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Egg yolk environment differentially influences physiological and morphological development of broiler and layer chicken embryos

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SUMMARY

Maternal effects are important in epigenetic determination of offspring phenotypes during all life stages. In the chicken (*Gallus gallus domesticus*), transgenerational transfer of egg yolk factors may set the stage for morphological and physiological phenotypic differences observed among breeds. To investigate the effect of breed-specific yolk composition on embryonic broiler and layer chicken phenotypes, we employed an *ex ovo*, xenobiotic technique that allowed the transfer of broiler and layer chicken embryos from their natural yolks to novel yolk environments. Embryonic day two broiler embryos developing on broiler yolk culture medium (YCM) had significantly higher heart rates than layer embryos developing on layer YCM (176±7 beats min^{-1} and 147±7 beats min^{-1}, respectively). Broiler embryos developing on layer YCM exhibited heart rates typical of layer embryos developing normally on layer YCM. However, layer embryo heart rates were not affected by development on broiler YCM. Unlike O_2 consumption, development rate and body mass of embryos were significantly affected by exposure to different yolk types, with both broiler and layer embryos displaying traits that reflected yolk source rather than embryo genotype. Analysis of hormone concentrations of broiler and layer egg yolks revealed that testosterone concentrations were higher in broiler yolk (4.63±2.02 pg mg^{-1} *vs* 3.32±1.92 pg mg^{-1}), whereas triiodothyronine concentrations were higher in layer yolk (1.05±0.18 pg mg^{-1} *vs* 0.46±0.22 pg mg^{-1}). Thus, a complex synergistic effect of breed-specific genotype and yolk environment exists early in chicken development, with yolk thyroid hormone and yolk testosterone as potential mediators of the physiological and morphological effects.

Key words: maternal effect, yolk hormone, chicken breed, epigenetics.

INTRODUCTION

Variation in physiological phenotype between animal populations has long been of interest to comparative physiologists (e.g. Bennett, 1987; Peterson et al., 1998; Williams, 2008) and continues to shape comparative developmental physiology (e.g. Peterson et al., 1998; Gibbs, 1999; Nijhout, 2003; Spicer and Burggren, 2003; Burggren and Warburton, 2005; Nikinmaa and Waser, 2007; Spicer and Rundle, 2007). Numerous animal models are available, but the chicken embryo, because of both its venerable history in developmental biology and extensive artificial selection for commercially profitable traits in domesticated breeds, is an excellent model for the study of phenotypic variation between animal populations. Part of the appeal of the model is that the poultry industry rigorously promotes two chicken breeds for different commercial purposes. The layer chicken is selected for prolific egg production, and the broiler chicken is selected for rapid growth for meat production. As an unfortunate consequence of their extremely rapid muscle growth, juvenile and adult broilers are predisposed to developing pulmonary hypertension, a condition that results when the energy demands of muscle tissue exceeds the capabilities of the cardiovascular system to supply adequate amounts of oxygen to these tissues (Olkowski, 2007). To compensate for muscle hypoxemia, the juvenile and adult broiler chicken cardiovascular system must perform at a higher capacity than that of the layer chicken to deliver sufficient oxygen to relatively under-perfused muscle tissue.

Interestingly, the phenotypic differences between the two chicken breeds arise as early as the first few days of embryonic development (Clum et al., 1995; Burggren et al., 2004; Boerjan, 2004), potentially setting the stage for the breed-specific differences observed in late-stage embryonic (Sato et al., 2006; Everaert et al., 2008), juvenile (Martinez-Lemus et al., 1999; Latimer and Brisbin, 1987; Odom et al., 2004) and adult (Martinez-Lemus et al., 1998; Koenen et al., 2002; Schreurs et al., 1995; McRae et al., 2006) phenotypes. Broiler embryos have faster overall development rates than layer embryos, which is first evident at 48 h of development (Boerjan, 2004; Everaert et al., 2008). Also, embryonic body mass differs significantly between layers and broilers throughout incubation, with differences becoming most apparent towards the end of egg incubation (Pal et al., 2002). This body mass difference persists post-hatch and well into adulthood, with broilers having masses up to four times greater than that of layers at 42 days post-hatch. More specifically, cardiovascular development of these two chicken breeds differs significantly early in embryonic development. At 40 h of incubation, broiler embryos show cardiac ventricular hypertrophy as compared with layer embryos (Boerjan, 2004). Also, heart rates of broiler embryos are higher than those of layer embryos, with the difference continuing well into the second day of post-hatch life (Yoneta et al., 2006; Yoneta et al., 2007).

Maternal effects have been acknowledged to be a major player in evolutionary dynamics by modulating offspring fitness *via* changes in early life traits (Bernardo, 1996; Mousseau and Fox, 1998; McAdam et al., 2002; Reinhold, 2002; Räsänen and Kruuk,

2007), and may serve to explain the breed-specific differences observed between broiler and layer chickens. Genetic differences between broiler and layer chicken will directly contribute to the phenotypic differences observed in embryos, juveniles and adults (Dunnington, 1994; Wong et al., 2004); however, maternally provided egg factors may also play a critically important role in determining the breed-specific traits of the offspring. The deposition of hormones, immunological factors (carotenoids and antibodies) and nutrients of maternal origin into the egg during ovulation in oviparous species influence offspring morphology, physiology and behavior in an epigenetic fashion (Groothuis and Schwabl, 2008; Ho and Burggren, 2010). Two maternally produced yolk hormones that have been intensely studied for their effects on offspring phenotype are thyroid hormones and testosterone. In birds, a decrease in yolk thyroid hormone results in smaller offspring (McNabb, 1998), whereas modulation of yolk testosterone and carotenoid levels contributes to altered embryonic and postnatal growth (Schwabl, 1996; Eising et al., 2001) and offspring immune function (Muller et al., 2005; Biard et al., 2007; Cucco et al., 2008). Additionally, thyroid hormones and testosterone have tachycardic effects, and can influence cardiovascular function by directly acting upon cardiomyocytes (Moussavi et al., 1985; Slotkin et al., 1992; Goldman-Johnson et al., 2008). The aforementioned hormones vary significantly among bird breeds and species, making them potential effectors of inter-breed variation in chickens.

