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Iron Status of the Late Term Broiler (*Gallus gallus*) Embryo and Hatchling

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Abstract: Our objective was to investigate and elucidate the iron status of the late term broiler (*Gallus gallus*) embryo and young chick. This would be vital for better understanding of the transition period that the hatchling experience immediately after hatch. For that, blood, liver and small intestinal samples from broiler embryos were taken on embryonic days 12 (12E), 14E, 17E, 19E, day of hatch and 3 days post hatch. Expression of transporters and enzyme involved in Fe uptake and transfer, ie. Divalent metal transporter-1 (DMT1; iron uptake transporter), ferroportin (iron transport across the enterocyte) and duodenal cytochrome-B reductase (DcytB; reduces iron at brush border membrane) were determined. In addition, liver tissue samples were analyzed for iron and ferritin (cellular iron storage protein) contents. Samples were also collected from the intestinal contents, yolk sac, amniotic fluid and intestinal content for iron concentration analysis. The intestinal mRNA abundance of DMT1 and DcytB were shown to increase as from day 12E until day 17E, whereas ferroportin expression was unchanged. As from 19E a graduate decrease in relative expression occurred. Liver iron and ferritin concentrations were shown to decrease as from day 12E and as day of hatch approaches. In addition, yolk iron concentrations decreased while iron concentrations in amniotic fluid have increased (days 12E through 17E). This was followed by iron concentrations increase in the intestinal content (day 17E trough day of hatch). In this study, we first document the late term broiler embryo iron related enzyme and transporters gene expression as well as the changes in liver ferritin and liver iron contents.

Key words: Broiler, embryo, iron, ferritin, brush border membrane, DMT1

INTRODUCTION

Iron (Fe) plays an essential role in a wide variety of biochemical processes and is a required cofactor for the function of over 300 different enzymes (Romanoff, 1967; Ludwiczek *et al*., 2004; Lozoff *et al*., 2006). The critical role of Fe in early embryonic development has been identified in studies indicating its presence in the chicken embryo (Romanoff, 1967; Sinclair *et al*., 1988). Iron participates in the synthesis of nucleic acids (Allen and Peerson, 2009) and is an important structural cofactor for many proteins, including DNA synthesis and oxygen transport (Whitnall and Richardson, 2006; Scott *et al*., 2008; Li and Zhao, 2009). It was suggested that poor Fe status will lead to embryonic malformations and delayed development and even death post hatch as heart hypertrophy was observed in Fe-deficient chicks (Aoyagi and Baker, 1995). In addition, Fe-deficiency was shown to decrease the intrinsic capacity of the liver for gluconeogenesis, hence to decrease liver glycogen stores (Klempa *et al*., 1989). Previous analyses of the broiler embryo liver, pectoral muscle and pipping muscle glycogen status indicated on significant reduction in glycogen levels prior to hatch (as from day 18 of incubation), a further reduction was measured at the day of hatch, this indicates on the poor energetic status and indirectly suggest a deficient Fe status of the broiler hatchling (Uni *et al*., 2005; Tako *et al*., 2005).

The intestinal Fe absorption is regulated, in part, by intracellular Fe concentrations in enterocytes (Ludwiczek *et al*., 2004). Iron ions (Fe^{2+} and Fe^{3+}) are reduced by DcytB to Fe^{2+} (unless already in the Fe^{2+} form) at the duodenal brush border membrane (BBM); which is then transported into the enterocyte via DMT1. Within the enterocyte, Fe is either stored as ferritin or trafficked to the Basolateral Membrane (BLM) and exported into the circulation. Transport across the BLM is accomplished by the coordinated action of ferroportin, an Fe transporter and hephaestin, which oxidizes Fe^{2+} to Fe^{3+}. Iron ions (Fe^{3+}) then bind to transferrin for distribution throughout the body via the plasma circulation (Ludwiczek *et al*., 2004). The rapid development of the gastrointestinal tract of post hatch broiler chick has been described (Uni *et al*., 2003a; Noy and Sklan, 2003); The late term broiler embryo digestive system is changing rapidly; it was previously shown that during the last 3 days of incubation (embryonic age 18E-hatch), the weight of the intestine, as a proportion of embryo weight, increased from approximately 1% on 17E to 3.5% at hatch. Also activities of maltase, aminopeptidase, Sodium-Glucose Transporter (SGLT)-1, ATPase and ZnT-1 (intestinal zinc exporter) began to increase on d 19E and further increased on the day of hatch. These major changes in the expression and localization of the functional BBM and BLM proteins provide the framework for ingestion of carbohydrate and protein rich exogenous feed post hatch (Uni *et al*., 2003b; Tako *et al*., 2005).

