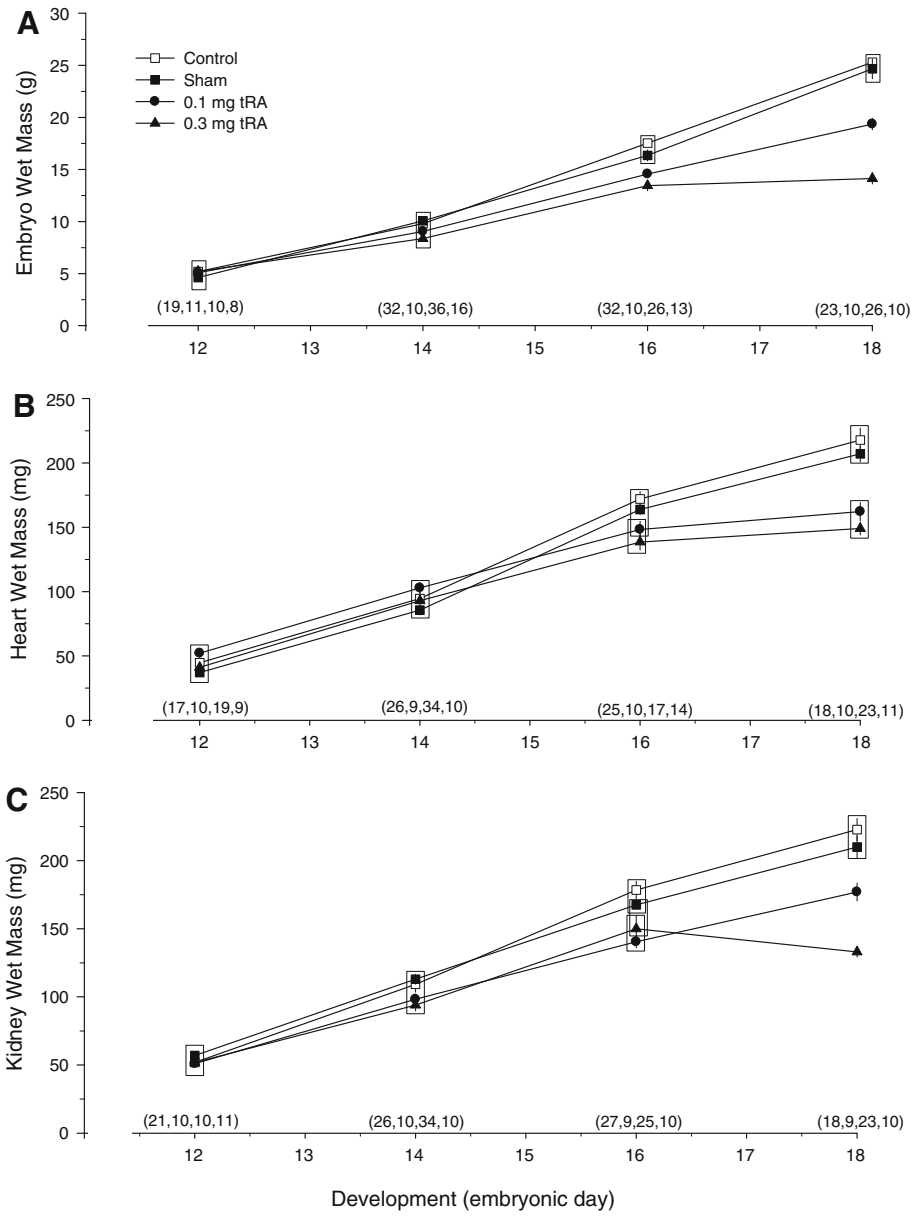

Fig. 2 Embryo and organ wet masses of control-, sham- and tRA-treated embryos. Wet mass of **a** whole embryo (g), **b** heart (mg), and **c** kidney (mg) measured at 12, 14, 16, and 18 days of development in the embryonic chicken. Statistically similar means are grouped within *boxes*. Sample sizes are given in *parenthesis* at the bottom of the plot in the following order: control embryos, sham embryos, embryos dosed with 0.1 mg tRA, embryos dosed with 0.3 mg tRA. Data are presented as mean ± SE

significant decrease in heart wet mass that was 34 % lower than control (217 ± 9 mg), and sham (207 ± 6 mg) (Fig. 2b, $p < 0.05$). Heart dry mass was decreased from control on day 18 ($p < 0.05$), with no concentration differences (Fig. 3b, $p > 0.05$). Sham-treated embryos exhibited lower heart dry mass compared with control embryos on day 18 (Fig. 3b, $p < 0.05$), but exhibited no differences over any time point in heart wet mass (Fig. 2b, $p > 0.05$).

Kidney mass

A significant interaction was seen between treatment and development for both wet and dry kidney mass (Figs. 2c, 3c, respectively, $p < 0.0001$). Kidney wet mass showed no difference from day 12 to 14. On day 16, both kidney wet and dry mass was reduced ($p < 0.05$) in embryos exposed to 0.1 mg tRA when compared to control embryos. However, kidney wet mass was not different from 0.3 mg tRA-treated embryos (Fig. 2c, $p > 0.05$). Kidney wet mass showed a dose-dependent response to tRA on day 18, with kidney wet mass reduced by 26 and 69 % from control upon exposure to 0.1 mg tRA and 0.3 mg tRA, respectively (Fig. 2c, $p < 0.05$). Kidney dry mass was reduced on day 16 in embryos treated with 0.1 mg tRA compared to control embryos (Fig. 3c, $p < 0.05$). No differences between control- and sham-treated embryos were observed over any point in development studied (Figs. 2c, 3c, respectively, $p > 0.05$).