Against this backdrop, we hypothesized that breed-specific embryonic phenotypes partly result from differences in maternally produced egg yolk factors, such as thyroid hormones and testosterone. To test this hypothesis, we utilized an *ex ovo*, xenobiotic technique that allowed complete alteration of the yolk environment of intact chicken embryos during a critical 48 h period in early development. The body mass, overall developmental stage, heart rate (f_H) and mass-specific oxygen consumption ($\dot{V}_{O_2}$) of layer and broiler chicken embryos that developed within the context of breed-specific yolk environments were investigated. Also, we quantified levels of triiodothyronine (T_3) and testosterone (TE) in the yolks of broiler and layer eggs to determine whether these physiologically relevant egg factors potentially contribute to the growth, development and cardiac chronotropic differences observed between broiler and layer chickens.

MATERIALS AND METHODS

Animals

White Leghorn layer and Cornish Rocks broiler chicken (*Gallus gallus domesticus* Linnaeus 1758) eggs were obtained from Texas A&M University (College Station, TX, USA), Ideal Poultry Breeding Farms, Inc. (Cameron, TX, USA) and Moyer's Chicks (Quakertown, PA, USA). Upon arrival, eggs were stored at room temperature (23–26°C) until the start of incubation. All eggs were incubated in commercial incubators (Circulated Air Hova-Bator Incubator, Model No. 1590, Savannah, GA, USA) at 37±0.5°C and 58±2% humidity. Eggs were automatically turned every hour during *in ovo* incubation.

Embryo cultures

Embryo cultures were prepared as described by Chapman et al. (Chapman et al., 2001). Embryos were removed from the yolk sac by first removing albumen from the area surrounding the embryo. A Whatman filter paper ring [outer diameter (o.d.)=38 mm, inner diameter (i.d.)=15 mm] was then positioned on the vitelline membranes so that the embryo was centrally framed by the ring, and the surrounding membranes tightly adhered to the filter paper ring. Embryos [~36 h old; Hamburger and Hamilton stage (HH) 9–14] and associated vitelline membranes were excised from the yolk by cutting the membrane along the outer edge of the filter paper ring. Embryos were washed free of residual yolk and albumen using 1× Tyrode's solution supplemented with penicillin and streptomycin. The embryo and associated vitelline membranes was placed on a second filter paper ring (o.d.=38 mm, i.d.=30 mm) so as to sandwich the vitelline membranes between the two filter paper rings. The entire embryo/filter paper unit was then placed onto prepared Parafilm culture dishes.

Parafilm culture dishes were prepared by covering the lids of 35 mm Petri dishes with Parafilm, and completely filling them with yolk culture medium (YCM) through a square opening (2×2 cm) cut out of the Parafilm. YCM consisted of one part fresh, homogenized yolk from layer or broiler eggs, and one part 1× Tyrode's solution (50:50 v/v) supplemented with 5 units penicillin–streptomycin ml^{-1} (Sigma T1788, St Louis, MO, USA). Embryos were cultured dorsal side down, and in direct contact with the YCM at the opening in the Parafilm dish.

Parafilm embryo cultures were placed into a seal-top plastic chamber (170×80 mm) fitted with inflow and outflow ports on the lid. Seven embryo cultures were placed in a single chamber. Chambers were flushed for 20 min with a gas mixture of 95% O_2 and 5% CO_2 (Wösthoff Gas Mixer, Calibrated Instruments, Inc., NY, USA). After flushing the chamber ports were sealed, and the chamber was maintained in a 37.5±0.2°C, clear-top Styrofoam incubator (Circulated Air Hova-Bator Incubator, Model No. 1590). Humidity was maintained by placing three wet Kimwipes® at the bottom of the chambers. The start of experiments was considered to be 1.5 h after the chambers had been sealed and placed in incubators.

A high-oxygen atmosphere (95% O_2, 5% CO_2) was used for the cultures in this study to prevent acidification of culture medium and increase survivability and proper development of cultured embryos (Susan C. Chapman, personal communication; Clemson University, Clemson, SC, USA). Heart rates and development of embryos incubated under this atmosphere were similar to those of windowed embryos incubated under normoxic conditions (Romanoff, 1960; Hamburger and Hamilton 1951). The actual percentage O_2 in the chambers declined over the period of Parafilm-culture incubation of the embryos. Initial readings were 90±5% O_2, decreasing to 47±8% O_2 after 24 h of incubation in a cohort of five chambers tested. The potential confounding factor of rate of O_2 decline of chambers was addressed by evenly distributing experimental culture groups among chambers.

There were four different types of cultures: broiler embryos on broiler YCM, layer embryos on layer YCM, broiler embryos on layer YCM, and layer embryos on broiler YCM.

Radioimmunoassays

Triiodothyronine

Fresh, preincubated broiler (N=3) and layer (N=4) chicken eggs were opened and yolks were separated from the surrounding albumen, homogenized and stored at –20°C for subsequent assays for yolk T_3. The methanol–chloroform extraction procedure of Wilson and McNabb was slightly modified to increase extraction efficiency (Wilson and McNabb, 1997). 0.5 g of homogenized yolk was placed in a 13 ml glass conical tube with 2 ml of methanol (supplemented with 1 mmol l^{-1} propylthiouracil; PTU), and vortexed for ~5 min to ensure a homogenous suspension. Radiolabeled T_3 (~74 Bq of [^{125}I]T_3; MP Biomedicals, New York, NY, USA) was added to each sample to estimate extraction efficiency. Subsequently, samples were

shaken (150 oscillations per minute on a shaker) for 10 min, and centrifuged for 10 min at 1184 ***g***. The supernatant was decanted into a 13 ml glass conical tube and the precipitate was resuspended in 1 ml methanol (1 mmol l^{-1} PTU), shaken, spun and decanted in another 13 ml glass conical tube. 5 ml of chloroform and 0.5 ml 2 mol l^{-1} NH_4OH was added to each tube, and the tubes were shaken and centrifuged. The upper phase in the tubes was removed and combined in a 13 ml glass tube. The suspension was dried overnight under a stream of filtered N_2. The dried samples were resuspended in 1 ml 2 mol l^{-1} NH_4OH, shaken, centrifuged and decanted into a 13 ml tube. Then 1 ml of chloroform was added to each tube, and the tubes were shaken, centrifuged, and the upper phase was removed and dried under a stream of filtered N_2. The samples were then resuspended in 150 µl phosphate-buffered saline (supplemented with Alizarin) and counted with a gamma counter (Beckman Gamma 5500 Liquid Scintillation Counter, Brea, CA, USA). The extraction efficiency was calculated for each sample by calculating the percentage of radioactivity that remained after the extraction procedure.

After counting for the extraction efficiency, the T_3 concentration of the resuspended sample (from the last step of extraction above) was analyzed using a competitive binding radioimmunoassay kit (Free T_3 Antibody Coated Tube-125I RIA Kit, MP Biomedicals). The manufacturer's instructions for the use of the hormone kit were followed exactly. To evaluate binding properties, yolks from the same strain were pooled and a serial dilution was completed prior to extraction (1.0–0.125 g yolk). The slopes of the dilution curve and the standard curve were equivalent (P>0.9).

