The profiling of amino acids in crop milk and plasma and mRNA abundance of amino acid transporters and enzymes related to amino acid synthesis in the crop tissue of male and female pigeons during incubation and chick-rearing periods

P. Xie,[*,†,‡,1] M. X. Han,[†] W. X. Chen,[†] X. P. Wan,[§] Y. G. Xu,[*,†] and D. Q. Gong[‡]

[*]*Jiangsu Collaborative Innovation Center of Regional Modern Agriculture & Environmental Protection, Huaiyin Normal University, Huaian 223300, China;* [†]*Jiangsu Key Laboratory for Eco-Agricultural Biotechnology around Hongze Lake, Huaiyin Normal University, Huaian 223300, China;* [‡]*College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China; and* [§]*Department of Physiology, Medical College of Georgia, Augusta University, Augusta, GA 30912, USA*

ABSTRACT The present study was carried out to investigate the changes in amino acid (**AA**) contents of crop milk and plasma and mRNA abundance of AA transporters and AA synthesis–related enzymes in the crop tissue of male and female pigeons during incubation and chick-rearing periods. Forty-two pairs of adult White King pigeons with 2 fertile eggs per pair were randomly divided into 7 groups by different breeding stages. The AA content of crop milk decreased from day 1 (**R1**) to day 25 (**R25**) of chick rearing ($P < 0.05$). In both male and female adult pigeons, the contents of Thr, Leu, Val, His, Asp, and Pro in plasma increased to maximum levels on R25. Parental sex effect and interaction between stage and sex were observed in the AA contents of pigeon plasma ($P < 0.05$). For AA transporters, the mRNA abundances of *SNAT2*, *ASCT1*, *LAT1*, and y^+LAT2 in the male crops reached the highest value on day 17 of incubation (I17), and the peak mRNA levels of *PAT-1*, *xCT*, $b^{0,+}AT$, and *CAT1* were found on R7 ($P < 0.05$). In females, the abundances of *ASCT1*, B^0AT1, *asc-1*, and *CAT1* mRNA peaked on R1, whereas the maximum levels of *LAT1*, *PAT-1*, $b^{0,+}AT$, and y^+LAT2 were observed on R7. For enzymes involved in AA synthesis, the highest gene expressions of glutamate dehydrogenase 1, acetolactate synthase in both parent pigeons, and L-threonine 3-dehydrogenase in female pigeon crops were attained on I17. The expressions of ornithine-δ-aminotransferase, glutamic-oxal(o)acetic transaminase 1, glutamic-oxal(o)acetic transaminase 2, asparagine synthetase, serine hydroxymethyltransferase 2, and glutamic-pyruvic transaminase 2 in both sexes and argininosuccinate lyase and L-threonine 3-dehydrogenase in males were the highest on R1. In conclusion, AA used for pigeon crop milk formation may originate from plasma and intracellular synthesis. The genes involved in AA transport and synthesis varied significantly with sexual effects, indicating that other factors should be considered in future explorations of the mechanism of protein formation in crop milk.

Key words: pigeon, crop milk, plasma, amino acid transporter, enzyme

2020 Poultry Science 99:1628–1642
https://doi.org/10.1016/j.psj.2019.10.057

INTRODUCTION

To provide optimal nutritional support for squabs, the crop sac of pigeon undergoes a dramatic series of changes in size, morphology, and function throughout the life cycle of epithelial cells, in response to prolactin and local growth factors (Horseman and Buntin, 1995; Gillespie et al., 2011; Xie et al., 2018). Crop milk contains predominantly protein (60% of dry matter) and lipids (30% of dry matter) at the beginning of regurgitation (Carr and James, 1931; Xie et al., 2017). This nourishing cheese-like substance was considered to be the major reason for squabs' much higher maturation rate compared with that of broilers, quails, and ostriches (Sales and Janssens, 2003). Crop milk is produced by both parental pigeons and cannot be stored by the organ; however, differences in the composition of crop

Received August 9, 2019.
Accepted October 23, 2019.

[1]Corresponding author: pengxiejqs@126.com

milk between male and female pigeons are rarely examined because of sampling difficulties, and to date, there has been a paucity of information regarding the protein synthesis of crop milk in the pigeon.

During lactation, the mammary gland creates a large demand for free amino acids (**AA**) from blood to feed milk production because more than 90% of milk protein is synthesized de novo in mammary tissues (Backwell et al., 1996). The uptake of AA is carried out by different transporters located on the cell membrane, and these AA transporter systems can be divided into neutral, cationic, anionic, and others; some of them shuttle multiple AA into and out of the cells (Bröer, 2008). However, inconsistent results concerning the expression patterns of AA transporter genes in the mammary gland from pregnancy to lactation have been reported in different mammals. Some have shown that the expression of L-type AA transporter 1 (***LAT1***), Na^+-dependent cationic AA transporter (***CAT1***), Na^+-coupled neutral AA transporter 2 (***SNAT2***), excitatory AA transporter 3 (***EAAT3***), and alanine/serine/cysteine/threonine transporter (***ASCT1***) in the mammary gland coincided with the peak of milk production in the rat and sow (Alemán et al., 2009; Chen et al., 2018), whereas Laspiur et al. (2004) found that *CAT1* and *ASCT1* expressions do not change, and $B^{0,+}$ mRNA abundance was lower during lactation in the pig. In pigeons, various AA transporters in the small intestine had been analyzed during the posthatch period (Gao et al., 2016; Zhang et al., 2017), and their expressions were considered to be representative of organ development; however, the potential role of these transporters in crop milk formation remains unknown.

Owing to insufficient uptake by mammary epithelial cells, in situ and de novo biosynthesis of nonessential AA (**NEAA**) is an important pathway supporting milk protein production, and it has been reported that some essential AA (**EAA**) can be taken up in excess for conversion to specific NEAA (Bröer, 2008). For example, branched-chain AA (**BCAA**) (valine, leucine, and isoleucine) are catalyzed to provide amino groups for the synthesis of glutamate and glutamine (DeSantiago et al., 1998; Lei et al., 2012), which are necessary for neonatal growth and digestive tract maturation (Cabrera et al., 2013). During the process of the intramammary metabolism of AA, key enzymes such as arginase, ornithine-δ-aminotransferase (***OAT***), and branched-chain alpha-keto acid dehydrogenase are indispensable and extensively studied (Yip and Knox, 1972; Basch et al., 1995; DeSantiago et al., 1998). An interesting question is whether these AA metabolic enzymes exist in pigeon crop tissues and are involved in crop milk formation. The author has presented the hypothesis that AA used for protein synthesis in crop milk probably originated from 2 pathways: one dependent on AA transport systems for targeting plasma AA and the other on de novo biosynthesis of AA in crop epithelial cells.

Therefore, the objective of the present study was to determine the changes in the AA composition of crop milk and plasma and gene expression of AA transporters and AA metabolic enzymes in crop tissues of male and female pigeons during incubation and chick rearing under artificial farming conditions.

MATERIALS AND METHODS

All procedures used in this study were approved by the Animal Care Advisory Committee of Yangzhou University.

Birds and Housing

Eighty-four (42 males and 42 females) adult White King pigeons of 60 wk of age were obtained from a commercial pigeon farm (Kunpeng Pigeon Co., Ltd., Xuzhou, China). All pigeons were paired after sexual maturity and chosen to have the same oviposition interval. Each pair was housed in an artificial aviary equipped with a nest and perch and subjected to a 50-D study, which included a 7-D acclimation and a 43-D experimental period, the latter consisting of 18-D incubation and 25-D chick-rearing periods. Birds were randomly divided into 7 groups according to their different breeding stages with 3 incubation times (4 [**I4**], 10 [**I10**], and 17 D [**I17**]) and 4 chick-rearing times (1 [**R1**], 7 [**R7**], 15 [**R15**], and 25 D [**R25**]). To maintain the broodiness of parental birds, plastic eggs were brought into cages only after the second egg was laid as described previously (Xie et al., 2018). Baby squabs hatched from the incubator were reared by parents after 18 D of incubation. To investigate the sexual effects on AA composition in male and female pigeons, the paired birds were separated into adjacent cages at night (21:00 pm) on the 18th D of incubation to avoid stress, with one squab fed by each parent (Xie et al., 2017). The birds were fed a pellet diet of 55% corn, 24.5% soybean meal (44.2% CP), 11% wheat, 1.2% dicalcium phosphate, 2% limestone, 0.25% salt, 0.5% vitamin and mineral premix, 2% soybean oil, 3.42% zeolite powder, 0.07% lysine, and 0.06% methionine (16.67% CP, 12.00 MJ/kg of ME, 1.13% calcium, 0.34% available P, 0.89% lysine, and 0.31% methionine). The nutrient data can be referenced from our previous study (Xie et al., 2016). The birds received feed, sand, and water ad libitum. Light was provided for 16 h daily throughout the experiment.

Sample Preparation

The pellet diets were ground to pass a 1-mm screen, and quintuple samples were used to analyze AA composition. Crop milk was produced by adult pigeons, but it cannot be stored by the organ, so milk of parents was often collected from young pigeons soon after being fed during the chick-rearing period (Bharathi et al., 1997). According to the method described previously (Xie et al., 2017), a surgical blade was used to carefully make slits on the crop of squabs after disinfection with povidone-iodine, and pigeon milk was collected and incision was immediately closed by double sutures. Two

samples from the same group were pooled, aliquoted, and stored at −80°C for AA analysis.

Both plasma AA composition and gene expressions in crop tissues were determined in adult pigeons. After a 12-h fast, blood from parental pigeons was collected via wing vein puncture into tubes containing EDTA, placed on ice, and centrifuged at 1,500 × *g* for 20 min at 4°C. Plasma from 2 adult birds of the same sex was pooled, aliquoted, and stored at −80°C for AA analysis. After blood sampling, all pigeons were euthanized by cervical dislocation, and their crop tissues were quickly frozen in liquid nitrogen and stored at −80°C for gene detection. After sampling, eggs and squabs were transferred to nests at the pigeon farm to be cared for by other pigeons.

AA Analysis

Amino acid analysis was done for assessing the correlation between AA in plasma and AA in crop milk during "lactation" in pigeons. AA concentrations in feed samples and crop milk were measured using HPLC with an Agilent 1,100 series system (Agilent Technologies, Santa Clara, CA) after hydrolysis in 6 mol HCl at 110°C for 24 h. AA concentrations in plasma were determined according to the method described by Sano et al. (2018). Briefly, sulfosalicylic acid was added to plasma to a final concentration of 5%, and then, the samples were placed on ice for 15 min followed by centrifugation to remove precipitated proteins. The extracts were filtered through 0.22 μm Millipore membranes (Millipore Corp., Bedford, MA) and analyzed using an AA analyzer (Hitachi L-8900, Tokyo, Japan). The following AAs were evaluated: Pro, Glu, Gly, Ala, Ser, Asp, Cys, Tyr, Phe, Met, Lys, Thr, Leu, Ile, Val, His, and Arg.

RNA Isolation and Real-Time Quantitative PCR

TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) was used for the isolation of total RNA from the crop tissue. Briefly, frozen samples were ground with liquid nitrogen, and tissue powder was immediately transferred into TRIzol reagent, then deproteinized by chloroform, precipitated with isopropanol, and washed in 75% ethanol; finally, the resulting RNA was resuspended in RNase-free water. RNA quality was confirmed by both native RNA electrophoresis and determination of the absorbance ratio 260 nm/280 nm. cDNA was synthesized by M-MLV reverse transcriptase at 42°C for 60 min with an oligo dT-Adaptor primer.