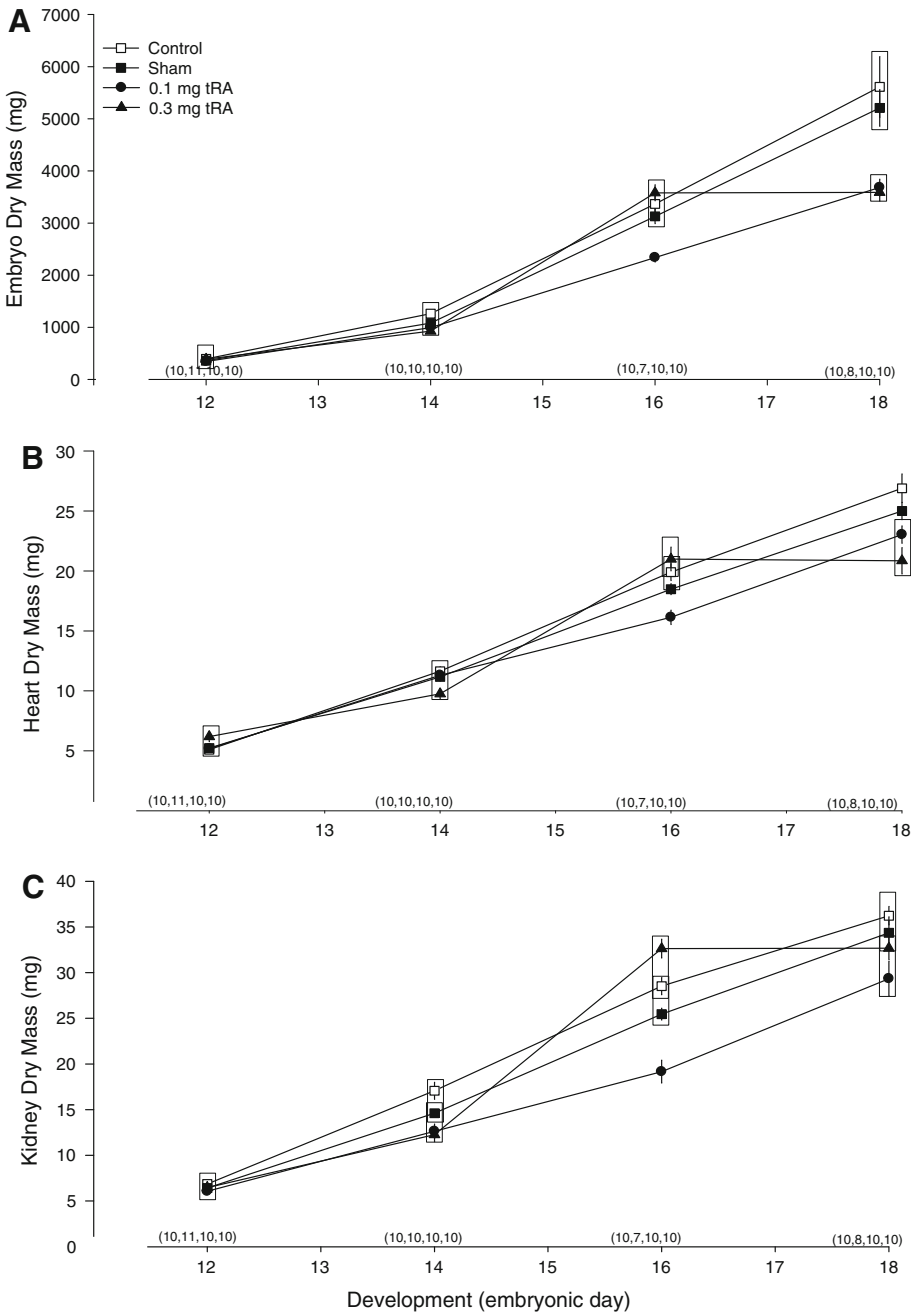

Fig. 3 Embryo and organ dry masses of control-, sham- and tRA-treated embryos. Dry mass of **a** whole embryo (mg), **b** heart (mg), and **c** kidney (mg) measured at 12, 14, 16, and 18 days of development in the embryonic chicken. Statistically similar means are grouped within *boxes*. Sample sizes are given in *parenthesis* as described in Fig. 2. Data are presented as mean ± SE

Yolk mass

On day 14, yolk wet mass was significantly reduced ($p < 0.05$) in embryos exposed to 0.3 mg tRA (9.7 ± 0.7 g) compared to sham embryos (11.4 ± 0.8 g), while no difference ($p > 0.05$) was observed between sham and embryos exposed to 0.1 mg tRA (Fig. 4a). The same trend was observed in yolk dry mass on day 14 with embryos injected with 0.3 mg tRA exhibiting decreased yolk dry mass (4.7 ± 0.3 g) when compared to sham embryos (5.5 ± 0.5 g) (Fig. 4b, $p < 0.05$). No other differences in wet or dry yolk mass were observed either between treatments or across the developmental time period studied.

Oxygen consumption

Control embryos showed a decrease in mass-specific oxygen consumption on day 18 when compared to days 14 or 16 ($p < 0.05$), with no difference between days 14 and 16 (Fig. 5, $p > 0.05$). A significant interaction was seen between day and treatment ($p < 0.001$). Embryos treated with 0.1 mg tRA exhibited a decrease in mass-specific oxygen consumption from day 14 (21.2 ± 0.3 μL^{-1} g^{-1} min^{-1}) to

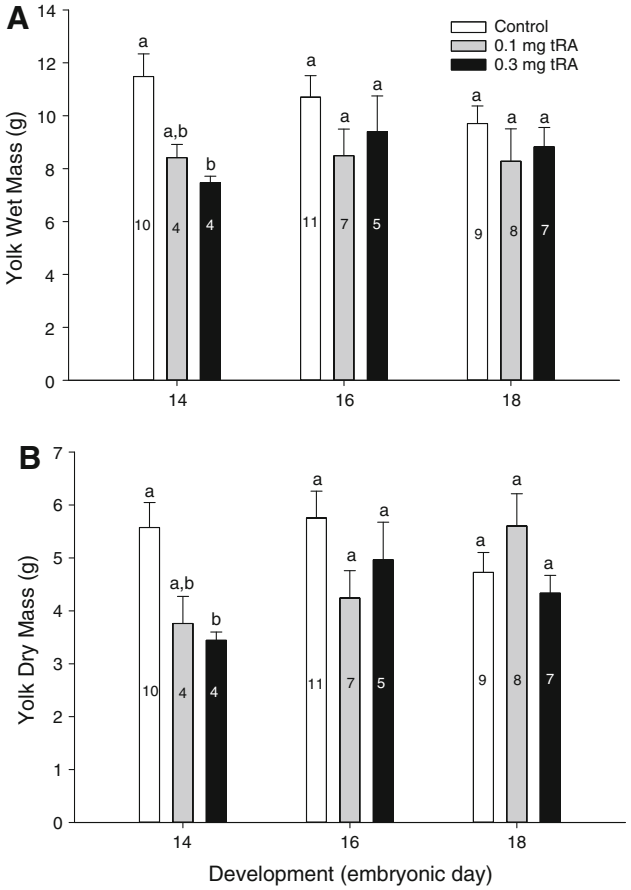

Fig. 4 Yolk masses of sham- and tRA-treated embryos. **a** Yolk wet mass (g) and **b** yolk dry mass (g) measured at 14, 16, and 18 days of development in the embryonic chicken. *Letters* indicate significance between treatments. Sample sizes are given within *each bar*. Data are presented as mean ± SE

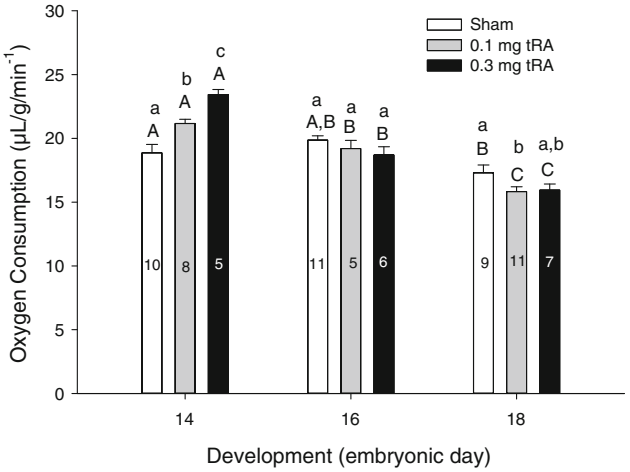

Fig. 5 Mass specific oxygen consumption (μL^{-1} g^{-1} min^{-1}) measured at 14, 16, and 18 days in sham and tRA-treated embryos. *Capital letters* indicate significance over development, while *small letters* indicate significance between treatments. Sample sizes are given within *each bar*. Data are presented as mean ± SE

day 18 (15.8 ± 0.4 μL^{-1} g^{-1} min^{-1}) (Fig. 5, $p < 0.05$), while embryos treated with 0.3 mg tRA showed a decrease from day 14 (23.4 ± 0.4 μL^{-1} g^{-1} min^{-1}) to day 18 (15.9 ± 0.5 μL^{-1} g^{-1} min^{-1}) (Fig. 5, $p < 0.05$). On day 14, tRA treatment increased mass-specific oxygen consumption over sham embryos 12 % in embryos injected with 0.1 mg and 24 % in embryos injected with 0.3 mg tRA (Fig. 5, $p < 0.05$).