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However, limited knowledge is available about the Fe status, Fe metabolism and the intestinal Fe related transporters and enzymes functionality in the late term broiler embryo.

Adequate Fe status of the broiler embryo and hatchling is crucial for its development and endurance in the first week post hatch (Aoyagi and Baker, 1995; NRC, 1994). Therefore, the present study examined the Fe status of the late term broiler embryo and hatchling. This was done by examining the relative expression of intestinal genes that code for BBM and BLM Fe related enzymes and transporters; Divalent metal transporter-1 (DMT1, the Fe uptake transporter); Duodenal cytochrome B (DcytB, reduces Fe at brush border membrane) and ferroportin (a protein involved in Fe transport across the enterocyte). Blood hemoglobin and liver ferritin amounts were measured along with determination of intestinal content, liver, yolk sac and amniotic fluid Fe concentrations.

The data shown and discussed in the current study is important for better understanding of the Fe and mineral metabolism in the developing broiler embryo.

MATERIALS AND METHODS

Embryonic tissue sampling: Forty-eight Cornish cross fertile broiler eggs were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA), from a maternal flock 35 weeks in lay (given adequate amounts of dietary Fe, >80 ppm) (NRC, 1994). Approximately 24 h after fertile eggs collection, the eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator. Six embryos/chicks were killed for each of the following days for analysis; 12, 14, 17, 19 d of incubation (similar egg weight was sampled at each time point, 12E, 14E, 17E, 19E, respectively), day of hatch (within 2 h of clearing the shell) and 72 h post hatch. For each embryo/hatchling and at each sampling day, blood samples were taken for hemoglobin analysis (directly from heart). Samples of the small intestine, yolk sac, liver and amniotic fluid were taken for Fe concentrations analysis. In addition, small intestine segments (1 cm long) were taken and were immediately frozen in liquid nitrogen and stored at -80°C for mRNA abundance analysis. Liver samples (1 gr) were isolated stored at -80°C for ferritin Fe concentration determination. All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

Hemoglobin measurements: As previously described (Tako *et al*., 2010; Tako and Glahn, 2011), blood samples were collected from the heart (n = 6, ~30 µL) using micro-hematocrit heparinized capillary tubes (Fisher, Pittsburgh, PA). Blood Hemoglobin (Hb) concentrations were determined spectrophotometrically using the cyanmethemoglobin method (H7506-STD, Pointe Scientific Inc. Canton, MI) following the kit manufacturer's instructions.

Isolation of total RNA: Total RNA was extracted from 30 mg of the small intestine tissue using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Briefly, tissues were disrupted and homogenized with a rotor-stator homogenizer in buffer RLT® (for lysis of cells/tissues before RNA isolation), containing β-mercaptoethanol. The tissue lysate was centrifuged for 3 min at 8,000xg in a micro centrifuge. An aliquot of the supernatant was transferred to another tube, combined with 1 volume of 70% ethanol and mixed immediately. Each sample (700 µL) was applied to an RNeasy mini column, centrifuged for 15 s at 8000xg and the flow through material was discarded. Next, the RNeasy columns were transferred to new 2-mL collection tubes and 500 µL of buffer RPE® was pipetted onto the RNeasy column followed by centrifugation for 15 s at 8000xg. An additional 500 µL of buffer RPE were pipetted onto the RNeasy column and centrifuged for 2 min at 8000xg. Total RNA was eluted in 50 µL of RNase free water. All steps were carried out under RNase free conditions. RNA was quantified by absorbance at $A_{260/280}$. Integrity of the 28S and 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. DNA contamination was removed using TURBO DNase treatment and removal kit from AMBION (Austin, TX, USA).

