Change of zinc mobilization and gene expression of key zinc transport proteins between the yolk sac membrane and liver of duck embryonic developing

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ABSTRACT Zinc (**Zn**) deposition in egg yolk is essential for the rapid growth and complete development of the avian embryo. Thus, it is crucial to obtain maximal Zn mobilization at an appropriate time during development in favor of the survival of avian embryos. The aim of this study was to study the developmental change of Zn mobilization and gene expression related to key Zn transport proteins between the yolk sac membrane and embryonic liver from the incubation d 17 (E17) to d 32 (E32) during duck embryonic developing. The weights of duck embryo, embryo without yolk sac, and embryonic liver increased as well as the yolk sac weight decreased linearly ($P < 0.0001$) when incubation day increased. The Zn concentration in the yolk sac did not change from E17 to E29 and only declined significantly from E29 to E32 of duck embryos, while hepatic Zn level decreased linearly as with the increased incubation time ($P < 0.01$). When the incubation day increased, the decreased Zn amount in the yolk sac and the increased Zn amount in the embryonic liver were observed ($P < 0.0001$). The calculated transfer-out rate of Zn in the yolk sac and transfer-in rate of Zn in livers were both increased from E23-26 to E29-32 ($P < 0.01$). Among E17, E23 and E29, the *solute carrier family 39 member (ZIP)* of *ZIP10, ZIP13*, and *ZIP14* genes mRNA expressions were increased in yolk sac membrane but were decreased in the embryonic liver, while *metallothionein 1* mRNA expression was increased both in the yolk sac membrane and liver ($P < 0.05$). In conclusion, yolk sac membrane and embryonic liver tissues displayed the similar developmental patterns of Zn mobilization and *metallothionein 1* mRNA expression from E17 to E32 during duck embryonic developing. The appropriate time of the maximal rate of Zn mobilization were observed between E29 and E32 of duck embryo, associated with the significant changes of gene expression related to some key Zn transport proteins on E29 in yolk sac membrane and liver tissues.

Key words: zinc mobilization, developmental change, zinc transport protein, duck embryo

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INTRODUCTION

Embryonic growth and development are dependent on the nutrients deposited in the egg (van der Wagt et al., 2020). Zinc (**Zn**) as an essential trace mineral was required in a small amount for the rapid growth and complete development of the avian embryo (Richards, 1997). Severe Zn deficiency resulted in the abnormal embryonic development and poor performing offspring in both hens (Blamberg et al., 1960) and broiler breeders (Zhu et al., 2017b). The disruption of embryonic development was associated with Zn function either as catalytic or structural cofactors in metal-containing enzymes and proteins as a function of development (Huang et al., 2019). Maternal Zn supply or in ovo Zn injection increased Zn deposition in egg yolk and then was transferred to the developing avian embryo from storage sites to promote the growth and development (Zhu et al., 2017a; Sun et al., 2018). Thus, it is crucial to study the developmental change of Zn mobilization to obtain an appropriate time during development in favor

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of the survival of avian embryos. A number of reports have been reported on the transfer of trace minerals (Zn, copper, manganese, etc) from the egg to the developing avian embryos (Ramsay, 1951; Sandrock et al., 1983; McCormick, 1991). Richards (1991b) showed that the Zn concentration declined in yolk sac of the chicken embryo and liver tissue of turkey embryo between d 14 and 28 of incubation (Richards, 1991), indicating that an active Zn mobilization occurred in the interorgan transport during development. However, it was unclear whether there was the parallel developmental change of Zn mobilization between yolk sac and liver tissues in avian species. The mobilization and uptake of yolk Zn stores is mediated by the yolk sac membrane principally; while the transferred Zn was transported and stored in embryonic liver, which is the most important organ for the storage and homeostatic regulation of trace mineral metabolism (Richards, 1997). The Zn homeostasis is regulated by the large number of transport proteins that are potentially dedicated to Zn^{2+} transport and storage, including members of the ZnT (Zn^{2+} transporter) family, members of the ZIP (i.e., Zn^{2+}-regulated metal transporter) family and metallothionein isoforms (Sekler et al., 2007). However, limited information was available for the gene expression of key Zn-binding protein on Zn homeostasis regulation. Hence, the aims of the present study were to determine the developmental change of Zn mobilization and gene expression related to key Zn transport proteins between yolk sac membrane and liver tissues from incubation d 17 to 32 during duck embryonic developing.

METHODS AND MATERIALS

Animals and Incubation

All animal protocols used in the present study were approved by the South China Agricultural University Institutional Animal Care and Use Committee.