Blood plasma osmolality

Blood plasma osmolality did not vary significantly ($p > 0.05$) over days 12–18 of development, remaining at ~268–270 (mmol L^{-1}) (Fig. 6). A significant ($p < 0.0001$) interaction occurred between treatment and development for blood plasma osmolality. Sham treatment had no significant effect on blood plasma osmolality at any point during development (Fig. 6, $p > 0.05$).

A dose of 0.3 mg tRA consistently and significantly ($p < 0.05$) reduced blood plasma osmolality compared to control over the entire developmental period (Fig. 6). A dose of 0.1 mg tRA transiently reduced blood plasma osmolality on day 12 and again on day 18 (Fig. 6, $p < 0.05$).

Allantoic fluid osmolalities

Allantoic fluid osmolality in control embryos decreased significantly ($p < 0.05$) between day 12 (229 ± 3 mmol L^{-1}) and day 18 (175 ± 4 mmol L^{-1}), with the steepest decrease occurring from day 14 (224 ± 4 mmol L^{-1}) to day 16 (186 ± 5 mmol L^{-1}) (Fig. 7a). A significant interaction occurred between treatment and development for allantoic fluid osmolality ($p < 0.0001$). Injection alone had no influence on allantoic fluid osmolality as no difference occurred between control and sham treatment over any point in development.

No difference in allantoic fluid osmolality was observed between control and tRA treatments from day 12 to day 14, with osmolality ranging from 210–230 mmol L^{-1} (Fig. 7a). However, on day 16, embryos treated with either 0.1 mg or 0.3 mg of tRA showed marked elevations over control values in allantoic fluid osmolality, caused primarily by the failure to decrease sharply at this point in development, a phenomenon that occurred in the untreated and sham populations. On day 18, allantoic fluid osmolality increased ~25 % between tRA treated (220 ± 6 mmol L^{-1}) and control (176 ± 4 mmol L^{-1}) embryos with no significant differences between tRA concentrations (Fig. 7a, $p < 0.05$).

Amniotic fluid osmolalities

Amniotic fluid osmolality remained relatively constant between 250 and 265 mmol L^{-1} over development ($p > 0.05$). The only significant difference induced by tRA

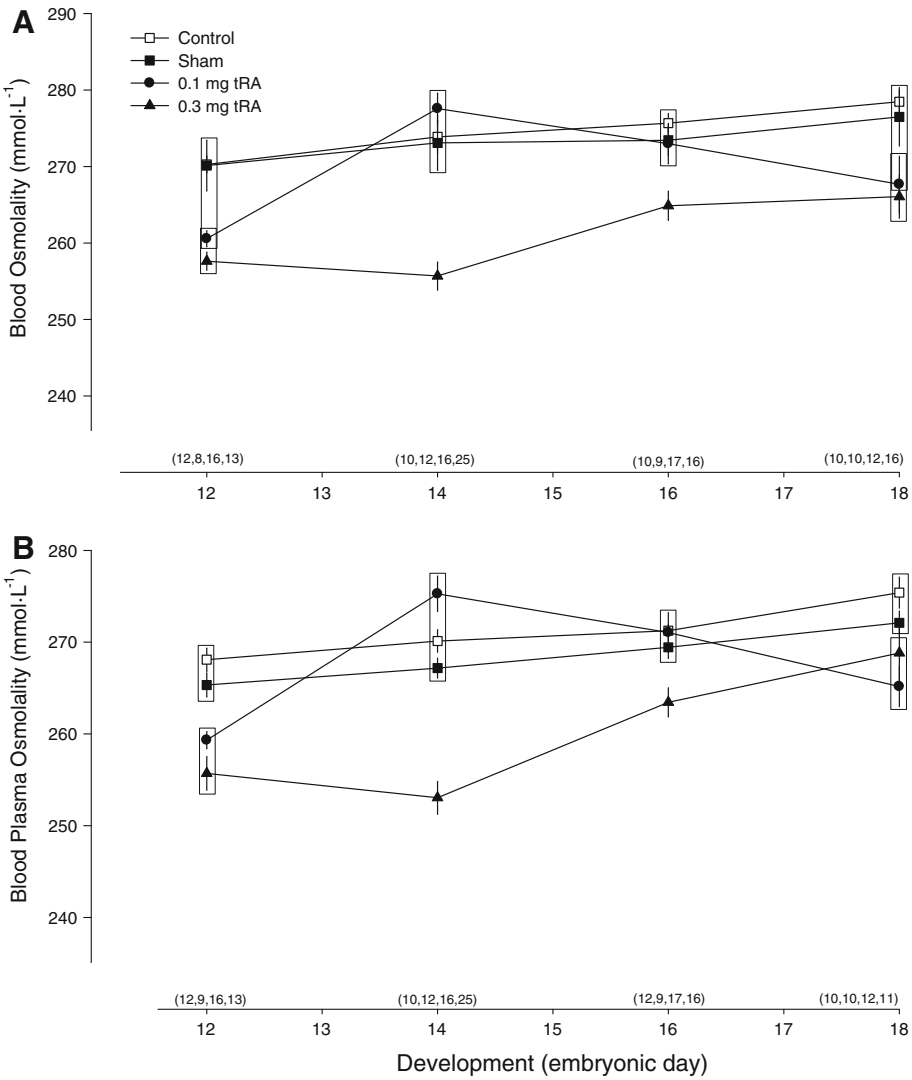

Fig. 6 Osmolality (mmol L^{-1}) of blood plasma measured at 12, 14, 16, and 18 days of development in control, sham- and tRA-treated embryos. Statistically similar means are grouped within *boxes*. Sample sizes are given in *parenthesis* as described in Fig. 2. Data are presented as mean ± SE

treatment occurred on day 16, when amniotic fluid osmolality decreased nearly 20 mmol L^{-1} at the highest dose of tRA. However, this difference was short lived since no difference existed on day 18 (Fig. 7b).

Plasma ion concentrations

Control embryo blood plasma [Na^+] remained constant over development, averaging 194 ± 3 mmol L^{-1} from day 12 to day 18 (Fig. 8a, $p > 0.05$). No significant difference was seen on any developmental day between control- and sham-treated embryos. On day 12, [Na^+] in embryos treated with 0.1 mg tRA (181 ± 3 mmol·L^{-1}) and 0.3 mg tRA (168 ± 1 mmol L^{-1}) had significantly reduced blood plasma [Na^+] compared with control embryos (197 ± 2 mmol L^{-1}) (Fig. 8a, $p < 0.05$), changes from control of 9 and 17 % for 0.1 and 0.3 mg, respectively. From day 14 to day 18, embryos treated with 0.1 mg tRA showed reduced blood plasma [Na^+] compared to control, but these values did not differ significantly from sham-treated embryos. On the other hand, embryos treated with 0.3 mg tRA showed markedly reduced blood plasma [Na^+] from control not only on day 12, but also over the entire developmental period studied. Average blood plasma [Na^+] was 194 ± 3 mmol L^{-1} for control embryos, while embryos treated with 0.3 mg tRA averaged 165 ± 2 mmol L^{-1} blood plasma [Na^+], a 19 % decrease over development (Fig. 8a).

Blood plasma potassium concentration [K^+] remained constant over development (Fig. 9a, $p > 0.05$). Control embryos showed an average blood plasma [K^+] of 2.5 ± 0.2 mmol L^{-1} from day 12 to day 18 (Fig. 9a). No significant difference was seen at any point in development between control, sham and treatment groups.

