Dynamic variations in serum amino acid and the related gene expression in liver, ovary, and oviduct of pigeon during one egg-laying cycle

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ABSTRACT The present study was carried to investigate dynamic variations in serum amino acid (**AA**) contents and the relative mRNA abundance of the AA transporters and AA synthesis-related enzymes in liver, ovary and oviduct of pigeons during one egg-laying cycle (**ELC**). In experiment 1, seventy laying pigeons (American Silver King) were randomly divided into 14 groups by different days of one ELC (**DELC**) and arranged as a 2 × 7 factorial design, which included 2 ages (6-mo-old or 12-mo-old) and 7 DELCs. For experiment 2, 35 six-mo-old laying pigeons (American Silver King) were randomly divided into 7 groups by different DELCs and immediately treated with a 12-h fasting. Dynamic variations in serum AAs were detected during one ELC, characterized by high levels of Lys, Met, Leu, Phe, Tyr, Asp, Ser, Glu, Ala, and TAA on day 1 (**D1**) of one ELC ($P < 0.05$). Fasting caused obvious decreases in serum levels of Leu, Ile, Val, Phe, Tyr, and TAA from day 2 **(D2**) to day 7 (**D7**) ($P < 0.05$). Relative organ weights of ovary and oviduct increased to the peak values on day 13 (**D13**) ($P < 0.05$). Serum calcium decreased to the lowest level on day 4 (**D4**) ($P < 0.05$) and serum total triglyceride was kept in a high level on D1, D7, day 10 (**D10**), and D13 ($P < 0.05$). Relative mRNA expression of the AA synthesis genes and the AA transport genes exhibited different variation patterns in liver, ovary and oviduct, but Pearson correlation test showed the percentage of positive r values with significant differences were much higher in oviduct than those in liver or ovary. In conclusion, dynamic variations of serum AAs during one ELC were positively related with the expression of the AA transport genes and AA synthesis genes in oviduct, suggesting the upregulated serum AAs might be necessary to meet the AAs requirement for egg white formation in pigeon.

Key words: amino acid transporter, egg-laying cycle, dynamic variation, laying pigeon, serum amino acid

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INTRODUCTION

Pigeon eggs not only provide the whole nutrient elements for embryo development, but also have long been recognized as one of good nutrient resources for humans (Sun, et al., 2019). Typically, pigeons have a two-egg clutch and there is a nearly 2-mo breeding cycle including nonbreeding, mating, hatching and squab raising, leading to a much lower egg yield (Goerlich, et al., 2009). On commercial farm for pigeon egg production, the laying pigeons are paired per cage by the female-female way and the laid eggs are continuously removed to minimize long interclutch period and increase the egg quantities (Bu, et al., 2015). Despite this, each pair of laying pigeons only produce about 60 eggs per year (Wang, et al., 2017; Chang, et al., 2018). This is partly because laying pigeons still require a laying interval of approximately 10 to 20 d until the next clutch (Dijkstra, et al., 2010). Previous studies reported the egg-laying interval of pigeon could be shorten to increase egg-laying rate as fed with diet containing 0.78% of lysine or the 0.90% of calcium (Ca) (Chang, et al., 2018; Chang, et al., 2019). Moreover, pigeon treated with a diet supplemented with sodium selenite displayed better reproductive performance, characterized by the increasing egg production and hatchability (Wang, et al., 2017). Thus, it's possible to improve the productivity of laying pigeons via the nutritional strategies.

Feed proteins are digested and hydrolyzed into many types of amino acids (**AAs**), which are absorbed into blood via the intestine and distributed into target tissues (Wu, 1998). Unlike the permanent elongation and shortening in glycogen, all intracellular proteins undergo continuous degradation into the constituent AAs and resynthesis by attaching free AAs one after the other

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accordingly (Wu, 2009). Thus, AAs coming from dietary proteins, along with those released by degradation of endogenous proteins, form a dynamic AAs pool in blood and tissues such as intestine, liver, muscle, kidney and so on. Serum AAs, part of free AAs pool, changed with age and fluctuated under different physiological conditions such as reproduction, growth, stress response and pathology in animals (Liao et al., 2018). In laying pigeon, egg formation is exactly regulated and carried out step-by-step. It starts from ovary for yolk formation and subsequently the egg white (ovalbumin) is formed along the magnum of oviduct. Finally, the production of the egg shell occurs in the uterus or shell gland before the egg is released from the oviduct (Birrenkott, et al., 1988; Goerlich, et al., 2010). This means the AAs requirements may change in a dynamic and real-time pattern along the egg formation. However, there is still no report describing their variation characteristics in serum of laying pigeon.

Pigeon fresh egg is composed of about 19% yolk and 74% white (or albumen), contained in a shell corresponding to 7% of the total egg weight (Sun, et al., 2019). Based on the protein contents in raw yolk, raw albumen and shell (Murphy, 1994), the total amount of protein per pigeon egg were accounted for 35.0% in yolk, 55.5% in albumen and 9.5% in shell including attached membranes, respectively. Thus, pigeon egg albumen contributed to half of the total synthesized proteins in the magnum of the oviduct, where the egg albumen layer was formed and the tubular glands synthesized the major egg albumen proteins (Chousalkar and Roberts, 2008). In laying pigeon, the expression of egg albumen protein-related genes was highest and protein transport activities were prominent in pigeon magnum at stage of 1 to 2 d before ovulation, suggested the raw material for albumen formation was gradually produced in advance and prepared to secrete during yolk passage along the oviduct (Lu, et al., 2020). The precursors for yolk formation were not synthesized in the ovary but produced by liver and then transported to ovary through bloodstream (Speake, et al., 1998; Nys and Guyot, 2011). Transcriptome analysis revealed that the mRNA levels of the synthesis-related genes of yolk precursors wereextremely abundant in the liver, but not in ovary in laying hens (Li, et al., 2015; Yin, et al., 2019). In addition, there were 409 genes identified and differentially expressed in pigeon ovary between pre- and postovulation (Xu, et al., 2016). Taken together, this study hypothesized that the raw materials used for pigeon yolk formation were mainly produced in liver and pigeon ovary also played a key role during egg yolk formation.

All tissues should be capable of nonessential AAs synthesis, remodeling and conversion of non-AA carbon skeletons into AAs. However, AAs across cell membranes or organelles must be carried by the AA transporters that are responsible for intracellular AAs concentrations (Hundal and Taylor, 2009). Thus, the status of tissue proteins biosynthesis can be reflected by both of the abundances of the AA synthesis-related enzymes and transporters. Taken together, the objectives of this study were to investigate the dynamic variations in serum AAs contents, the relative mRNA abundance of the AA transporters and AA synthesis-related enzymes in liver, ovary and oviduct. The correlations among these parameters were also analyzed to elucidate the possible mechanism underlying these variations.

MATERIALS AND METHODS

Ethics Approval

All animal procedures in this study were approved by the committee of Animal Research Institute (Certification No. SYXK (Su) 2011-0036), Nanjing Agricultural University, China.

Pigeon Rearing

On one commercial pigeon farm (Nanjing Dongchen Pigeon Industry Co. Ltd., Nanjing, China), 2 experiments were conducted in a windowed poultry house including about 200 pairs of 6-mo laying pigeons (American Silver King) and 500 pairs of 12-mo laying pigeons (American Silver King). All birds were paired per cage (50 × 60 × 45 cm) by the female-female way and the cages were equipped with a perch and a nest. Both trials used a corn-soybean meal-based diet and the ingredients and nutrition levels were shown in Table 1. Diets were fed in pellet form produced by high temperature extrusion process and formulated to meet or exceed the nutrient levels, which were recommended by the local producer and referred to previous studies (Chang, et al., 2018; Chang, et al., 2019). Birds were housed under a 16L:8D lighting cycle and given free access to water and food. The values of mean daily temperature and relative humidity were 20 ± 5°C and 55 ± 15%, respectively.