Testosterone

Fresh, preincubated broiler (N=12) and layer (N=12) chicken eggs were opened and yolks were separated from the surrounding albumen, homogenized and stored at –20°C for subsequent assays for yolk TE. TE concentrations in collected yolk samples were determined using radioimmunoassay following the protocol for steroid hormones in egg yolks established by Schwabl (Schwabl, 1993) and modified for use with broiler and layer chicken yolks. The protocol was modified by using a phosphate-buffered saline containing yolk stripped of hormones [modified from Wingfield et al. (Wingfield et al., 1984)] as the background buffer for the standard curves as described by Boonstra et al. (Boonstra et al., 2009). In brief, samples were first diluted by adding 30 mg of egg yolk in 1 ml of double distilled H_2O and a small amount of [^{3}H]TE (~74 Bq) to estimate extraction efficiency. The samples were incubated overnight (4°C) and then 4 ml petroleum ether:diethyl ether (30:70 v/v) was added to samples, vortexed for 1 min, the aqueous phase was snap-frozen and the ether phase decanted into a 12 ml test tube. The ether extraction was repeated twice more and the three ether phases were combined and dried under a stream of N_2. Samples were resuspended in 90% ethanol and incubated at –20°C overnight. Then 2 ml of hexane was added to the ethanol, the samples were vortexed gently and the lower phase collected. The hexane wash was repeated and both ethanol phases were combined and centrifuged at 650 ***g*** for 5 min. The liquid was decanted into a 7 ml test tube and dried under a stream of N_2. These samples were resuspended in 550 µl phosphate-buffered saline, and incubated at 4°C overnight. Duplicate samples were analyzed in a single radioimmunoassay (Schwabl, 1993). TE antibody was obtained from Esoterix Laboratory Sciences, Inc. (T3-125, Carlsbad, CA, USA). Dihydrotestosterone (20% cross reactive) and delta-1-testosterone (52% cross reactive) cross react significantly with the antibody (Esoterix Laboratory Sciences). To evaluate binding properties, yolks were pooled and a serial dilution was completed prior to extraction (200 mg yolk to 12.5 mg yolk). The slopes of the dilution curve and the standard curve were equivalent (P>0.9).

Wet and dry body mass

Live embryos were anesthetized by exposure to –20°C for 4 min. To obtain wet body mass, embryos were dissected from surrounding vitelline membranes in 1× Tyrode's solution, blotted with Whatman filter paper for 20 s, and weighed (mg) using a microbalance. Dry embryo mass was obtained by placing embryos into a 60°C oven for 48 h prior to weighing. Embryo wet mass was assessed at 24 h and 48 h after the start of the experiment, and dry mass was assessed at the end of 48 h only.

Developmental staging

Embryonic development was determined using HH staging at the time of Parafilm embryo culture and then again at 12, 24 and 48 h after transfer to culture dishes. Immediately before embryos (HH9–HH14) were placed on Parafilm culture dishes, they were staged based on somite number using light microscopy. Staging after 12, 24 and 48 h of incubation was primarily based on the degree of spinal curvature (≥HH14) because embryos could not be magnified to assess somite number or limb development. For example, forebrain and hindbrain forming a 45 deg angle was indicative of HH14 embryos, whereas HH15 embryos were characterized by a 67.5 deg curvature of the forebrain and hindbrain.

Heart rate

The heart beat of embryos maintained at 37.5±0.5°C was counted by direct observation over the course of 1 min at 12, 24 and 48 h after the start of the experiments. f_H is reported as beats min^{-1}. Owing to high f_H variation per embryo, f_H for each embryo was an average of three successive counts.

Mass-specific oxygen consumption

Oxygen consumption ($\dot{V}_{O_2}$) of embryos was assessed by closed-system respirometry (N=42). Metabolic respirometers were constructed from 118 ml glass jars with metal screw-top lids. The bottom of the respirometer was filled with polyester casting resin to reduce the respirometer air volume to 27 ml. The lid of the respirometer was fitted with a syringe port constructed from the hub of a 25 gauge needle, allowing for the attachment of a metal stopcock and a 5 ml glass syringe (Proper MFG Co., Inc., New York, NY, USA) for air sampling. Individual embryo cultures were placed in the center of each respirometer, and the lids were sealed to the mouth of the respirometer with the aid of petroleum jelly. The respirometers were then placed into an acrylic incubator (Model: RL 1&2, Lyon Electric Company, Inc., Chula Vista, CA, USA) set at 37.5±2°C, and allowed to equilibrate for 30 min with the syringe port open for free exchange of air between the respirometer and the ambient air.

After 30 min, a three-way metal stopcock attached to a 5 ml glass syringe was inserted into the syringe port of the lid, and a 1.5 ml sample of air was drawn from the respirometer into the syringe and injected into a flow-through O_2 electrode (Model 16-730 Microelectrodes, Inc., Bedford, NH, USA) to obtain the initial oxygen partial pressure (P_{O_2}) of gas in the respirometer. The voltage output of the electrode was recorded and converted into partial pressure (mmHg) by Chart software (Power Lab: Chart 5, ADInstruments, Colorado Springs, CO, USA). The stopcock and syringe were returned to the port of the respirometer, and the

respirometers were sealed by setting the stopcock to prevent passage of air between the respirometer and the syringe. After 60 min, 1.5 ml of respirometer gas was drawn into the syringe and injected into the flow-through O_2 electrode to obtain the final P_{O_2} value.

Mass-specific oxygen consumption ($\dot{V}_{O_2}$; in ml O_2 g^{-1} h^{-1}) of transfer embryos was calculated using the following equation:

$$\dot{V}_{O_2} = \frac{\Delta P_{O_2} \cdot v}{\Delta t \cdot m},$$

where ΔP_{O_2} is the change in P_{O_2} of the chamber gas (mmHg) over the time interval, Δt, between readings (h), v is the total volume of air in the respirometer (ml), and m is the wet mass (g) of the embryo. Total gas volume was determined for each metabolic respirometer by subtracting the mass of the empty respirometer from the mass of the chamber filled with distilled water. An additional 5 g was subtracted from the final weight to account for the volume displaced by the Parafilm culture dish, assuming that 1 g of distilled water occupied a volume of 1 ml. Respirometers containing yolk culture dishes without embryos were sampled during each trial run to account for non-biological fluctuations in oxygen concentration of respirometer gas. Mass-specific $\dot{V}_{O_2}$ of embryos was adjusted accordingly.