Allantoic fluid ion concentrations

Allantoic fluid [Na^+] in control embryos was steady at ~155 mmol L^{-1} from 12–14 days of development, but

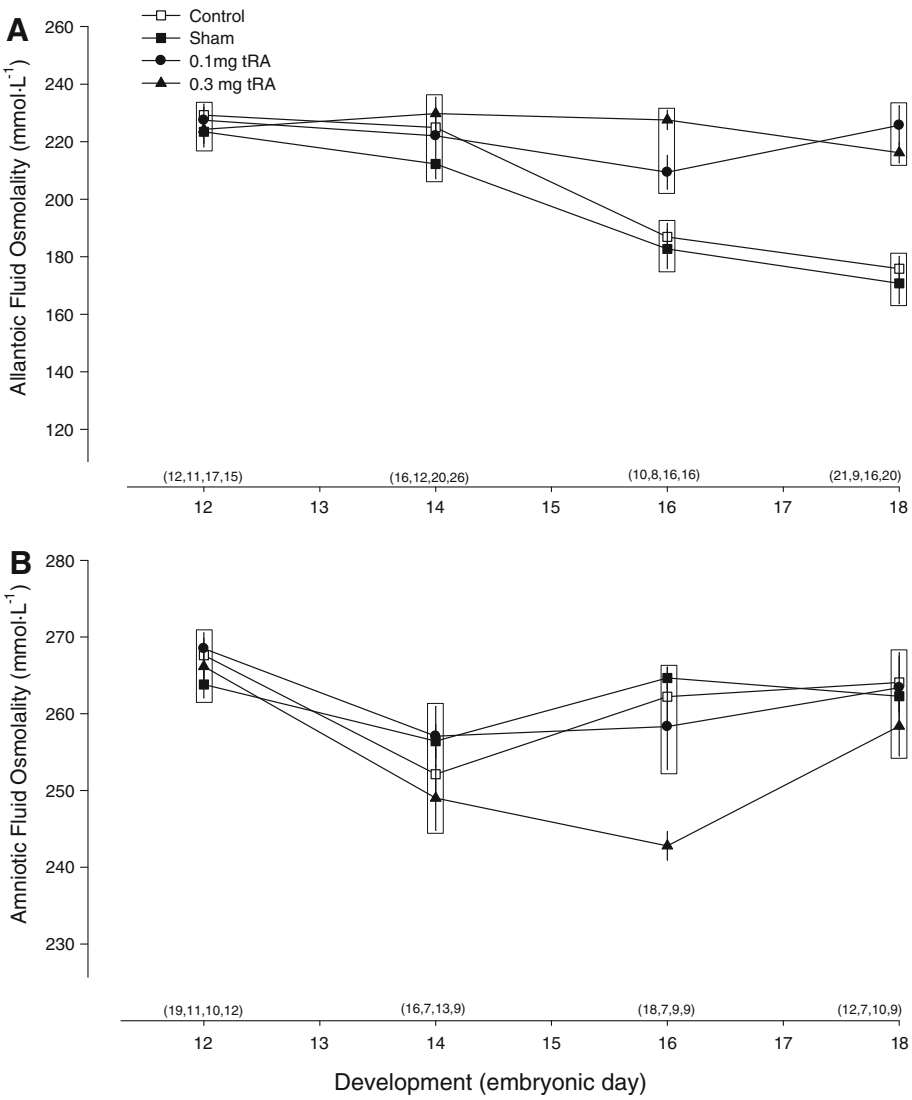

Fig. 7 Osmolality (mmol L^{-1}) of (**a**) allantoic and (**b**) amniotic fluid measured at 12, 14, 16, and 18 days of development in control-, sham- and tRA-treated embryos. Statistically similar means are grouped within *boxes*. Sample sizes are given in *parentheses* as described in Fig. 2. Data are presented as mean ± SE

then decreased significantly ($p < 0.05$) from 154 ± 5 mmol L^{-1} at day 12 down to 69 ± 4 mmol L^{-1} at day 18 (Fig. 8b).

No difference was seen from day 12 to day 14 among any groups. Sham-treated embryos showed no difference from control embryos with the exception of day 18, when sham-treated embryos showed a slightly reduced allantoic fluid [Na^+] (45 ± 10 mmol L^{-1}) compared to control embryos (69 ± 4 mmol L^{-1}). However, a significant interaction occurred between treatment and development for allantoic fluid [Na^+] (Fig. 8b, $p < 0.05$). Day 16 embryos treated with 0.1 mg tRA (156 ± 3 mmol L^{-1}) showed increased allantoic fluid [Na^+] compared to control embryos (94 ± 16 mmol L^{-1}) (Fig. 8b, $p < 0.05$). No difference was seen between concentrations of tRA. On day 18, allantoic fluid [Na^+] in embryos treated with 0.1 mg tRA (141 ± 5 mmol L^{-1}) and 0.3 mg tRA (124 ± 5 mmol L^{-1}), while not different from one another, was elevated compared to control (69 ± 4 mmol L^{-1}) (Fig. 8b, $p < 0.05$).

Allantoic fluid [K^+] increased significantly over development, with day 18 (23.8 ± 2.3 mmol L^{-1}) being higher compared to day 12 (6.6 ± 1.9 mmol L^{-1}) in control embryos (Fig. 9b, $p < 0.05$). A significant interaction was seen between treatment and development for allantoic fluid [K^+] (Fig. 9b, $p < 0.05$). No difference was seen between control- and sham-treated embryos at any point during the study period. Allantoic fluid [K^+] was not altered with tRA from day 12 to day 14. On day 16, embryos dosed with 0.1 mg tRA (13.8 ± 2.9 mmol L^{-1}) and 0.3 mg tRA (6.1 ± 1.3 mmol L^{-1}) showed markedly lower allantoic fluid [K^+] compared to control (23.1 ± 1.9 mmol L^{-1}) (Fig. 9b, $p < 0.05$). These large differences were not sustained because no difference was seen among any treatment on day 18 (Fig. 9b, $p > 0.05$).