Sample Preparation

The egg-laying interval or clutch-to-clutch interval was always accounted from the day after the second egg laid (**SEL**) to the day before the first egg laid (**FEL**) of the subsequent clutch (Dijkstra, et al., 2010). In order to study the dynamic changes of serum AAs during the whole egg-laying process, one egg-laying cycle (**ELC**) in the present study was accounted from the day as the FEL to the day before the next FEL. Before experiment, 187 pigeons were randomly selected to count for the average ELC by tracing the egg-laying records and the values of ELC ranged from 8 to 17 d. The number of birds with the same ELC was shown in brackets as follows: 8 (1), 9 (14), 10 (38), 11 (51), 12 (27), 13 (27), 14 (10), 15 (9), 16(5), and 17 (5). The average ELC was 11.71 ± 0.14 d and the average body weight of the birds was 501.99 ± 16.62 g. Considering the obvious difference of ELC among birds and the average ELC of approximately 11-12 days, two time points of day 10 (D10) and day 13 (D13) in the same ELC were designed to detect

Table 1. Ingredients and nutrient levels of basal diet for laying pigeons.[1]

Items	Content
Ingredients composition (%)	
Corn	51.00
Soybean meal	30.00
Soybean	2.60
Wheat	10.00
Limestone	1.80
Calcium hydrogen phosphate	1.50
Vegetable oil	1.00
Salt	0.50
Lysine-HCl	0.10
DL-Methionine	0.15
Acidifier[2]	0.20
Mould remover[3]	0.10
Mineral/vitamin premix[4]	1.05
Total	100.00
Calculated nutrient (%)[5]	
Dry matter	82.51
ME (Mcal/kg)	2.86
Crude protein	21.47
Total calcium	1.05
Total phosphorus	0.40
Methionine	0.34
Methionine+cysteine	0.71
Lysine	1.10
Arginine	1.35
Isoleucine	0.84
Leucine	1.74
Phenylalanine	1.01
Threonine	0.78
Tyrosine	0.70
Valine	0.98

[1]Diet was formulated to meet or exceed the nutrient levels, which were recommended by the local producer (Nanjing Dongchen Pigeon Industry Co., Ltd, Nanjing, China) and referred to previous studies (Chang, et al., 2018; Chang, et al., 2019).

[2]Acidifier (Shanghai Sueneng Aisaiwu Biotechnology Co., Ltd, Shanghai, China) consisted of 12% water, 20% lactic acid, 10% citric acid and 58% silica as carrier.

[3]Mould remover (Bio-Nutrition International Inc., Madison, USA) was composted of Saccharomyces cerevisiae (> 1.0×10^{10} cfu/kg) and hydrated calcium sodium aluminosilicate (carrier).

[4]The premix provided the following per kg of diet: Vitamin A, 20000 IU; Vitamin D_3, 2000 IU; Vitamin E, 15 IU; Vitamin K_3, 40 mg; Vitamin B_1, 5.5 mg,Vitamin B_2, 6 mg, Vitamin B_6, 1.5 mg; Vitamin B_{12}, 5 mg; Vitamin C, 3 mg; Folic acid, 10 mg; Biotin, 0.2 mg; Niacin, 4 mg; Calcium pantothenate, 70 mg; Choline chloride, 100 mg; Potassium chloride, 300 mg; Allicin, 300 mg; Cu (as quinocetone), 75 mg; Fe (as ferrous sulfate), 20 mg; Mn (as manganese sulfate), 30 mg; Zn (as zinc sulfate), 30 mg.

[5]Nutrient values were calculated from data provided by Feed Database in China (2018).

the changes of serum AAs level during the end of one ELC. Thus, all 7 different days of one ELC (**DELC**) for sample collection were set as follows: 1) D1, the day as the FEL; 2) D2, the day after the FEL day; 3) D3, the day as the SEL; 4) D4, 1 d after the SEL; 5) D7, 4 d after the SEL; 6) D10, 7 d after the SEL; 7) D13, 10 d after the SEL.

Experiment 1. In order to compare the variations of serum AAs levels between the 6-mo and 12-mo laying pigeons, seventy birds were randomly divided into 14 groups distinguished by 2 different ages (6-mo-old or 12-mo-old) and 7 DELCs (D1, D2, D3, D4, D7, D10, and D13). There were 5 birds in each group. Immediately, blood samples were collected from a wing vein, centrifuged to separate the serum and kept at −80 °C until analysis.

Experiment 2. Serum AAs pool is impacted by exogenous AAs obtained from diet and endogenous AAs from cellular proteolysis and de novo AA synthesis (Liao, et al., 2018). In order to elucidate the dynamic variations of food consumption and its effects on the serum AA levels, 12 pairs of pigeons (6 mo) were selected to trace the values of food intake and body weight at 10 time points across 2 ELCs, as follows: FD1, the first egg-laying day of the first-detected ELC; FD2, the day after FD1; FD3, 2 d after FD1; FD4-6, the period from 3 d to 5 d after FD1; FD7-9, the period from 6 d to 8 d after FD1; FD10-S, the period from 9 d after FD1 to the day before the second-detected ELC; SD1, the first egg-laying day during the second-detected ELC; SD2, the day after SD1; SD3, 2 d after SD1; SD4, 3 d after SD1. Birds were fed ad libitum throughout the experiment. Pigeons and residual diets were weighed every evening (19:00−20:00 pm) to calculate the feed consumption.

In order to study the relationship between serum AAs and the AAs requirement for egg formation inside of the body, another 35 six-mo laying pigeons were randomly divided into 7 groups distinguished by the 7 DELCs (D1, D2, D3, D5, D7, D10, and D13) to detect dynamic variations of serum AAs levels. There were 5 birds in each group. Immediately, these birds were treated with a 12-h feed deprivation to eliminate the effect of dietary AAs on serum AAs pool. And then, all birds were sacrificed to collect blood samples and the isolated serum samples were stored at −80 °C until determine. Organs (heart, liver, kidney, pancreas, gizzard, abdominal fat, ovary, and oviduct) were dissected and weighed. The relative organ weight was calculated as (organ weight/body weight) × 100%. For gene detection, liver, ovary, and oviduct magnum were quickly frozen in liquid nitrogen and stored at −80 °C.

Serum AA Analysis

Analysis of the serum free AAs concentration was performed using an automated Automatic Amino Acid Analyzer (Hitachi L-8900, Tokyo, Japan). Serum sample (100 μL) was precipitated by addition of a 3-fold volume (300 μL) of 5% sulfosalicylic acid. Then, the sample was mixed and placed on ice for 30 min followed by centrifugation at 20,000 rpm for 20 min at 4°C to remove precipitated proteins. The supernatant was filtered through a 0.22 μm Millipore membrane (Millipore Corp., Bedford, MA) and the filtrate was collected for the subsequent analysis. The following AAs were measured: Asp, Ala, Arg, Cys, Glu, Gly, Ile, Leu, Lys, Met, Phe, Ser, Tyr, Thr, and Val. Total amino acids (**TAA**) was calculated by adding these 15 AAs.

Serum Biochemical Parameters

The concentrations of serum total protein (**TP**), albumin (**ALB**), total cholesterol (**TC**), triglyceride (**TG**), low density lipoprotein cholesterol (**LDL**), and Ca were assayed using standard commercial kits following the protocols recommended by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum glucose (**GLU**) was measured using the Glucose Assay Kit (Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China). The serum globulin (**GLB**) content was calculated by subtracting ALB from TP content.

RNA Isolation and Real-time Quantitative PCR

Total RNA was isolated from frozen liver (liver lobe), ovary (ovary cortex containing ovarian small follicles) and oviduct (magnum) using TRIzol reagent (Invitrogen, Carlsbad, CA). For each sample, 5 μg of total RNA was reverse transcribed to cDNA by oligonucleotide primers and M-MLV reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China). The mRNA abundances of Na^+-coupled neutral amino acid transporter 2 (*SNAT2*), L-type amino acid transporter 4 (*LAT4*), Na^+-dependent neutral amino acid transporter (B^0AT1), excitatory amino acid transporter 3 (*EAAT3*), Na^+-dependent cationic amino acid transporter (*CAT1*), Na^+-dependent cationic and Na^+-dependent neutral amino acid transporter 2 (y^+LAT2), argininosuccinate lyase (*ASL*), glutamate dehydrogenase 1 (*GDH1*), serine hydroxymethyltransferase 2 (*SHMT2*), phosphoglycerate dehydrogenase (*PDH*), and β-actin were measured using the real-time PCR method on an ABI 7300 system. Primers were designed with Primer 5.0 software (Premier Biosoft, Palo Alto, CA), and β-actin was used as the internal control gene. The primer sets used were shown in Table 2. PCR reactions were run in triplicates in a 20-μL reaction volume (consisting of SYBR Premix Ex Taq, ROX Reference Dye, 200 nM primer, and 100 ng cDNA template). The amplification conditions were as follows: DNA polymerase activation at 95°C for 30 s, followed by 42 amplification cycles of denaturation at 95°C for 5 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The relative mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

All data were presented as mean ± SEM. To study the dynamic variations in serum AAs, two-way ANOVA was performed to determine the effects of pigeon ages and DELCs and their interaction using the GLM procedure of SPSS 17.0 software (SPSS Inc., Chicago, IL). Means were separately analyzed according to post-hoc Duncan's honestly significant difference test. One-way ANOVA was used to analyze the effects of DELCs on serum AAs, the relative organ weight, serum biochemical parameters and the relative mRNA expression of the AA transport-related and synthesis-related genes in pigeon treated with the 12-h fasting. The statistical significance of differences among 7 groups was evaluated by Duncan's honestly significant difference post hoc test. Statistical t test was performed to compare serum AAs between the fasting-treated and no-fasting-treated laying pigeons. Pearson correlation coefficient was tested between serum AAs and the mRNA expression of the related genes in liver, ovary and oviduct. Probability values less than 0.05 were considered statistically significant.

