

Growth of embryo and gene expression of nutrient transporters in the small intestine of the domestic pigeon (*Columba livia*)^{*}

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Abstract: The objective of this study was to investigate the relationship between gene expression of nutrient (amino acid, peptide, sodium and proton) transporters in the small intestine and embryonic growth in domestic pigeons (*Columba livia*). One hundred and twenty-five fertilized eggs were randomly assigned into five groups and were incubated under optimal conditions (temperature of 38.1 °C and relative humidity of 55%). Twenty embryos/birds from each group were sacrificed by cervical dislocation on embryonic day (E) 9, 11, 13, 15 and day of hatch (DOH). The eggs, embryos (without yolk sac), and organs (head, brain, heart, liver, lungs, kidney, gizzard, small intestine, legs, and thorax) were dissected, cleaned, and weighed. Small intestine samples were collected for RNA isolation. The mRNA abundance of intestinal nutrient transporters was evaluated by real-time reverse transcription-polymerase chain reaction (RT-PCR). We classified these ten organs into four types according to the changes in relative weight during embryonic development. In addition, the gene expression of nutrient transporters was differentially regulated by embryonic day. The mRNA abundances of $b^{0+}AT$, $EAAT3$, y^+LAT2 , $PepT1$, $LAT4$, $NHE2$, and $NHE3$ increased linearly with age, whereas mRNA abundances of $CAT1$, $CAT2$, $LAT1$, $EAAT2$, $SNAT1$, and $SNAT2$ were increased to higher levels on E9 or E11 and then decreased to lower levels until DOH. The results of correlation analysis showed that the gene expressions of $b^{0+}AT$, $EAAT3$, $PepT1$, $LAT4$, $NHE2$, $NHE3$, and y^+LAT2 had positive correlations with body weight ($0.71 < \text{correlation coefficient (CC)} < 0.82$, $P < 0.0001$), while $CAT1$, $CAT2$, $EAAT2$, $SNAT1$, and $SNAT2$ had negative correlations with body weight ($-0.86 < \text{CC} < -0.64$, $P < 0.0001$). The gene expressions of $b^{0+}AT$, $EAAT3$, $LAT4$, $PepT1$, $NHE2$, $NHE3$, and y^+LAT2 showed positive correlations with intestinal weight ($0.80 < \text{CC} < 0.91$, $P < 0.0001$), while $CAT1$, $CAT2$, and $EAAT2$ showed negative correlations with intestinal weight ($-0.84 < \text{CC} < -0.67$, $P < 0.0001$). It was concluded that the differences between growth trajectories of organs and gene expression of nutrient transporters in small intestine were due to their functional and physiological properties, which provided a comprehensive study of amino acid and peptide transporter mRNA in the small intestine during embryonic growth of pigeons.

Key words: Embryonic growth, Nutrient transporters, Gene expression, Small intestine, Pigeons

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1 Introduction

Embryonic growth in precocial birds has been widely investigated, for instance duck (Tangara *et al.*, 2010), turkey (Stepińska *et al.*, 2012), and chicken (Li *et al.*, 2014). In contrast to precocial poultry, young pigeons, a typical representative of altricial birds, are

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feed with pigeon milk from the crop by parents for a few days. However, there is limited information available on the development of embryo and organs in this species.

The embryonic growth and development in poultry not only directly affect the embryonic quality and hatchability, but also affect the ontogeny of the young after hatch. Unlike mammals, avian embryo development is dependent upon the finite nutrient deposits in the fertile egg (Finkler *et al.*, 1998). Embryonic development of chicks is divided into three phases. At the first third of incubation (first week of incubation), the germ, chorionic sac, and allantoic cavity are established. Embryo completion is formed through the second third (from Day 8 to Day 14), and the embryo development is prepared for emergence and hatching in the final stage of incubation (from Day 15 to the day of hatch (DOH)) (Moran, 2007). In the last 2 to 3 d of incubation, the residual yolk, as a nutritional source, reaches the small intestine via the yolk stalk (Noy and Sklan, 1998). Therefore, the embryo would impair absorption of nutrients from the yolk sac, and then nutrient absorption occurs primarily through the small intestine close to hatch (Noy *et al.*, 1996; Speier *et al.*, 2012). Thus, it is necessary to understand the development patterns of intestinal absorptive capacity because of its key function in nutrient intake.

In the small intestine, the absorption of nutrients is mediated by transporter proteins expressed in the enterocyte. The nutrient passed through the epithelium of the small intestine and into the blood stream via special transporters. According to Poncet and Taylor (2013), different nutrient transporters have different gene expression patterns and functions. For example, amino acids are transported into the intestinal epithelial cells as di- or tri-peptides by the H⁺-dependent peptide transporter 1, *PepT1* (Leibach and Ganapathy, 1996) or a variety of amino acid transporters that have specificity for anionic, cationic, or neutral amino acids (Hyde *et al.*, 2003). Most of the studies are focused on the gene expressions of intestine nutrient transporters in precocial poultry, such as chicken (Mott *et al.*, 2008; Sun *et al.*, 2015; Miska *et al.*, 2014) and turkey (de Oliveira *et al.*, 2009). Our laboratory has studied gene expressions of cationic and neutral amino acid (Zeng *et al.*, 2011), excitatory amino acid (Li *et al.*, 2011), small peptide, and sodium and proton (Li *et al.*, 2012) transporters in the

small intestine of chick embryos. Furthermore, these studies found that the expression levels of the *b⁰⁺AT*, *EAAT3*, and *PepT1* in the small intestine had strong correlations with chick embryo development, which could be used as indicators for the chick embryo growth of the small intestine (Li *et al.*, 2013). The gene expression level of intestinal fatty acid (Xie *et al.*, 2012; 2013) and sodium glucose (Dong *et al.*, 2012b) transporters in pigeons, and the activity of digestive enzyme in pigeon yolk sac membrane during pre- and post-hatch development have been reported (Dong *et al.*, 2012a). However, the underlying relationship of embryonic growth and gene expression of nutrient transporters in the small intestine has not been evaluated in pigeons. Therefore, the objective of this study was to investigate: (1) the growth of embryo and organs in the stage of embryonic development and (2) the underlying relationship between gene expression of nutrient (amino acid, peptide, sodium and proton) transporters in the small intestine and embryonic growth of pigeons.