Mass-specific $\dot{V}_{O_2}$ was assessed at 12, 24 and 48 h after the start of the experiment in order to capture a full range of developmental stages and body masses for each experimental group.

The masses of preincubation egg components

Egg components include egg yolk, albumen and shell, with total egg mass being the sum of these components. Masses of egg components were obtained by first weighing the intact egg using a balance (Denver Instrument Company, Bohemia, NY, USA), and then separating yolk and shell components for weighing. Yolks were individually placed in plastic dishes and cleaned of residual albumen using Kimwipes®. Shells were washed of residual albumen and allowed to dry for at least 24 h before weighing. Because significant amounts of albumen were permanently lost in the process of separating egg components, albumen mass was obtained by subtracting shell and yolk mass from total egg mass. 58 layer chicken eggs and 32 broiler chicken eggs were used to obtain masses.

Statistical analyses

Changes in wet and dry body mass, heart rate and yolk hormone concentrations were assessed using either factorial two-way or three-way analysis of variance (ANOVA) with respect to embryo type, egg yolk type and time, depending on the number of contributing factors, followed by Tukey's *post hoc* comparisons (SigmaStat v3.5, Systat, Inc.). Because the design of ANOVA analyses do not permit *post hoc* comparison between embryos cultured on their native yolk (broiler embryos cultured on broiler YCM and layer embryos cultured on layer YCM) and between broiler embryos cultured on layer YCM and layer embryos cultured on broiler YCM, additional ANOVAs with respect to group (defined by embryo type and yolk type) and time were performed. HH stage data were considered to be non-discrete, thus non-parametric two-way ANOVAs and subsequent Kruskal–Wallis *post hoc* analyses were used to detect statistical significance among experimental groups. Yolk hormone concentrations were normalized by logarithmic transformations prior to statistical analyses. Data were transformed back for graphical presentation.

Embryonic mass-specific $\dot{V}_{O_2}$ was analyzed using linear regression analyses. Comparison of group regression slopes and elevations was performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

The relationship between egg component mass (yolk, albumen or shell) and total egg mass was assessed using linear regression analysis. Additionally, the allometry of egg component mass to egg mass was investigated by regression of the logarithm of the component mass of each egg on the logarithm of egg mass (Dzialowski and Sotherland, 2004). The slope (b) of the regression line was used to determine whether the relationship was isometric (b=1) or allometric (b<1 or b>1). Regressions were considered isometric when the 95% confidence intervals of the slope included 1, and allometric when not including 1. Isometry indicated a linear relationship between egg component mass and egg mass, whereas allometry indicated a non-linear relationship between the two variables. Slope data were reported as b±95% confidence interval.

RESULTS

Egg component masses

Egg albumen, yolk and shell mass were directly related to total egg mass for broiler and layer breeds (Fig. 1). Although the relationship between the mass of the egg component and total egg mass (represented by b) did not significantly differ between the two breeds for each egg component mass, the elevations (y-intercept) of the regressions significantly differed between broilers and layers for each egg component. Over the range of total egg mass, broiler albumen mass (a) and shell mass (s) were significantly lower than layer albumen mass and shell mass ($F_{a(1,87)}$=31.73, P_a<0.0001; $F_{s(1,87)}$=7.33, P_s=0.008), whereas yolk mass (y) of broiler eggs was greater than that of layer eggs ($F_{y(1,87)}$=75.33, P_y<0.0001). However, total egg mass was not different between broiler and layer eggs [58.47±0.39 g (N=32) and 58.62±1.15 g (N=58), respectively]. Collectively, these data show that although broiler eggs contained

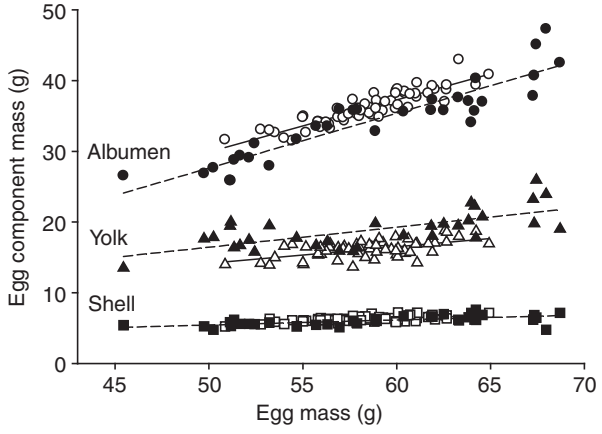

Fig. 1. Egg components mass of broiler (N=32) and layer (N=58) eggs as a function of egg mass. Filled symbols represent broiler egg components and unfilled symbols represent layer egg components. Broken lines (broiler eggs) and solid lines (layer eggs) through data points indicate best fit regression of the mass of each egg component and egg mass. Regression models for layer egg components and egg mass are as follows: M_{yl}=0.24M_{el}+2.43; r^2=0.33; M_{al}=0.74M_{el}–6.97; r^2=0.82; M_{sl}=0.08M_{el}+1.79; r^2=0.31. Regression models for broiler egg components and egg mass are as follows: M_{yb}=0.28M_{eb}+2.21; r^2=0.48; M_{ab}=0.78M_{eb}–11.13; r^2=0.084; M_{sb}=0.07M_{eb}+1.85; r^2=0.41. M_{ab}, broiler albumen mass; M_{al}, layer albumen mass; M_{eb}, broiler egg mass; M_{el}, layer egg mass; M_{sb}, broiler egg shell mass; M_{sl}, layer egg shell mass; M_{yb}, broiler egg yolk mass; M_{yl}, layer egg yolk mass.

more yolk, they also contained less albumen and less shell than layer eggs. This contributed to the lack of difference in total egg mass between the two types of eggs.

Not only is there a difference in egg yolk, albumen and shell mass between the two breeds, but the nature of the total egg mass-dependent change in these egg components is quite different for each component. The comparison of b of the log–log regressions between the mass of each egg component and total egg mass indicated that the relative contribution of yolk mass to total egg mass changed in direct proportion to total mass of broiler eggs (b=0.85±0.16, r^2=0.48) and layer eggs (b=0.85±0.16, r^2=0.33). However, the relative contribution of albumen mass and shell mass to egg mass increased allometrically with egg mass for both broiler (b_a=1.32±0.10, r_a^2=0.85; b_s=0.67±0.15, r_s^2=0.39) and layer eggs (b_a=1.19±0.07, r_a^2=0.82; b_s=0.73±0.14, r_s^2=0.32). Essentially, regardless of breed type, egg yolk mass varied proportionately with total egg mass, whereas the relative contribution of albumen and egg shell mass to the eggs varied with total egg mass.