To investigate whether intracellular availability of AA was controlled by coordinated activity of AA carrier proteins and enzymes responsible for de novo AA synthesis, fourteen AA transporters grouped in 3 categories (neutral, cationic, and anionic AA transporters) and 13 enzymes were analyzed in crops during incubation and chick rearing. Specific primers (Table 1) were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA), and β-actin was used as the internal control gene. The mRNA abundance of these genes was detected with real-time quantitative PCR. Real-time quantitative PCR was performed using SYBR Premix Ex Taq (Takara, Dalian, China) in a C1000 Touch Thermal Cycler equipped with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and evaluated with CFX Manager 3.1 software (Bio-Rad). The PCR program was 95°C for 30 s followed by 39 cycles of 95°C for 5 s and 60°C for 30 s. Each sample was analyzed in triplicate. Melting curve analysis was used to verify amplification specificity. The relative expression quantity was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

All data are presented as means ± SE. Data were statistically evaluated using SPSS 17.0 (SPSS Inc., Chicago, IL) and analyzed using the GLM procedure. The model included the main effects of stage, sex, and their interactions. Differences among breeding stages were estimated by Duncan's post hoc test. All statements of significance are based on $P < 0.05$.

RESULTS

AA Content

As shown in Tables 2 to 4, the breeding stage had a significant effect on the AA contents of crop milk and pigeon plasma ($P < 0.05$). All EAA and NEAA in the crop milk decreased markedly from day 1 to day 25 of chick rearing ($P < 0.05$) (Table 3), which is close to the AA levels of the pigeons' diet (Table 2). The contents of Val, Glu, and Ala in the male crop milk tended to be higher than those in the females ($P = 0.060$–0.066), but there was no significant difference in all AA contents between the male and female crop milk. An interaction between stage and sex was also not significant ($P > 0.05$) (Table 5). In male pigeon plasma, Thr, Leu, Val, Phe, Arg, His, Asp, and Pro increased to their maximum levels on R25, whereas Met, Cys, Gly, Ser, and Glu peaked on R1 ($P < 0.05$) (Table 4). In female pigeon plasma, Met, Thr, Ile, Leu, Val, His, Tyr, Asp, Glu, Ala, and Pro increased to their maximum levels on R25, whereas Cys and Arg peaked on R1, and the highest levels of Lys, Phe, Gly, and Ser were found on R7 ($P < 0.05$) (Table 4). A parental sex effect was observed in the AA contents of pigeon plasma, except for Cys, Phe, and Arg ($P < 0.05$) (Table 6). Concentrations of Met, Thr, Leu, His, Gly, Asp, Ser, and Ala in the female pigeons were higher than those in the male pigeons from R1 to R25 ($P < 0.001$) (Table 4; 6). For most plasma AA, except for Phe, Gly, and Asp, there was a significant interaction between stage and sex ($P < 0.05$) (Table 6).

Table 1. Primers used in the present study.

Target gene	Nucleotide sequence (5'→3')	Accession No.	Size (bp)
Genes involved in amino acid transport			
$b^{0,+}AT$	F: ATTATTGGAATCCCCTTGGTTAC	XM_005514428	80
	R: AGTTCTGTTGAAGTCATTACGGTG		
y^+LAT2	F: ACATTTCCCTCGCACTCT	XM_005504755	215
	R: CACTGCCACAGCATCACT		
CAT1	F: CTTCGCCGTGGTGATA	XM_005501421	108
	R: CCGAGGACGAGGATGT		
SNAT2	F: AAAGCCGAAGCCGTAAAAGAA	XM_005499916	92
	R: GTATCCAAAGAGAGCAGCCAATAAA		
ASCT1	F: TCGTTCAATGAGGCGACTATG	XM_013367937	142
	R: CGAAGATGTATTTTCCCAGGCT		
LAT1	F: TGTTTCCTTCTGGATGACGC	XM_021290674	197
	R: TCTCTGCTTTTCCTGCTTATGA		
LAT4	F: GACCTTCACATCGCTTACG	XM_005502005	163
	R: TGCCCAGACAAGCAGAAT		
B^0AT1	F: TGGCATAGCAGCAATGTCGG	XM_005509991	95
	R: CTTGGAAGGAGTTGAAGAAATACC		
$ATB^{0,+}$	F: AGCGTGGCAACTGGTCAA	XM_005500482	110
	R: TCCGCCGTTCTGGTAGGT		
asc-1	F: ACCTCCTCGTCCCCATCACTTA	XM_013371151	296
	R: CCTCGTGGTTGTCTTGCTTGTG		
EAAT3	F: ACAGGTGTTGCTGCTTTG	XM_021294088	171
	R: GGTGCTGCCCACTCTAT		
xCT	F: ATCTTCATCTCTCCCAAAGGCAT	XM_005499885	101
	R: AGGGCACCAAAGAGCGAGAG		
$IMINO^B$	F: AACATAGCTCCTCCGCCTTTC	XM_021300732	98
	R: GCCTTCCTGCCTTCACTCTTT		
PAT-1	F: TTGGGGAAGGCGTGTAGTG	XM_021288996	204
	R: ACAAAAGGCAGCAGGGAAAG		
Genes involved in amino acid synthesis			
GDH1	F: TGCCCTGCGACATCCTCATC	XM_021288950	217
	R: GGTTCAGGTTCTTCAGCCACTCA		
GS	F: CACCGAGGAGATGAGGAAAGAG	XM_013371526	242
	R: CAAAGTAGCCGCAGCCGTC		
OAT	F: CTTGGCGAATACGAGGAGATG	XM_013368105	222
	R: GCTGGATGGGTCGGTAGAA		
ASL	F: AGGAGGCTGTCTTTGATGTTGTGG	XM_021286946	245
	R: CAGGCTGAGATTATTGAGGGTGA		
ASN	F: ATTCCCATATCTGTGGCTGTGTTAC	XM_021290989	202
	R: CACTTTTCTGTTGGTGGTGTCC		
GOT1	F: AGAACTTTGGGCTCTACAATGAAC	XM_013368474	257
	R: GGATTCCAGGCGAGACCGA		
GOT2	F: CGGTTCTTCAAGTCCAGTCG	XM_005504788	116
	R: TTGGGGTCATAGTAGCGGTAA		
TDH	F: GTTTCCAACCCCAATCCCTC	XM_021294171	279
	R: CTTGGCGAATACGAGGAGATG		
PDH	F: CGATAGCACTACTGGCTTGTTG	XM_005498831	296
	R: CAGACAGGGGGGCTGAGAG		
SHMT1	F: ATCGCCGACGCCAACAGTG	XM_005504475	97
	R: CGCAGTGGTCAAAGGGGGA		
SHMT2	F: GGTGGATAAGAAGACGGGCAA	XM_005514134	77
	R: GCAGGGAGGGGAAAACAGC		
GPT2	F: GCTTTCCGTTCGCATTTGTC	XM_005504680	276
	R: GGTGCCATTTTGTGAGCCTTTG		
ACSN	F: CCGAGGTTTGGGTCCTTTAT	XM_005513927	126
	R: CAATCTGGCTCCAACACGC		
Internal control			
β-actin	F: TCAGGGTGTGATGGTTGGTAT	XM_005504502	159
	R: TCATTGTAGAAAGTGTGGTGCC		

Abbreviations: 3PGDH, phosphoglycerate dehydrogenase; *ACSN*, acetolactate synthase; *asc-1*, asc-type amino acid transporter 1; *ASCT1*, Na^+-dependent neutral amino acid transporter; *ASL*, arginino-succinate lyase; *ASN*, asparagine synthetase; *$ATB^{0,+}$*, Na^+- and Cl^--dependent neutral and cationic amino acid transporter; *$b0^+AT$*, Na^+-dependent cationic and zwitterionic amino acid transporter; *B^0AT1*, Na^+-dependent neutral amino acid transporter; *CAT1*, Na^+-dependent cationic amino acid transporter; *EAAT3*, excitatory amino acid transporter 3; F, forward; *GDH1*, glutamate dehydrogenase 1; *GOT1*, glutamic-oxal(o)acetic transaminase 1; *GOT2*, glutamic-oxal(o)acetic transaminase 2; *GPT2*, glutamic-pyruvic transaminase 2; *GS*, glutamine synthetase; *$IMINO^B$*, Na^+- and Cl^--dependent proline IMINO transporter; *LAT1*, L-type amino acid transporter 1; *LAT4*, L-type amino acid transporter 4; *OAT*, ornithine-δ-aminotransferase; *PAT-1*, proton-coupled amino acid transporter 1; R, reverse; *SHMT1*, serine hydroxymethyltransferase 1; *SHMT2*, serine hydroxymethyltransferase 2; *SNAT2*, Na^+-coupled neutral amino acid transporter 2; *TDH*, L-threonine 3-dehydrogenase; *xCT*, cystine/glutamate exchange transporter; *y^+LAT2*, Na^+-dependent cationic and Na^+-dependent neutral amino acid transporter 2.

Table 2. Analyzed concentrations of amino acids in the diet.[1]

Amino acids	Concentration (%)
Essential amino acids (EAA)	
Lysine	0.88
Methionine	0.32
Cystine	0.33
Threonine	0.63
Isoleucine	0.64
Leucine	1.38
Valine	0.75
Phenylalanine	0.81
Arginine	1.13
Histidine	0.35
Glycine	0.67
Tyrosine	0.33
Nonessential amino acids (NEAA)	
Asparagine	1.38
Serine	0.74
Glutamine	2.58
Alanine	0.77
Proline	0.51

[1]Values were presented as the means of triplicate per sample.

Gene Expression of Neutral AA Transporters

mRNA abundances of *SNAT2*, *ASCT1*, and *LAT1* in the male crops reached maximum levels at I17 ($P < 0.05$) (Figure 1, A–C), whereas abundances of *ASCT1* and B^0AT1 mRNA in the female crops and *LAT4* mRNA in the male crops peaked on R1 ($P < 0.05$) (Figure 1, B, D and E). In the females, gene expression of *LAT1* reached a significant 10-fold increase at day 7 of chick rearing ($P < 0.05$) (Figure 1C), and the peak value for *asc-1* expression was attained on R1, where an approximate 70-fold increase was observed compared with I4 ($P < 0.05$) (Figure 1G). Expressions of *LAT4* and $ATB^{0,+}$ genes in the female crops and *asc-1* gene in the male crops decreased significantly after I17 (Figure 1, D, F and G). A significant interaction of stage and sex for gene expression of *ASCT1*, *LAT4*, B^0AT1, $ATB^{0,+}$, and *asc-1* was observed in the study ($P < 0.05$) (Table 7).

Gene Expression of Anionic AA and Imino Acid Transporters

EAAT3 gene expression changed significantly with stage, sex, and their interaction ($P < 0.05$) (Table7) and reached the highest level at I10 in both the male and female pigeon crops ($P < 0.05$) (Figure 2A). *xCT* gene expression in the male pigeon crops increased 6.6-fold on day 7 of chick rearing ($P < 0.05$) (Figure 2B), and similarly, *PAT-1* gene expression in both the male and female pigeons gradually reached maximum values on R7 with a 7.4- to 11.2-fold increase compared with the beginning of incubation and then sharply decreased ($P < 0.05$) (Figure 2D). $IMINO^B$ gene expression peaked on R25 in the females, whereas no significant change was observed in the males ($P > 0.05$) (Figure 2C).

Gene Expression of Cationic AA Transporters

The peak mRNA levels of $b^{0,+}AT$ in both male and female pigeons, *CAT1* in the male pigeons and y^+LAT2 in the female pigeons were attained on R7, where a 3.2- to 22-fold increase was observed compared with day 4 of incubation ($P < 0.05$) (Figure 3, A–C). mRNA levels of both *CAT1* in the female pigeons and y^+LAT2 in the male pigeons increased to their highest level on R1 ($P < 0.05$) (Figure 3, B and C).