DMT1, DcytB and Ferroportin mRNA abundance analysis: As previously described (Tako *et al*., 2010; Tako and Glahn, 2011); First strand cDNAs were synthesized from 5 µg of total RNA from each bird using oligo $(dT)_{18}$ as primers in the presence of MLV reverse transcriptase (Fermentase), for 1 h at 42°C. PCR was carried out with primers chosen from the fragment of the chicken duodenal DMT1 gene (GI 206597489) (forward: 5'-AGC CGT TCA CCA CTT ATT TCG-3'; reverse: 5'-GGT CCA AAT AGG CGA TGC TC-3'), DcytB gene (GI 219943161) (forward: 5'-GGC CGT GTT TGA GAA CCA CAA TGT T-3'; reverse: 5'-CGT TTG CAA TCA CGT TTC CAA AGA T-3') and Ferroportin gene (GI 61098365) (forward: 5'-GAT GCA TTC TGA ACA ACC AAG GA'; reverse: 5'-GGA GAC TGG GTG GAC AAG AAC TC-3'). Ribosomal 18S was used to normalize the results, with primers from the *Gallus gallus* 18S ribosomal RNA (GI 7262899) (forward: 5'- CGA TGC TCT TAA CTG AGT-3'; reverse: 5'- CAG CTT TGC AAC CAT ACT C-3'). Determination of the linear phase of the PCR amplification was performed with Tfi- DNA polymerase

(A-125, Access RT-PCR system, Promega) with pooled aliquots removed at 15, 20, 25, 30, 35, 40, 45, 50 and 55 cycles. Amplification of the chicken duodenal DMT1, DcytB and Ferroportin genes were performed for 32, 33 and 30 cycles respectively, which consisted of denaturation (95°C, 30 s), annealing (48°C, 1 min) and extension (72°C, 1 min); ribosomal 18S was amplified at 32 cycles under identical conditions in a different tube. Ribosomal 18S (426 bp) and chicken duodenal DMT1/Ferroportin/DcytB PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide and quantified using the Quantity One 1-D analysis software (BioRad, Hercules, CA).

Ferritin and Fe in the liver: Liver samples were treated as described by (Mete *et al*., 2005). Briefly, the frozen tissue samples were thawed on ice for approximately 30 min. One gram of sample was diluted into 1 mL of 50 mM Hepes buffer, pH 7.4 and homogenized on ice using an Ultra-Turrax homogenizer at maximum speed (13000 g) for 2 min. One mL of each homogenate was subjected to heat treatment for 10 min at 75°C to aid isolation of ferritin since other proteins are not stable at that temperature (Mete *et al*., 2005; Passaniti and Roth, 1989; Tako and Glahn, 2011). After heat treatment, the samples were immediately cooled down on ice for 30 min. Thereafter, samples were centrifuged at 13000 g for 30 min at 4°C until a clear supernatant was obtained and the pellet containing most of the insoluble denaturated proteins was discarded. All tests were conducted in duplicates for each animal.

Electrophoresis and staining gels: Native polyacrylamide gel electrophoresis was conducted using a 6% separating gel and a 5% stacking gel. Samples were run at a constant voltage of 100V. After electrophoresis, the gels were treated with either of the two stains: Coomasie blue G-250 stain, specific for proteins, or potassium ferricyanide ($K_3Fe(CN)_6$) stain, specific for iron. The corresponding band found in the protein and iron stained gel was considered to be ferritin (Passaniti and Roth, 1989; Mete *et al*., 2005; Tako and Glahn, 2011).

Gels measurements: The gels were scanned with Bio-Rad densitometer. Measurements of the bands were conducted using the Quantity-One 1-D analysis program (Bio-Rad, Hercules, CA). The local background was subtracted from each sample. Horse spleen ferritin (Sigma Aldrich Co., St. Louis, MO) was used as a standard for calibrating ferritin protein and iron concentrations of the samples. Dilutions of the horse spleen ferritin were made and treated similarly to the liver supernatant samples in order to create a reference line for both protein and iron-stained gels (Mete *et al*., 2005; Pietrangelo *et al*., 1995).

Statistical analysis: One tailed student's *t*-tests were performed to compare differences between means using the JMP software (SAS Institute, Cary, NC). Values were considered significantly different at $p<0.05$. Values in the text are means±SEM.

RESULTS

Blood hemoglobin (Hb): Hb concentration increased gradually as day of hatch approaches and were 6.3 ± 0.55^{d}; 7.2 ± 0.61 ; ^{cd} 8.55 ± 0.69 ; ^{bc} 9.72 ± 0.65 ; ^{ab} 10.55 ± 0.82^{a} and 10.93 ± 0.78^{a} g/dL for embryonic days 12E, 14E, 17E, day of hatch and 3d post hatch, respectively.