2 Materials and methods

All experimental methods and management procedures were approved by the Animal Care Committee of South China Agricultural University (Guangzhou, China).

2.1 Experimental design and sample collection

A total of 125 fertilized pigeon (*Columba livia*) eggs were obtained from Brother Economic Development Co. Ltd. (Guangdong, China). Eggs were weighed ((21.08±0.12) g) and randomly assigned into five groups. The egg weights in each group did not show a significant difference ($P>0.90$). The eggs were then incubated at 38.1 °C and 55% relative humidity in an incubator (Keyu, Guangzhou, China). At embryonic day (E) 7 of incubation, all the eggs were candled for the detection of unfertilized eggs; these were removed from the incubator. On E9, E11, E13, E15, and DOH, the eggs were opened and embryos/birds were freed from the allantois and yolk. At any given age, twenty embryos/birds from each group were sacrificed by cervical dislocation. The eggs, embryos (without yolk sac), and organs (head, brain, heart, liver, lung, kidney, gizzard, small

intestine, leg, and thorax) were dissected, lightly blotted and drained of blood or any fluid contents with phosphate-buffered saline (NaCl, 8 g/L; KCl, 0.2 g/L; Na₂HPO₄, 1.42 g/L; KH₂PO₄, 0.27 g/L) at 4 °C, and weighed. All of the small intestine samples were placed in 0.5-ml microcentrifuge tubes, frozen in liquid nitrogen, and stored at -80 °C for mRNA analysis.

2.2 Total RNA isolation and cDNA synthesis

The total RNA was isolated from the small intestine tissue of pigeon embryos using TRIzol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The RNA quality was checked by 1.5% agarose gel electrophoresis, stained with 10 µg/ml ethidium bromide. The optical density (OD) of the RNA was measured by a NanoDrop 2000 UV-Vis spectrophotometer (Thermo, Wilmington, MA, USA) and OD₂₆₀/OD₂₈₀ ratio was between 1.8 and 2.0. Reverse transcription (RT) reaction mixtures (20 µl) comprised 2 µg of total RNA, N10 primers (Sangon, Shanghai, China), M-MLV reverse transcriptase (Promega, Madison, USA), RNase inhibitor (TaKaRa, Shiga, Japan), and dNTP. The complementary DNA (cDNA) products were diluted 1:3 using DEPC water and stored at 20 °C for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

2.3 Quantification of mRNA by qRT-PCR

The abundance of mRNA was measured by qRT-PCR with a Stratagene MxPro 3005P thermocycler (Agilent Technologies, Santa Clara, USA). The qRT-PCR mixture contained 2 µl of cDNA template, 10 µl of SYBR Green Real-Time PCR Master Mix (TOYOBO, Tokyo, Japan), 7 µl of DEPC water, and 1 µl of each gene-specific primer. The PCR program was 1 cycle at 95 °C for 60 s, 35 cycles of 95 °C for 15 s, 58 °C/63 °C for 40 s, and 72 °C for 40 s, 95 °C for 60 s, 58 °C/63 °C for 15 s, and 72 °C for 30 s. The melting curve analysis was performed by 1 cycle at 95 °C for 60 s, 58 °C/63 °C for 15 s, and 72 °C for 30 s, starting at 58 °C with a heating rate of 0.2 °C per second up to 95 °C. The cycle threshold (C_T) values for each gene were averaged. The following nutrient transporters were investigated in our study: solute carrier (SLC) family 1 member 2 (*EAAT2*), SLC family 1 member 1 (*EAAT3*), SLC family 7 member 1 (*CAT1*), SLC family 7 member 2 (*CAT2*), SLC family 7 member 6 (*y⁺LAT2*), SLC family 7

member 9 (*b^{0,+}AT*), SLC family 7 member 5 (*LAT1*), SLC family 43 member 2 (*LAT4*), SLC family 38 member 1 (*SNAT1*), SLC family 38 member 2 (*SNAT2*), SLC family 15 member 1 (*PepTI*), SLC family 9 member 2 (*NHE2*), and SLC family 9 member 3 (*NHE3*). Six housekeeping genes, *α-tubulin*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *β-actin*, TATA box binding protein (*TBP*), ribosomal protein L13 (*RPL13*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were also evaluated. All the gene-specific primers were designed by Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). Table 1 shows the primer sequences used.

2.4 Statistical analysis

The relative weight of embryo was absolute embryo weight to set egg weight, and the relative weight of organs (head, brain, heart, liver, gizzard, small intestine, kidney, lung, leg, and thorax) was measured by the absolute organ weight to set yolk-free embryo weight.

The obtained C_T values of housekeeping genes were quantified and entered into the geNorm application according to the method described by Vandesompele *et al.* (2002). The average expression stability (M) values of control genes calculated by geNorm showed that *GAPDH*, and *β-actin*, *TBP*, and *HPRT1* were the most stable genes among *GAPDH*, *β-actin*, *HPRT1*, *TBP*, *RPL13*, and *α-tubulin*. In addition, the pair-wise variation value V_{4/5} was below 0.15 and the normalization factors utilized *GAPDH*, *β-actin*, *TBP*, and *HPRT1* as housekeeping genes. The relative mRNA expression levels were calculated by the method of 2^{-ΔC_T} and revised through normalization factors. Data were processed by SAS version 9.1.3 statistical software package (SAS Institute Inc., Cary, NC, USA). The correlation coefficients (CCs) were derived from the Pearson product-moment correlation using a CORR Procedure analysis. The regression analysis was performed by the REG Procedure. A linear model, power function or logarithmic function was decided according to the scatter diagram plot, which used a least-square regression analysis method of the REG procedure by a stepwise variable filter method. Values were expressed as the mean ± standard error (SE). Differences with P < 0.05 and P < 0.01 were considered statistically significant and highly significant, respectively.