Body mass and yolk environment

Yolk environment affected layer and broiler embryos to quite different extents. Layer embryos showed significant changes in breed-specific morphology in response to culturing on broiler YCM, but not *vice versa*. As anticipated, embryo wet mass significantly increased from ~12.70±5.10 mg (N=44) to ~22.29±7.75 mg (N=88) over the course of 24 h of development in culture ($F_{(1,131)}$=59.06, P<0.001; Fig. 2A). During this early period of development (60–84 h old), layer embryos cultured on broiler YCM for 24 and 48 h were significantly heavier, in terms of wet mass, than layer embryos cultured on layer YCM (Tukey's, q=3.0, P=0.040, and Tukey's, q=3.08, P=0.03, respectively). However, these embryos were not different from broiler embryos cultured on broiler YCM. After 48 h of development in culture, dry mass of layer embryos showed the same significant trends as wet mass, with layer embryos cultured on broiler YCM having heavier mass than those cultured on layer YCM (Tukey's, q=3.38, P=0.03; Fig. 2B). Yolk type did not significantly alter broiler embryo wet or dry mass at any time during the course of the experiment.

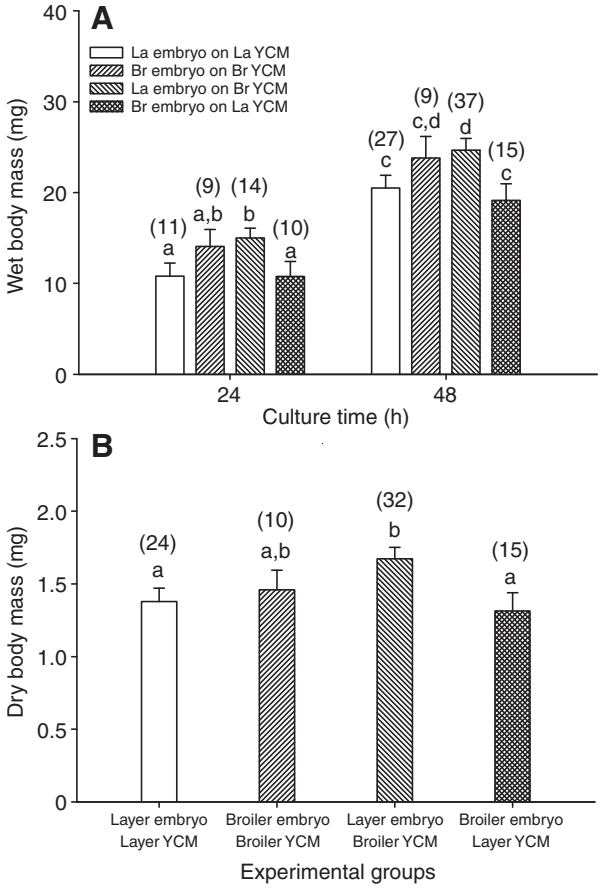

Fig. 2. (A) Wet and (B) dry body mass of broiler (Br) and layer (La) embryos cultured on broiler or layer yolk culture medium (YCM) for a period of 24 and/or 48 h after the start of experiments. Wet body mass was assessed in embryos cultured for 24 and 48 h after the start of experiments, whereas dry body mass was assessed in embryos cultured for 48 h after the start of experiments. Values are means ± s.e.m. (N=9–37; see numbers above the bars); different letters indicate significant difference (P<0.05).

Development rate and yolk environment

At the end of 36 h *in ovo* incubation, broiler embryos were significantly less developed than layer embryos ($F_{(1,130)}$=4.04, P=0.046; Tukey's, q=2.84, P=0.044; Fig. 3). Subsequent random assignment of embryos to experimental groups resulted in a significant difference in developmental stage between layer embryos cultured on broiler YCM and broiler embryos cultured on broiler YCM at the outset of the experiment [layer embryos on broiler YCM (N=75) were more developed than broiler embryos on broiler YCM (N=27); Tukey's, q=2.97, P=0.04]. However, after 12 h incubation on YCM, layer and broiler embryos were at the same developmental stage, and there were no differences among any of the groups (N=7–13). After 24 h of development on YCM, embryo stage was significantly affected by yolk type ($F_{(1,148)}$=5.39, P=0.022), but not embryo type. At this time, layer embryos cultured on layer YCM (N=43) were significantly less developed than layer embryos growing on broiler YCM (N=61; Tukey's, q=3.40, P=0.016). After 48 h from the time of culture, layer embryos generally were more developed than broiler embryos ($F_{(1,77)}$=4.15, P=0.045), but *post hoc* comparisons did not reveal any significant differences between culture groups (N=9–26). Similar to the effects on body mass, breed-specific change in yolk environment significantly altered the development rate of layer embryos, but not that of broiler embryos.

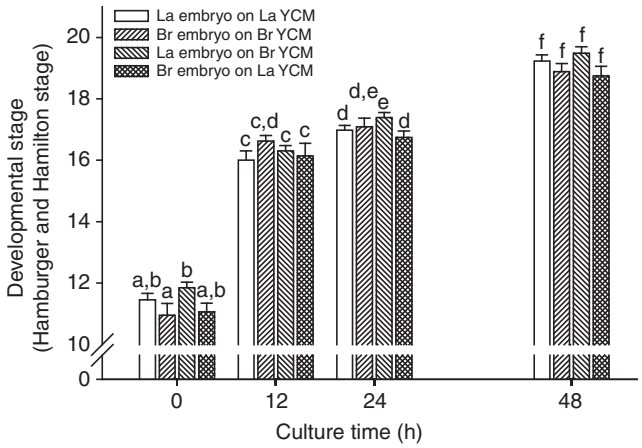

Fig. 3. Developmental stage of broiler (Br) and layer (La) embryos cultured on broiler or layer YCM for a period of 12, 24 and 48 h after the start of experiments. Values are means ± s.e.m. (N=7–75; refer to text for specific sample sizes); different letters indicate significant difference (P<0.05).