RESULTS

Variations in Anionic AAs, Cationic AAs and Neutral Hydrophilic AAs in Pigeon Serum Under Normal Condition

As shown in Table 3, based on two-way ANOVA analysis, the DELC had obvious effect on the contents of Lys ($P < 0.001$), Tyr ($P = 0.006$), Asp ($P = 0.012$), Ser ($P < 0.001$), and Glu ($P = 0.026$). In both of 2 ages, the values of Lys, Tyr, and Ser peaked at D2, decreased

Table 2. Primers used in the present study.

Target gene	Primer sequence (5'/3')	Tm (°C)	Accession No.	Size (bp)
Genes involved in amino acid transport				
SNAT2	F: GTGGCACCTTTGGTAGATG R: AATCGCAGGATGGCAGAC	56	XM_005499916	129
LAT4	F: GACCTTCACATCGCTTACG R: TGCCCAGACAAGCAGAAT	56	XM_005502006.1	163
B^0AT1	F: TGGCATAGCAGCAATGTCGG R: CTTGGAAGGAGTTGAAGAAATACC	59	XM_005509991	95
EAAT3	F: ACAGGTGTTGCTGCTTTG R: GGTGCTGCCCACTCTAT	56	XM_005507165.1	171
CAT1	F:CCCAGCAACACTGTCAGCTA R:GACCTTTGTGAGGCCTGGTT	60	XM_005501421.3	151
y^+LAT2	F: TATAAGGAGAACGGGGCAACCA R:CAAGCTAAACAGAACACGTGGC	60	XM_021290806.1	199
Genes involved in amino acid synthesis				
ASL	F: AGGAGGCTGTCTTTGATGTTGTGG R: CAGGCTGAGATTATTGAGGGTGA	61	XM_021286946	245
GDH1	F:GGCTAGTGCGTCAGAGAAGG R:AGGCCCAGGTTGTATCTTGC	60	XM_021288950.1	105
SHMT2	F: CGTCAACGTTCAGCCCTACT R: TTGACGTCCGACATGTACCC	60	XM_005499916	129
PDH	F:ACTGGGAACAGCCTTAGTGC R:GTCTTCCCGTTCAGCTCCAT	60	XM_005498831.2	143
Internal control gene				
β-actin	F: ATTGTCCACCGCAAATGCTTC R:AAATAAAGCCATGCCAATCTCGTC	60	XM_005504502.2	115

Abbreviations: *ASL*, argininosuccinate lyase; B^0AT1, Na^+-dependent neutral amino acid transporter; *CAT1*, Na^+-dependent cationic amino acid transporter; *EAAT3*, excitatory amino acid transporter 3; *GDH1*, glutamate dehydrogenase 1; *LAT4*, L-type amino acid transporter 4; y^+LAT2, Na^+-dependent cationic and Na^+-dependent neutral amino acid transporter 2; *PDH*, phosphoglycerate dehydrogenase; *SHMT2*, serine hydroxymethyltransferase 2; *SNAT2*, Na^+-coupled neutral amino acid transporter 2.

Table 3. Anionic amino acids, cationic amino acids and neutral hydrophilic amino acids in pigeon serum during one egg-laying cycle.[1]

Items	Anionic amino acids, μg/mL		Cationic amino acids, μg/mL		Neutral hydrophilic amino acids, μg/mL			
	Asp	Glu	Arg	Lys	Thr	Ser	Tyr	Cys
DELC								
D1	7.15 ± 0.58^{abc}	22.21 ± 2.51^{ab}	65.43 ± 4.78	68.27 ± 3.66^{bc}	149.49 ± 5.60	46.13 ± 2.63^{b}	35.26 ± 2.65^{b}	11.76 ± 1.72
D2	8.71 ± 0.73^{a}	23.78 ± 3.63^{a}	70.74 ± 6.57	100.81 ± 7.15^{a}	162.49 ± 7.12	54.94 ± 3.90^{a}	48.33 ± 2.56^{a}	13.43 ± 0.69
D3	7.28 ± 0.94^{abc}	20.31 ± 1.95^{ab}	66.04 ± 5.81	65.19 ± 6.80^{bc}	149.32 ± 10.34	47.51 ± 2.78^{ab}	40.31 ± 2.82^{b}	14.11 ± 1.48
D4	7.16 ± 0.43^{abc}	22.96 ± 1.87^{a}	67.68 ± 5.46	61.79 ± 7.72^{c}	140.98 ± 8.60	40.13 ± 2.44^{b}	37.65 ± 1.93^{b}	13.36 ± 1.26
D7	7.83 ± 0.64^{ab}	23.69 ± 1.71^{a}	81.90 ± 4.30	69.08 ± 9.40^{bc}	135.81 ± 11.83	42.03 ± 3.33^{b}	39.91 ± 2.89^{b}	12.29 ± 1.13
D10	5.82 ± 0.63^{c}	17.61 ± 1.54^{b}	63.08 ± 5.16	54.52 ± 5.80^{c}	137.82 ± 8.53	31.09 ± 2.66^{c}	37.35 ± 2.56^{b}	12.10 ± 0.92
D13	6.27 ± 0.38^{bc}	20.83 ± 2.26^{ab}	70.38 ± 6.22	84.18 ± 6.29^{ab}	151.42 ± 11.77	39.96 ± 2.70^{b}	42.24 ± 1.75^{ab}	11.19 ± 0.85
Age								
6 mo	7.42 ± 0.34	16.60 ± 0.71^{b}	66.51 ± 2.73	79.74 ± 4.25^{a}	141.36 ± 5.55	42.40 ± 1.57	42.00 ± 1.65	12.80 ± 0.62
12 mo	6.95 ± 0.41	27.10 ± 1.12^{a}	72.28 ± 3.34	65.78 ± 4.05^{b}	154.30 ± 4.10	44.86 ± 2.32	38.69 ± 1.14	12.42 ± 0.69
P-value								
DELC	0.012	0.026	0.233	<0.001	0.471	<0.001	0.006	0.503
Age	0.334	<0.001	0.120	0.016	0.072	0.178	0.117	0.658
D × A	<0.001	0.011	0.100	0.785	0.811	0.088	0.013	0.072

Means with different superscript lowercase letters within the same column differ according to two-way ANOVA ($P < 0.05$).

Abbreviations: D1, the day as the first-egg laying; D2, the day after the first-egg laying day; D3, the day as the second-egg laying (SEL); D4, one day after the SEL; D7, four days after the SEL; D10, seven days after the SEL; D13, ten days after the SEL; DELC, different days of one egg-laying cycle; D × A, interaction between DELC and Age.

[1]Values are means ± SEM; n = 4-5.

at D3 and maintained the low level until D13. A parental age effect was observed in the contents of Lys ($P = 0.016$) and Glu ($P < 0.001$). In the 6-mo pigeons, the Lys content was significantly higher than that in the 12-mo pigeons, but the Glu content were significantly lower. There was a significant interaction between DELC and age for the contents of Tyr, Asp, and Glu.

Variations in Neutral Hydrophobic AAs and TAA in Pigeon Serum Under Normal Condition

As shown in Table 4, serum TAA variation was evident during one ELC. In both of the 6-mo and 12-mo pigeons, serum TAA reached to the peak values on D2, decreased after the second egg laid on D3, and maintained the low level until D13. The average values of serum TAA from 7 time points were 645.99 ng/mL in the 6-mo pigeons and 660.08 ng/mL in the 12-mo pigeons. Based on two-way ANOVA analysis, DELC had obvious effect on the contents of Met ($P = 0.016$), Leu ($P = 0.005$), Phe ($P = 0.004$), Ala ($P = 0.002$), and TAA ($P = 0.001$). In both of two ages, the values of Met, Leu, Phe, and Ala peaked at D2, decreased at D3 and maintained the low level until D13. A parental age effect was observed in the contents of Met ($P < 0.001$), Ile ($P = 0.042$), Leu ($P < 0.001$), Val ($P = 0.003$), Phe ($P < 0.001$), and Ala ($P < 0.001$), but not in the contents of TAA. The contents of Ile, Leu, Val, Phe in the 6-mo pigeons were significantly higher than those in the 12-mo pigeons, whereas the contents of Met and Ala were significantly lower. There was a significant interaction between DELC and age for the Leu content.