Table 1 Primers used for real-time PCR

Gene	GenBank ID	Sense (S)/antisense (AS) primer (5'→3')	PCR product length (bp)
Transporter gene			
<i>b^{0,+}AT</i>	XM_005514428.1	S: AATCATTGGCTCAGGTATCTT AS: TTTGTGACGAGTAGGCTTG	237
<i>CAT1</i>	XM_005501421	S: CTTCGCCGTGGTGATA AS: CCGAGGACGAGGATGT	108
<i>CAT2</i>	XM_005501186	S: TCAAGCGTAGCAAGAGC AS: CGAAGATTAGGACCAAGAT	232
<i>EAAT2</i>	NM_001282830.1	S: GCCTAAACAAGTAGAAGTGC AS: CCAGGATGACACCGAATA	140
<i>EAAT3</i>	XM_005507165.1	S: ACAGGTGTTGCTGCTTTG AS: GGTGCTGCCACTCTAT	171
<i>LAT1</i>	XM_005504728	S: GCAAAGGGTGATGTGACGAG AS: AGAGGCAGGTTTCTGTAGGGA	170
<i>LAT4</i>	XM_005502006.1	S: GACCTTCACATCGCTTACG AS: TGCCCAGACAAGCAGAAT	163
<i>NHE2</i>	XM_005504658	S: ATTGAGCCACTCTTTGTCTT AS: TGGTTCTTCCCAACTGTAG	236
<i>NHE3</i>	XM_418895.2	S: ATTGCCTCTTTTACCCTTAC AS: CTGTCCAGGTGGCCATTA	215
<i>PepT1</i>	XM_005504628.1	S: CTATTCAGATTCAGCCAGACC AS: ATTACTTTGATTTGGGATTGC	250
<i>SNAT1</i>	XM_005499915	S: TGGGCGGTTTCTTGTTGT AS: GATGGTGGGCAAAGCGTA	277
<i>SNAT2</i>	XM_005499916	S: GTGGCACCTTTGGTAGATG AS: AATCGCAGGATGGCAGAC	129
<i>y⁺LAT2</i>	XM_001231336	S: GCTGAACCTGGAACCACTATTAC AS: AAGTGAGGTCCACAAACGAA	102
Housekeeping gene			
<i>α-tublin</i>	NM_001080860	S: GAGAACACGGATGAAACCTACT AS: TGGTGGCTGACACTAAATGG	118
<i>β-actin</i>	NM_205518.11	S: ATTGTCCACCGCAAATGCTTC AS: AAATAAAGCCATGCCAATCTCGTC	113
<i>GAPDH</i>	NM_001282835.1	S: TCTGCCAAAGTCCAAGTG AS: ACTTTATGTTGCTGGGAT	187
<i>HPRT1</i>	XM_005500563.1	S: TACGACCTGGACTTGTCT AS: ATTTATAGCCACCCTTGAG	172
<i>RPL13</i>	XM_005504844.1	S: TGTCCAACCATCCGATACCA AS: GTCGCCATCTTCAGTTCCTCA	263
<i>TBP</i>	XM_005508625.1	S: CAGCCCATTTACCAGG AS: CAACCAAGATTCACCGT	142

b^{0,+}AT: solute carrier (SLC) family 7 member 9; *CAT1*: SLC family 7 member 1; *CAT2*: SLC family 7 member 2; *EAAT2*: SLC family 1 member 2; *EAAT3*: SLC family 1 member 1; *LAT1*: SLC family 7 member 5; *LAT4*: SLC family 43 member 2; *NHE2*: SLC family 9 member 2; *NHE3*: SLC family 9 member 3; *PepT1*: SLC family 15 member 1; *SNAT1*: SLC family 38 member 1; *SNAT2*: SLC family 38 member 2; *y⁺LAT2*: SLC family 7 member 6; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *HPRT1*: hypoxanthine phosphoribosyltransferase 1; *RPL13*: ribosomal protein L13; *TBP*: TATA box binding protein

3 Results

3.1 Growth of embryo and organs

The absolute weight of yolk-free embryo increased from 1.27 g on E9 to 11.47 g on DOH (Fig. 1). The relative embryo weight increased significantly throughout development, with a sharp increase from E13 to E15. According to the changes of the relative weight, ten organs were classified into four groups during the embryonic developmental stage. The head and brain were classified as the first type of organs with a gradually decreasing relative weight (Fig. 2), whereas the kidney, gizzard, and leg were classified as the second type of organ with a continuously rising relative weight (Fig. 3). The heart, small intestine, and liver were classified as the third type of organ with generally increasing relative weight from E9 to DOH but a more rapid increase near the end of incubation (Fig. 4). The lung and thorax were classified as the

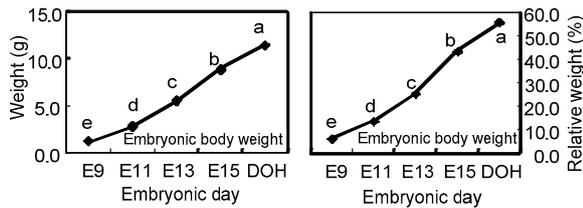


Fig. 1 Absolute weight and relative weight of embryonic body

Relative weight is calculated as absolute weight normalized to fresh egg weight (%). Values are expressed as mean±SE ($n=20$). Data with no common letters (a–e) indicate significant difference among embryonic days ($P<0.05$)

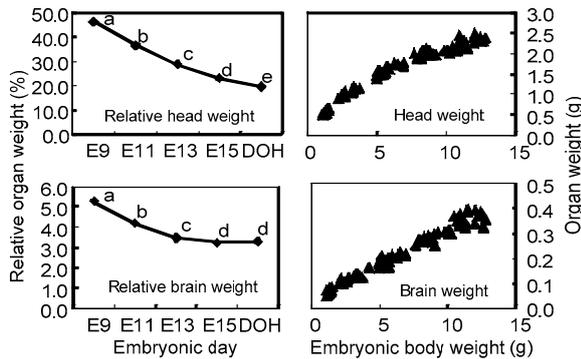


Fig. 2 Relative organ (head and brain; $n=20$) weight and organ (head and brain; $n=112$) weight plotted as a function of embryonic body weight for pigeons

Relative organ weight is calculated as absolute weight normalized to embryonic body weight without yolk (%). Values are expressed as mean±SE. Data with no common letters (a–e) indicate significant difference among embryonic days ($P<0.05$)