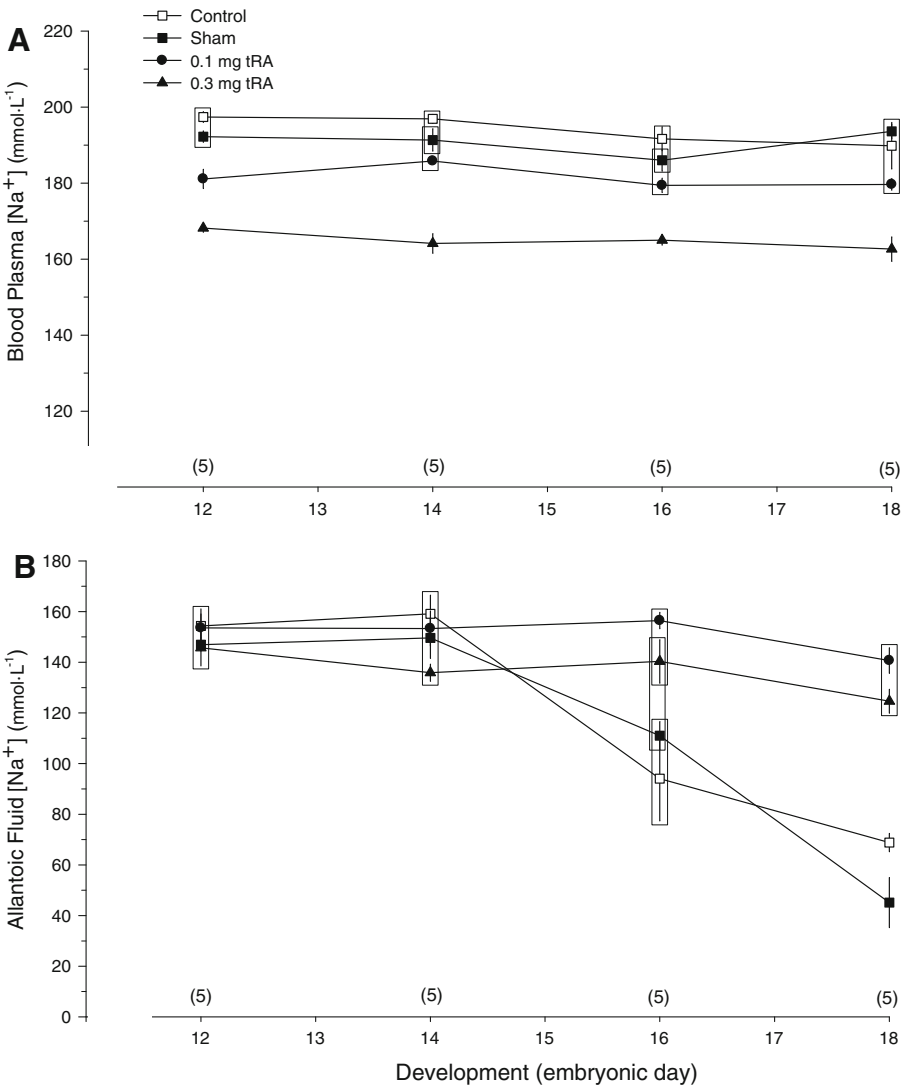

Fig. 8 [Na^+] (mmol L^{-1}) in (**a**) blood plasma and (**b**) allantoic fluid measured at 12, 14, 16, and 18 days of development in control-, sham- and tRA-treated embryos. Statistically similar means are grouped within *boxes*. Sample sizes are given in *parentheses* as described in Fig. 2. Data are presented as mean ± SE

Hematology

Hematocrit in control embryos increased significantly over development from 21 ± 6 % on day 12 to 36 ± 1 % on day 18 (Fig. 10a, $p < 0.05$). Similarly, red blood cell count increased significantly over development from 1.32 ± 0.03 × 10^6 μL^{-1} on day 12 to 2.28 ± 0.6 × 10^6 μL^{-1} on day 18 (Fig. 10b, $p < 0.05$). Neither red blood cell count nor Hct differed between control and sham treatment at any point during development.

Red blood cell count as well as Hct was elevated above control in embryos treated with 0.1 mg tRA on day 12, while this dose of tRA produced no significant differences when compared to control after day 12 for both red blood cell count (Fig. 10b) and Hct (Fig. 10a, $p > 0.05$). Treatment with 0.3 mg tRA consistently elevated red blood cell count over the course of development compared to control, with a mean average difference of 27 % (1.80 ± 0.04 vs. 2.26 ± 0.08 × 10^6 μL^{-1}) (Fig. 10b). Similarly, Hct was elevated in embryos treated with 0.3 mg tRA from days 12 to 16 (26 ± 2 vs. 34 ± 2 %). However, these differences did not continue to day 18 (Fig. 10a).

Control hemoglobin (Hb, g dL^{-1}) concentration increased slightly over development with day 18 (10.6 ± 0.2 g dL^{-1}) being significantly higher compared to day 12 (6.5 ± 0.2 g dL^{-1}) (Fig. 10c, $p > 0.05$). A significant interaction was seen between treatment and development ($p < 0.0001$). No difference was observed between control and sham Hb concentration at any point during development ($p > 0.05$). On day 12, both tRA treatments produced an elevated Hb concentration compared to control, although no difference was seen between the two tRA concentrations. From day 14 to day 18, treatment with 0.3 mg tRA produced elevated Hb concentrations averaging 30 % with the largest difference between control (8.7 ± 0.2 g dL^{-1}) and 0.3 mg tRA (12.1 ± 0.4 g dL^{-1}) on day 16 (Fig. 10c). Finally, an increased Hb concentration was observed on day 18 when embryos were treated with 0.1 mg tRA (11.8 ± 0.7 g dL^{-1})

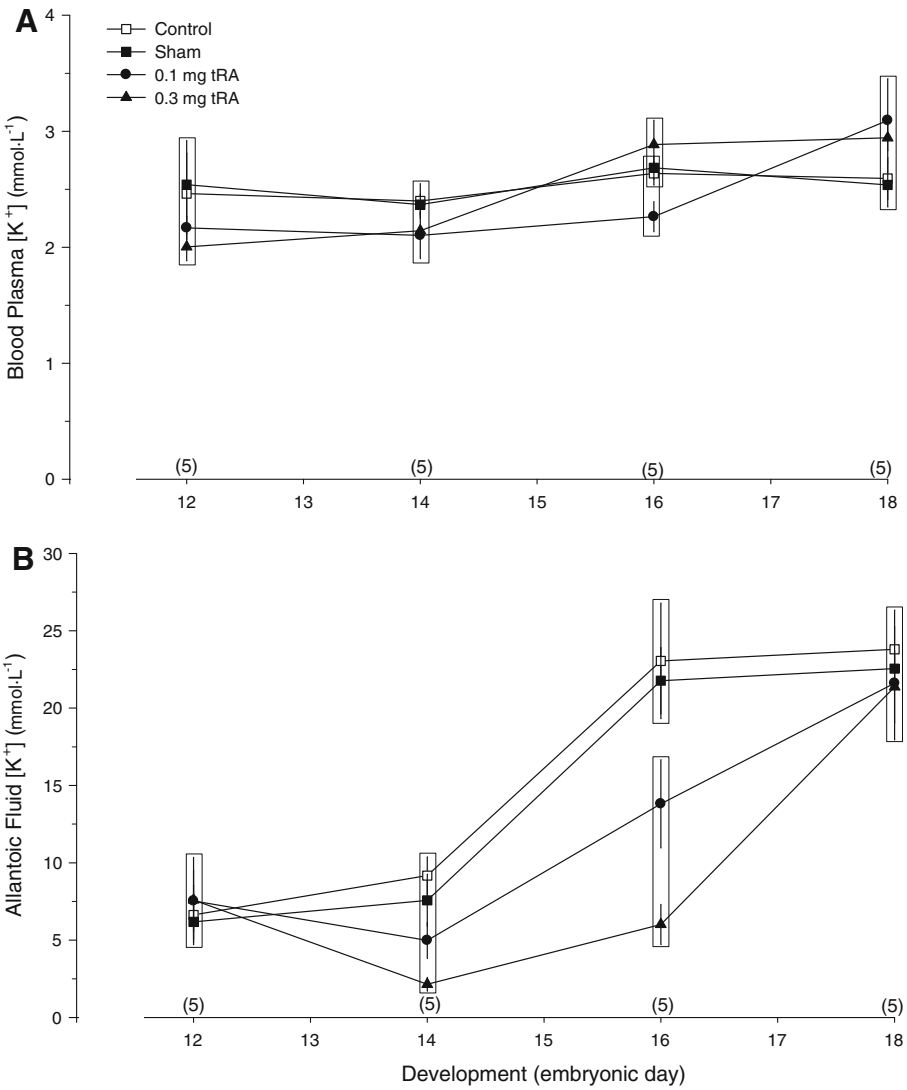

Fig. 9 $[K^+]$ (mmol L^{-1}) in (**a**) blood plasma and (**b**) allantoic fluid measured at 12, 14, 16, and 18 days of development in control-, sham- and tRA-treated embryos. Statistically similar means are grouped within *boxes*. Sample sizes are given in *parentheses* as described in Fig. 2. Data are presented as mean ± SE

compared to control (10.6 ± 0.2 g dL^{-1}) (Fig. 10c, $p > 0.05$).