Eighty 33-wk-old laying duck breeders were selected from a commercial breeder farm (WENS Group, Yunfu, Guangdong, China) and were fed restrictively (160 g/d/bird) with a commercial feed at the nutritional level (11.82 MJ ME/kg, 180 g CP/kg, 8.0 g lysine/kg, 7.2 g methionine + cysteine/kg, 24.0 g calcium/kg, 3.8 g available phosphorus/kg, 87.4 mg zinc/kg). All birds were reared in the caged system under a temperature of 22 ± 2°C and a humidity of 55 ± 5%. Water was available ad libitum and a lighting program 16L: 8D was provided. On the last 2 d of 33 wk of age, a total of 80 hatched eggs were selected to study the developmental changes of weights and Zn mobilizations in embryos. All eggs were placed in 2 trays at the middle of the trolley, which were maintained at an incubation temperature of 37.5 ± 0.5°C and a relative humidity of 55 ± 5% until embryonic d 30 (**E30**). Eggs were turned at an angle of 90° every hour from the start of incubation until E30. Then, all eggs were transferred to hatching crates and moved to hatchers. The hatcher was set at a temperature of 37.0 ± 0.5°C, which declined to 36.0 ± 0.5°C at the end of incubation. Male duck breeders were fed the same diet formulated to meet the nutritional requirements throughout the experimental period. The practice of semen collection started at 25 wk of age, and the quality of semen was determined by the volume and numbers of semen and sperm motility. During the experimental feeding period, semen was collected and mixed from male duck breeders at 33 wk of age. Artificial insemination was performed every twice one week.

Sample Collections and Analyses

Ten egg embryos, representing the weight distribution of the eggs at set, were selected on E17, E20, E23, E26, E29, and E32, respectively. Two embryos were killed by cervical dislocation and then the egg embryos, yolk sac, yolk sac-free embryo and liver were weighed to calculate the average values per replicate from each incubation time. The yolk sac content was separated from the yolk sac and then homogenized. The yolk sac and liver samples were stored at −20°C for Zn analyses. Small pieces of yolk sac membrane and embryonic liver samples on E17, E23, and E29 were rinsed in a 0.9% autoclaved-saline solution and placed in microcentrifuge tubes at −80°C for analysis of mRNA expression of genes related to Zn absorption and transport. Equal weight subsamples from the 2 embryos in each replicate were pooled into one sample for the measurements of Zn content and gene expression.

Zinc contents in yolk sac and embryonic liver samples were measured using an inductively coupled plasma emission spectroscope (IRIS Intrepid II, Thermal Jarrell Ash, Waltham, MA) after wet digestions with HNO_3 and $HClO_4$ as described by (Zhu et al., 2017b). The total Zn contents of the yolk sac and embryonic liver were calculated by multiplying Zn concentration and weight. The relative Zn mobilization rate was calculated as the ratio of Zn content change expressed as the absolute values in yolk sac or embryonic liver per day between E17-20, E20-23, E23-26, E26-29, and E29-32, respectively. For determination of gene mRNA expression, total RNA extraction, reverse-transcription, and RT-qPCR for gene mRNA expression were conducted as described previously (Zhu et al., 2015). The primer sequences are listed as following: *GAPDH* (forward) GGTGCTAAGC GTGTCATCATCTC, (reverse) CCCCCTCAGCT-GATGCTCCCATGA; *metallothionein 1* (forward) AAAGGCTGCTGCTCCTGCT, (reverse) AGCTG-CACTTGGCGGAGG; ZIP6 (forward) ACGCAGAT-CATCAGCAGAACTTGG, (reverse) GACCTAACC GAGCAACCGACTTG; ZIP8 (forward) AACCAC-CATCATCCAGCAACG-G, (reverse) ACGGCAT-CACTCAGTGTTACCATC; ZIP10 (forward) GCCA CAACCACAGCCACCAC, (reverse) AATGCCTC-CAAGTGCCACAAGAC; ZIP13 (forward) TGCAGT GCAACAACGGA GAAGG, (reverse) TCTAGCGT-CAGGAAGGTCAGGAAG; ZIP14 (forward) TGCTA CTGGCTGAAGGAGGTGAG, (reverse) TGGAA-GACGGAGACGGTGAAGG. The *GAPDH* was used

to normalize the expressions of the targeted genes. The $2^{-\triangle\triangle Ct}$ was used to calculate the mRNA level of each target gene.