Table 3. Analyzed concentrations of amino acids (% dry matter) in crop milk during 25 D of chick rearing.[1]

	Chick-rearing period[2]							
	R1		R7		R15		R25	
Item	Male	Female	Male	Female	Male	Female	Male	Female
Essential amino acids (EAA)								
Lys	4.02 ± 0.14^{A}	3.63 ± 0.36^{a}	1.60 ± 0.05^{B}	1.23 ± 0.13^{b}	0.97 ± 0.04^{C}	0.93 ± 0.06^{b}	0.86 ± 0.01^{C}	0.86 ± 0.01^{b}
Met	1.10 ± 0.02^{A}	1.00 ± 0.16^{a}	0.40 ± 0.01^{B}	0.35 ± 0.01^{b}	0.37 ± 0.003^{B}	0.36 ± 0.01^{b}	0.36 ± 0.001^{B}	0.37 ± 0.003^{b}
Cys	0.85 ± 0.03^{A}	0.84 ± 0.06^{a}	0.31 ± 0.01^{B}	0.33 ± 0.003^{b}	0.32 ± 0.004^{B}	0.32 ± 0.003^{b}	0.32 ± 0.001^{B}	0.32 ± 0.001^{b}
Thr	2.71 ± 0.07^{A}	2.48 ± 0.23^{a}	1.16 ± 0.03^{B}	0.96 ± 0.05^{b}	0.86 ± 0.04^{C}	0.78 ± 0.07^{b}	0.66 ± 0.01^{D}	0.65 ± 0.002^{b}
Ile	2.04 ± 0.04^{A}	1.88 ± 0.16^{a}	0.88 ± 0.02^{B}	0.75 ± 0.05^{b}	0.71 ± 0.004^{C}	0.64 ± 0.02^{b}	0.61 ± 0.01^{D}	0.61 ± 0.002^{b}
Leu	4.98 ± 0.13^{A}	4.62 ± 0.37^{a}	2.31 ± 0.04^{B}	1.98 ± 0.11^{b}	1.78 ± 0.01^{C}	1.64 ± 0.15^{b}	1.39 ± 0.01^{D}	1.34 ± 0.01^{b}
Val	2.56 ± 0.04^{A}	2.34 ± 0.19^{a}	1.16 ± 0.03^{B}	0.97 ± 0.06^{b}	0.80 ± 0.01^{C}	0.78 ± 0.02^{b}	0.75 ± 0.01^{C}	0.75 ± 0.01^{b}
Phe	2.18 ± 0.04^{A}	2.11 ± 0.12^{a}	1.38 ± 0.03^{B}	1.15 ± 0.11^{b}	0.88 ± 0.01^{C}	0.86 ± 0.03^{b}	0.81 ± 0.004^{C}	0.83 ± 0.01^{b}
Arg	3.39 ± 0.08^{A}	3.13 ± 0.27^{a}	1.57 ± 0.04^{B}	1.36 ± 0.05^{b}	1.31 ± 0.01^{C}	1.21 ± 0.05^{b}	1.13 ± 0.002^{D}	1.16 ± 0.01^{b}
His	1.02 ± 0.01^{A}	0.95 ± 0.06^{a}	0.59 ± 0.01^{B}	0.51 ± 0.04^{b}	0.40 ± 0.01^{C}	$0.39 \pm 0.02^{b,c}$	0.35 ± 0.003^{D}	0.36 ± 0.001^{c}
Gly	2.47 ± 0.06^{A}	2.25 ± 0.20^{a}	1.16 ± 0.02^{B}	0.96 ± 0.07^{b}	0.83 ± 0.03^{C}	0.76 ± 0.04^{b}	0.65 ± 0.01^{D}	0.69 ± 0.02^{b}
Tyr	1.55 ± 0.06^{A}	1.46 ± 0.12^{a}	0.71 ± 0.02^{B}	0.56 ± 0.06^{b}	0.46 ± 0.01^{C}	0.41 ± 0.05^{b}	0.32 ± 0.01^{D}	0.33 ± 0.01^{b}
Nonessential amino acids (NEAA)								
Asp	4.63 ± 0.12^{A}	4.28 ± 0.35^{a}	2.36 ± 0.06^{B}	2.03 ± 0.10^{b}	1.86 ± 0.02^{C}	1.69 ± 0.15^{b}	1.38 ± 0.02^{D}	1.39 ± 0.003^{b}
Ser	2.59 ± 0.07^{A}	2.41 ± 0.19^{a}	1.25 ± 0.03^{B}	1.09 ± 0.04^{b}	0.97 ± 0.03^{C}	$0.90 \pm 0.09^{b,c}$	0.74 ± 0.002^{D}	0.41 ± 0.34^{c}
Glu	6.88 ± 0.12^{A}	6.42 ± 0.42^{a}	4.12 ± 0.08^{B}	3.57 ± 0.27^{b}	3.04 ± 0.07^{C}	2.80 ± 0.11^{b}	2.54 ± 0.04^{D}	2.57 ± 0.004^{b}
Ala	3.02 ± 0.07^{A}	2.80 ± 0.23^{a}	1.39 ± 0.03^{B}	1.16 ± 0.06^{b}	1.05 ± 0.06^{C}	0.87 ± 0.06^{b}	0.76 ± 0.01^{D}	0.76 ± 0.01^{b}
Pro	1.00 ± 0.04^{A}	1.03 ± 0.01^{a}	0.80 ± 0.02^{B}	0.77 ± 0.04^{b}	0.64 ± 0.03^{C}	$0.59 \pm 0.09^{b,c}$	0.51 ± 0.003^{D}	0.50 ± 0.001^{c}

[A–D, a–c]Mean values within the same row not sharing a common superscript letter are significantly different ($P < 0.05$).
[1]Data are shown as means of 3 pooled determinations of 2 crop milk samples each.
[2]The stages included day 1 (R1), 7 (R7), 15 (R15), and 25 (R25) of the chick-rearing period.

Table 4. Analyzed concentrations of amino acids (μg/mL) in pigeon plasma during 25 D of chick rearing.[1]

	Chick-rearing period[2]							
	R1		R7		R15		R25	
Item	Male	Female	Male	Female	Male	Female	Male	Female
Essential amino acids (EAA)								
Lys	67.30 ± 0.34^{B}	69.93 ± 0.07^{a}	67.82 ± 0.08^{B}	70.13 ± 0.08^{a}	70.30 ± 0.08^{A}	68.71 ± 0.46^{b}	69.85 ± 0.13^{A}	69.57 ± 0.04^{b}
Met	8.61 ± 0.04^{A}	9.18 ± 0.03^{c}	8.55 ± 0.24^{A,B}	9.41 ± 0.08^{b}	8.20 ± 0.79^{B}	8.72 ± 0.07^{d}	8.43 ± 0.11^{C}	10.02 ± 0.03^{a}
Cys	3.08 ± 0.17^{A}	2.99 ± 0.02^{a}	3.02 ± 0.44^{A}	2.98 ± 0.03^{a}	2.81 ± 0.09^{B}	2.87 ± 0.01^{b}	2.54 ± 0.07^{C}	2.64 ± 0.02^{c}
Thr	101 ± 0.45^{B}	108 ± 0.37^{b}	102 ± 0.17^{A,B}	108 ± 0.67^{b}	102 ± 0.37^{B}	106 ± 0.08^{c}	103 ± 0.35^{A}	111 ± 0.50^{a}
Ile	14.76 ± 0.09^{B}	14.14 ± 0.05^{b}	14.65 ± 0.02^{B}	14.26 ± 0.04^{b}	15.06 ± 0.04^{A}	14.30 ± 0.04^{b}	14.65 ± 0.03^{B}	14.76 ± 0.09^{a}
Leu	50.30 ± 0.17^{C}	51.69 ± 0.15^{c}	51.12 ± 1.11^{B}	52.11 ± 0.06^{b}	52.05 ± 0.10^{A}	52.30 ± 0.08^{b}	52.08 ± 0.19^{A}	52.75 ± 0.14^{a}
Val	26.30 ± 0.12^{B}	26.71 ± 0.11^{b}	26.61 ± 0.15^{B}	28.00 ± 0.02^{a}	26.56 ± 0.06^{B}	26.12 ± 0.05^{c}	28.01 ± 0.03^{A}	28.22 ± 0.13^{a}
Phe	11.99 ± 0.03^{B}	12.38 ± 0.09^{a,b}	12.28 ± 0.15^{A,B}	12.61 ± 0.13^{a}	11.98 ± 0.04^{B}	11.96 ± 0.19^{b}	12.44 ± 0.14^{A}	12.37 ± 0.06^{a,b}
Arg	68.35 ± 0.16^{B}	70.37 ± 0.06^{a}	69.95 ± 0.19^{A}	70.26 ± 0.20^{a}	70.09 ± 0.16^{A}	69.27 ± 0.42^{b}	70.38 ± 0.17^{A}	68.64 ± 0.36^{b}
His	31.69 ± 0.17^{B}	33.22 ± 0.20^{bc}	31.99 ± 0.05^{B}	33.72 ± 0.13^{b}	31.28 ± 0.04^{C}	32.60 ± 0.03^{c}	32.43 ± 0.02^{A}	34.67 ± 0.30^{a}
Gly	5.64 ± 0.06^{A}	6.16 ± 0.09^{b}	5.69 ± 0.05^{A}	6.42 ± 0.02^{a}	5.23 ± 0.06^{B}	5.89 ± 0.05^{c}	5.21 ± 0.06^{B}	6.00 ± 0.03^{b,c}
Tyr	13.67 ± 0.07^{B}	12.94 ± 0.04^{c}	14.02 ± 0.03^{A}	13.26 ± 0.03^{b}	13.03 ± 0.05^{C}	12.80 ± 0.03^{d}	13.08 ± 0.04^{C}	13.98 ± 0.05^{a}
Nonessential amino acids (NEAA)								
Asp	12.79 ± 0.08^{B}	13.58 ± 0.15^{a,b}	13.01 ± 0.07^{A,B}	13.86 ± 0.13^{a,b}	12.81 ± 0.08^{B}	12.96 ± 0.09^{b}	13.28 ± 0.16^{A}	14.26 ± 0.56^{a}
Ser	53.04 ± 0.29^{A}	56.01 ± 0.13^{a}	52.71 ± 0.23^{A,B}	56.08 ± 0.10^{a}	52.07 ± 0.09^{B}	55.63 ± 0.20^{a}	50.15 ± 0.09^{C}	54.60 ± 0.33^{b}
Glu	32.10 ± 0.07^{A}	29.52 ± 0.06^{b}	31.45 ± 0.13^{B}	29.89 ± 0.15^{a}	30.31 ± 0.17^{C}	28.58 ± 0.04^{c}	28.16 ± 0.19^{D}	30.19 ± 0.11^{a}
Ala	42.00 ± 0.03	44.99 ± 0.17^{c}	42.50 ± 0.75	45.62 ± 0.05^{b}	42.55 ± 0.11	42.86 ± 0.10^{d}	42.41 ± 0.28	48.15 ± 0.10^{a}
Pro	21.06 ± 0.49^{B}	22.87 ± 0.10^{b}	22.31 ± 0.10^{A}	22.98 ± 0.03^{b}	22.15 ± 0.06^{A}	22.05 ± 0.03^{c}	22.85 ± 0.22^{A}	24.04 ± 0.01^{a}

$^{A–D, a–d}$Mean values within the same row not sharing a common superscript letter are significantly different ($P < 0.05$).
[1]Data are shown as means of 3 pooled determinations of 2 pigeon plasma samples each.
[2]The stages included day 1 (R1), 7 (R7), 15 (R15), and 25 (R25) of the chick-rearing period.

Gene Expression of GDH1, GS, OAT, and ASL

Gene expression of glutamate dehydrogenase 1 (***GDH1***), glutamine synthetase (***GS***), *OAT*, and argininosuccinate lyase (***ASL***) varied significantly with stage and sex ($P < 0.001$) (Table 8). *GDH1* expression in both parent pigeon crops increased to their highest values on I17, and there was a sharp 100-fold increase in *GS* expression on R25 compared with I4 ($P < 0.05$) (Figure 4, A and B). Gene expressions of *OAT* in both males and females and *ASL* in males were highest on R1, whereas female *ASL* gene expression peaked on R10 ($P < 0.05$) (Figure 4, C and D).

Gene Expression of GOT1, GOT2, and ASN

Glutamic-oxal(o)acetic transaminase 1 (***GOT1***), glutamic-oxal(o)acetic transaminase 2 (***GOT2***), and asparagine synthetase (***ASN***) all maintained relatively higher levels of gene expression in both the male and female pigeon crops from I17 to R7, and their maximum values were observed on R1 with a 3- to 11.6-fold increase compared with day 4 of incubation ($P < 0.05$) (Figure 5). A significant interaction of stage and sex for gene expression of *GOT2* and *ASN* was observed ($P < 0.05$) (Table 8).