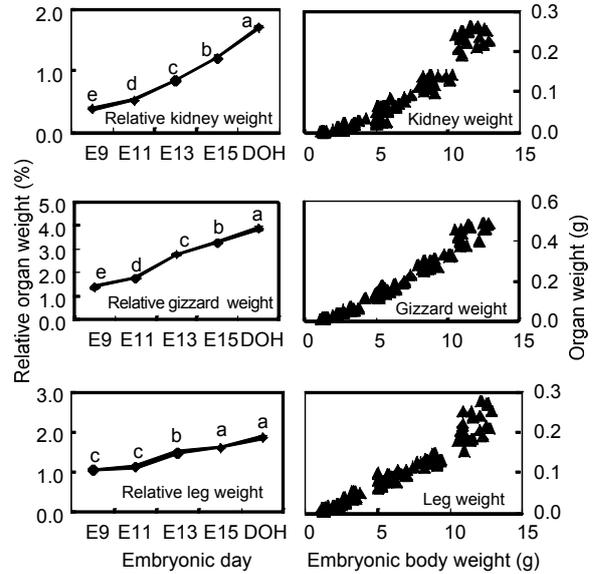


Fig. 3 Relative organ (kidney, gizzard, and leg; $n=20$) weight and organ (kidney, gizzard, and leg; $n=112$) weight plotted as a function of embryonic body weight for pigeons

Relative organ weight is calculated as absolute weight normalized to embryonic body weight without yolk (%). Values are expressed as mean±SE. Data with no common letters (a–e) indicate significant difference among embryonic days ($P<0.05$)

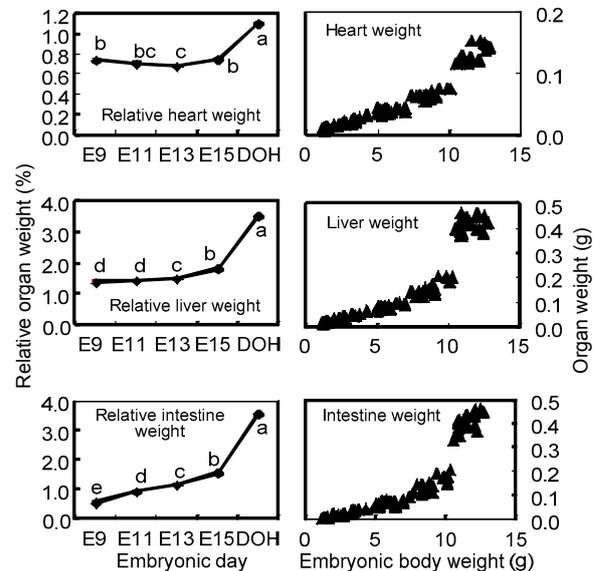


Fig. 4 Relative organ (heart, liver, and small intestine; $n=20$) weight and organ (heart, liver, and small intestine; $n=112$) weight plotted as a function of embryonic body weight for pigeons

Relative organ weight is calculated as absolute weight normalized to embryonic body weight without yolk (%). Values are expressed as mean±SE. Data with no common letters (a–e) indicate significant difference among embryonic days ($P<0.05$)

fourth type of organ with initially increasing relative weight, peaking on E11 and E13, respectively, and then decreasing (Fig. 5).

3.2 Correlation of organ and body weight

Correlation analysis of organ and body weight is shown in Table 2. During embryonic development, the weights of head, brain, heart, liver, lung, kidney, gizzard, small intestine, thorax, and legs were strongly positively correlated with body weight ($CC>0.8$, $P<0.0001$). Yolk was negatively correlated with body weight ($CC=-0.637$, $P<0.0001$). In addition, we performed regression analysis on organ and body weight (Table 2).

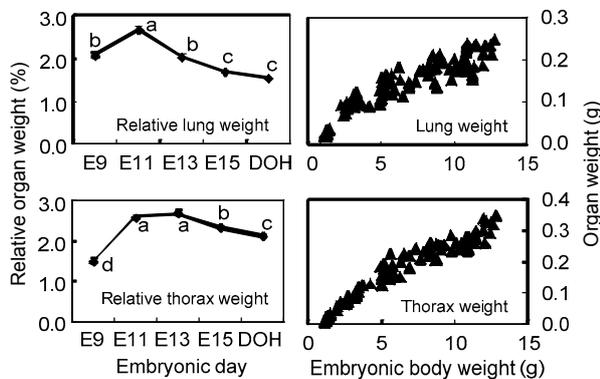


Fig. 5 Relative organ (lung and thorax; $n=20$) weight and organ (lung and thorax; $n=112$) weight plotted as a function of embryonic body weight for pigeons

Relative organ weight is calculated as absolute weight normalized to embryonic body weight without yolk (%). Values are shown as mean \pm SE. Data with no common letters (a–e) indicate significant difference among embryonic days ($P<0.05$)

A linear model, power function or logarithmic function with organ weight was represented as a function of embryo weight according to the scatter diagram plot. The results indicated that organs have their special development patterns.

3.3 Correlation of embryo weight and embryonic day

On Day 16 of incubation, eggs were checked every 3 h to determine time of external pipping and hatching, and the DOH was on E17.5. The correlation of embryo weight and embryonic day was measured. The result showed that the body weights had linearly correlated with the embryonic day ($CC=0.978$, $P<0.0001$) and the equation of linear regression was $Y=-10.59+1.26X$ ($R^2=0.9606$; Y was embryo weight of pigeon, and X was embryonic day).