Statistical Analyses

Data were analyzed by one-way ANOVA using the PROC GLM procedure of the SAS (SAS Inst. Inc., Cary, NC). Orthogonal polynomials were applied for linear and quadratic effects on the parameters of both embryonic development and tissue Zn mobilization in response of incubation time. All data were presented as mean ± SEM. The replicate served as the experimental unit. Differences among means were tested by the Fisher's Least Significance Difference test method, and statistical significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

The development and growth of poultry embryos are dependent upon the trace minerals deposits in the eggs (Torres and Korver, 2018; Hopcroft et al., 2019). Zinc is an essential nutrient required in small amounts for normal growth and development of the avian embryo functioning as catalytic or structural cofactors in metal-containing enzymes (Huang et al., 2019). When Zn level was increased in the egg and yolk sac, the hatchability was increased primarily due to the decrease incidence of middle and latter embryonic mortality (Zhu et al., 2017a). Therefore, it is crucial to achieving an appropriate time of Zn mobilization exerting its biological functions to promote embryonic growth and development (Richards and Steele, 1987). The developmental processes and Zn mobilization between the yolk sac and liver tissues have been studied in duck embryos from E17 to E32. When incubation day increased, the weights of duck embryo, embryo without yolk sac, and embryonic liver increased linearly but the yolk sac weight decreased linearly ($P < 0.0001$; Figure 1). The weights of the embryo, embryo without yolk sac, and embryonic liver increased from 32.84 g, 6.88 g, and 0.057 g on E17 to 49.17 g, 45.67 g, and 1.188 g on E32, increased by 15.33 g, 38.79 g, and 1.13 g between E17 and E32, respectively. The weight of the yolk sac decreased from 26.7 g to 3.51 g from E17 to E32. A similar pattern was observed in chick embryos reported previously (Yadgary et al., 2010; Yadgary and Uni, 2012). The Zn concentration in the yolk sac did not change from E17 to E29 and only declined significantly from E29 to E32 of duck embryos ($P > 0.05$, Figure 2). It was not agreed with the declined Zn concentration in yolk sac between incubation reported in the developing chick embryo (Dewar et al., 1974). Hepatic Zn level decreased linearly as with the increased incubation time, which was consistent with that observed in turkey embryo during the incubation period (Richards and Weinland, 1985). The Zn amount in the yolk sac decreased linearly when the incubation day increased, while the Zn amount in the embryonic liver increased linearly as with the increased incubation time ($P < 0.0001$; Figure 2). Also, our results showed that the calculated transfer-out rate of Zn in the yolk sac and transfer-in rate of Zn in livers were both increased from E23−26 to E29−32. The parallel developmental changes of Zn mobilization rate in the interorgan transport during the latter half incubation implied that the maximal rate of Zn mobilization during E29 to E32 could be conducted to serve a wide range of biological reactions, such as functioning as an antioxidant defense system. It was indicated that close to hatching, the greater Zn demand may be required from the yolk sac to the target tissues to maintain the rapid growth and development (Richards, 1997). Because that the yolk sac and liver tissues were developing to its full function during incubation, it is possible that the transfer of Zn to the embryo is upregulated by the yolk sac during the latter time of incubation. Additionally, the developmental change of Zn mobilization suggested that transfer mechanisms might be involved in some key Zn transport proteins in duck embryos at the different developmental stage.

The complexity and importance of Zn homeostasis is regulated by the large number of proteins that are