Table 5. *P*-values for the effects of stage, sex, and their interaction in analyzed concentrations of amino acids in pigeon crop milk during 25 D of chick rearing.

	P-value		
Item	Stage	Sex	Stage × sex
Essential amino acids (EAA)			
Lys	<0.001	0.096	0.459
Met	<0.001	0.331	0.755
Cys	<0.001	0.868	0.950
Thr	<0.001	0.106	0.542
Ile	<0.001	0.084	0.572
Leu	<0.001	0.099	0.600
Val	<0.001	0.066	0.316
Phe	<0.001	0.138	0.455
Arg	<0.001	0.111	0.572
His	<0.001	0.112	0.154
Gly	<0.001	0.103	0.360
Tyr	<0.001	0.182	0.732
Nonessential amino acids (NEAA)			
Asp	<0.001	0.098	0.526
Ser	<0.001	0.104	0.734
Glu	<0.001	0.060	0.472
Ala	<0.001	0.066	0.629
Pro	<0.001	0.816	0.933

Table 6. *P*-values for the effects of stage, sex, and their interaction in analyzed concentrations of amino acids in pigeon plasma during 25 D of chick rearing.

	P-value		
Item	Stage	Sex	Stage × sex
Essential amino acids (EAA)			
Lys	<0.001	<0.001	<0.001
Met	<0.001	<0.001	<0.001
Cys	<0.001	0.86	0.046
Thr	<0.001	<0.001	0.015
Ile	<0.001	<0.001	<0.001
Leu	<0.001	<0.001	0.004
Val	<0.001	<0.001	<0.001
Phe	0.003	0.075	0.13
Arg	0.036	0.75	<0.001
His	<0.001	<0.001	0.045
Gly	<0.001	<0.001	0.13
Tyr	<0.001	<0.001	<0.001
Nonessential amino acids (NEAA)			
Asp	0.008	<0.001	0.30
Ser	<0.001	<0.001	0.015
Glu	<0.001	<0.001	<0.001
Ala	<0.001	<0.001	<0.001
Pro	<0.001	<0.001	0.001

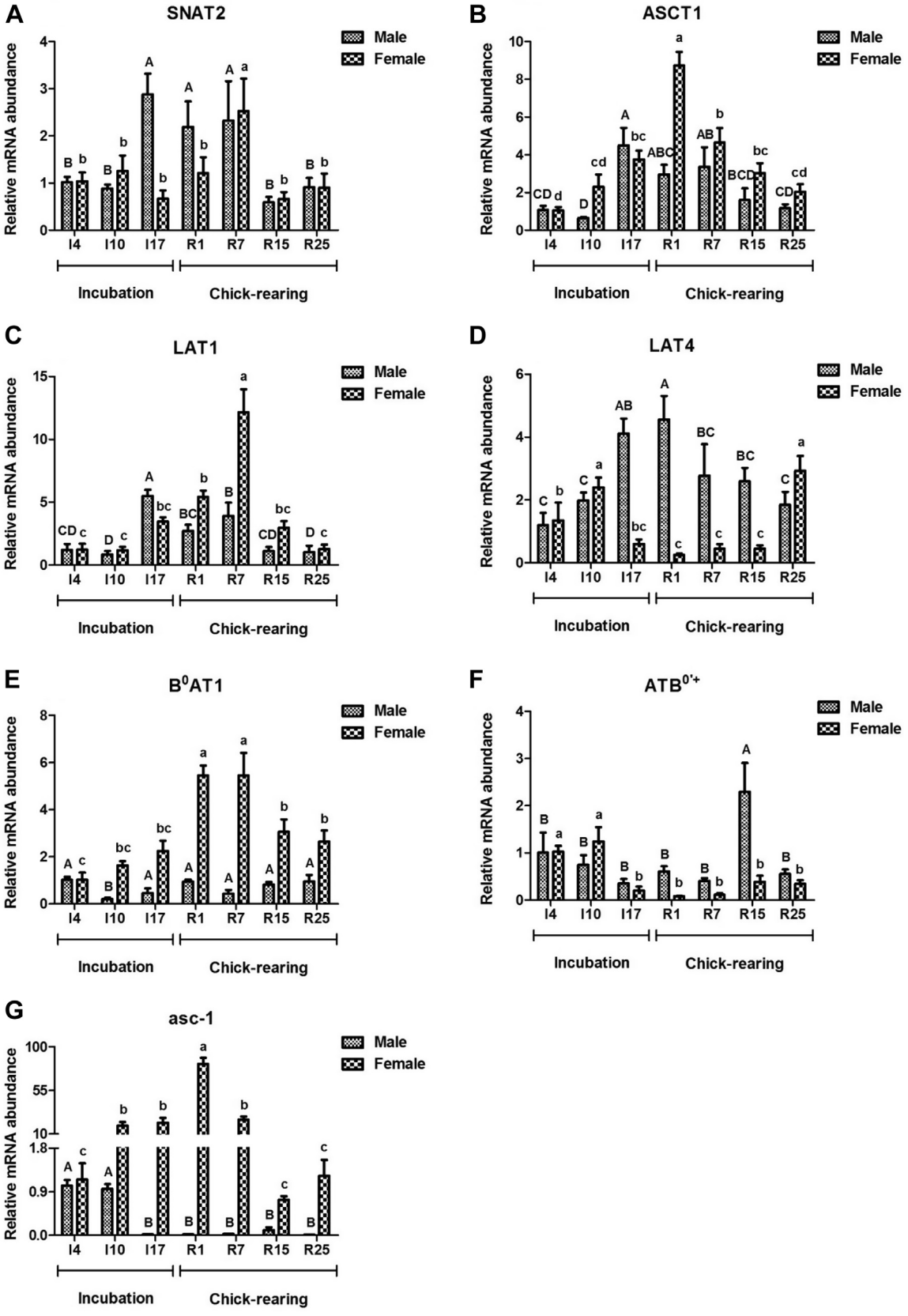

Figure 1. Changes in relative abundance of mRNA for Na^+-coupled neutral amino acid transporter 2 (*SNAT2*) (A), Na^+-dependent neutral amino acid transporter (*ASCT1*) (B), L-type amino acid transporter 1 (*LAT1*) (C), L-Type amino acid transporter 1 (*LAT4*) (D), Na^+-dependent neutral amino acid transporter (B^0AT1) (E), Na^+- and Cl^--dependent neutral and cationic amino acid transporter ($ATB^{0'+}$) (F), and asc-type amino acid transporter 1 (*asc-1*) (G). The stages included incubation periods of I4, I10, and I17 and the chick-rearing periods of R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A–D) or lowercase letters (a–d) are significantly different ($P < 0.05$).

Table 7. *P*-values for the effects of stage, sex, and their interaction in expressions of genes involved in amino acid transport in male and female pigeons during incubation and chick-rearing period.

	Genes involved in amino acid transport													
Item	*SNAT2*	*ASCT1*	*LAT1*	*LAT4*	B^0AT1	$ATB^{0,+}$	*asc-1*	*EAAT3*	*xCT*	$IMINO^B$	*PAT-1*	$b^{0,+}AT$	*CAT1*	y^+LAT2
P-value														
Stage	<0.001	<0.001	0.001	0.002	0.001	<0.001	<0.001	<0.001	0.010	0.021	<0.001	0.002	<0.001	<0.001
Sex	0.427	<0.001	0.081	0.757	<0.001	<0.001	<0.001	0.014	0.169	0.570	<0.001	0.970	<0.001	0.929
Stage × sex	0.612	<0.001	0.122	0.015	<0.001	<0.001	<0.001	<0.001	0.340	0.061	0.749	0.008	<0.001	0.881

Abbreviations: *asc-1*, asc-type amino acid transporter 1; *ASCT1*, Na^+-dependent neutral amino acid transporter; $ATB^{0,+}$, Na^+- and Cl^--dependent neutral and cationic amino acid transporter; B^0AT1, Na^+-dependent neutral amino acid transporter; $b^{0,+}AT$, Na^+-dependent cationic and zwitterionic amino acid transporter; *CAT1*, Na^+-dependent cationic amino acid transporter; *EAAT3*, excitatory amino acid transporter 3; $IMINO^B$, Na^+- and Cl^--dependent proline IMINO transporter; *LAT1*, L-type amino acid transporter 1; *LAT4*, L-type amino acid transporter 4; *PAT-1*, proton-coupled amino acid transporter 1; *SNAT2*, Na^+-coupled neutral amino acid transporter 2; *xCT*, cystine/glutamate exchange transporter; y^+LAT2, Na^+-dependent cationic and Na^+-dependent neutral amino acid transporter 2.

Gene Expression of TDH, 3PDGH, SHMT1, and SHMT2

Gene expression of L-threonine 3-dehydrogenase (***TDH***) in the male and female pigeon crops reached maximum values on R1 and I17, respectively ($P < 0.05$) (Figure 6A). Gene expressions of serine hydroxymethyltransferase 1 (***SHMT1***) and serine hydroxymethyltransferase 2 (***SHMT2***) varied significantly with sex and interaction of stage and sex ($P < 0.05$) (Table 8). 3-Phosphoglycerate dehydrogenase (***3PDGH***) gene expressions in the females and *SHMT1* gene expression in the males increased to the highest level on R7 ($P < 0.05$) (Figure 6, B and C), whereas *SHMT2* gene expression in both male and female pigeons peaked on R1 ($P < 0.05$) (Figure 6D).

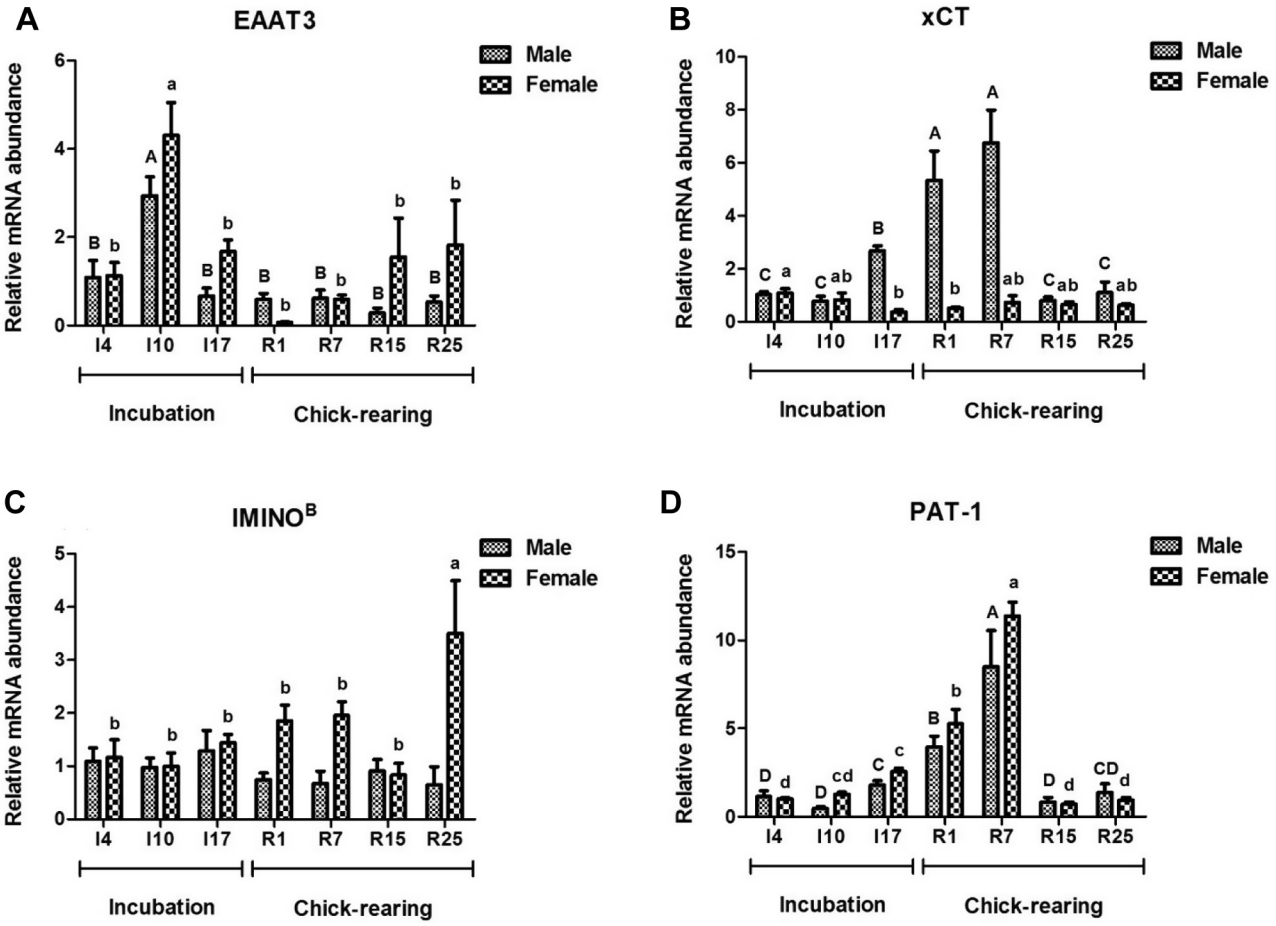

Figure 2. Changes in relative abundance of mRNA for excitatory amino acid transporter 3 (*EAAT3*) (A), cystine/glutamate exchange transporter (*xCT*) (B), Na^+- and Cl^--dependent proline IMINO transporter ($IMINO^B$) (C), and proton-coupled amino acid transporter 1 (*PAT-1*) (D). The stages included incubation periods of I4, I10, and I17 and chick-rearing periods of R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A–D) or lowercase letters (a–d) are significantly different ($P < 0.05$).