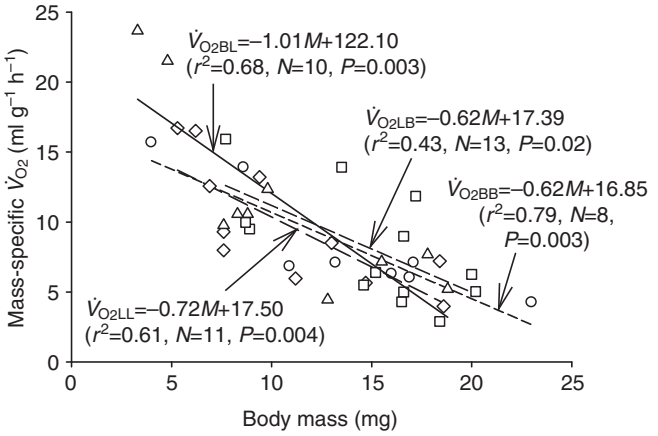

Fig. 4. Mass-specific oxygen consumption of cultured embryos as a function of body mass (M). Group designations [BB (circle), BL (triangle), LB (square) and LL (diamond)] refer to the embryo type and YCM type (B, broiler; L, layer), respectively, for each group.

Mass-specific oxygen consumption and yolk environment

The relationship between mass-specific $\dot{V}_{O_2}$ and wet body mass (mg) of each culture group is shown in Fig. 4. Mass-specific $\dot{V}_{O_2}$ of cultured embryos ranged from ~5 to 24 ml g^{-1} h^{-1} for embryo masses of ~3–23 mg (N=42), and on average, mass-specific $\dot{V}_{O_2}$ decreased two- to threefold over this body mass range. Comparisons of the regression models revealed that neither the slopes nor elevations of the regression lines were significantly different from one another ($F_{(3,34)}$=0.85, P=0.48; $F_{(3,37)}$=0.53, P=0.66, respectively). Thus the relationship between mass-specific $\dot{V}_{O_2}$ and wet body mass (M) of cultured chicken embryos can be expressed as $\dot{V}_{O2}$=–0.75M±18.79 (F=62.184, r^2=0.61, P<0.001).

Heart rate and yolk environment

Three-way ANOVA revealed significant main effects of embryo type ($F_{(1,154)}$=6.80, P=0.01), yolk type ($F_{(1,154)}$=10.35, P=0.002) and time post-culture ($F_{(2,154)}$=13.42, P<0.001) on heart rate, but no interactions among factors. Heart rate increased by ~10–30 beats min^{-1} during the 36 h (culture time 12–48 h) of development in culture (Fig. 5), as expected based on previous studies (see Romanoff, 1960). During this period, broiler embryos showed elevated f_H compared with layer embryos. Mean f_H of layer embryos (La) cultured on their native YCM were significantly lower than mean f_H of broiler embryos (Br) on their native YCM after 12 h [f_{HBr}=176±7 beats min^{-1} (N=6), f_{HLa}=147±7 beats min^{-1} (N=7); Tukey's, q=4.27, P=0.004] and 24 h in culture [f_{HBr}=185±5 beats min^{-1} (N=11), f_{HLa}=167±3 beats min^{-1} (N=31); Tukey's, q=4.22, P=0.004]. However, this difference between broiler and layer f_H was no longer statistically significant at 48 h in culture [f_{HBr}=205±10 beats min^{-1} (N=4), f_{HLa}=187±5 beats min^{-1} (N=10)].

During development on culture dishes, the increase in f_H of broiler embryos cultured on layer YCM was significantly less than that of broiler embryos cultured on broiler YCM. After 24 h of incubation, mean f_H of broiler embryos cultured on broiler YCM (185±5 beats min^{-1}, N=11) was significantly higher than that of all other groups, except for layer embryos cultured on broiler YCM (Tukey's, all P<0.034; Fig. 5). However, after 48 h incubation, mean f_H of broiler embryos on layer YCM and mean f_H of layer embryos on broiler YCM were no longer statistically different, whereas mean f_H of broiler embryos continued to be significantly affected by yolk type (Tukey's, q=3.16, P=0.04). These results indicate that broiler embryonic f_H was more sensitive to a breed-specific change in yolk environment than was layer f_H, but only after 24 h of exposure to the foreign yolk environment.

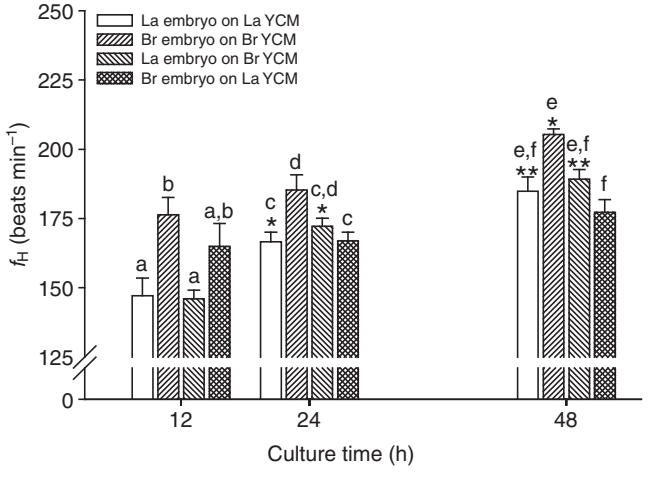

Fig. 5. Mean heart rate of broiler (Br) and layer (La) embryos cultured on broiler or layer YCM for 12, 24 and 48 h. Values are means ± s.e.m. (N=6–41, except BB at 48 h, N=4; and BL at 48 h, N=3). *Significantly different from 12 h mean heart rate within culture groups; **significantly different from 12 and 24 h mean heart rate within culture groups; different letters indicate significant difference between culture group mean heart rates within culture times of 12, 24 or 48 h (P<0.05).

Yolk hormones

An interaction of breed and yolk hormone was detected ($F_{(1,30)}$=10.5, P=0.003), indicating that breed differences in hormone levels varies depending on the specific hormone under investigation. The average yolk T_3 concentration of preincubated broiler chicken eggs (0.46±0.22 pg mg^{-1}; N=3) was significantly lower than the yolk T_3 concentration detected in preincubated layer chicken eggs (1.05±0.18 pg mg^{-1}; N=4; Tukey's, q=3.61, P=0.02; Fig. 6). In contrast, the average yolk TE concentration in broiler eggs (4.63±2.02 pg mg^{-1}; N=12) was significantly higher than the concentrations detected in layer eggs (3.32±1.92 pg mg^{-1}; N=12; Tukey's, q=2.99, P=0.04). Intra-assay variation for yolk TE was 2.4% and for T_3 was 2.0%.