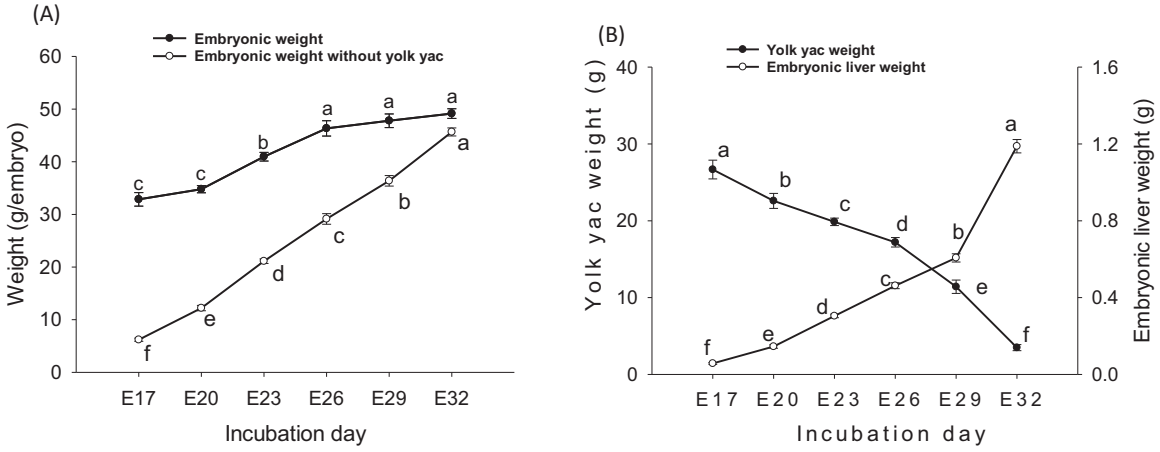

Figure 1. Developmental changes of weights in the embryo, embryo without yolk sac, yolk sac, and embryonic liver from E17 to E32. All values are expressed as means ± SE. Means with different letters (A-F) differ significantly ($P < 0.05$) between incubation days. Mean represented the average value of 5 replicates ($n = 5$).

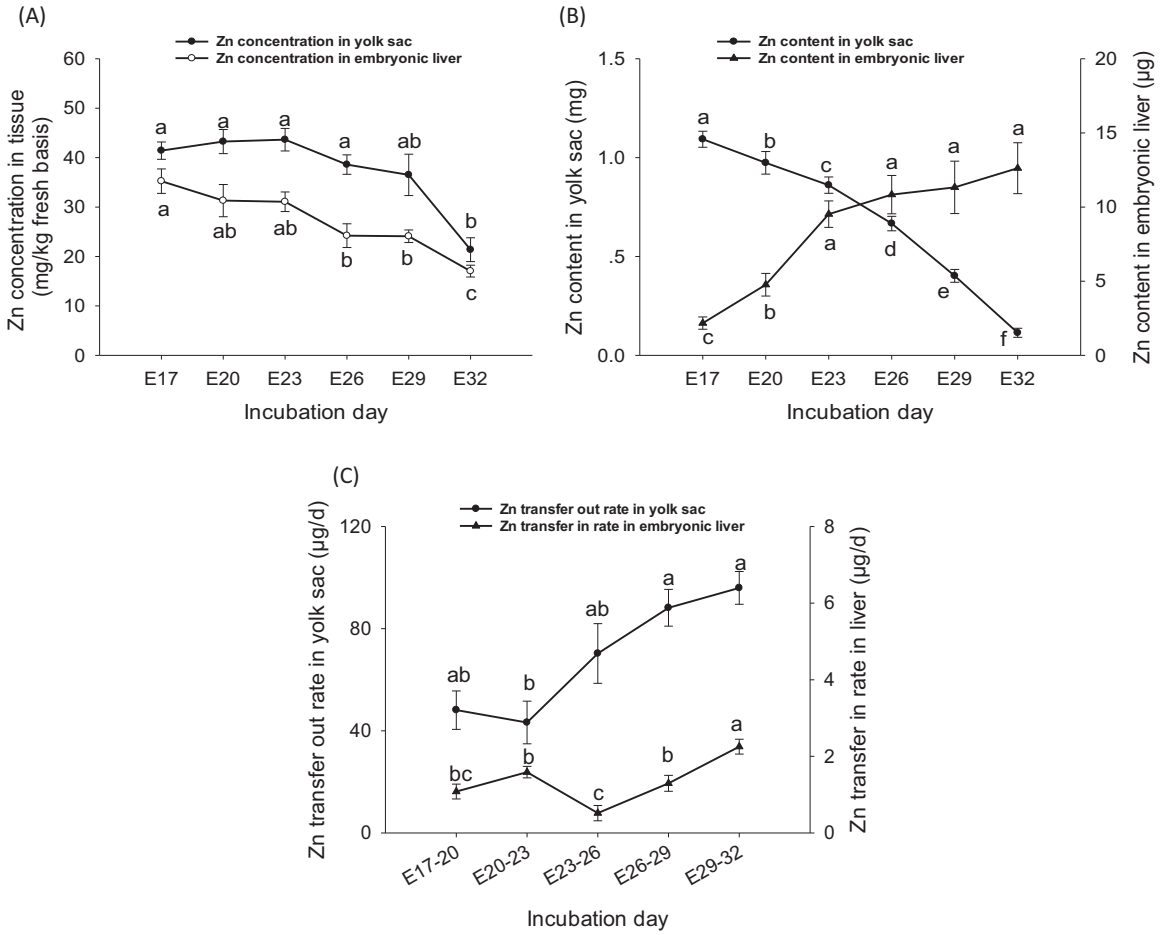

Figure 2. The developmental changes of Zn concentration (A) and Zn amount (B) in yolk sac as well as Zn mobilization rates (C) in yolk sac and embryonic liver. All values are expressed as means ± SE. Means with different letters (A-F) differ significantly ($P < 0.05$) between incubation days. Mean represented the average value of 5 replicates (n = 5). The total Zn contents in yolk sac and embryonic liver were calculated by multiplying Zn concentration and weight. Then, the relative Zn mobilization rates were calculated as the ratio of Zn content change in yolk sac or embryonic liver per day during E17−20, E20−23, E23−26, E26−29, and E29−32, respectively.