Changes in Feed Intake and Body Weight of Pigeon During ELC

Figure 1 showed there were evident variations in food intake and body weight of pigeons, but no significance among these time points. Pigeon laid the first egg on

Table 4. Neutral hydrophobic amino acids and the total amino acids in pigeon serum during one egg-laying cycle.[1]

Items	Ala	Gly	Ile	Leu	Val	Met	Phe	T-AA
DELC								
D1	62.08 ± 4.64^{abc}	77.27 ± 5.83	13.55 ± 1.18	26.53 ± 2.09^{b}	34.15 ± 3.06	12.67 ± 1.17^{ab}	16.88 ± 1.00^{bc}	648.83 ± 32.73^{bc}
D2	72.56 ± 5.56^{a}	86.92 ± 5.37	15.87 ± 1.00	31.56 ± 1.62^{a}	40.40 ± 1.95	14.54 ± 0.89^{a}	18.90 ± 1.21^{a}	763.98 ± 33.37^{a}
D3	61.43 ± 4.04^{abc}	77.02 ± 3.23	13.67 ± 1.45	27.03 ± 2.69^{ab}	35.77 ± 3.12	11.34 ± 1.00^{b}	16.10 ± 1.30^{c}	652.42 ± 25.73^{bc}
D4	51.88 ± 4.86^{c}	73.73 ± 2.36	11.19 ± 1.80	21.51 ± 2.77^{c}	31.12 ± 3.84	10.76 ± 1.06^{b}	14.93 ± 1.55^{c}	606.83 ± 22.81^{bc}
D7	57.36 ± 3.38^{bc}	76.08 ± 3.41	13.47 ± 1.32	27.17 ± 3.24^{ab}	36.52 ± 2.94	11.23 ± 1.00^{b}	16.07 ± 0.90^{c}	650.45 ± 32.32^{bc}
D10	50.89 ± 3.88^{c}	68.44 ± 3.31	10.46 ± 0.92	22.53 ± 2.68^{bc}	29.46 ± 1.99	11.28 ± 0.96^{b}	16.81 ± 1.48^{bc}	566.58 ± 21.48^{c}
D13	65.62 ± 4.64^{ab}	80.76 ± 5.03	13.56 ± 1.20	26.00 ± 1.96^{bc}	34.20 ± 2.15	11.89 ± 1.08^{b}	18.26 ± 1.50^{ab}	676.75 ± 32.38^{a}
Age								
6-mo	55.13 ± 1.81^{b}	74.71 ± 2.06	14.20 ± 0.64^{a}	31.38 ± 0.94^{a}	37.65 ± 1.36^{a}	10.15 ± 0.37^{b}	20.13 ± 0.44^{a}	652.16 ± 15.22
12-mo	67.30 ± 3.00^{a}	80.76 ± 2.77	12.22 ± 0.75^{b}	20.78 ± 1.05^{b}	31.61 ± 1.50^{b}	14.10 ± 0.54^{a}	13.50 ± 0.37^{b}	661.90 ± 21.24
P-value								
DELC	0.002	0.092	0.071	0.005	0.105	0.016	0.004	0.001
Age	<0.001	0.052	0.042	<0.001	0.003	<0.001	<0.001	0.533
D × A	0.135	0.106	0.123	0.035	0.122	0.525	0.129	0.236

Means with different superscript lowercase letters within the same column differ according to two-way ANOVA ($P < 0.05$).

Abbreviations: D1, the day as the first-egg laying; D2, the day after the first-egg laying day; D3, the day as the second-egg laying (SEL); D4, one day after the SEL; D7, four days after the SEL; D10, seven days after the SEL; D13, ten days after the SEL; DELC, different days of one egg-laying cycle; D × A, interaction between DELC and Age; TAA, total amino acid.

[1]Values are means ± SEM; n = 4−5. Unit of serum AAs content was μg/mL.

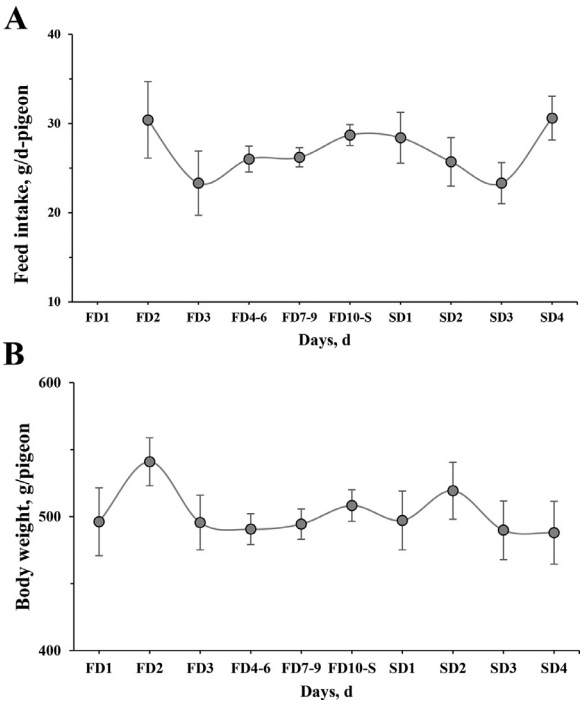

Figure 1. Variations in feed intake (A) and body weight (B) of the 6-mo pigeon during one egg-laying cycle. FD1, the first egg-laying day of the first-detected ELC; FD2, the day after FD1; FD3, two days after FD1; FD4-6, the period from 3 d to 5 d after FD1; FD7-9, the period from 6 d to 8 d after FD1; FD10-S, the period from 9 d after FD1 to the day before the second-detected ELC; SD1, the first egg-laying day during the Second-detected ELC; SD2, the day after SD1; SD3, 2 d after SD1; SD4, 3 d after SD1. Values are means ± SEM (n = 12 pairs of female-female pigeons).

FD1 of the first-detected ELC and SD1 of the second-detected ELC, and the second eggs were laid on FD3 of the first-detected ELC and SD3 of the second-detected ELC, respectively. Obviously, average feed intake decreased to the lower level on FD3 and SD3, when the second eggs were laid during the first-detected and the second-detected ELCs, respectively. After the day pigeon laid the second egg, average feed intake gradually increased and kept at a high level until the second ELC. For body weight, there was a remarkable increase at the FD2 and SD2 time points before the day of the second egg laid. Moreover, the increased body weight was also observed on FD10-S before the first egg laid in the second-detected ELC.

Variations in Serum AAs of Pigeons Treated With a 12-h Fasting

As shown in Table 5, there was a significant difference in all serum AAs contents except for Met, Thr and Ser among different DELCs. Like the variation pattern of pigeons without fasting, serum TAA also peaked at D2, sharply decreased at D3 and gradually recovered back to the peak value at D13 in the 6-mo pigeons treated with a 12-h fasting ($P = 0.014$). The contents of Ile, Val, Arg, Gly, Tyr, Asp, Glu, and Ala increased significantly at D2 and there was a significant decrease in the contents of Lys, Ile, Leu, Try, Gly, Val, Phe, Asp, Glu, and Ala at D3.

Compared with the pigeons with free access to feed, the contents of TAA evidently decreased in the pigeons treated with a 12-h fasting at each DELCs. In Table 6, statistical t tests showed significant differences in 126 event-pairs, in which the P-values of 63 (50%) were < 0.05 and 16 (13%) were < 0.001. The numbers of the P-values less than 0.05 at different time points were shown as follows: D1 (4), D2 (12), D3 (12), D4 (10), D7 (13), D10 (8), and D13 (4). During the period from D2 to D7, there was a significant increase in serum Ile, Leu, Val, Phe, Tyr and TAA between two groups. It's noteworthy that pigeons with fasting showed higher Cys content than pigeons without fasting at D1, D2, D7, D10 and D13 ($P < 0.05$).

Relative Organ Weights During One ELC

As Table 7 showed, relative organ weight of pancreas and gizzard decreased to the lowest level at D2 ($P < 0.05$). Moreover, relative organ weight of oviduct was increased at D2 and D13 ($P < 0.05$), and relative organ weight of ovary only increased at D13 ($P < 0.05$). There was no difference in body weight, and relative organ weights of heat, liver, kidney, and abdominal fat among the 7 DELCs.

Serum Biochemical Parameters During One ELC

In Table 8, serum TG reached the highest value of 20.08 at D13 and was reduced to lowest value of 1.08 at D4 ($P < 0.05$). Serum glucose and Ca were kept at higher levels at D1 and D13 and decreased to lower level at D4 ($P < 0.05$). There was no difference in the parameters of TP, ALB, GLB, TC, and LDL among the 7 DELCs.