Blood pH and bicarbonate

Blood pH decreased significantly ($p < 0.05$) from 7.72 ± 0.01 on day 12 to 7.52 ± 0.02 on day 18 (Fig. 11a). Concurrently, bicarbonate ion concentration $[HCO_3^-]$ (mEq L^{-1}) slightly increased over development with day 18 (25 ± 1 mEq L^{-1}) significantly higher compared to day 12 (13 ± 2 mEq L^{-1}) (Fig. 11b, $p < 0.05$). No significant differences in blood pH or $[HCO_3^-]$ were observed between control and sham treatment at any point in development (Fig. 11a and b respectively).

Treatment with 0.1 mg and 0.3 mg tRA significantly lowered $p < 0.05$ blood pH in day 12 embryos to a pH 7.69 ± 0.01 and 7.52 ± 0.01, respectively, compared to values of 7.72 ± 0.01 for controls and 7.73 ± 0.1 for sham (Fig. 11a). On day 16, 0.1 mg tRA produced a significant decrease in blood pH when compared to control. No differences in blood pH were observed on day 18 between any groups (Fig. 11a, $p > 0.05$). Blood $[HCO_3^-]$ only differed on day 12 with embryos treated with 0.3 mg tRA (25.2 ± 1.3 mEq L^{-1}) exhibiting higher $[HCO_3^-]$ compared to control (12.7 ± 1.1 mEq L^{-1}) (Fig. 11b, $p < 0.05$).

Discussion

This study examined the effects of exogenous administration of trans-retinoic acid (tRA), on the developing chicken embryo, specifically attempting to target a potential critical window in metanephric kidney development. Overall, the findings demonstrate that exogenous administration of tRA can modify embryonic development, kidney development, and alter the hydromineral balance during the later third of embryonic development.

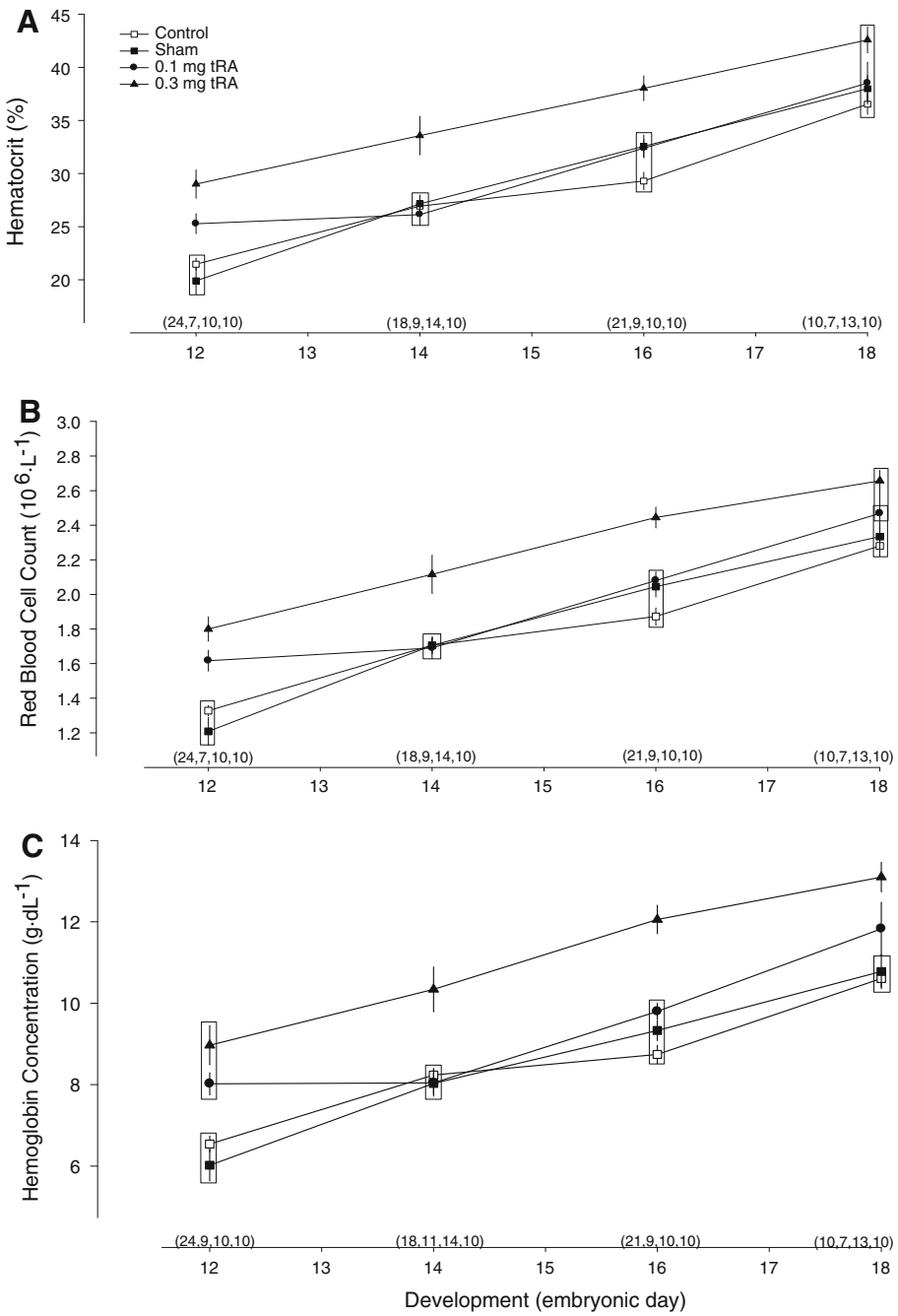

Fig. 10 Hematological variables measured at 12, 14, 16, and 18 days of development in control-, sham- and tRA-treated embryos. **a** Hematocrit (%), **b** red blood cell count (10^6 μL^{-1}), and **c** blood hemoglobin (Hb, g dL^{-1}). Statistically similar means are grouped within *boxes*. Sample sizes are given in parentheses as described in Fig. 2. Data are presented as mean ± SE

Embryo mortality

Mortality of control- and sham-treated embryos tripled in embryos injected with 0.3 mg tRA (Fig. 1). While cause of mortality was not determined in the current study, the increase in mortality measured on day 10 in the higher tRA-treated group, can be attributed to the increased tRA concentration, and not the mode of injection, as neither the sham or 0.1 mg tRA-treated embryos exhibited elevated mortality when compared with control mortality.

Embryo and organ growth

Early in development, tRA acts as a morphogen influencing early vertebrate organogenesis and cell differentiation (Means and Gudas 1995). Retinoic acid is important during early embryogenesis (De Luca 1991). To date, no such study has examined how retinoic acid influences growth and development later in the incubation period for avian embryos. In the current study, as anticipated, both embryo wet and dry masses increased throughout development with all organ masses studied, being significantly larger later in