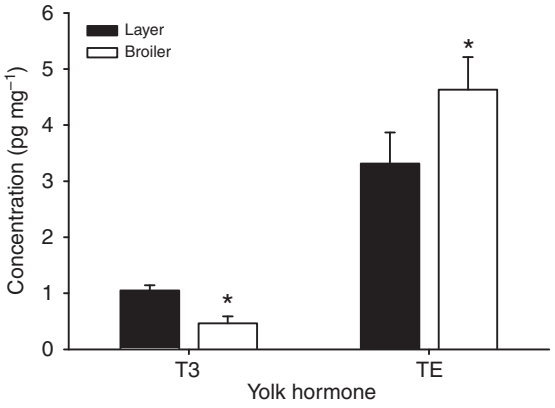

Fig. 6. Yolk triiodothyronine (T_3) and yolk testosterone (TE) concentrations of broiler and layer eggs prior to incubation. *Significantly different from corresponding layer chicken egg yolk hormone concentration. Values are means ± s.e.m.; N=3–12.

DISCUSSION

During early embryonic development, chicken embryos rely exclusively on egg components for growth and development, which provides the potential for maternal effects to impact development of breed-specific phenotypes. However, we are unaware of any studies that have addressed the significance of maternally provided yolk environment in determining broiler and layer chicken phenotypes during the first few days of embryonic development. In the present study, we demonstrate that when embryos and egg yolk environments are switched between the broiler and layer breeds of chicken, egg yolk environment, *per se*, has a significant effect on embryonic body mass, heart rate and development rate, thus revealing potential epigenetic mechanisms in phenotype determination in chickens [see Ho and Burggren for review of epigenetic processes in physiology (Ho and Burggren, 2010)]. Interestingly, mass-specific oxygen consumption is unaffected in these xenobiotic experiments.

Previous studies indicate that embryonic body mass of broiler embryos and layer embryos differ during development, with broiler embryos attaining significantly heavier wet and dry body masses than layer embryos from embryonic days 12–20 (Pal et al., 2002; Sato et al., 2006; Everaert et al., 2008). We significantly extend these findings by showing that the embryonic mass difference between the two breeds occur as early as 60 and 84 h of development on native yolk environments, with broiler embryos being ~3 mg (wet body mass) heavier than layer embryos (Fig. 2A). Remarkably, the switching of embryos and egg yolk environments between the two breeds causes significant changes in the natural growth rate of layer embryos, but less dramatic changes in broiler embryo growth rate over this early period of development (Fig. 2A,B). Sato et al. suggested that, during late embryonic development (days 12–18), the difference in growth rate between the two breeds is probably the result of genetically driven differential rates of yolk lipid absorption and metabolism between the two breeds (Sato et al., 2006). The present data indicate that, prior to embryonic day 4, genotypic differences that determine lipid absorption rates and/or growth rates in broiler and layer embryos are not the sole determining factor in the breed-specific mass differences. Rather, our data show that during this early stage in development, egg yolk factors play a large role in growth rate determination.

Perturbations in yolk environment affect development rate of broiler and layer embryos in the same fashion as growth rate. Layer embryos in this study were significantly more mature (~HH12) than broiler embryos (~HH11) after 36 h of development, *in ovo* (Fig. 3). Importantly, we did not detect any significant correlation between initial HH stage and the parameters that we measured (χ^2, $P>0.5$). This eliminates the possibility of initial HH stage driving the effects observed in this study. When layer embryos were cultured on broiler YCM and allowed to develop for 24 h, their development rate was accelerated significantly compared with layer embryos cultured on layer YCM. This phenomenon parallels that observed for body mass (Fig. 2), thus indicating that the increase in body mass of layer embryos cultured on broiler YCM is probably a function of an acceleration in development rate rather than an increase in tissue mass *per se*. However, this effect is short-lived, disappearing by 48 h in culture (as opposed to body mass differences that were still detected after 48 h of development in culture). The brevity of the yolk effect on development rate may be because there is a sensitive period between 48 and 60 h in chicken development when yolk environment has a large impact on embryonic development rate, but has no obvious long-lasting role in shifting the trajectory of this trait during early development. An alternative explanation is that using the Hamburger and Hamilton method of stage determination does not provide the resolution required to capture subtle shifts in embryonic development rate that may be present at 48 h of development on YCM (Hamburger and Hamilton, 1951). Interestingly, the nature of the effects induced by perturbations in egg yolk environment is breed-specific. That is, broiler yolk accelerates development rate of layer embryos, whereas layer yolk has no apparent effect on the development rate of broiler embryos. Clearly, there exists a complex interaction between genotypic and egg yolk differences between the breeds.

Many environmental factors markedly influence heart rate during development. Abiotic factors such as temperature, oxygen availability and light intensity affect heart rate in chicken embryos, with the nature of the effects being age-dependent (Gimeno et al., 1966; Tazawa and Nakagawa, 1985; Chan and Burggren, 2005). In late-stage avian embryos, heart rate is, in part, a function of P_{O_2} within the air cell of the egg. This pressure is largely determined by eggshell conductance under normoxic ambient conditions (Okuda and Tazawa, 1988; Tazawa et al., 1988; Christensen et al., 2006). However, differences between broiler and layer chicken embryo heart rates at embryonic days 10–14 are not attributed to differences in shell conductance between the two breeds (Yoneta et al., 2006). This indicates that egg factors other than shell conductance may influence early cardiac development. Presently, we show that in the absence of maternally provided egg shell and albumen, egg yolk plays a direct role in ontogeny of heart rate in early-stage chicken embryos. Remarkably, layer yolk composition decelerates the heart rate of broiler embryos, whereas the composition of broiler egg yolk sufficiently abolishes the breed-specific difference in heart rate (Fig. 5). It is likely that the difference in yolk composition plays a role (or at least sets the stage) for the previously reported late-stage differences in heart rate between layer and broiler chicken breeds (Yoneta et al., 2006).

This study provides the first comparison of mass-specific $\dot{V}_{O_2}$ between broiler and layer embryos during the first few days of embryonic development. We found that breed-specific egg yolk environment did not have a great impact on the mass-specific $\dot{V}_{O_2}$ of 48–84 h broiler or layer chicken embryos (Fig. 4). Mass-specific $\dot{V}_{O_2}$ recorded for early stage embryos (HH14–HH21) in the present study are comparable to those of layer embryos found by Burggren et al. (Burggren et al., 2000). Late-stage broiler embryos (embryonic days 12–18) have lower mass-specific $\dot{V}_{O_2}$ than do layer embryos of the same age (Sato et al., 2006), but results of the present study examining early-stage embryos suggest that layer and broiler embryos cultured on native YCM do not differ in embryonic mass-specific $\dot{V}_{O_2}$.