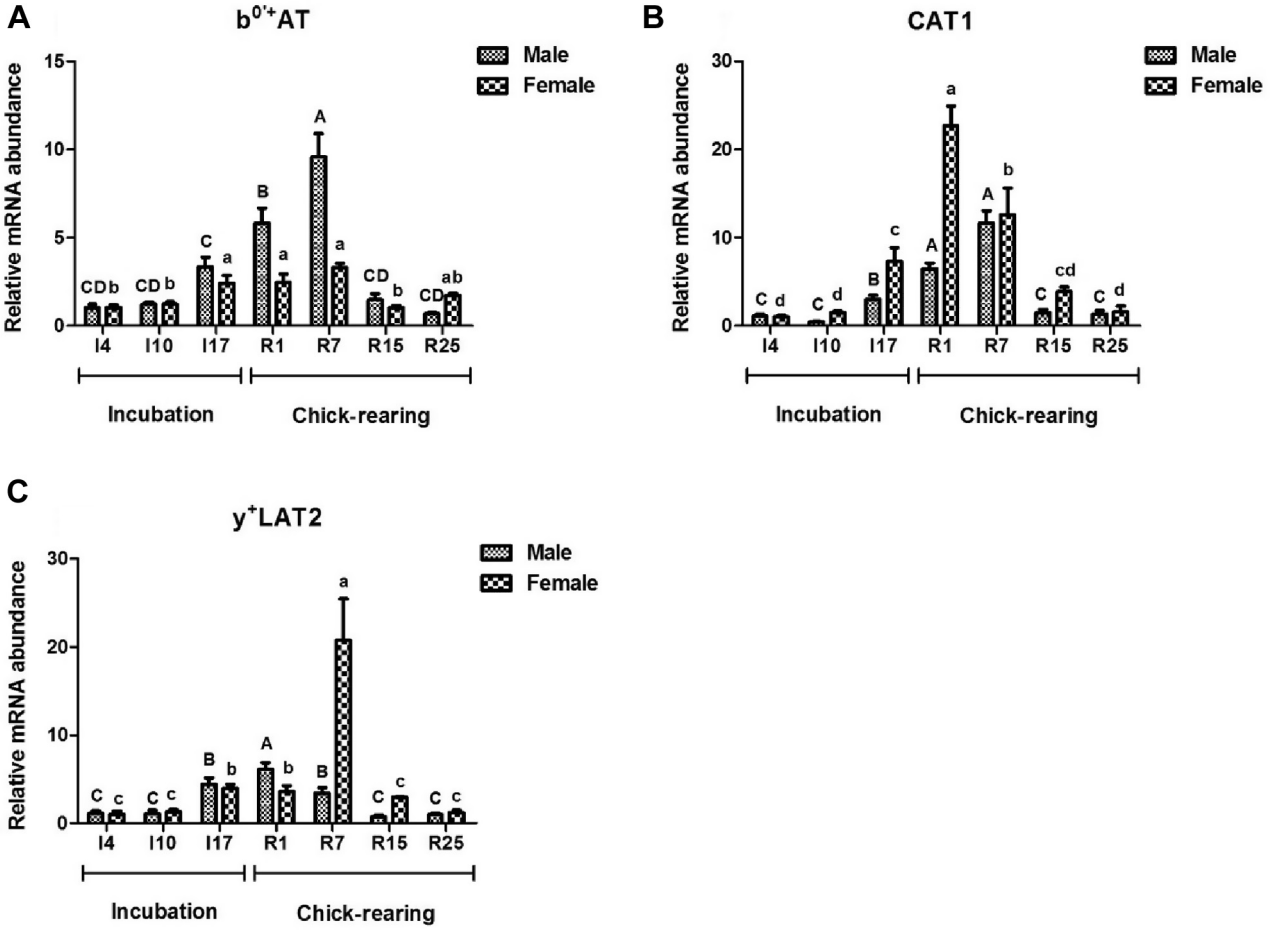

Figure 3. Changes in relative abundance of mRNA for Na^+-dependent cationic and zwitterionic amino acid transporter ($b^{0,+}AT$) (A), Na^+-dependent cationic amino acid transporter (*CAT1*) (B), and Na^+-dependent cationic and Na^+-dependent neutral amino acid transporter 2 (y^+LAT2) (C). The stages included incubation periods of I4, I10, and I17 and chick-rearing periods of R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A–D) or lowercase letters (a–d) are significantly different ($P < 0.05$).

Gene Expression of GPT2 and ACSN

mRNA abundance of glutamic-pyruvic transaminase 2 (***GPT2***) in the male and female pigeons reached a peak level on R1, with a 3.1- to 9.4-fold increase, whereas the maximum value for acetolactate synthase (***ACSN***) mRNA abundance was attained on I17 ($P < 0.05$) (Figure 7), and expression of the 2 genes was also affected by the interaction of stage and sex ($P < 0.05$) (Table 8).

DISCUSSION

Owing to the extreme deficiency of carbohydrates, protein in crop milk is fundamental for the growth and development of pigeon squabs (Hu et al., 2016). AA are the building blocks of proteins that control various metabolic pathways important for whole-body homeostasis (Wu, 2003). In pigeon milk, approximately 17% of the protein is composed of free AA, and these proportions increase during the first week of production

Table 8. *P*-values for the effects of stage, sex, and their interaction in expressions of genes involved in amino acid synthesis in male and female pigeons during incubation and chick-rearing period.

	Genes involved in amino acid synthesis												
Item	*GDH1*	*GS*	*OAT*	*ASL*	*GOT1*	*GOT2*	*ASN*	*TDH*	*3PDGH*	*SHMT1*	*SHMT2*	*GPT2*	*ACSN*
Stage	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
Sex	0.001	<0.001	<0.001	<0.001	0.217	<0.001	0.195	0.126	0.023	<0.001	0.006	<0.001	0.244
Stage × sex	0.077	<0.001	0.001	<0.001	0.156	0.002	<0.001	<0.001	0.086	<0.001	<0.001	<0.001	0.004

Abbreviations: 3PGDH, phosphoglycerate dehydrogenase; *ACSN*, acetolactate synthase; *ASL*, argininosuccinate lyase; *ASN*, asparagine synthetase; *GDH1*, glutamate dehydrogenase 1; *GOT1*, glutamic-oxal(o)acetic transaminase 1; *GOT2*, glutamic-oxal(o)acetic transaminase 2; *GPT2*, glutamic-pyruvic transaminase 2; *GS*, glutamine synthetase; *OAT*, ornithine-δ-aminotransferase; *SHMT1*, serine hydroxymethyltransferase 1; *SHMT2*, serine hydroxymethyltransferase 2; *TDH*, L-threonine 3-dehydrogenase.

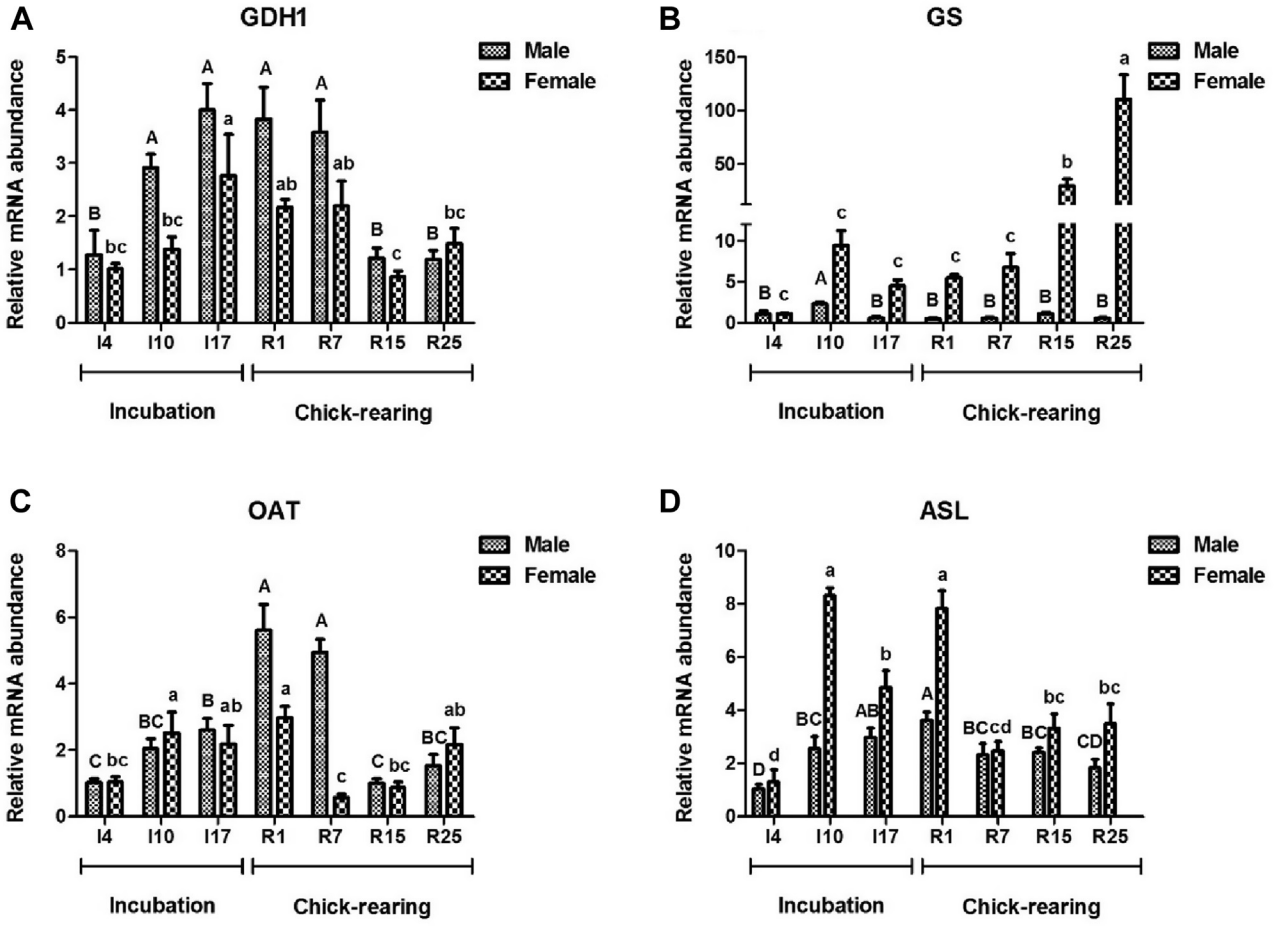

Figure 4. Changes in relative abundance of mRNA for glutamate dehydrogenase 1 (*GDH1*) (A), glutamine synthetase (*GS*) (B); ornithine-δ-aminotransferase (*OAT*) (C), and argininosuccinate lyase (*ASL*) (D). The stages included incubation periods of I4, I10, and I17 and chick-rearing periods of R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A–D) or lowercase letters (a–d) are significantly different ($P < 0.05$).