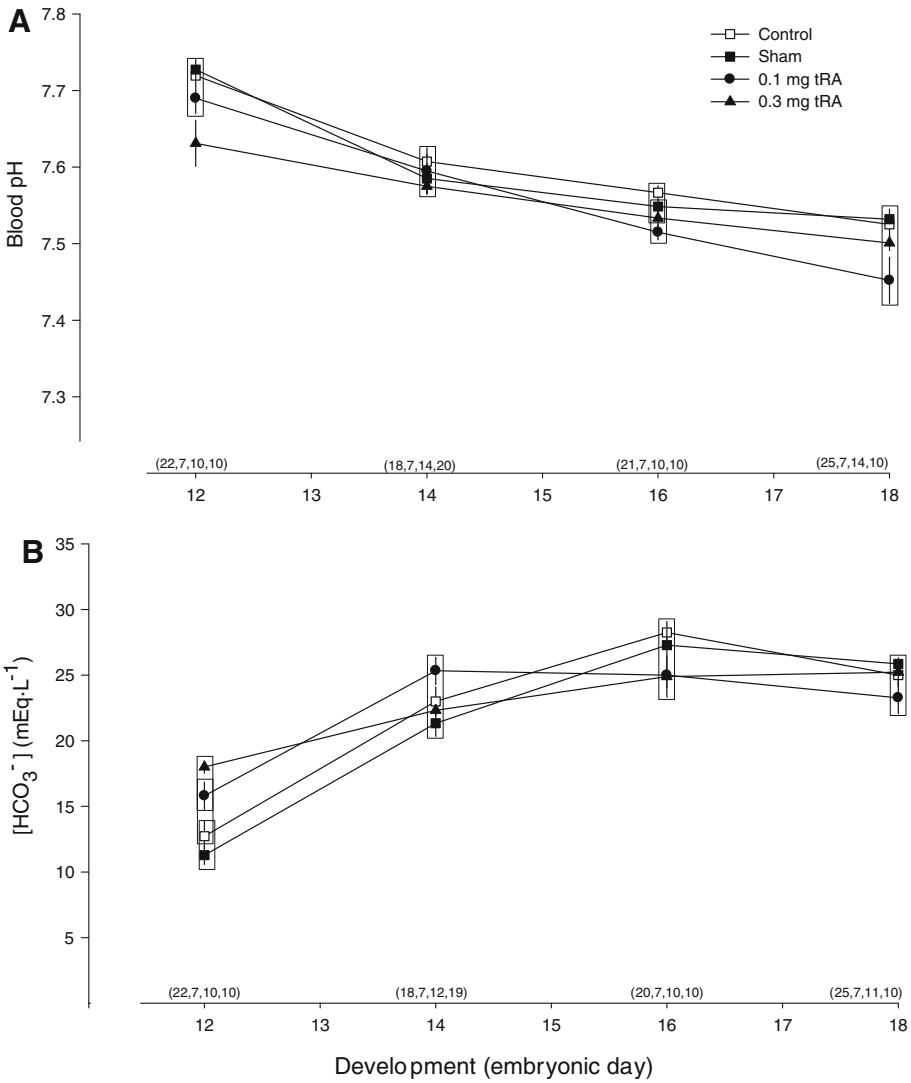

Fig. 11 **a** Blood pH and **b** bicarbonate ion (HCO_3^-) concentration (mEq L^{-1}) measured at 12, 14, 16, and 18 days of development in control-, sham- and tRA-treated embryos. Statistically similar means are grouped within *boxes*. Sample sizes are given in *parentheses* as described in Fig. 2. Data are presented as mean ± SE

development (day 18) compared to early in development (day 12) (Figs. 2, 3). Measured mass data and growth trends in the current study are consistent with other studies examining embryo and organ growth over the later third of development (e.g., Dzialowski et al. 2002; Black and Burggren 2004a; Chan and Burggren 2005). The greatest increase in mass for all organs, including embryo mass, appeared to occur early in the developmental timeframe we examined (days 12–16). During this time period, the large increases in organ mass may be a function of increased organogenesis (organ mass increase) and cell proliferation. Later in development (day 18), organ maturation could be occurring as organ and embryo mass increase occurred at a slower rate when compared to days 12–16.

Embryos exposed to tRA appeared to exhibit a delayed growth trajectory, especially at the end of the developmental time frame studied (Figs. 2, 3). Interestingly, it appears that embryo and kidney wet mass were more sensitive to exogenous administration of tRA compared to dry mass as evident from dose-dependent decreases in both embryo and kidney wet mass on day 18 (Fig. 2a, c).

Yolk mass

Metabolic rate, and potentially yolk sac utilization, is elevated for a short period after tRA injection as the embryo continues to develop, the embryo may face a shortage of nutrients necessary for normal growth as the development progresses. Yolk mass data suggests that embryos exposed to tRA exhibit smaller yolk sacs on day 14, but no differences are observed as development progresses (Fig. 4a, b). Embryos that have undergone surgical removal of the pituitary gland (hypophysectomy) fail to reabsorb extra embryonic compartments, including the yolk sac and its associated nutrients (Woods et al. 1971; Murphy et al. 1982). While embryos exposed to tRA exhibited lower yolk mass on day 14, this trend does not continue to day 18, suggesting that the rate of yolk sac reabsorption may be

decreased in tRA-treated embryos. At present, it is unclear to what degree, if any; the content of the yolk compartment was altered upon exogenous exposure to tRA. As a consequence of impaired metabolic rate, the water content (Betz 1975), and lipid and protein content of the yolk compartment have shown to be altered (Betz 1967). Examining the contents of the yolk compartment is a necessary step to address if administration of exogenous tRA may have altered the hydration state of the embryo, as indicated by a dose-dependent decrease in embryo wet mass, but not embryo dry mass, or directly influenced the contents of the yolk compartment.

Oxygen consumption

Oxygen consumption determined in the current study compare with reported values for embryos incubated and measured under similar conditions at days 14, 16, and 18 (Black and Burggren 2004a). Oxygen consumption exhibited a dose-dependent increase on day 14 in embryos dosed with 0.1 and 0.3 mg tRA when compared with sham embryos (Fig. 5). The general trend for decreasing mass-specific oxygen consumption was observed in all three groups, with oxygen consumption rates on day 14 being higher when compared to the rate of oxygen consumption on day 18 (Fig. 5). Currently, it is unclear how tRA would increase the rate of oxygen consumption, but if embryos are only exposed to tRA over a short window following injection on day 8, metabolic rate may be temporarily elevated but returns to control levels 8 days after tRA exposure (day 16).

Fluid osmolalities

Blood plasma osmolality remained relatively constant over normal development in both control- and sham-treated embryos (Fig. 6). These values in the current study of $\sim$275 $mOsmol^{-1}$ l are consistent with those reported in embryonic chickens under the same incubation conditions and developmental time period (Hoyt 1979; Davis et al. 1988; Braun 1999; Andrewartha et al. 2011; Burggren et al. 2012; Bolin and Burggren 2013). Blood plasma osmolality was significantly reduced in embryos treated with the highest dose of tRA (0.3 mg tRA) compared to both control and sham treatment. Allantoic fluid osmolality decreased over development in both the control- and sham-treated embryos with the lowest osmolality observed on day 18 (Fig. 7a). This decrease in allantoic fluid osmolality across normal chicken embryo development results from sodium and chloride movement across the allantois into the blood (Stewart and Terepka 1969). Consequently, the allantoic fluid osmolality becomes progressively more hyposmotic over development compared to whole blood. On the other hand, embryos treated with tRA exhibited persistently high allantoic fluid osmolality towards the end of incubation at day 16 and day 18 (Fig. 7a), suggesting that the normal movement of sodium and chloride across the allantois into the blood compartment may have been disturbed by developmental disruptions induced by tRA treatment.

Fluid in the amniotic compartment accumulates during development as a result of chloride being actively pumped into this compartment with water passively following by osmosis (Murphy et al. 1991). Amniotic fluid osmolality remained largely constant over development in control- and sham-treated embryos in our study (Fig. 7b). Embryos treated with 0.1 mg tRA also maintained relatively stable amniotic fluid osmolality over development (not different from control at any day). However, 0.3 mg tRA injection produced a significant decrease in amniotic fluid osmolality on day 16. This was the only day during the study in which amniotic fluid was affected by treatment with tRA (Fig. 7b). At the present time it is unclear as to why a transient decrease in amniotic fluid osmolality was observed in embryos treated with the higher dose of tRA.