Although intriguing, it is not surprising that breed-specific changes in yolk environment do not affect embryonic $\dot{V}_{O_2}$, whereas growth, development and heart rate are altered by yolk environment changes. Discordant effects of environmental stress on $\dot{V}_{O_2}$ and morphological traits early in embryonic development have been reported (see Altimiras and Phu, 2000; Sharma et al., 2006). Early in development, chicken embryo growth (characterized by eye diameter and vascular growth) and metabolic rate is not related to cardiac output or heart rate (Burggren et al., 2000; Burggren et al., 2004). We show that the heart rate of broiler embryos is significantly altered by breed-specific changes in yolk environment, and that the chronotropic cardiac change in broiler embryos due to a change in yolk environment is not accompanied by any significant changes in broiler body mass, development rate or $\dot{V}_{O_2}$. In contrast, body mass and development rate of layer chicken embryos are altered, whereas heart rate remains unchanged, by breed-specific changes

in yolk environment. Thus, in the present study, it can be assumed that yolk-induced perturbations in heart rate, mass-specific $\dot{V}_{O_2}$ and growth rate are independent of one another. However, further investigation is needed to address whether changes in heart rate early in development contributes to heart rate regulation, metabolic rate, development rate or growth rate later in development.

We assessed yolk mass and total mass of broiler and layer chicken eggs because these two factors significantly predict hatchling mass, hatchling maturity and offspring performance in oviparous species (Sotherland and Rahn, 1987; Sinervo and Huey, 1990; Dzialowski and Sotherland, 2004). Similar to Sato et al. the current results show that broiler eggs, although not different in mean total mass to layer eggs, possess significantly larger yolk mass than layer eggs (Sato et al., 2006). This difference in egg yolk mass between the two breeds is compensated by significantly smaller albumen mass in broiler eggs than layer eggs (Fig. 1). In this study, it appears that the larger yolk mass of broiler eggs is related to larger body mass of those embryos cultured on broiler yolk, and the relatively smaller yolk mass of layer eggs related to the relatively smaller body mass of those embryos cultured on layer yolk (Fig. 2). This suggests that yolk mass may be useful in predicting embryo mass during early embryonic development (HH14–21), a period when yolk volume *per se* does not play a role in limiting body mass.

Yolk environment is made up of a multitude of macromolecules (hormones, lipids, carbohydrates, carotenoids, antibodies) that are physiologically relevant to the developing offspring (Schwabl, 1993; McNabb and Wilson, 1997; Royle et al., 2001; Saino et al., 2007; Newbrey and Reed, 2009). Yolk hormones, which are highly variable among and within avian species (Schwabl, 1993; Reed and Vleck 2001; Groothuis et al., 2005; Gil et al., 2007; Martin and Schwabl, 2008; Gil, 2008), are candidates for the observed physiological and morphological effects on early embryo development. We have characterized the profiles of two important yolk hormones, T_3 and TE, in broiler and layer eggs to elucidate the potential underlying mechanisms of yolk-induced changes in early embryo morphology and physiology. Analyses indicate that yolk T_3 concentration of broiler embryos is significantly lower than that of layer eggs, by approximately twofold (Fig. 6). Given the previously observed tachycardic and cardiomyocyte proliferative effects of thyroid hormones in developing animals (Moussavi et al., 1985; Schjeide et al., 1989; Slotkin et al., 1992; Flamant and Samarut, 1998), it is unlikely that yolk T_3 is directly and solely driving the cardiac and developmental effects of layer yolk and broiler yolk observed in this study. In other words, if yolk thyroid hormones were to play a key role in modulating early embryonic heart rate in this study, then we would have observed a tachycardic, rather than a bradycardic effect of layer YCM on broiler embryos. Our data is contrary to what is expected of yolk thyroid hormones based on existing literature, and may suggest a non-traditional role of yolk thyroid hormone in embryonic cardiac regulation.

In contrast to yolk T_3 concentration, yolk TE concentration was significantly greater in broiler egg yolks than layer egg yolks. Because yolk TE increases overall hatching body size and hatching muscle mass in birds (Lipar and Ketterson, 2000; Eising et al., 2001), and differentiation of embryonic stem cells into beating cardiomyocytes (Goldman-Johnson et al., 2008), it is highly possible that the significant difference in yolk TE between the two breeds is responsible for the chronotropic responses and larger body masses observed in layer embryos cultured on broiler yolk. These results are consistent with literature from other bird species showing that higher yolk TE concentrations positively affect prenatal and postnatal growth and development (Schwabl, 1996; Navara et al., 2006; Schwabl et al., 2007). It is probable that egg yolk effects detected in the current study result from a complex interaction of breed-specific egg yolk factors, involving yolk T_3 and yolk TE as well as other yolk components, and breed-specific genotype. For example, inter-molecular interactions of yolk T_3 and yolk TE, and numerous yolk hormones not assessed in this study have been implicated in modulating avian development (Groothuis et al., 2005).

Overall, our study suggests that artificial selection for the broiler phenotype (i.e. rapid growth rate and large adult body size), and layer phenotype (i.e. prolific egg production) is responsible for breed-specific differences in yolk factors that play a part in defining the broiler and layer phenotype. Furthermore, selection for these different adult phenotypes is likely to have resulted in the concomitant selection for embryos that differ in their sensitivity and response to maternal egg yolk factors. Thus, complete analyses of yolk hormones and other yolk factors (e.g. lipid concentrations, carotenoids, antibodies) and complementary studies examining the dose-dependent effects of these yolk factors in early developing embryos of different chicken breeds are required to better understand the mechanisms underlying the current findings.

Conclusion

During early embryonic development (i.e. in the first 4 days of incubation), chicken embryos rely heavily on egg components for growth and development, which strongly implicates maternal effects in the development of breed-specific phenotypes. The current study sheds light on the role of the yolk environment in determining the well-characterized phenotypes of the layer chicken and the broiler chicken, each of which have been artificially selected for traits serving different purposes, and show genotypic differences. Moreover, this study reveals a complex interaction of breed-specific genotype and breed-specific yolk environment in early chicken development. These novel findings using xenobiotic approaches reinforce the significance of maternal effects and epigenetic processes on early embryonic heart rate, growth rate, oxygen consumption and development rate of vertebrates.

LIST OF ABBREVIATIONS

Br	broiler chicken
HH	Hamburger and Hamilton chicken stage
f_H	heart rate
La	layer chicken
M	wet body mass (mg)
m	wet body mass (g)
P_{O_2}	oxygen partial pressure
T_3	triiodothyronine
TE	testosterone
$\dot{V}_{O_2}$	oxygen consumption
YCM	yolk culture medium

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