(Vandeputte-Poma and van Grembergen, 1959; Vandeputte-Poma, 1980). Consistent with results found previously (Hedge, 1972; Zhang, et al., 2017), our data showed that Glu, Asp, and Leu were the 3 most abundant AA. All AA concentrations in the present study decreased significantly on R7 and reached their lowest levels on R25, which is close to the AA levels of the pigeons' diet. With the end of 1-wk peak lactation, there was a feed transition for pigeon squabs (Shetty et al., 1992). Pellet diets or grains appeared in the crop milk, and nearly half of crop milk has been substituted for whole grains up to 14 D of squab age. On day 28 of chick rearing, "lactation" has completely ceased (Vandeputte-Poma, 1980), and crop milk was fully changed into parental pigeon feed. However, young pigeons older than 20 D still lacked the self-feeding capacity, so adults "regurgitated" almost entirely pigeon feed to the young. Therefore, high contents of AA in crop milk at the beginning of "lactation" were gradually "diluted." This can well explain why the AA concentrations continuously decreased.

During lactation in mammals, the uptake of AA from circulation into the mammary gland is enhanced (Rezaei et al., 2016), so AA concentrations of plasma were often measured as an indicator of milk protein synthesis. Sciascia et al. (2015) reported that an increase in milk protein concentration is accompanied by elevated concentrations of intracellular AA in mammary glands, but a decrease in the AA concentration of plasma was observed, suggesting inconsistent AA metabolism in different tissues. However, Chen et al. (2018) found that in sows, the plasma concentrations of most AA were greater on day 1 of lactation compared with those on day 17. Our results showed that not all plasma AA exhibit a similar pattern change; about half were higher late in chick rearing than that at the beginning, and the rest were higher on R1 or R7. In addition, our previous study showed that whatever feeding strategy is adopted, body weight loss of parental pigeons seemed to be inevitable upon the completion of rearing, and the breast and thigh muscles, which function as an AA reserve, quickly reduced in weights (Xie et al., 2017; 2018). Therefore, as found in mammals (Trottier, 1995; Chen et al., 2018), it is probable that AA originating from the feed and muscle tissues through protein catabolism will provide substrates for milk synthesis in pigeons. Interestingly, parental pigeon sex has no effect on AA concentrations in crop milk in our study, but 8 types of

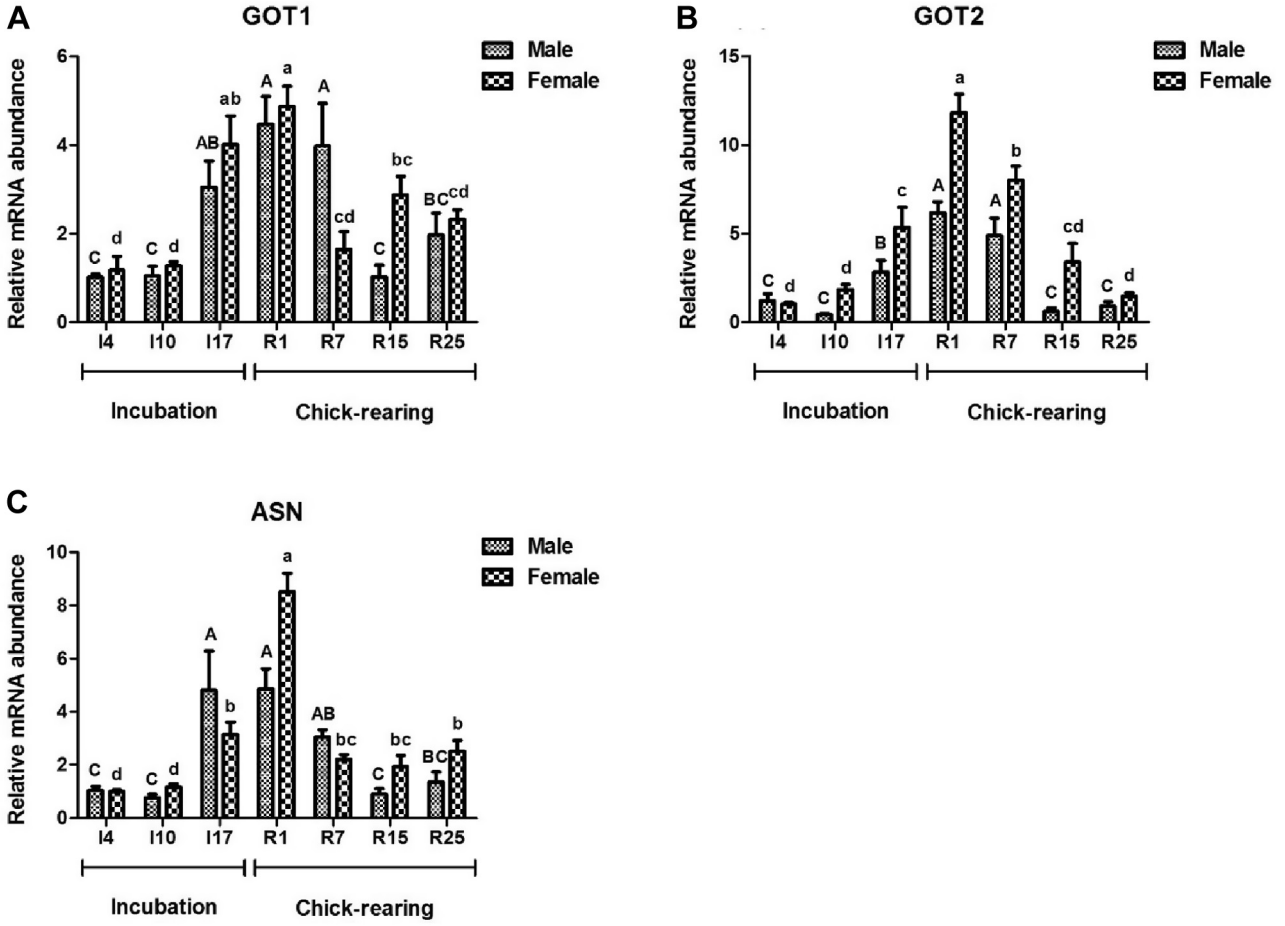

Figure 5. Changes in relative abundance of mRNA for glutamic-oxal(o)acetic transaminase 1 (*GOT1*) (A), glutamic-oxal(o)acetic transaminase 2 (*GOT2*) (B), and asparagine synthetase (*ASN*) (C). The stages included incubation periods of I4, I10, and I17; chick-rearing periods of R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A–C) or lowercase letters (a–d) are significantly different ($P < 0.05$).

plasma AA in the female pigeons were higher than those in the male pigeons. The different uptake efficiency of AA from blood into crop milk between male and female pigeons may be an important reason. Moreover, plasma AA were also used for self-sustainment in animals. Different breeding behaviors (Lea et al., 1986) and liver metabolism (Wan et al., 2018) between male and female pigeons indicated their potential different AA requirement during the breeding period.

In pigeons, AA transporters have not been studied in any other tissues except the small intestine. During peak lactation in mammals, gene expressions of *SNAT2*, *LAT1*, *LAT2*, *ASCT1*, *CAT1*, *EAAT3*, $b^{0,+}AT$, and $y^{+}LAT2$ have all been reported to increase significantly, suggesting their potential role in milk protein synthesis (Shennan et al., 2002; Alemán et al., 2009; Manjarin et al., 2011; Chen et al., 2018). *SNAT2*, mainly expressed in epithelial cells, recruits neutral AA. Its gene expression can be activated by prolactin, and a previous study found that prolactin was a main reason for crop milk formation; in addition, its level in pigeon serum maintained a high level from day 17 of incubation to day 4 of chick rearing (Dumont, 1965; Xie et al., 2018). The uptake of Ala, Ser, and Cys by *ASCT1* in multiple types of cells was independent of pH and energy but dependent on Na^{+} (Katragadda et al., 2005). The ASC system was not reported to be responsive to hormones, but its changing pattern suggested that prolactin regulation might be involved. *LAT1* and *LAT4* are charged with the transportation of aromatic and large BCAA in various types of cells. *LAT1* is an obligatory antiporter and cannot mediate the net uptake or efflux of its targets, whereas *LAT4* is shown to mediate the Na^{+}-independent uniport of AA despite its narrow substrate specificity (Babu et al., 2003; Bodoy et al., 2005). Higher levels of *LAT4* in male pigeons possibly enhanced AA transport efficiency from the plasma AA reserve. Different *LAT4* expressions between male and female pigeons showed that a sexual effect exists.

$B^{0}AT1$ transports almost all neutral AA. Its relatively higher expression in female crops during peak secretion suggests its importance only in female pigeons. $ATB^{0,+}$ shows a high affinity for both neutral and cationic AA; however, it may be nonessential to milk formation because of lower expression. A consistent result was the observation that system $B^{0,+}$ ($ATB^{0,+}$) decreased from early to peak lactation in mammals (Laspiur

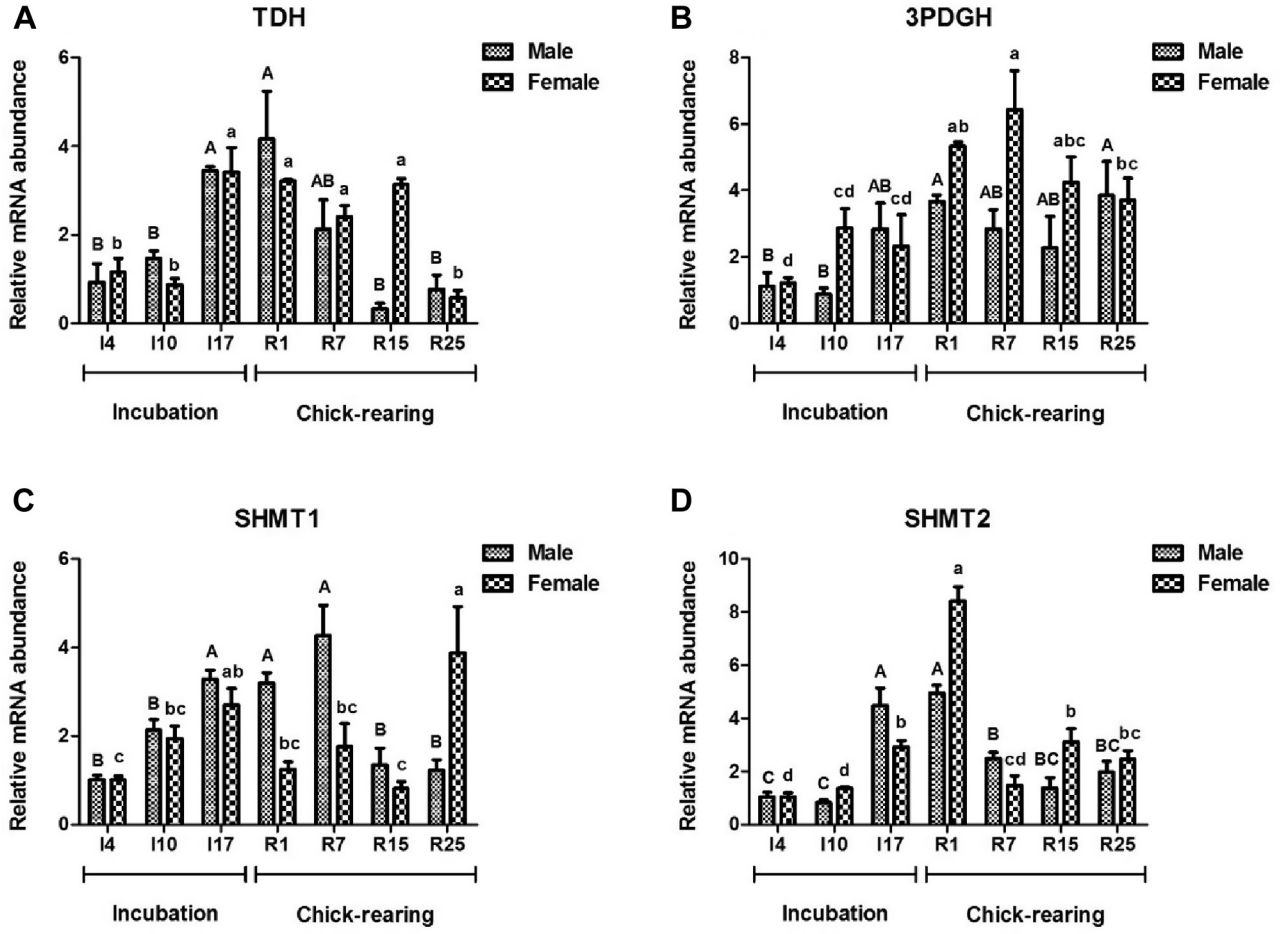

Figure 6. Changes in relative abundance of mRNA for L-threonine 3-dehydrogenase (*TDH*) (A), 3-phosphoglycerate dehydrogenase (*3PDGH*) (B), serine hydroxymethyltransferase 1 (*SHMT1*) (C), and serine hydroxymethyltransferase 2 (*SHMT2*) (D). The stages included incubation periods of I4, I10, and I17 and chick-rearing periods of R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A–C) or lowercase letters (a–d) are significantly different ($P < 0.05$).

et al., 2004). *asc-1* conveys the Na^+-dependent high-affinity transport of short-chain neutral AA. AA efflux could be strongly transstimulated under the regulation of this transporter when intracellular and extracellular AA concentrations need to be balanced (Nakauchi et al., 2000). Sharp increases in *asc-1* in female pigeons indicated its special role in AA transport compared with its depressed expression in male pigeons.