3.4 Gene expression of nutrient transporters in small intestine

In our study, gene expressions for ten amino acid transporters (*EAAT3*, *EAAT2*, *b^{0,+}AT*, *y⁺LAT2*, *CAT1*, *CAT2*, *LAT1*, *LAT4*, *SNAT1*, and *SNAT2*), two sodium and proton transporters (*NHE2* and *NHE3*), and a peptide transporter (*PepT1*) were measured. Compared with the housekeeping genes, *GAPDH*, *β -actin*, *TBP*, and *HPRT1*, the nutrient transporter expression patterns were analyzed on E9, E11, E13, E15, and DOH in the small intestine of embryonic pigeons. The results indicated that genes of *EAAT3*, *b^{0,+}AT*, *y⁺LAT2*, *PepT1*, *LAT4*, *NHE2*, and *NHE3* showed similar expression profiles in the small intestine with

Table 2 Correlation of organ weight and embryonic body weight

Weight of organs (Y)	Embryonic body weight of pigeon (X , g)	
	Correlation coefficient (P -value)	Regression model (R^2)
Head	0.973 (<0.0001)	$Y=0.54X^{0.61}$ (0.9874)
Brain	0.987 (<0.0001)	$Y=54.71\times 10^{-3}X^{0.76}$ (0.9819)
Yolk	-0.637 (<0.0001)	$Y=1.14\ln X$ (0.8164)
Thorax	0.968 (<0.0001)	$Y=0.19\ln X$ (0.9861)
Leg	0.971 (<0.0001)	$Y=16.41\times 10^{-3}X$ (0.9815)
Heart	0.946 (<0.0001)	$Y=6.27\times 10^{-3}X^{1.13}$ (0.9544)
Liver	0.903 (<0.0001)	$Y=15.30\times 10^{-3}\times 1.33^X$ (0.9661)
Lung	0.925 (<0.0001)	$Y=0.07\ln X$ (0.9860)
Kidney	0.952 (<0.0001)	$Y=3.02\times 10^{-3}X^{1.66}$ (0.9731)
Gizzard	0.981 (<0.0001)	$Y=12.26\times 10^{-3}X^{1.47}$ (0.9920)
Small intestine	0.894 (<0.0001)	$Y=4.01\times 10^{-3}X^{1.74}$ (0.9655)

A linear model, power function or logarithmic function with organ weight was represented as a function of embryonic body weight according to the scatter diagram plot ($n=112$), which used a least-square regression analysis method of the REG Procedure by a stepwise variable filter method

a gradual increase in the intestine from E9 to DOH and a rapid rise during late embryogenesis (Table 3). The gene expressions of *CAT1*, *CAT2*, *LAT1*, *EAAT2*, *SNAT1*, and *SNAT2* exhibited similar profiles in the small intestine, with higher levels on E9 or E11, which then decreased to lower levels until the end of incubation (Table 3).

3.5 Correlation of gene expression and weight of body and small intestine

The results indicated that the gene expression of *EAAT3* and *PepT1* had a strong positive correlation

with body weight ($CC > 0.8$, $P < 0.0001$), but *CAT1* had a strong negative correlation with body weight and it had a CC of -0.860 . The gene expressions of $b^{0,+}AT$, *LAT4*, *NHE2*, *NHE3*, and y^+LAT2 showed positive correlations with body weight ($0.6 < CC < 0.8$, $P < 0.0001$), whereas *CAT2*, *EAAT2*, *SNAT1*, and *SNAT2* had negative correlations with body weight ($-0.8 < CC < -0.6$, $P < 0.0001$). Although the *LAT1* gene expression in the embryonic small intestine was not at a low level during the embryonic developmental stage, it had a low correlation with body weight ($CC = -0.381$, $P = 0.0035$) (Table 4).

Table 3 Effect of embryonic day on gene expression of nutrient transporters in small intestine

Gene	Gene expression ($\times 10^{-3}$)				
	E9	E11	E13	E15	DOH
$b^{0,+}AT$	2.76 \pm 0.26 ^b	9.12 \pm 1.71 ^b	8.75 \pm 0.79 ^b	58.43 \pm 8.29 ^b	603.21 \pm 52.28 ^a
<i>CAT1</i>	358.37 \pm 18.00 ^b	460.62 \pm 30.62 ^a	350.16 \pm 22.65 ^b	163.62 \pm 12.66 ^c	53.24 \pm 5.50 ^d
<i>CAT2</i>	449.87 \pm 45.35 ^b	651.16 \pm 71.66 ^a	362.22 \pm 22.33 ^b	151.00 \pm 14.48 ^c	72.42 \pm 5.63 ^c
<i>EAAT2</i>	403.76 \pm 48.53 ^a	429.48 \pm 38.33 ^a	374.95 \pm 32.88 ^a	136.05 \pm 7.77 ^b	67.81 \pm 4.64 ^b
<i>EAAT3</i>	35.95 \pm 4.83 ^d	65.77 \pm 4.91 ^d	135.10 \pm 18.10 ^c	222.63 \pm 14.53 ^b	363.88 \pm 40.61 ^a
<i>LAT1</i>	353.45 \pm 42.27 ^a	287.8 \pm 21.51 ^{ab}	246.56 \pm 29.82 ^b	148.05 \pm 17.3 ^c	72.71 \pm 5.44 ^c
<i>LAT4</i>	52.58 \pm 2.44 ^d	72.76 \pm 5.15 ^{cd}	99.97 \pm 5.06 ^{bc}	121.78 \pm 13.06 ^b	597.92 \pm 30.59 ^a
<i>NHE2</i>	37.25 \pm 4.58 ^b	55.68 \pm 7.13 ^b	28.09 \pm 3.22 ^b	64.48 \pm 10.43 ^b	703.42 \pm 46.41 ^a
<i>NHE3</i>	22.40 \pm 3.02 ^b	33.43 \pm 8.71 ^b	25.55 \pm 2.75 ^b	78.89 \pm 8.66 ^b	420.75 \pm 58.39 ^a
<i>PepT1</i>	4.96 \pm 0.70 ^c	4.42 \pm 0.60 ^c	16.96 \pm 1.22 ^c	88.12 \pm 6.58 ^b	385.96 \pm 33.79 ^a
<i>SNAT1</i>	288.51 \pm 23.14 ^a	54.86 \pm 9.88 ^b	59.22 \pm 4.02 ^b	40.96 \pm 2.44 ^b	28.79 \pm 4.09 ^b
<i>SNAT2</i>	851.41 \pm 56.95 ^a	286.70 \pm 51.87 ^b	185.37 \pm 6.49 ^c	172.85 \pm 12.02 ^c	153.12 \pm 8.22 ^c
y^+LAT2	82.92 \pm 6.79 ^c	74.02 \pm 4.04 ^c	88.30 \pm 3.26 ^c	152.20 \pm 13.87 ^b	624.61 \pm 30.72 ^a

E: embryonic day. *GAPDH*, *β -actin*, *TBP* and *HPRT1* are used for normalization as housekeeping genes. Data are shown as mean \pm SE ($n=12$). Different letters (a–e) within the same row indicate significant differences among embryonic day ($P < 0.05$).