Fluid ion concentrations

Plasma [Na^+] very slightly declined over development in both the control and sham treatment, as also reported by Murphy et al. (1982). Our measured plasma Na^+ values for embryos ($\approx$170–195 mmol L^{-1} (Fig. 8a) are higher than values previously reported for chicken embryos ($\sim$110–140 mmol L^{-1}—Howard 1957; Stewart and Terepka 1969; Murphy et al. 1982, 1986), though they are closer to the range of 150–175 reported for adult chickens (e.g., Koike et al. 1979; Árnason et al. 1986; Simaraks et al. 2004).

Allantoic fluid [Na^+] decreased over development in both the control and sham treatments (Fig. 8b). While our reported values are slightly higher when compared to the literature, the trend in the current study is consistent with other studies that demonstrate a decrease in allantoic fluid [Na^+] over development (Stewart and Terepka 1969; Murphy et al. 1982). The volume of the allantoic fluid reaches its maximum on days 12–13 of development, and thereafter declines. This reabsorption of sodium and chloride occurs against large concentration gradients for each ion (Stewart and Terepka 1969; Murphy et al. 1982), as well as against an electrical driving force for sodium (Graves et al. 1984).

Interestingly, allantoic fluid [Na^+] in embryos treated with tRA was significantly elevated from control on days 16 and 18 of embryonic development—this effect was dose-independent (Fig. 8b). Between days 12 and 18, the allantoic epithelium appears to transport sodium actively,

generating the short circuit current (SSC) source across the epithelium (Graves et al. 1986). Furthermore, as the allantoic epithelium matures, the SCC (essentially, active sodium transport) increases substantially. This serves as an important function of the allantoic sac in the late-stage embryo, namely, the reabsorption of a hyperosmotic fluid into the embryo's circulation (Stewart and Terepka 1969; Murphy et al. 1982; Graves et al. 1986). Perhaps embryos dosed with tRA are unable to actively pump Na^+ out of the allantoic fluid. If this is the case, the driving force that normally powers a net absorption from the allantoic sac into the embryonic circulation may be altered.

The composition of the allantoic fluid over development is a product of the gut, chorioallantoic membrane, and the kidneys. Exogenous administration of tRA could have altered the metanephric kidney sodium–potassium–ATPase activity. The embryonic kidney has been shown to conserve Ca^{2+}, Na^+, and sulfates as early as day 9, and continues with the maturation of the metanephric kidney as development progresses (Clark et al. 1993). Salt loading experiments demonstrate that $^{24}Na^+$- labeled administered to the amniotic fluid of 7-day-old chick embryos moves to the embryonic blood and passes through the embryonic kidney into the allantoic fluid (Zemanová and Babický 1990). Both mesonephros and metanephros demonstrate sodium–potassium–ATPase activity at 16.5 days of embryonic development with lower ATPase activity in the metanephros (Doneen and Smith 1982). Further analysis is required to determine if exogenous administration of tRA can modify the sodium–potassium–ATPase activity in the developing kidney. Blood plasma $[K^+]$ in the present experiment was 2.0– $\sim$2.5 $mmol^{-1}$ l across development, which is equivalent to or slightly lower than previously reported values for chicken embryos between days 9 and 17 of development (Howard 1957; Stewart and Terepka, 1969). Plasma $[K^+]$ in the present study was largely unaffected by tRA treatment (Fig. 9a). Allantoic fluid $[K^+]$ increased from day 14 to day 16 and then plateaued for the remainder of the study period (Fig. 9b), reflecting a similar allantoic fluid concentration and developmental pattern of change as reported by Stewart and Terepka (1969). Treatment with tRA decreased allantoic fluid $[K^+]$ on day 16 when compared to control. However no difference was seen between tRA doses. On day 18, no difference existed between control and treatments.

Collectively, then, these data suggest that Na^+ is the major ion contributing to both blood plasma and allantoic fluid osmolality. Elevated allantoic fluid $[Na^+]$ in tRA-treated embryos, as well as decreased blood plasma osmolality, indicates that sodium may be unable to move out of the allantoic fluid into the blood compartment as the development progresses. The allantoic membrane possesses a sodium pump (Moriarty and Terpeka 1969; Moriarty and Hogben 1970; Kyriakides and Simkiss 1975) that facilitates the movement of sodium from the allantoic fluid to the blood plasma. Although ion contents of the amniotic fluid were not determined, it is likely that the mechanisms that regulated ion and fluid movement were not affected in embryos treated with tRA, thus not altering the amniotic osmolality much over development. Importantly, fluid volume was not measured in any of the compartments; therefore, a change in fluid volume might contribute to the observed changes in compartment osmolality and ion composition.

Hematology

Values obtained in the current study for hematocrit, red blood cell count and hemoglobin concentration align well with previously published hematological values (Tazawa 1980; Dzialowski et al. 2002; Black and Burggren 2004b; Khorrami et al. 2008; Tazawa et al. 2012). Hematocrit and red blood cell count were both significantly elevated from day 12 to day 16 in embryos treated with 0.3 mg tRA (Fig. 10a, b). Collectively, these data suggest that the Hct increase was from the addition of new RBCs rather than from an increase in mean corpuscular volume. In addition, 0.1 mg tRA elevated both red blood cell count and hematocrit on day 12, but not on any later day studied. The exact mechanism by which tRA influenced both the hematocrit and red blood cell count was not assessed in the current study. However, oral administration of retinoic acid to vitamin A depleted rats elevated the erythropoietin serum concentration as a result of the accumulation of erythropoietin mRNA (Okano et al. 1994). Embryos treated with 0.3 mg of tRA had elevated hemoglobin concentration over the course of development (Fig. 10c). Elevated hemoglobin concentration would also lend support for the rise in hematocrit, in addition to the increased red blood cell count. Thus, it appears that tRA has some erythrocyte-stimulating properties that could produce an increased hematocrit through stimulation of red blood cells in tRA-treated embryos. Further studies are required to tease apart the possible mechanisms that would cause the results seen in this current study.

Blood pH and $[HCO_3^-]$

Embryonic components of acid base balance, specifically pH and $[HCO_3]$, were maintained for the duration of development (Fig. 11a, b). The developing embryo most likely does not rely on renal compensation to prevent acid base imbalances as the renal system (metanephric development) continues to develop after hatching (Romanoff 1960; Carretero et al. 1995). The extent to which the normal contribution of the kidney maintains acid–base balance

in the developing chicken embryo requires far more investigation. However, the current experiments have demonstrated the potential of tRA to disrupt, albeit modestly, blood pH in the developing embryo, and may subsequently prove to be an important tool in subsequent investigations.

Summary

The current study evaluated the extent to which exogenous administration of tRA disrupts the kidney development, assessed through disruptions in fluid compartment homeostasis, and whole embryo development. Embryos exposed to exogenous tRA appeared to be developmentally delayed when compared to control and sham embryos as indicated by the dose-dependent decrease in whole embryo and kidney wet mass on day 18. In addition, metabolic rate, and yolk utilization was altered upon exposure to tRA, although this difference manifested itself early (day 14), in contrast to the reduction in mass (day 16–18). Altered renal function was inferred from decreased plasma [Na^+] and elevated allantoic [Na^+] in experimental embryos. Further studies are needed to clarify if the disruption in internal homeostasis is a product of impaired renal function, or a secondary consequence of impaired metabolism or delayed rate of development. Since the metanephric kidney continues to develop post-hatching, and eventually becomes the adult kidney, future studies should be extended into the post-hatching period, perhaps even giving insights in the phenomenon of fetal programming.

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