The transcript abundance of *EAAT3* in crop tissues maintained a low level after I10. The uptake of Glu

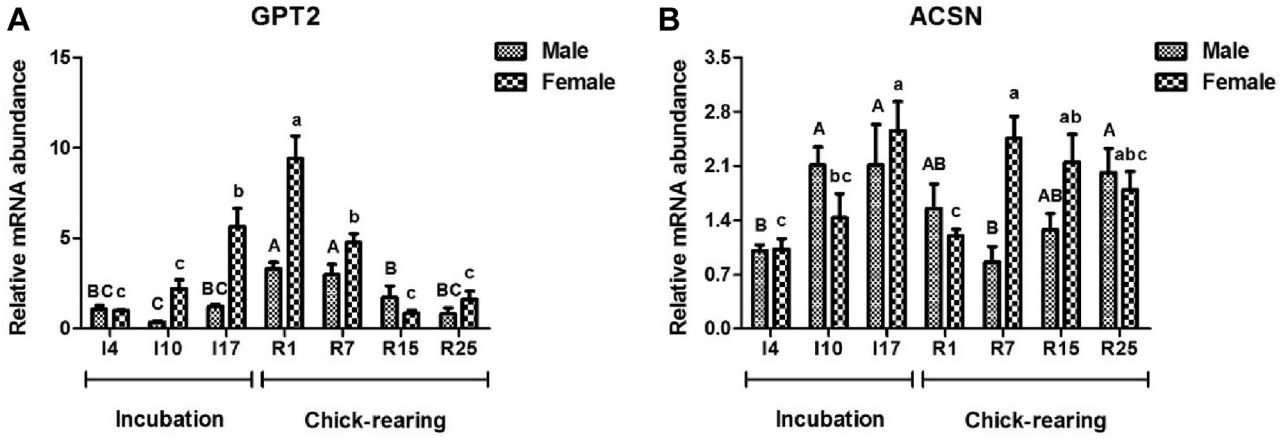

Figure 7. Changes in relative abundance of mRNA for glutamic-pyruvic transaminase 2 (*GPT2*) (A) and acetolactate synthase (*ACSN*) (B). The stages included incubation periods of I4, I10, and I17 and chick-rearing periods of R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A–C) or lowercase letters (a–c) are significantly different ($P < 0.05$).

and Asp is far less than their output in mammalian milk (Trottier et al. 1997). These 2 AA are NEAA, and their contents were higher than other AA in crop milk. Glu can be produced through catalysis of alpha-ketoglutarate and transamination during the intracellular AA metabolism because activities of *GDH1*, *GOT1*, *GOT2*, and *GPT2* were all enhanced. Lipid content of crop milk produced by the males was higher during the early stages of chick rearing than that produced by the females. More lipid accumulation often causes more oxidative stress and can induce *xCT* activity (Bannai et al., 1991), facilitating the exchange of extracellular cystine for the synthesis of intracellular glutathione, an antioxidant that plays an important role in maintaining redox balance (Patel et al., 2004).

According the changing patterns of gene expression in the present study, proline uptake by crop cells was mainly dependent on *PAT-1* instead of $IMINO^B$. However, a previous study found that short-chain fatty acids, not just an amino group, are also PAT1 substrates (Foltz et al., 2004). Owing to the acidic microenvironment and abundant active microorganisms in the crop sac (Shetty et al., 1990), it is speculated that short-chain fatty acids will be increased with the process of milk formation.

The expression of cationic AA transporters, $b^{0,+}AT$ and *CAT1*, was found to increase from I17 to I7 in the present study. In mammals, the absorption of NEAA from circulation by mammary cells is insufficient to support milk protein production (Mepham, 1982). EAA, especially cationic AA, are taken up in excess by lactating cells, and they can be converted to NEAA or intermediate products for the Krebs cycle (Menzies et al., 2009). For example, excessive arginine in the mammary gland can be converted into proline by *OAT* and pyrroline-5-carboxylate reductase (Basch et al., 1995; 1996). A similar metabolic process may exist in pigeon crop cells. *OAT* gene expression was observed to be higher during the peak of crop milk formation. y^+LAT2 is charged for cationic AA efflux, and the activated gene, also found in the present study, may play a role in the balance of intracellular and extracellular content of relevant AA (Bröer et al., 2000).

Neutral (*SNAT2*, *ASCT1*, and *LAT1*) and cationic ($b^{0,+}AT$, *CAT1*, and y^+LAT2) AA transporters in crop tissues of both parental sexes analyzed in our study showed significant higher expressions from I17 to R7, and they could be an important reason for lower concentrations of responsive AA in plasma at the beginning of "lactation" in pigeons. However, the fluctuation of anionic AA transporters (*EAAT3*, *xCT* and $IMINO^B$) did not coincide well with the changing pattern of Asp, Glu, and Pro and biosynthesis in situ of these 3 NEAA because the high expression of related enzymes from I17 to R7 should be taken into consideration. Meanwhile, some AA showed different changing patterns between male and female pigeons. It suggests that AA transporter was not the only determinant for plasma AA concentration, and other potential factors, such as self-sustainment, AA metabolism, and hormones, may be involved, which still needs further research.

The genes involved in AA synthesis in the present study were all detected by their expressions, which showed active AA metabolism in the crop sacs of pigeons. Glutamine synthetase is a key enzyme for glutamine production in most animal cells (Wu, 2013), but its activity is absent in mammary glands (Li et al., 2009), differing from pigeon crops. Our data showed that *GS* expression in crop tissue increased dramatically in the females in the late stages of chick rearing, whereas in the males, it maintained a low level over the entire breeding stage, suggesting that a sexual effect exists in glutamine synthesis. *ASL* converts citrulline to arginine under the regulation of glucocorticoids (Flynn et al., 1999). The higher levels of *ASL* gene expression from I10 to I17 suggest that de novo synthesis of arginine was possible as an advance reserve before crop milk secretion.

Asparagine is synthesized by *ASN* through a transfer of the amide nitrogen of glutamine to aspartate in an ATP-dependent reaction (Richards and Schuster, 1998). In our study, *ASN* increased significantly from I17 to R7, and we speculated that asparagine is mainly dependent on de novo biosynthesis as is the case in mammals (Trottier et al., 1997) because of lower expression in relative transporter.

The conversion of the L-threonine to 2-amino-3-ketobutyrate by *TDH* is the first step of glycine synthesis. In "lactating" pigeons, serum insulin increases significantly (Hu et al., 2016) and was shown to enhance the glycine synthesis (Menzies et al., 2009), so *TDH* may be involved in insulin regulation. The biosynthesis reaction of serine from a glycolytic intermediate 3-phosphoglycerate was initiated by *3PDGH* (Yamasaki et al., 2001), which was shown to maintain relatively high levels after day 10 of incubation. Serine hydroxymethyltransferase exists as cytosolic (*SHMT1*) and mitochondrial isozyme (*SHMT2*), which catalyze the transfer of a beta carbon from serine to tetrahydrofolate (**THF**) to form glycine and 5,10-methylene-THF (Hebbring et al., 2012). The changing pattern of *3PDGH*, *SHMT1*, and *SHMT2* and higher levels of serine in pigeon plasma at the beginning of chick rearing suggested that serine may be a very important origination for glycine.

2-Acetolactate is converted from pyruvate by *ACSN* as the initial step in the valine and leucine pathway or subsequently with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate as the intermediate in the isoleucine pathway (Gollop et al., 1988). Concentrations of BCAA, Ile, Leu, and Val in pigeon plasma are observed to be significantly lower on R1, suggesting that their influxes in crop cells finally lead to the depressed *ACSN* expression responsible for BCAA synthesis.

In the current study, most of enzymes related in AA synthesis showed maximum expression levels from I17 to R7, which is the peak of crop milk secretion. Therefore, de novo biosynthesis of related AA in epithelial cells, such as Glu, Pro, Arg, Asp, Ser, and Gly, could

be an important pathway for crop milk formation. The final changing pattern of AA concentrations in pigeon milk was probably dependent on the combined action of AA transporters and enzymes related in AA synthesis or decomposition in crop tissues.

CONCLUSIONS

The present study speculates that the AA used for crop milk formation in pigeons may partially originate from free AA in plasma, and their uptake into crop cells may be dependent on the efficiency of various transporters. Changes in the gene expression of AA synthesis–related enzymes suggested an active intracellular AA metabolism, providing an important source for NEAA in crop milk. In addition, although the final AA composition in male and female pigeons has exhibited no differences, the genes involved in AA transport and synthesis vary significantly with sexual effects, which indicate that other factors, such as self-sustainment, AA metabolism, and hormones, should be considered in future explorations of the mechanisms of crop milk formation.

ACKNOWLEDGMENTS

The authors thank all the members in the school for their generous technical suggestions. The research was supported by National Natural Funds of China (No. 31501974), Natural Science Foundation of Jiangsu Province, China (BK20150462), and China Postdoctoral Science Foundation, China (2017M621839).

REFERENCES

Alemán, G., A. López, G. Ordaz, N. Torres, and A. R. Tovar. 2009. Changes in messenger RNA abundance of amino acid transporters in rat mammary gland during pregnancy, lactation, and weaning. Metabolism 58:594–601.

Babu, E., Y. Kanai, A. Chairoungdua, D. K. Kim, Y. Iribe, S. Tangtrongsup, P. Jutabha, Y. Li, N. Ahmed, S. Sakamoto, N. Anzai, S. Nagamori, and H. Endou. 2003. Identification of a novel system L amino acid transporter structurally distinct from heterodimeric amino acid transporters. J. Biol. Chem. 278:43838–43845.

Backwell, F. R., B. J. Bequette, D. Wilson, J. A. Metcalf, M. F. Franklin, D. E. Beever, G. E. Lobley, and J. C. MacRae. 1996. Evidence for the utilization of peptides for milk protein synthesis in the lactating dairy goat in vivo. Am. J. Physiol. 271:955–960.

Bannai, S., H. Sato, T. Ishii, and S. Taketani. 1991. Enhancement of glutathione levels in mouse peritoneal macrophages by sodium arsenite, cadmium chloride and glucose/glucose oxidase. Biochim. Biophys. Acta 1092:175–179.

Basch, J. J., E. D. Wickham, and H. M. Farrell. 1995. Ornithine-delta-aminotransferase in lactating bovine mammary glands. J. Dairy Sci. 78:825–831.