potentially dedicated to Zn^{2+} transport and buffering, such as ZIP member family and metallothionein isoforms (Sekler et al., 2007). In mammals, ZIP transporters increase intracellular cytoplasmic Zn by promoting extracellular and, perhaps, vesicular Zn transport into cytoplasm. The expressions of Zn transporter protein genes have been proved to be sensitive to the changes of Zn status during embryonic development (Andrews et al., 2004; Dufner−Beattie et al., 2006). However, the role of ZIP transporter has not been fully revealed in avian species. In response to the increased incubation day, *ZIP10, ZIP13*, and *ZIP14* mRNA expressions were increased in yolk sac membrane but were decreased in the embryonic liver ($P < 0.05$), while *ZIP8* mRNA expression was increased in yolk sac membrane from E17 to E23 and then decreased from E23 to E29 ($P < 0.01$; Table 1). It is assumed that the different synthetic abilities of specific Zn-binding protein between yolk Zn mobilization and hepatic Zn transportation and storage. Between E17 and E29, *metallothionein 1* mRNA expression in both the yolk sac membrane and embryonic liver was increased with the increased incubation time ($P < 0.05$; Table 1). Similar findings were confirmed by the greater hepatic *metallothionein 1* mRNA expression in the chick embryo at the latter incubation stage. As reported previously, hepatic Zn level of cytoplasmic Zn could induce metallothionein expression as a function of development in embryo. Moreover, maternal Zn supply or *in ovo* Zn injection could induce *metallothionein* mRNA expression in chick embryos during the latter half of incubation (Zhu et al., 2017a; Sun et al.,

Table 1. The developmental change of gene expressions related to key Zn transport proteins in yolk sac membrane and embryonic liver tissues.

Item[1,2]	Incubation day	*MT1*	*ZIP6*	*ZIP8*	*ZIP10*	*ZIP13*	*ZIP14*
YCM	E17	0.41^{b}	1.40	1.31	0.68^{b}	0.75^{b}	0.68^{b}
	E23	2.78^{a}	1.20	1.07	1.43^{a}	0.76^{b}	1.00^{b}
	E29	3.56^{a}	1.00	1.08	1.41^{a}	1.44^{a}	1.56^{a}
	SEM	0.67	0.24	0.17	0.13	0.15	0.16
	P value	0.01	0.54	0.55	0.002	0.001	0.008
Liver	E17	0.44^{b}	1.27	0.56^{b}	1.85^{a}	2.25^{a}	3.41^{a}
	E23	1.81^{ab}	1.04	1.59^{a}	1.19^{b}	1.14^{b}	0.59^{b}
	E29	4.86^{a}	1.02	0.63^{b}	0.76^{b}	0.73^{b}	0.74^{b}
	SEM	0.87	0.21	0.17	0.18	0.31	0.43
	P value	0.04	0.68	0.003	0.006	0.03	0.002

YCM = yolk sac membrane; *GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MT1 = metallothionein 1; ZIP 6, 8, 10, 13, 14 = solute carrier family 39 member 6, 8, 10, 13, 14.*

The *GAPDH* expression was used to normalize the expressions of the targeted genes.

[1]Mean represented the average value of 5 replicates (n = 5).

[2]Lacking common letters (a or b) significant differences at $P < 0.05$.

2018). De et al. (1991) reported that primary cultures of chick embryo hepatocytes and fibroblasts in response to Zn supplementation of culture medium by upregulation of *metallothionein* mRNA expression. The significant changes of *metallothionein 1* and *ZIP10, ZIP13*, and *ZIP14* mRNA expressions in tissues on E29 confirmed that the mobilization and uptake of Zn homeostasis was strengthened between the yolk sac membrane and target embryonic liver at this appropriate time.

In conclusion, yolk sac membrane and embryonic liver tissues displayed the similar developmental patterns of Zn mobilization and *metallothionein 1* mRNA expression of duck embryo. The appropriate time of the maximal rate of Zn mobilization were observed between E29 and E32 of duck embryo, associated with the significant changes of genes expression related to some key Zn transport proteins on E29 in yolk sac membrane and embryonic liver tissues.

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DISCLOSURES

The authors declare that there is no conflict of interest related to the preparation and publication of this paper.

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