The mRNA Expression of the AA-Related Genes in Liver, Ovary and Oviduct

As shown in Table 9, the relative mRNA expression of the AA synthesis genes and the AA transport genes exhibited different variation patterns in liver, ovary and oviduct. There were 7 significant P-values detected from the 10 selected genes in liver, 9 in ovary and 4 in oviduct, respectively. For the genes encoding the AA transporters, the *SNAT2* expression was up-regulated in oviduct at D2 ($P < 0.05$), but not in liver and ovary. Both of liver and ovary exhibited a significant increase in the *LAT4* expression at D1, and B^0AT1 expression of reached to the highest level in liver at D13 and in ovary at D3 ($P < 0.05$). The gene expression of *EAAT3* had higher level at D1 in liver and at D2 in ovary ($P < 0.05$). There was a significant increase in the expression of *CAT1* and y^+LAT2 at D3 in ovary. For the genes encoding the AA synthesis enzymes, the *ASL* expression was kept at higher level at D10 and D13 ($P < 0.05$), while *GDH1* and *PDH* were upregulated ($P < 0.05$) at D3 in ovary. The gene expression of *SHMT2* exhibited significant difference among 7 DELCs in all 3 organs.

Table 5. Serum amino acids (μg/mL) in the 6-mo laying pigeon at different time points after 12 h-fasting during one egg-laying cycle.[1]

Item	One egg-laying cycle							*P*-value
	D1	D2	D3	D4	D7	D10	D13	
Anionic amino acids								
Asp	6.74 ± 0.49^{a}	5.59 ± 1.28^{ab}	3.33 ± 0.26^{cd}	2.08 ± 0.49^{d}	4.10 ± 0.53^{bc}	3.84 ± 0.39^{bcd}	5.76 ± 0.57^{ab}	<0.001
Glu	19.79 ± 1.84^{ab}	21.72 ± 3.52^{a}	15.08 ± 0.71^{bc}	12.52 ± 1.57^{c}	19.06 ± 1.28^{ab}	21.32 ± 1.49^{a}	21.86 ± 0.86^{a}	0.002
Cationic amino acids								
Lys	58.89 ± 5.88^{ab}	51.90 ± 5.88^{bc}	34.10 ± 4.83^{c}	37.07 ± 6.65^{c}	47.71 ± 3.44^{bc}	57.77 ± 9.30^{ab}	72.37 ± 3.67^{a}	0.001
Arg	41.36 ± 3.28^{b}	58.65 ± 5.70^{a}	51.24 ± 2.73^{ab}	60.75 ± 2.97^{a}	56.18 ± 3.67^{a}	52.15 ± 7.14^{ab}	48.42 ± 2.00^{ab}	0.020
Neutral hydrophilic amino acids								
Cys	16.77 ± 1.89^{ab}	17.86 ± 1.04^{ab}	16.78 ± 1.52^{ab}	15.78 ± 2.33^{b}	23.21 ± 2.72^{a}	23.03 ± 1.31^{a}	23.20 ± 0.96^{a}	0.016
Ser	29.42 ± 2.04	38.13 ± 4.16	28.55 ± 2.06	24.92 ± 4.31	24.64 ± 3.04	25.03 ± 1.51	28.21 ± 3.34	0.142
Thr	89.83 ± 6.92	113.01 ± 6.69	91.51 ± 6.36	87.88 ± 10.42	100.63 ± 6.35	108.30 ± 16.92	122.67 ± 10.39	0.101
Tyr	30.36 ± 1.83^{a}	29.54 ± 1.14^{a}	20.21 ± 3.27^{b}	20.58 ± 2.46^{b}	20.90 ± 2.38^{b}	19.30 ± 0.94^{b}	21.78 ± 1.86^{b}	0.004
Neutral hydrophobic amino acids								
Met	7.98 ± 0.56	9.38 ± 1.01	7.20 ± 0.67	7.45 ± 0.37	8.36 ± 0.43	8.43 ± 0.97	9.32 ± 0.63	0.155
Ala	52.06 ± 5.08^{ab}	56.67 ± 4.70^{a}	42.01 ± 1.94^{ab}	36.48 ± 6.39^{b}	52.58 ± 4.77^{ab}	53.67 ± 4.64^{a}	58.91 ± 5.17^{a}	0.039
Ile	8.59 ± 0.88^{ab}	10.29 ± 0.70^{a}	7.66 ± 0.46^{bc}	6.07 ± 0.59^{c}	10.19 ± 0.99^{a}	9.75 ± 0.53^{ab}	9.94 ± 0.50^{ab}	0.001
Leu	19.87 ± 1.20^{a}	20.16 ± 1.64^{a}	14.39 ± 0.76^{bc}	12.08 ± 1.78^{c}	20.90 ± 1.71^{a}	18.12 ± 0.98^{ab}	20.16 ± 1.34^{a}	0.001
Val	26.15 ± 1.93^{ab}	31.49 ± 1.85^{a}	22.49 ± 1.22^{bc}	18.91 ± 1.57^{c}	27.52 ± 2.15^{ab}	26.30 ± 1.06^{ab}	26.46 ± 1.14^{ab}	0.001
Phe	16.70 ± 0.53^{ab}	16.66 ± 1.04^{ab}	11.16 ± 0.41^{c}	10.82 ± 1.79^{c}	14.40 ± 1.14^{bc}	14.68 ± 1.19^{abc}	18.97 ± 1.67^{a}	0.001
Gly	76.25 ± 9.93^{bc}	97.50 ± 5.91^{a}	69.99 ± 4.25^{bc}	82.62 ± 3.56^{ab}	59.34 ± 5.47^{c}	66.72 ± 3.87^{bc}	62.61 ± 5.29^{c}	0.002
TAA	500.75 ± 30.95^{ab}	578.54 ± 28.06^{a}	435.69 ± 19.05^{b}	435.68 ± 39.71^{b}	489.72 ± 24.83^{ab}	508.40 ± 17.81^{ab}	550.64 ± 21.82^{a}	0.014

Means with different superscript lowercase letters within the same row differ according to one-way ANOVA ($P < 0.05$).

Abbreviations: D1, the day as the first-egg laying; D2, the day after the first-egg laying day; D3, the day as the second-egg laying (SEL); D4, one day after the SEL; D7, four days after the SEL; D10, seven days after the SEL; D13, ten days after the SEL; TAA, total amino acid.

[1]Values are means ± SEM; n = 4-5.

Table 6. *P*-values from *t* tests comparing serum amino acid in the 6-mo pigeons treated with and without a 12-h fasting at different time points during one egg-laying cycle.[1]

Item	One egg-laying cycle						
	D1	D2	D3	D4	D7	D10	D13
Anionic amino acids							
Asp	0.986	0.246	0.001	<0.001	0.001	0.047	0.647
Glu	0.087	0.083	0.480	0.045	0.090	0.004	0.007
Cationic amino acids							
Lys	0.279	0.004	0.010	0.063	0.087	0.556	0.030
Arg	0.003	0.894	0.062	0.310	0.010	0.821	0.080
Neutral hydrophilic amino acids							
Cys	0.033	0.029	0.295	0.966	0.012	0.001	<0.001
Ser	0.008	0.027	0.005	0.136	0.006	0.010	0.136
Thr	0.002	<0.001	0.063	0.013	0.197	0.207	0.553
Tyr	0.719	<0.001	0.003	0.006	<0.001	0.004	<0.001
Neutral hydrophobic amino acids							
Met	0.174	0.022	0.031	0.202	0.260	0.373	0.799
Ala	0.925	0.273	0.074	0.629	0.521	0.989	0.891
Ile	0.076	0.030	<0.001	0.025	0.026	0.204	0.039
Leu	0.082	<0.001	<0.001	0.002	0.002	<0.001	0.004
Val	0.246	0.036	<0.001	0.009	0.006	0.064	0.025
Phe	0.121	0.001	<0.001	0.012	0.035	0.008	0.140
Gly	0.331	0.064	0.168	0.047	0.045	0.324	0.193
TAA	0.116	<0.001	<0.001	0.027	0.006	0.052	0.077

Abbreviations: D1, the day as the first-egg laying; D2, the day after the first-egg laying day; D3, the day as the second-egg laying (SEL); D4, one day after the SEL; D7, four days after the SEL; D10, seven days after the SEL; D13, ten days after the SEL; TAA, total amino acid.

[1]Data were analyzed using *t* tests method of SPSS.