Table 4 Correlation of embryo weight and gene expression

Gene expression (X)	Embryo weight (Y)	
	Correlation coefficient (P -value)	Regression model (R^2)
$b^{0,+}AT$	0.743 (< 0.0001)	$Y = 1.80 \ln X + 12.80$ (0.9021)
<i>CAT1</i>	-0.860 (< 0.0001)	$Y = -3.77 \ln X + 0.46$ (0.8009)
<i>CAT2</i>	-0.745 (< 0.0001)	$Y = -3.68 \ln X + 1.16$ (0.8346)
<i>EAAT2</i>	-0.790 (< 0.0001)	$Y = -4.14 \ln X - 0.14$ (0.8372)
<i>EAAT3</i>	0.820 (< 0.0001)	$Y = 24.43 X^{0.78}$ (0.8488)
<i>LAT1</i>	-0.381 (0.0035)	
<i>LAT4</i>	0.791 (< 0.0001)	$Y = 3.91 \ln X + 13.77$ (0.8854)
<i>PepT1</i>	0.821 (< 0.0001)	$Y = 1.98 \ln X + 13.35$ (0.9585)
<i>NHE2</i>	0.736 (< 0.0001)	$Y = 2.47 \ln X + 12.17$ (0.7050)
<i>NHE3</i>	0.711 (< 0.0001)	$Y = 2.57 \ln X + 14.06$ (0.8279)
<i>SNAT1</i>	-0.636 (< 0.0001)	$Y = 9.30 \times (5.59 \times 10^{-3})^X$ (0.8279)
<i>SNAT2</i>	-0.656 (< 0.0001)	$Y = 1.15 X^{-1.06}$ (0.8341)
y^+LAT2	0.782 (< 0.0001)	$Y = 3.75 \ln X + 13.49$ (0.7702)

A linear model, power function or logarithmic function with embryo weight was represented as a function of gene expression according to the scatter diagram plot ($n=60$), which used a least-square regression analysis method of the REG Procedure by a stepwise variable filter method

The gene expressions of $b^{0+}AT$, $EAAT3$, $LAT4$, $PepT1$, $NHE2$, $NHE3$, and y^+LAT2 showed positive correlations with intestinal weight ($CC > 0.8$, $P < 0.0001$), whereas $CAT1$, $CAT2$, and $EAAT2$ showed negative correlations, with CC ranging from -0.841 to -0.672 (Table 5). However, there was no significant correlation between the gene expression of other genes ($LAT1$, $SNAT1$, and $SNAT2$) and intestinal weight. Based on regression analysis, it was shown that different nutrient transporters had different gene expression patterns. This might be attributed to the demand of physiological function.

4 Discussion

4.1 Development of embryos

It was well known that avian embryo growth is affected by breeder age, egg weight, and incubation temperature (Tona et al., 2004; Ipek et al., 2014). With larger fresh eggs, there are more resources available within the egg's closed environment for the embryo to use during development. Thus, larger eggs have larger embryos (Al-Murrani, 1978). During embryonic development, the absolute weight of pigeon embryo increases constantly with age. The average embryo mass of pigeon on DOH was only (13.36 ± 0.17) g. Compared with precocial poultry, pigeons, as an altricial poultry, had small embryos for

the lower energy. One report has stated that growth of embryos varies in direct proportion to egg mass during incubation (Filippi et al., 2012). However, to elucidate the changes in embryo, the relative embryo weight was measured during embryonic development. The relative embryo weight increased significantly with age, and the embryo growth in relative terms was increasing approximately up to 60% from E9 to DOH. In addition, to measure the correlation of embryonic day and embryo weight, we presented the data of embryo weight as a function of embryonic day. The result showed that the growth of embryos was linearly correlated with the embryonic day, which indicated that the development of embryos was affected by embryonic day.

4.2 Development of organs

Different organs have different growth trajectories (Figs. 2–5). However, some organs have similar growth patterns when normalized by body weight. The head and brain, when normalized by body weight, showed a similar pattern as the first kind of organ with a gradual decrease during embryonic development (Fig. 2). It is well known that the head and the brain, among the ten organs we measured, are a section of the body renowned for dropping their relative weight during development in many species (Azzam and Mortola, 2007). The increased absolute weights of those two organs but their decreased relative weights

Table 5 Correlation of small intestinal weight and gene expression

Gene expression (X)	Small intestinal weight (Y)	
	Correlation coefficient (P -value)	Regression model (R^2)
$b^{0+}AT$	0.907 (<0.0001)	$Y=0.07\ln X+0.39$ (0.8972)
$CAT1$	-0.841 (<0.0001)	$Y=-0.16\ln X-0.12$ (0.8952)
$CAT2$	-0.672 (<0.0001)	$Y=-0.15\ln X-0.08$ (0.7738)
$EAAT2$	-0.748 (<0.0001)	$Y=-0.17\ln X-0.12$ (0.8167)
$EAAT3$	0.801 (<0.0001)	$Y=1.10X^{1.34}$ (0.8559)
$LAT1$	-0.104 (0.4408)	
$LAT4$	0.943 (<0.0001)	$Y=0.18\ln X+0.49$ (0.9378)
$PepT1$	0.946 (<0.0001)	$Y=0.92X+0.03$ (0.9378)
$NHE2$	0.914 (<0.0001)	$Y=0.11\ln X+0.43$ (0.9056)
$NHE3$	0.864 (<0.0001)	$Y=0.11\ln X+0.45$ (0.8845)
$SNAT1$	-0.455 (0.0004)	
$SNAT2$	-0.469 (0.0003)	
y^+LAT2	0.942 (<0.0001)	$Y=0.75X+0.01$ (0.9471)

A linear model, power function or logarithmic function with small intestinal weight was represented as a function of gene expression according to the scatter diagram plot ($n=60$), which used a least-square regression analysis method of the REG Procedure by a stepwise variable filter method

may be accounted for by their priority development. Li *et al.* (2013) and Azzam *et al.* (2007) observed similar growth trajectories in the head during chick embryonic development. In contrast to the head and brain, the kidney, gizzard, and leg were classified as the second kind of organ because their relative weight had similar growth patterns, initially low and increasing gradually during embryonic development. The relative weight of kidney was similar to our previous study on chicken, while the gizzard, as one of the digestive organs, showed different development trajectories as described by Li *et al.* (2013).