Gene expression of iron enzyme and transporters in the small intestine: The relative mRNA abundance of Brush Border Membrane (BBM) Fe enzyme (DcytB); Fe transporter (DMT1) and basolateral Fe transporter (Ferroportin) was determined for embryonic days 12E, 14E, 17E and 19E, day of hatch (within 2 h of clearing the shell) and on day 3 post hatch (72 h of clearing the shell) in the small intestine. Expression of DcytB, DMT1 and Ferroportin genes were detected from d 12E. Determined quantities relative to 18S indicated that expression of all the parameters examined was low on days 12E and 14E, increased 3- to 7 -fold on day 17E and all decreased again on the day 19E. Further reduction in relative expression occurs on day 3 post hatch (Fig. 1).

Ferritin in the liver: Liver ferritin amounts were calculated as previously described (Passaniti and Roth, 1989; Mete *et al*., 2005; Tako and Glahn, 2011). The mean values of the relative ferritin protein amounts of the liver samples of all animals are presented in Fig. 2. Ferritin relative amounts significantly decreased after d 12E and remained low as day of hatch approaches and on day 3 post hatch.

Liver Fe concentrations decreased between days 14E and day 17E, then continue to decrease through hatch (Table 1).

Iron concentrations in yolk sac, amniotic fluid and intestinal content: Iron concentration analysis of the yolk sac content showed that Fe concentration is declining as from day 12E through day 17E ($p<0.05$). A further reduction in Fe concentration occurs as day of hatch approaches ($p<0.05$). In contrast Fe concentration in the amniotic fluid gradually increases as from day 12E through day 17E (just prior to its consumption, $p<0.05$). This follows Fe concentration increase of the intestinal content, that show to increase as from day 17E (Table 1).

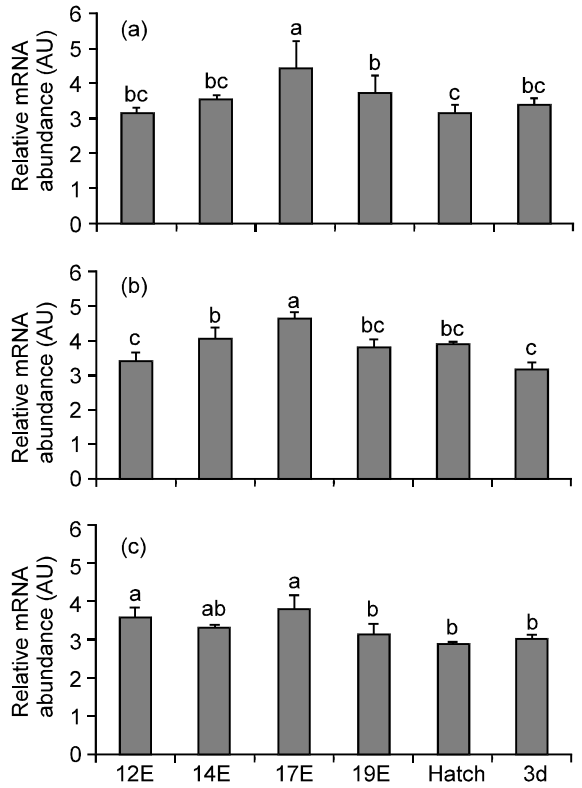

Fig. 1: Changes in chicken duodenal mRNA abundance of (A) duodenal cytochrome B (DcytB) (B) divalent transporter 1 (DMT1) and (C) ferroportin in broiler embryos (incubation days 12E, 14E, 17E, 19E), day of hatch and 3 days post hatch. Changes in mRNA abundance were measured by semi quantitative reverse transcription-PCR and expressed relative to abundance of 18S rRNA in Arbitrary Units (AU). Values are means±SEM, n=6.
[a,b]Mean values within genes tested with unlike letters were significantly different ($p<0.05$)

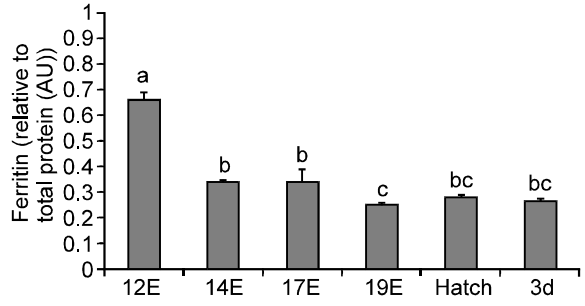

Fig. 2: Relative ferritin protein amounts of liver samples of broiler embryos (incubation days 12E, 14E, 17E, 19E), day of hatch and 3 days post hatch (values are mean±SEM, n = 6)