Correlation Between Serum AA Contents and the AA-Related Gene Expression in Liver, Ovary and Oviduct

Figure 2 was converted from Table S1 and showed heat map of Pearson correlation coefficient (r) between serum AAs and the mRNA expression of the related genes in liver, ovary and oviduct. Obviously, the number of positive r values with significant difference (**NPRS**) were 27 (18.8%) of the 144 event-pairs in oviduct, whereas NPRS were 13 (8.1%) of the 160 event-pairs in liver and 5 (3.2%) in ovary, respectively. Moreover, the number of negative r values with significant difference (**NNRS**) were 11 (7.6%) of the 144 event-pairs in oviduct, whereas NNRS were 5 (3.1%) of the 160 event-pairs in liver and 16 (10%) in ovary, respectively. It is worthy to notice that all 11 NNRS were observed between *LAT4* and 11 AA items in oviduct, whereas there were 11 NPRS between *SHMT2* and 11 AA items. For pigeon ovary, there were 5 NNRS in the *PDH* band, 6 in the *GDH1* band, and 3 in the *CAT1* band. In pigeon liver, 6 of NPRS were observed in the $y^{+}LAT2$ band and 3 in the *GDH1* band.

DISCUSSION

Unlike the 24-h egg-laying cycle in laying hens, pigeons take much more time to form an egg. For the yolk formation of laying hen, the ovary follicles grew rapidly and more than 90% of egg yolk was deposited into ovarian follicular during the final 7 days before ovulation. And then, the oviduct magnum synthesized all proteins, which were stored in magnum granules and then rapidly secreted to form egg albumen during about 4-h passage of the yolk (Nys and Guyot, 2011). Based on the incorporation of dietary Sudan dyes into the yolk of laying pigeon, an estimate of the period of rapid yolk deposition and the interval from the end of yolk deposition until ovipositon were 6.5 d and 1.5 d, respectively (Birrenkott, et al., 1988). This implied pigeon needed about 6.5 d to finish the rapid follicular development for yolk formation and then took about 1.5 d to form the egg white and the shell in the oviduct before oviposition.

Table 7. The relative organ weights in the 6-mo pigeons treated with a 12-h fasting at different time points during one egg-laying cycle.[1]

Items[2]	One egg-laying cycle							*P*-Value
	D1	D2	D3	D4	D7	D10	D13	
Weight, kg	0.49 ± 0.01	0.51 ± 0.06	0.48 ± 0.02	0.50 ± 0.01	0.47 ± 0.02	0.46 ± 0.03	0.51 ± 0.02	0.689
Heart, g	5.25 ± 0.30	5.97 ± 0.29	6.07 ± 0.21	5.73 ± 0.26	5.44 ± 0.36	5.57 ± 0.08	5.71 ± 0.37	0.586
Heart index, %	1.08 ± 0.06	1.18 ± 0.05	1.27 ± 0.02	1.14 ± 0.03	1.14 ± 0.03	1.22 ± 0.07	1.11 ± 0.04	0.233
Liver, g	7.45 ± 0.30	7.56 ± 1.12	6.80 ± 0.22	7.42 ± 0.47	7.55 ± 0.36	7.37 ± 0.75	8.12 ± 0.86	0.897
Liver index, %	1.53 ± 0.07	1.46 ± 0.08	1.42 ± 0.02	1.48 ± 0.06	1.60 ± 0.05	1.61 ± 0.13	1.57 ± 0.12	0.770
Kidney, g	2.28 ± 0.18	2.69 ± 0.17	2.61± 0.16	2.57 ± 0.15	2.52 ± 0.16	2.15 ± 0.10	2.45 ± 0.19	0.392
Kidney index, %	0.47 ± 0.04	0.53 ± 0.02	0.55 ± 0.04	0.51 ± 0.02	0.53 ± 0.03	0.47 ± 0.01	0.48 ± 0.04	0.609
Pancreas, g	0.79 ± 0.08	1.05 ± 0.09	1.00 ± 0.06	1.13 ± 0.08	1.07 ± 0.06	1.15 ± 0.11	1.05 ± 0.10	0.124
Pancreas index, %	0.16 ± 0.02^{b}	0.21 ± 0.01^{ab}	0.21 ± 0.02^{ab}	0.23 ± 0.01^{a}	0.23 ± 0.01^{a}	0.25 ± 0.02^{a}	0.20 ± 0.01^{ab}	0.028
Gizzard, g	8.26 ± 0.19	11.07 ± 0.61	10.47 ± 0.59	10.90 ± 0.36	11.16 ± 0.60	10.74 ± 1.32	10.26 ± 0.65	0.050
Gizzard index, %	1.70 ± 0.05^{b}	2.19 ± 0.09^{a}	2.19 ± 0.12^{a}	2.18 ± 0.07^{a}	2.35 ± 0.05^{a}	2.32 ± 0.16^{a}	2.01 ± 0.14^{ab}	0.007
AF, g	5.06 ± 1.13	4.29 ± 1.31	6.99 ± 2.21	5.54 ± 0.52	3.57 ± 0.67	5.24 ± 2.60	4.51 ± 0.81	0.663
AF index, %	1.03 ± 0.19	0.79 ± 0.13	1.41 ± 0.37	1.10 ± 0.08	0.73 ± 0.10	1.06 ± 0.40	0.87 ± 0.14	0.470
Ovary, g	0.93 ± 0.03^{b}	1.45 ± 0.73^{b}	1.39 ± 0.45^{b}	0.86 ± 0.12^{b}	1.81 ± 0.54^{b}	3.14 ± 1.39^{ab}	5.02 ± 1.23^{a}	0.008
Ovary index, %	0.19 ± 0.01^{b}	0.25 ± 0.08^{b}	0.23 ± 0.09^{b}	0.17 ± 0.02^{b}	0.41 ± 0.13^{b}	0.69 ± 0.27^{ab}	0.98 ± 0.22^{a}	0.010
Oviduct, g	9.23 ± 0.47^{b}	14.98 ± 1.51^{a}	5.65 ± 0.77^{bc}	4.79 ± 0.44^{c}	5.70 ± 1.10^{bc}	7.89 ± 2.44^{bc}	15.82 ± 0.96^{a}	<0.001
Oviduct index, %	1.88 ± 0.10^{b}	2.93 ± 0.21^{a}	1.18 ± 0.17^{bc}	0.95 ± 0.06^{c}	1.25 ± 0.29^{bc}	1.72 ± 0.53^{bc}	3.02 ± 0.11^{a}	<0.001

Means with different superscript lowercase letters within the same row differ according to one-way ANOVA ($P < 0.05$).

Abbreviations: AF, abdominal fat; D1, the day as the first-egg laying; D2, the day after the first-egg laying day; D3, the day as the second-egg laying (SEL); D4, one day after the SEL; D7, four days after the SEL; D10, seven days after the SEL; D13, ten days after the SEL.

[1]Values are means ± SEM; n = 4-5.

[2]Relative organ weights (Organ index) was calculated as (organ weight/body weight) × 100%.

Table 8. Serum biochemical parameters in the 6-mo laying pigeons treated with a 12-h fasting at different time points during one egg-laying cycle.[1]

Item	One egg-laying cycle							*P*-value
	D1	D2	D3	D4	D7	D10	D13	
Protein metabolism-related parameters, mg/mL								
TP	27.41 ± 5.69	25.40 ± 1.60	24.42 ± 2.10	30.38 ± 2.05	27.88 ± 3.18	23.18 ± 0.89	34.50 ± 4.53	0.315
ALB	14.64 ± 1.62	15.82 ± 0.67	15.54 ± 1.18	15.65 ± 2.19	19.55 ± 2.47	16.30 ± 1.39	18.94 ± 0.85	0.339
GLB	12.78 ± 4.52	9.58 ± 1.70	8.88 ± 2.22	14.74 ± 2.58	8.33 ± 2.06	6.45 ± 2.29	18.64 ± 3.39	0.103
Lipid metabolism-related parameters, μmol/mL								
TC	12.60 ± 2.10	12.92 ± 1.45	16.20 ± 1.77	13.59 ± 1.03	15.00 ± 2.23	13.22 ± 2.16	12.52 ± 1.39	0.763
LDL	2.29 ± 0.35	2.29 ± 0.42	2.47 ± 0.46	1.95 ± 0.31	2.68 ± 0.56	2.39 ± 0.54	2.46 ± 0.37	0.928
TG	8.57 ± 1.99^{bc}	3.28 ± 0.80^{cd}	1.59 ± 0.27^{d}	1.08 ± 0.15^{d}	8.14 ± 2.68^{bc}	10.40 ± 2.93^{b}	20.08 ± 1.74^{a}	<0.001
Carbohydrate metabolism-related parameters, μmol/mL								
Glucose	23.78 ± 1.41^{a}	18.66 ± 0.90^{ab}	18.15 ± 1.22^{ab}	16.92 ± 0.64^{b}	22.11 ± 2.85^{ab}	18.18 ± 1.38^{ab}	24.23 ± 2.53^{a}	0.047
Egg-laying mineral, μmol/mL								
Ca	2.37 ± 0.13^{b}	2.19 ± 0.08^{bc}	2.07 ± 0.08^{bc}	1.91 ± 0.13^{c}	2.32 ± 0.05^{b}	2.21 ± 0.17^{bc}	2.70 ± 0.07^{a}	<0.001

Means with different superscript lowercase letters within the same row differ according to one-way ANOVA ($P < 0.05$).