Basch, J. J., E. D. Wickham, and H. M. Farrell. 1996. Pyrroline-5-carboxylate reductase in lactating bovine mammary glands. J. Dairy Sci. 79:1361–1368.

Bharathi, L., K. B. Shenoy, and S. N. Hegde. 1997. Biochemical differences between crop tissue and crop milk of pigeons (*Columba Livia*). Comp. Biochem. Physiol. A. 116:51–55.

Bodoy, S., L. Martín, A. Zorzano, M. Palacín, R. Estévez, and J. Bertran. 2005. Identification of LAT4, a novel amino acid transporter with system L activity. J. Biol. Chem. 280:12002–12011.

Bröer, S. 2008. Amino acid transport across mammalian intestinal and renal epithelia. Physiol. Rev. 88:249–286.

Bröer, A., C. Wagner, F. Lang, and S. Bröer. 2000. Neutral amino acid transporter ASCT2 displays substrate-induced Na^+ exchange and a substrate-gated anion conductance. Biochem. J. 346:705–710.

Cabrera, R. A., J. L. Usry, C. Arrellano, E. T. Nogueira, and J. Odle. 2013. Effects of creep feeding and supplemental glutamine or glutamine plus glutamate (Aminogut) on pre- and post-weaning growth performance and intestinal health of piglets. J. Anim. Sci. Biotechnol. 4:29.

Carr, R. H., and C. M. James. 1931. Synthesis of adequate protein in the glands of the pigeon crop. Am. J. Physiol. 97:227–231.

Chen, F., S. H. Zhang, Z. X. Deng, Q. Q. Zhou, L. Cheng, S. W. Kim, J. Chen, and W. T. Guan. 2018. Regulation of amino acid transporters in the mammary gland from late pregnancy to peak lactation in the sow. J. Anim. Sci. Biotechnol. 9:103–114.

DeSantiago, S., N. Torres, A. Suryawan, A. R. Tovar, and S. M. Hutson. 1998. Regulation of branched-chain amino acid metabolism in the lactating rat. J. Nutr. 128:1165–1171.

Dumont, J. N. 1965. Prolactin-induced cytologic changes in the mucosa of the pigeon crop during crop-"milk" formation. Z. Zellforsch. Mikrosk. Anat. 68:755–782.

Flynn, N. E., C. J. Meininger, K. Kelly, N. H. Ing, S. M. Morris, and G. Wu. 1999. Glucocorticoids mediate the enhanced expression of intestinal type II arginase and argininosuccinate lyase in post-weaning pigs. J. Nutr. 129:799–803.

Foltz, M., M. Boll, L. Raschka, G. Kottra, and H. Daniel. 2004. A novel bifunctionality: PAT1 and PAT2 mediate electrogenic proton/amino acid and electroneutral proton/fatty acid symport. FASEB J. 18:1758–1760.

Gao, C. Q., J. X. Yang, M. X. Chen, H. C. Yan, and X. Q. Wang. 2016. Growth curves and age-related changes in carcass characteristics, organs, serum parameters, and intestinal transporter gene, expression in domestic pigeon (*Columba livia*). Poult. Sci. 95:867–877.

Gillespie, M. J., V. R. Haring, K. A. McColl, P. Monaghan, J. A. Donald, K. R. Nicholas, R. J. Moore, and T. M. Crowley. 2011. Histological and global gene expression analysis of the 'lactating' pigeon crop. BMC Genomics 12:452–461.

Gollop, N., Z. Barak, and D. M. Chipman. 1988. Assay of products of acetolactate synthase. Meth. Enzymol. 166:234–240.

Hebbring, S. J., Y. Chai, Y. Ji, R. P. Abo, G. D. Jenkins, B. Fridley, J. Zhang, B. W. Eckloff, E. D. Wieben, and R. M. Weinshilboum. 2012. Serine hydroxymethyltransferase 1 and 2: gene sequence variation and functional genomic characterization. J. Neurochem. 120:881–890.

Hedge, S. N. 1972. The amino-acid composition of pigeon milk. Curr. Sci. 41:23–24.

Horseman, N. D., and J. D. Buntin. 1995. Regulation of pigeon cropmilk secretion and parental behaviors by prolactin. Annu. Rev. Nutr. 15:213–238.

Hu, X. C., C. Q. Gao, X. H. Wang, H. C. Yan, Z. S. Chen, and X. Q. Wang. 2016. Crop milk protein is synthesised following activation of the IRS1/Akt/TOR signalling pathway in the domestic pigeon (*Columba livia*). Br. Poult. Sci. 57:855–862.

Katragadda, S., R. S. Talluri, D. Pal, and A. K. Mitra. 2005. Identification and characterization of a Na^+-dependent neutral amino acid transporter, ASCT1, in rabbit corneal epithelial cell culture and rabbit cornea. Curr. Eye Res. 30:989–1002.

Laspiur, J. P., J. L. Burton, P. S. Weber, R. N. Kirkwood, and N. L. Trottier. 2004. Short communication: amino acid transporters in porcine mammary gland during lactation. J. Dairy Sci. 87:3235–3237.

Lea, R. W., D. M. Vowles, and H. R. Dick. 1986. Factors affecting prolactin secretion during the breeding cycle of the ring dove (*Streptopelia risoria*) and its possible role in incubation. J. Endocrinol. 110:447–458.

Lei, J., D. Feng, Y. Zhang, F. Q. Zhao, Z. Wu, A. San Gabriel, Y. Fujishima, H. Uneyama, and G. Wu. 2012. Nutritional and regulatory role of branched-chain amino acids in lactation. Front. Biosci. 17:2725–2739.

Li, P., D. A. Knabe, S. W. Kim, C. Lynch, S. Hutson, and G. Wu. 2009. Lactating porcine mammary tissue catabolized branched-chain

amino acids for glutamine and asparate synthesis. J. Nutr. 139:1502–1509.
Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408.
Manjarin, R., J. P. Steibel, V. Zamora, N. Am-In, R. N. Kirkwood, C. W. Ernst, P. S. Weber, N. P. Taylor, and N. L. Trottier. 2011. Transcript abundance of amino acid transporters, β-casein, and α-lactalbumin in mammary tissue of periparturient, lactating, and postweaned sows. J. Dairy Sci. 94:3467–3476.
Mepham, T. B. 1982. Amino acid utilization by lactating mammary gland. J. Dairy Sci. 65:287–298.
Menzies, K. K., C. Lefèvre, K. L. Macmillan, and K. R. Nicholas. 2009. Insulin regulates milk protein synthesis at multiple levels in the bovine mammary gland. Funct. Integr. Genomics 9:197–217.
Nakauchi, J., H. Matsuo, D. K. Kim, A. Goto, A. Chairoungdua, S. H. Cha, J. Inatomi, Y. Shiokawa, K. Yamaguchi, I. Saito, H. Endou, and Y. Kanai. 2000. Cloning and characterization of a human brain Na^+-independent transporter for small neutral amino acids that transports D-serine with high affinity. Neurosci. Lett. 287:231–235.
Patel, S. A., B. A. Warren, J. F. Rhoderick, and R. J. Bridges. 2004. Differentiation of substrate and non-substrate inhibitors of transport system xc(-): an obligate exchanger of L-glutamate and L-cystine. Neuropharmacology 46:273–284.
Rezaei, R., Z. Wu, Y. Hou, F. W. Bazer, and G. Wu. 2016. Amino acids and mammary gland development: nutritional implications for milk production and neonatal growth. J. Anim. Sci. Biotechnol. 7:20.
Richards, N. G., and S. M. Schuster. 1998. Mechanistic issues in asparagine synthetase catalysis. Adv. Enzymol. Relat. Areas Mol. Biol. 72:145–198.
Sales, J., and G. P. J. Janssens. 2003. Nutrition of the domestic pigeon (*Columba livia domestica*). World Poult. Sci. J. 59:221–232.
Sano, A., E. Kakazu, T. Morosawa, J. Inoue, T. Kogure, M. Ninomiya, T. Iwata, T. Umetsu, T. Nakamura, S. Takai, and T. Shimosegawa. 2018. The profiling of plasma free amino acids and the relationship between serum albumin and plasma-branched chain amino acids in chronic liver disease: a single-center retrospective study. J. Gastroenterol. 53:978–988.
Sciascia, Q. L., D. Pacheco, and S. A. Mccoard. 2015. Administration of exogenous growth hormone is associated with changes in plasma and intracellular mammary amino acid profiles and abundance of the mammary gland amino acid transporter SLC3A2 in mid-lactation dairy cows. PLoS One 10:e0134323.
Shennan, D. B., D. T. Calvert, M. T. Travers, Y. Kudo, and C. A. R. Boyd. 2002. A study of L-leucine, L-phenylalanine and L-alanine transport in the perfused rat mammary gland: possible involvement of LAT1 and LAT2. Biochim. Biophys. Acta 1564:133–139.
Shetty, S., K. R. Sridhar, K. B. Shenoy, and S. N. Hegde. 1990. Observations on bacteria associated with pigeon crop. Folia. Microbiol. 35:240–244.
Shetty, S., L. Bharathi, K. B. Shenoy, and S. N. Hegde. 1992. Biochemical properties of pigeon milk and its effect on growth. Comp. Biochem. Physiol. B. 162:632–636.
Trottier, N. 1995. Protein Metabolism in the Lactating Sow. PhD Diss. Univ Illinois, Urbana.
Trottier, N. L., C. F. Shipley, and R. A. Easter. 1997. Plasma amino acid uptake by the mammary gland of the lactating sow. J. Anim. Sci. 75:1266–1278.
Vandeputte-Poma, J., and G. van Grembergen. 1959. Freie Aminosaure in der Kropfmilch der Taube. Naturwissenschafen 46:329–330.
Vandeputte-Poma, J. 1980. Feeding, growth and metabolism of the pigeon, Columba livia domestica: duration and role of crop milk feeding. J. Comp. Physiol. 135:97–99.
Wu, G. 2003. Amino acids: metabolism, functions, and nutrition. Amino Acids 37:1–17.
Wu, G. 2013. Amino Acids: Biochemistry and Nutrition. CRC Press, Boca Raton.
Wan, X. P., P. Xie, Z. Bu, and X. T. Zou. 2018. Changes in hepatic glucose and lipid metabolism-related parameters in domestic pigeon (*Columba livia*) during incubation and chick rearing. J. Anim. Physiol. Anim. Nutr. 102:e558–e568.
Xie, P., X. Y. Jiang, Z. Bu, S. Y. Fu, S. Y. Zhang, and Q. P. Tang. 2016. Free choice feeding of whole grains in meat-type pigeons:I. effect on performance, carcass traits, and organ development. Br. Poult. Sci. 57:699–706.
Xie, P., X. P. Wang, Z. Bu, and X. T. Zou. 2017. Differential expression of fatty acid transporters and fatty acid synthesis-related genes in crop tissues of male and female pigeons (*Columba livia domestica*) during incubation and chick rearing. Br. Poult. Sci. 58:594–602.
Xie, P., X. P. Wan, Z. Bu, E. J. Diao, D. Q. Gong, and X. T. Zou. 2018. Changes in hormone profiles, growth factors and mRNA expression of the related receptors in crop tissue, relative organ weight, and serum biochemical parameters in the domestic pigeon (*Columba livia*) during incubation and chick-rearing periods under artificial farming conditions. Poult. Sci. 97:2189–2202.
Yamasaki, M., K. Yamada, S. Furuya, J. Mitoma, Y. Hirabayashi, and M. Watanabe. 2001. 3-Phosphoglycerate dehydrogenase, a key enzyme for l-serine biosynthesis, is preferentially expressed in the radial glia/astrocyte lineage and olfactory ensheathing glia in the mouse brain. J. Neurosci. 21:7691–7704.
Yip, M. C. M., and W. E. Knox. 1972. Function of arginase in lactating mammary gland. Biochem. J. 127:893–899.
Zhang, X. Y., N. N. Zhang, X. P. Wan, L. L. Li, and X. T. Zou. 2017. Gene expression of amino acid transporter in pigeon (*Columbia livia*) intestine during post-hatch development and its correlation with amino acid in pigeon milk. Poult. Sci. 96:1120–1131.