Table 1: Iron concentrations (µg/g sample)[1,2] in amniotic fluid, yolk, intestinal content and liver tissue of broiler embryos (incubation days 12E, 14E, 17E, 19E), day of hatch and 3 days post hatch (values are mean±SEM, n = 6)

Day	Amnion	Yolk	Intestinal content	Liver
12E	1.319±0.13[b]	37.75±4.5[a]	-	105.56±8.5[a]
14E	1.125±0.08[b]	36.95±4.0[a]	-	97.17±8.8[a]
17E	5.62±0.5[a]	11.05±0.85[b]	2.59±0.26[c]	50.29±4.5[b]
19E	-	5.95±0.56[c]	3.00±0.28[c]	50.24±8.07[b]
Hatch	-	4.84±0.37[c]	5.80±0.63[b]	42.27±7.58[b]
3 d	-	3.56±0.24[cd]	8.92±1.4[a]	24.42±1.46[c]

[1]Iron concentrations in samples (0.5 g) were determined by an inductively-coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co. Franklin, MA) following wet ashing.
[2]For each organ, means without a common superscript are significantly different ($p<0.05$)

DISCUSSION

Iron is an essential micro nutrient for the normal growth and development of the avian embryo (Savage, 1968). It was previously shown that Fe deficiency can be readily induced in developing avian embryos by feeding insufficient amounts of the mineral to laying hens, which, in turn, produce eggs that are depleted in trace minerals (Richards and Steele, 1987). Iron deficiency has been found to cause impaired growth, abnormal development of all of the major organ systems and in extreme deficiencies, death of the embryo (Savage, 1968; Richards and Steele, 1987). In addition, heart hypertrophy was observed in Fe-deficient chicken hatchlings (Aoyagi and Baker, 1995).

Thus, it is crucial to the survival of the broiler embryo that the adequate amount of each essential trace mineral be available at the appropriate time during its growth and development within the egg. Unlike the mammalian fetus, which derives nutrients from maternal circulation via the placenta, the avian embryo develops *in ovo* without access to a constant circulating maternal nutrient supply. Instead, all of the nutrients, including Fe, required for the rapid growth and complete development of the embryo are deposited by the hen into the egg at the time of its formation (White, 1991). Therefore, it is important to emphasize that in the current study, the broiler hens were given the common commercial diet with adequate amount of Fe (>80 µg/g diet) (NRC, 1994). It was established that at hatch the yolk represents the major nutrient supply to the hatchling until transition to utilization of exogenous feed occurs. It was shown that prior to hatch part of the yolk is transported to the small intestine via the yolk stalk and is conveyed to the upper small intestine by antiperistaltic contractions of the small intestine where digestion initially by pancreatic and finally by brush border hydrolases takes place (Geyra *et al*., 2001; Uni *et al*., 2003a; Tako *et al*., 2004; Tako *et al*., 2005).

The above suggests that the yolk sac content and membranes play an important role in the embryonic development and metabolism. Hence, they have been extensively studied and a number of functions, including the mobilization of yolk nutrient stores, have been attributed to the endodermal cells that line the inner surface of this membrane and are in direct contact with yolk (Nitsan *et al*., 1995; Noy and Sklan, 1998). Vitellogenin, the yolk precursor, serves as a trace mineral transporting proteins that mediates the transfer of essential nutrients from stores within the liver of the hen to the ovary and developing oocyte and hence, the yolk of the egg. The mobilization and uptake of egg trace mineral stores is mediated by the extra-embryonic membranes, principally the yolk sac membrane. The yolk sac also serves as a short term storage site for trace minerals. Because it is an important site of plasma protein synthesis, the yolk sac has the ability to regulate the export of trace minerals to the embryo during development. within the embryo, specific metaloproteins function in the interorgans transport, cellular uptake and intracellular storage of trace minerals. Thus, embryonic trace mineral homeostasis is established through the coordinated actions of the yolk sac, which mobilizes and exports trace minerals derived from the egg stores; the vitelline circulation, which transports them to the embryo; and the liver, which accumulates trace minerals and distributes them to the rest of the tissues of the embryo via the embryonic circulation.

Previously, the distribution of zinc, iron and copper in turkey eggs was characterized (Richards, 1997). Both zinc and iron showed similar distribution, with the majority of these two minerals deposited in the yolk granule fraction (88.8% and 84.6% of total egg Zn and Fe, respectively). During the turkey embryonic development and similar to the findings of the current study, yolk and liver tissue Fe concentrations declined as from day 14E to 26E (i.e. as day of hatch approaches) (Richards, 1991).