Abbreviations: ALB, albumin; D1, the day as the first-egg laying; D2, the day after the first-egg laying day; D3, the day as the second-egg laying (SEL); D4, one day after the SEL; D7, four days after the SEL; D10, seven days after the SEL; D13, ten days after the SEL; GLB, globin; LDL, low-density lipoprotein; TAA, total amino acid; TC, total cholesterol; TG, total triglyceride; TP, total protein.

[1]Values are means ± SEM; n = 4-5.

About 90% of the dry matter of pigeon egg albumen was proteins, synthesized in a much faster process (<2 d) than the synthesis of pigeon yolk constituents (>6 d), indicating a deficiency in dietary nutrients would impact egg albumen much faster than the yolk (Xu, et al., 2016; Lu, et al., 2020). In this study, there was an evident dynamic variation in serum TAA and the most of serum AAs in both 6-mo and 12-mo laying pigeons during one ELC. Considering the spatial-temporal dynamic change of egg formation, the peak values of these AAs observed around of the first and second ovipositions might be attributed to the nutritional requirement for the fast formation of the egg albumen in oviduct of the laying pigeons.

Serum free AAs pool at any time is affected by the appearance rates of exogenous and endogenous AAs into blood and by their disappearance rates from the blood (Liao, et al., 2018). The endogenous AAs were referred to those coming from cellular proteolysis and de novo AA synthesis inside of the body, while the exogenous AAs were referred to those obtained from the dietary supply outside of the body. In laying hens, the increasing methionine supplementation induced a linear increase in plasma concentrations of Ala, Tyr, Val, Gly, and Ser (Wan, et al., 2017), and serum albumin level increased significantly in response to supplemental L-Val (Azzam, et al., 2015). The disappearance of AAs from blood pool was mainly influenced by the absorption from blood by cells for synthesis of new proteins or by utilization as fuel for body energy requirement (Liao, et al., 2018). Previous study reported AAs have the capability of supplying glucose needs during fasting period via gluconeogenesis, and plasma TAA trended to increase via protein catabolism if a long-term

Table 9. The mRNA expression of genes related to amino acid transporter and synthesis in liver, ovary and oviduct of the 6-mo laying pigeons treated with a 12-h fasting at different time points during one egg-laying cycle.[1]

	One egg-laying cycle							
Item	D1	D2	D3	D4	D7	D10	D13	*P*-value
Liver, Amino acid transport								
SNAT2	1.00 ± 0.18	1.22 ± 0.24	1.39 ± 0.35	1.42 ± 0.06	1.12 ± 0.27	1.22 ± 0.12	1.03 ± 0.34	0.721
LAT4	1.00 ± 0.23^{a}	0.80 ± 0.06^{ab}	0.69 ± 0.15^{ab}	0.75 ± 0.10^{ab}	0.57 ± 0.12^{bc}	0.55 ± 0.08^{bc}	0.24 ± 0.06^{c}	0.019
B^{0}AT1	1.00 ± 0.14^{bc}	0.43 ± 0.08^{c}	1.05 ± 0.15^{bc}	1.26 ± 0.25^{bc}	1.68 ± 0.38^{b}	2.81 ± 0.71^{a}	3.05 ± 0.18^{a}	<0.001
EAAT3	1.00 ± 0.10^{a}	0.53 ± 0.16^{b}	0.37 ± 0.09^{b}	0.54 ± 0.10^{b}	0.32 ± 0.11^{b}	0.40 ± 0.05^{b}	0.20 ± 0.04^{b}	0.004
CAT1	1.00 ± 0.20	0.65 ± 0.18	0.54 ± 0.12	0.36 ± 0.11	0.90 ± 0.28	0.46 ± 0.08	0.39 ± 0.10	0.094
y$^+$LAT2	1.00 ± 0.15^{b}	7.42 ± 0.69^{a}	1.29 ± 1.00^{b}	1.33 ± 0.65^{b}	2.37 ± 0.28^{b}	6.86 ± 0.34^{a}	6.09 ± 0.29^{a}	<0.001
Amino acid synthesis								
ASL	1.00 ± 0.22	1.86 ± 0.56	1.49 ± 0.09	1.06 ± 0.25	0.93 ± 0.11	0.89 ± 0.25	1.19 ± 0.33	0.373
GDH1	1.00 ± 0.14^{e}	2.53 ± 0.61^{e}	7.40 ± 0.35^{cd}	4.12 ± 0.75^{de}	9.99 ± 2.57^{c}	17.55 ± 0.74^{b}	28.30 ± 1.45^{a}	<0.001
SHMT2	1.00 ± 0.18^{a}	0.96 ± 0.29^{a}	0.67 ± 0.18^{ab}	0.51 ± 0.09^{b}	0.27 ± 0.04^{b}	0.36 ± 0.07^{b}	0.39 ± 0.07^{b}	0.006
PDH	1.00 ± 0.26	1.16 ± 0.17	0.94 ± 0.21	0.98 ± 0.10	0.58 ± 0.13	0.64 ± 0.13	0.54 ± 0.09	0.066
Ovary, Amino acid transport								
SNAT2	1.00 ± 0.34	1.81 ± 0.13	1.25 ± 0.19	1.05 ± 0.29	1.35 ± 0.30	0.76 ± 0.18	1.11 ± 0.35	0.383
LAT4	1.00 ± 0.22^{a}	0.53 ± 0.11^{bc}	0.83 ± 0.08^{ab}	0.45 ± 0.14^{bc}	0.50 ± 0.09^{bc}	0.51 ± 0.09^{bc}	0.29 ± 0.03^{c}	0.018
B^{0}AT1	1.00 ± 0.33^{b}	3.54 ± 1.27^{b}	12.56 ± 2.83^{a}	3.08 ± 0.45^{b}	2.61 ± 1.06^{b}	1.91 ± 0.26^{b}	1.89 ± 0.37^{b}	<0.001
EAAT3	1.00 ± 0.24^{ab}	1.64 ± 0.12^{a}	0.55 ± 0.38^{b}	0.83 ± 0.15^{b}	0.72 ± 0.30^{b}	0.33 ± 0.15^{b}	0.81 ± 0.20^{b}	0.042
CAT1	1.00 ± 0.36^{ab}	0.64 ± 0.10^{abc}	1.19 ± 0.30^{a}	0.61 ± 0.16^{abc}	0.33 ± 0.10^{c}	0.42 ± 0.19^{bc}	0.29 ± 0.05^{c}	0.021
y$^+$LAT2	1.00 ± 0.35^{b}	1.44 ± 0.11^{b}	2.21 ± 0.13^{a}	1.04 ± 0.34^{b}	0.69 ± 0.17^{b}	0.89 ± 0.24^{b}	0.66 ± 0.07^{b}	0.005
Amino acid synthesis								
ASL	1.00 ± 0.47^{b}	1.79 ± 0.38^{b}	1.14 ± 0.13^{b}	1.35 ± 0.36^{b}	1.73 ± 0.63^{b}	5.63 ± 1.78^{a}	5.02 ± 1.67^{a}	0.012
GDH1	1.00 ± 0.18^{b}	1.30 ± 0.24^{b}	1.06 ± 0.32^{b}	3.02 ± 1.02^{a}	0.07 ± 0.03^{b}	0.14 ± 0.08^{b}	0.17 ± 0.03^{b}	0.002
SHMT2	1.00 ± 0.25^{b}	1.72 ± 0.21^{b}	3.78 ± 0.77^{a}	1.45 ± 0.28^{b}	1.61 ± 0.36^{b}	2.14 ± 0.64^{b}	1.02 ± 0.30^{b}	0.007
PDH	1.00 ± 0.64^{b}	1.03 ± 0.10^{b}	2.17 ± 0.26^{a}	1.13 ± 0.10^{b}	0.72 ± 0.07^{b}	0.45 ± 0.25^{b}	0.63 ± 0.12^{b}	0.005
Oviduct, Amino acid transport								
SNAT2	1.00 ± 0.08^{ab}	1.35 ± 0.47^{a}	0.72 ± 0.20^{b}	0.52 ± 0.13^{b}	0.66 ± 0.10^{b}	0.41 ± 0.15^{b}	0.62 ± 0.04^{b}	0.034
LAT4	1.00 ± 0.10^{bc}	0.58 ± 0.15^{cde}	1.19 ± 0.11^{ab}	1.51 ± 0.20^{a}	0.68 ± 0.09^{cd}	0.17 ± 0.02^{e}	0.31 ± 0.11^{de}	<0.001
B^{0}AT1	1.00 ± 0.26	1.10 ± 0.23	0.87 ± 0.39	0.20 ± 0.07	0.69 ± 0.18	0.84 ± 0.34	0.55 ± 0.15	0.111
EAAT3	1.00 ± 0.23	1.34 ± 0.36	0.79 ± 0.23	0.91 ± 0.13	0.78 ± 0.20	0.63 ± 0.22	1.28 ± 0.07	0.231
CAT1	1.00 ± 0.32	2.21 ± 0.66	1.00 ± 0.35	0.85 ± 0.13	1.68 ± 0.38	2.07 ± 0.44	1.75 ± 0.36	0.102
y$^+$LAT2	1.00 ± 0.08	1.14 ± 0.12	0.84 ± 0.24	0.46 ± 0.07	1.06 ± 0.26	1.04 ± 0.30	1.13 ± 0.27	0.219
Amino acid synthesis								
ASL	1.00 ± 0.25	0.92 ± 0.38	0.79 ± 0.11	0.44 ± 0.04	0.84 ± 0.28	0.97 ± 0.13	0.81 ± 0.34	0.679
GDH1	—	—	—	—	—	—	—	—
SHMT2	1.00 ± 0.22^{abc}	1.85 ± 0.52^{a}	0.57 ± 0.17^{bc}	0.45 ± 0.09^{c}	0.94 ± 0.23^{bc}	1.43 ± 0.26^{ab}	0.92 ± 0.41^{bc}	0.028
PDH	1.00 ± 0.29^{bc}	2.64 ± 0.79^{a}	0.51 ± 0.17^{bc}	0.30 ± 0.03^{c}	1.11 ± 0.28^{bc}	1.59 ± 0.36^{b}	1.36 ± 0.16^{bc}	0.003