In this study, the heart, liver, and small intestine were included as the third kind of organ for the similar growth pattern with a slight increase from E9 to E15 but a rapid rise from E15 to DOH in terms of their relative weights. During embryonic development, nutrition from yolk, digestive tract or blood was stored, absorbed and transformed in the liver and then passed into other organs to utilize. However, in the final stage of incubation, β -oxidation of fatty acids cannot provide the energy needed for embryonic development (Tazawa *et al.*, 1983; Moran, 2007). Therefore, the development of embryos was mainly driven by glucose metabolism, such as gluconeogenesis, glycogen synthesis, and glycogenolysis, which can explain the reason for the relative liver weight increasing rapidly near the end of incubation. This result demonstrated that the liver, as one organ of metabolism, might play an important role in the last 2 to 3 d of incubation. It is necessary to mention that the small intestine, as another digestive organ, plays a key role in the avian because of its key function in absorbing nutrients. In our study, both the absolute weight and relative weight of small intestine exhibited a gradual increase throughout the hatching process with a sharp rise before hatch. Similar results were also reported by Dong *et al.* (2012b). This change, as has also been observed in precocial poultry, such as chickens (Li *et al.*, 2013) and turkeys (de Oliveira *et al.*, 2009). A rapid increase in intestinal weight and relative weight from E15 to DOH was to prepare for enteral nutrition. In addition, the lung and thorax were classified as the fourth type of organ. During chick embryonic development, the formation of the vascular system of the lung was on E17, and the respiratory function of lung began close to hatching. The reasons why the development of lung showed

such different patterns between precocial and altricial species required further investigation.

To test the growth pattern of organs, correlations were observed in body weight and organ weight. In addition to the yolk, ten other organs (head, brain, heart, liver, lung, kidney, gizzard, small intestine, leg, and thorax) were positively correlated with embryo body weight. When these organ weights were plotted as a function of embryonic body weight, a logarithmic, linearly or power model was used according to the scatter diagram plot. The results indicated that the ontogeny of organs was not linear with that of the embryo. Furthermore, organs had their own special growth patterns.

4.3 Gene expression

During the last stage of chick embryonic development, the major nutrient source for embryos not only derived from the yolk sac membrane, but also via the yolk stalk into the small intestine (Noy and Sklan, 1998). Dong *et al.* (2012b) had reported that nutrient absorption shifted from the sac membrane to the small intestine between E16 to DOH for pigeons. Thus, the small intestine plays a central role in nutrient absorption at the end of the incubation period. To our knowledge, the absorptive capacity of small intestine can be evaluated by studying the nutrient transporter gene expression pattern. Therefore, we investigated the gene expression of 13 nutrient transporters normally expressed in the small intestine that were responsible for nitrogen nutrient uptake. The mRNA expressions of *EAAT3*, *b⁰⁺AT*, *y⁺LAT2*, *PepT1*, *LAT4*, *NHE2*, and *NHE3* showed similar patterns in the small intestine, with a slight increase or no change from E9 to E15 and a rapid rise from E15 to DOH, suggesting an importance in the last 2 or 3 d before hatching. According to Hirst (1993), intestinal amino acid transporter expression is known to be induced by raised levels of dietary protein. This suggested that provision of nutrients via the yolk stalk from E16 to DOH may provide a nutrient signal for induction of transporter mRNA. On the contrary, the gene expressions of *CAT1*, *CAT2*, *LAT1*, *EAAT2*, *SNAT1*, and *SNAT2* exhibited similar profiles in the small intestine, with higher levels on E9 or E11 and lower levels toward the end of incubation; this indicated that those six genes played an important role in the second stage of incubation (from E8 to E14).

At the brush border membrane, peptides and free amino acids are taken into cells by peptide and amino acid transporters. Reports from our laboratory on chicken suggested that $b^{0,+}AT$, *EAAT3*, and *PepT1*, which are expressed on the brush border membrane, could be used as indicators of the small intestinal growth of chick embryos (Li *et al.*, 2013). In this study, the mRNA levels of those three transporters increased significantly ($P < 0.0001$) with embryonic day, especially $b^{0,+}AT$, which increased approximately 220-fold from E9 to DOH, suggesting that $b^{0,+}AT$ was more important during late embryogenesis. The $b^{0,+}AT$ is a Na^+ -independent amino acid transporter, with a high extracellular affinity for cationic amino acids. *EAAT3*, as an excitatory amino acid transporter, has a high affinity for glutamate and aspartate. Glutamate, as an oxidative fuel source, is involved in a variety of metabolic sequences in enterocytes, increasing the amounts of energy needed to meet the needs of the rapidly growing embryos. Thus, it is reasonable that mRNA expression of *EAAT3* increased significantly ($P < 0.0001$) from E11 to DOH in our study. Gilbert *et al.* (2007) found that levels of *EAAT3* mRNA increased linearly ($P = 0.001$) from E20 to D14 in broiler small intestine, and the most important site for free amino acid assimilation was the ileum. These changes suggested that *EAAT3* served as an important role in the growth of the small intestine for its key function in nutrient uptake.

Amino acids are transported into the enterocyte as di- or tri-peptides through *PepT1* (Leibach and Ganapathy, 1996), which is more efficient than through the free amino acid transporters (Daniel, 2004). Dong *et al.* (2012b) stated that the mRNA levels of *PepT1* increased linearly ($P < 0.0001$) from E12 to D14 in pigeon. The mRNA levels of *PepT1* were greatest in the duodenum compared with the ileum, but were of middle levels in jejunum; this was consistent with studies in chicken by Gilbert *et al.* (2007). In this study, expression of *PepT1* mRNA increased significantly from E13 to DOH, suggesting an importance in peptide transport in pigeon embryo development. In addition, the quantities of *NHE2* and *NHE3* expressed at the brush border membrane were also evaluated in this study. The *NHE2* and *NHE3* belong to the family of antiporters, known generically as Na^+/H^+ exchangers (*NHEs*). It is well known that Na^+/H^+ exchange is crucial for the regulation of in-

tracellular H^+ in exchange for external Na^+ . However, Na^+/H^+ exchange is up- and down-regulated as a part of digestion and diarrheal diseases via intestinal neutral NaCl absorption (Orlowski and Grinstein, 1997). In our study, the mRNA expression of *NHE2* and *NHE3* increased significantly ($P < 0.0001$) on the day of hatching, which was similar to the expression of $b^{0,+}AT$ mRNA, suggesting it becomes more important in the late stage of incubation.