In addition, it was previously reported that whole chicken embryo concentrations of zinc, copper, iron and manganese were initially high on Day 5 of incubation and that they had declined sharply by Day 10 (Dewar *et al*., 1974). The initial high concentrations of these trace minerals probably reflect different transfer mechanisms depending on the stage of development. Between days 5 and 10 of incubation, the yolk sac is still developing to its full function and it is possible that the transfer of trace minerals to the embryo is not as highly regulated by the yolk sac as it is during the latter half of incubation. It is also possible that in the early stages of development of the chick embryo, higher trace mineral concentrations are required to support the very rapid expansion of embryo mass (Marchaim and Kulka, 1967). A possible reason for the sharp decline in yolk sac Fe towards the end of incubation, is the diminished erythroopoietic activity and subsequent accumulation of nonheme Fe in the embryo. The nonheme Fe stores was proposed to be utilized by the chick during early post hatch period (Ramsay, 1951).

Theil and Tosky reported on the importance of primitive red cell ferritin as an early intra-embryonic iron storage site (Theil and Tosky, 1979). They suggested that up to 30% of the total Fe reserves of the early developing chick embryo can be accounted for as red cell ferritin-bound Fe. The decline in this store of Fe corresponds with the replacement of the primitive red cells by red cells of the definitive (adult) lineage. Thus, primitive red cell ferritin was presumed to contribute its Fe to the synthesis of hemoglobin contained in the definitive red cells. In the early developing chick embryo, ontogenetic changes in erythropoiesis are apparently linked with red cell ferritin synthesis, Fe storage and hemoglobin synthesis (Theil, 1980). Bauminger *et al*. (Bauminger *et al*., 1982) detected relatively high concentrations of ferritin-bound Fe in chick embryo fibroblasts cultured from day 12 chick embryos, indicating an ability of these cells to accumulate and store Fe. Ramsay (Ramsay, 1951) studied changes in heme Fe in the embryo and the yolk sac membrane and related the changes in yolk sac Fe levels with growth of the embryo. Toward the end of incubation, there is a sharp decline in yolk sac heme iron due to diminished erythropoietic activity and a subsequent accumulation of nonheme Fe in the embryo. The nonheme Fe store was proposed to be utilized by the chick during the early post hatching period. However, in none of these studies liver ferritin was determined, nor, the expression of the relevant Fe uptake related enzymes and transporters of the intestinal enterocyte and in different stages of embryonic development.

In the current study, the relative expression of DMT1, DcytB and ferroportin showed a similar pattern of expression; the relative expression gradually increases as from day 12E through day 17E. At day 19E the relative gene expression decreases and further decrease (DcytB) or remain stable (DMT1, Ferroportin) at hatch. The additional reduction in mRNA abundance could be explained by the amniotic fluid oral consumption (primarily water and albumen protein) prior to pipping of the air cell. After hatch (day 3 post hatch) when the chick is exposed to the external feed, the relative gene expression further decreases, due to high Fe concentration in the starter diet.

In this study, Fe concentration analysis of the yolk sac content showed that Fe concentration is declining as from day 12E to hatch. A further reduction in Fe concentration occurs at day 3 post hatch. These results are in agreement with previous observations (Ramsay, 1951). In contrast, Fe concentration in the amniotic fluid increases as day from 14E to 17E (just prior for its consumption). This follows detectable Fe in the intestinal content as from day 17E. In addition and as

previously shown (Wei *et al*., 2007; Liu *et al*., 2009), blood hemoglobin concentrations gradually increase as day of hatch approaches, further increase occurs at day 3 post hatch.

In the current study and to further investigate the Fe status of the broiler embryo, we measured ferritin relative amounts in the embryonic liver and hatchling. Liver ferritin relative amounts decreases significantly ($p<0.05$) from 12E to 19E.

In addition, liver Fe concentrations gradually decrease as from embryonic day 14E and as day of hatch approaches. Further reduction occurs post hatch ($p<0.05$), as the transition to external feed occurs.

The current study suggests that the limited Fe stores that are available to the broiler's embryo are rapidly exploited in the final stages of embryonic development and as day of hatch approaches. Upon hatch the hatchling's Fe status is poor and without the appropriate diet (i.e. sufficient Fe in the starter diet) the hatchling's growth, normal development, energetic status and survival during the first week post hatch may be affected.

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