Gene expression of the amino acid transporters on the basolateral membrane is different from that on the brush border membrane (Gilbert *et al.*, 2007). In our study, the genes for *EAAT2*, *CAT1*, *CAT2* and *LAT1* are expressed in the basolateral membrane with higher levels on E9 or E11, then decrease with age, suggesting an important role in pigeon embryonic growth. In the early stages of embryonic development, nutrients are absorbed into enterocytes from the blood through the basolateral membrane. Thus, the four genes may be expressed with higher levels on E9 or E11. It is well known that CAT proteins transport cationic amino acids in a Na^+ -independent way (Closs *et al.*, 2006). However, the system y^+ cationic amino acid transport (*CAT*) family undergoes adaptive up-regulation during amino acid starvation (Humphrey *et al.*, 2006) and thus might be repressed by nutrient provision from the yolk stalk. Zeng *et al.* (2011) found that the level of *CAT1* in broiler intestine increased linearly with age ($P < 0.0001$) but changed little during embryonic development, while *CAT2* mRNA increased from E9 to E17, then decreased on DOH. In our study, the *CAT1* and *CAT2* exhibit similar expression patterns, peaking on E11, then decreasing gradually until the end of incubation. Gilbert *et al.* (2007) reported similar findings in chicken, with the expressions of *CAT1* and *CAT2* decreasing with age. *LAT1* was investigated widely on the basis of its function to transport large neutral amino acids into *Xenopus* oocytes when expressed with *4F2hc* (Kanai *et al.*, 1998; Mastroberardino *et al.*, 1998). In our study, *LAT1* expression in the intestine was at a high level on E9 and gradually decreased in expression from E11 to DOH.

The sodium-coupled neutral amino acid transporter 1 (SNAT1) is a membrane protein, which plays a crucial role in the uptake of glutamine into cells in the central nervous system (Chaudhry *et al.*, 2002). SNAT1 is also responsible for the transport of neutral

amino acids into or out of cells in mammals (McGivan and Bungard, 2007). So far, *SNATI* is recognized as a target for oncogene action because the amount of its mRNA is increased in human hepatocarcinoma cells. In this study, the mRNA level of *SNATI* was relatively high on E9 and decreased to a low level from E11 to DOH, suggesting an important role during incubation. A similar gene expression pattern was observed in *SNAT2*. Our study found that the gene expression of *SNAT2* was greater than that of *SNATI* throughout development, which suggested that *SNAT2* might be more important than *SNATI* in the small intestine during embryonic development. This phenomenon can be explained by the fact that *SNAT2* belongs to the system A transporter and is expressed in the intestine and most likely reflects the activity of system A (Bröer, 2008). However, *SNAT2* appears adaptive upregulation during the amino acids starvation (Tanaka *et al.*, 2005). Therefore, the gene expression of *SNAT2* might be repressed by nutrient provision from the yolk stalk. Furthermore, we demonstrated that gene expression of y^+LAT2 and *LAT4* increased slowly from E9 to E15, and then increased rapidly near DOH, suggesting an important role in the post-hatch pigeons. Similarly, the results for y^+LAT2 were also reported in chicken to increase significantly from E18 to DOH (Gilbert *et al.*, 2007). A similar result for *LAT4* was observed in chickens by Zeng *et al.* (2011).

To test the relationship between gene expression of nutrient transporters and embryo and small intestinal weight, we used regression analysis. Our study demonstrated that there was a strong correlation between gene expressions of *EAAT3* and *PepT1* and embryo weight, while there was a strong negative correlation between *CAT1* gene and body weight. This suggested those three genes were more important in embryonic development. Furthermore, we found that most of those gene expressions detected in our study were strongly correlated with small intestinal weight, such as b^0+AT , y^+LAT2 , *CAT1*, *LAT4*, *EAAT3*, *PepT1*, *NHE2*, and *NHE3*. This indicated gene expression of those nutrient transporters might facilitate the digestive capacity of the small intestine in pigeon during embryonic development. However, whether the expression of those genes can be regarded as indicators for the growth of the small intestine requires further research.

5 Conclusions

In our study, we classified ten organs into four types according to changes in relative weight during embryonic development. These results provide a comprehensive study of amino acid and peptide transporter mRNA in the small intestine of pigeons. Future studies will involve transporter proteins and their functionality to understand nutrient absorption capacity of small intestine in the pigeon.

Compliance with ethics guidelines

Ming-xia CHEN, Xiang-guang LI, Jun-xian YANG, Chun-qi GAO, Bin WANG, Xiu-qi WANG, and Hui-chao YAN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题目: 家鸽胚胎的生长与其小肠营养素转运载体基因表达

目的: 探索家鸽胚胎期器官生长发育及其小肠营养素转运载体基因表达规律, 为家鸽胚胎期的营养调控提供理论基础。

创新点: 第一次较全面地研究了家鸽胚胎期小肠氨基酸转运载体基因表达规律。

方法: 利用实时荧光定量逆转录聚合酶链反应 (real-time RT-PCR) 技术, 同时结合了多内标校正方法对小肠营养素转运载体基因表达模式进行分析。

结论: 在家鸽胚胎期发育的过程中, 不同的器官生长模式不完全一致。营养素转运载体 $b^{0+}AT$ 、 $EAAT3$ 、 $LAT4$ 、 $PepT1$ 、 $NHE2$ 、 $NHE3$ 和 $y^{+}LAT2$ 基因的表达与小肠重量呈正相关关系, $CAT1$ 、 $CAT2$ 和 $EAAT2$ 基因的表达与小肠重量呈负相关关系。

关键词: 胚胎生长; 营养素转运载体; 基因表达; 小肠; 家鸽