Means with different superscript lowercase letters within the same row differ according to one-way ANOVA ($P < 0.05$).

Abbreviations: ASL, argininosuccinate lyase; B^{0}AT1, Na$^+$-dependent neutral amino acid transporter; CAT1, Na$^+$-dependent cationic amino acid transporter; D1, the day as the first-egg laying; D2, the day after the first-egg laying day; D3, the day as the second-egg laying (SEL); D4, one day after the SEL; D7, four days after the SEL; D10, seven days after the SEL; D13, ten days after the SEL; EAAT3, excitatory amino acid transporter 3;*GDH1*, glutamate dehydrogenase 1; *LAT4*, L-type amino acid transporter 4; *y$^+$LAT2*, Na$^+$-dependent cationic and Na$^+$-dependent neutral amino acid transporter 2; *PDH*, phosphoglycerate dehydrogenase; *SHMT2*, serine hydroxymethyltransferase 2; *SNAT2*, Na$^+$-coupled neutral amino acid transporter 2.

[1]Values are means ± SEM; n = 4-5. The values are normalized to D1.

deprivation occurred (Costas, et al., 2011). Under some conditions, the endogenous AAs contributed to about two-thirds of the total AAs used for the whole body (Wu, et al., 2013), suggesting optimal dietary AAs was necessary to avoid utilization of the endogenous AAs to compensate the normal serum AAs pool in animals for production. In the present study, compared with pigeons without fasting, the sharply decreased serum AAs were mostly attributed to the 12-h fasting, implying the feed-derived AAs played a major role in the serum AA pool. Impressively, being similar with the pigeons without fasting, serum TAA still had a much higher values around the egg-laying time in pigeons treated with the 12-h fasting, suggesting the up-regulated serum AAs should be obtained from endogenous protein degradation and that was necessary for pigeon to form eggs.

Pigeon gizzard is highly muscular that acts the grinding machine to crush the food and responds rapidly to dietary changes. Relative gizzard weight increased in a linear manner as the amount of coarse corn was upregulated in diet, indicating the increased corn particles stimulated gizzard function and increased gizzard weight (Singh, et al., 2014). In this study, pigeons showed an evident change in feed intake and body weight as free access to feed and water (Figure 1), whereas there was no significant difference in body weight and gizzard weight after the 12-h fasting. Thus, the reduced relative gizzard weight at D1 might be related with the changed feed amount in gizzard, but further study should be carried to elucidate the reason why the sharp variation of relative gizzard weight occurred around the FEL time. Pigeon pancreas can secret pancreatic juice into duodenum to help digest fats, carbohydrates and proteins as soon as feed reaches the stomach. With the increased fasting-treated time, there were progressive reduction in total protein, water content and organ weight of pancreas in pigeons with free access to water, whereas these reductions could be recovered following the refeeding (Webster, et al., 1972). In the present study, relative pancreas weight obviously decreased at D1 and then recovered gradually with the increasing feed intake. This might be because the

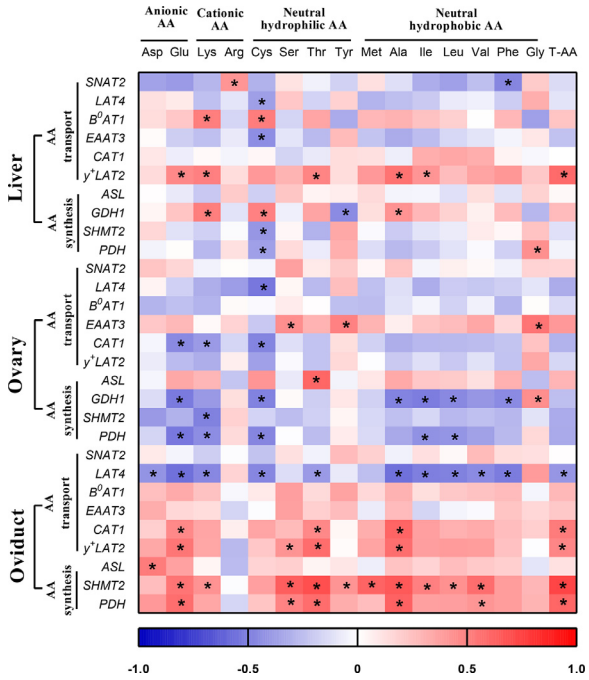

Figure 2. Heat map of Pearson correlation coefficients between serum amino acids and the mRNA expression of the related genes in liver, ovary and oviduct. Gradient color barcodes at the bottom indicates the minimum value in blue and the maximum in red. The asterisk means a significant correlative ($P < 0.05$).

changed feed intake impacted pancreatic protein synthesis, resulting in the variations in pancreas weight of pigeon. The relative organ weights of ovary and oviduct observed with the largest values at D13 were mainly attributed to the increasing follicular size with bigger yolk in ovary for yolk formation and the increased protein contents in magnum for egg albumen formation.

Similar with serum AAs, serum TG consisted of exogenous sources obtained from dietary supply and endogenous sources mainly coming from the TG-rich lipoproteins in form of very low density lipoprotein (**VLDL**). Due to the little or no solubility in blood, the synthesized TG in liver are mainly packed together with hydrophilic proteins to form VLDL, which are in charge of carrying endogenous TG via blood circulation to other tissue or organs (Yao and Wang, 2012). All egg TGs are contained in the egg yolk, produced in liver, and then transported to the ovary in the form of VLDL (Nys and Guyot, 2011). The weight of total lipids in fresh pigeon yolk was about 29.3% and TG was the major lipid components of the yolk, averaging 58% of the total lipids (Vanheel, et al., 1981). During the yolk formation, the rate of the VLDL released from liver into blood was always faster than its absorption by ovarian follicles, causing a 2 to 10-fold increase in circulating TG (Zaefarian, et al., 2019). In the present study, serum TG was kept in a high level during the period from D7 to D13 in pigeons treated with the 12-h fasting, indicating the liver might increase the endogenous TG synthesis and its release into blood during the stage of rapid yolk deposition in ovarian follicular in the final 7 days. Calcium was directly provided by ionic blood Ca for shell formation and no Ca was stored in the shell gland of oviduct (Nys and Guyot, 2011). For eggshell formation, about two-thirds of Ca is directly supplied by the hen's diet and one-third (30−40%) by mobilization of bone Ca (Bouvarel, et al., 2011). This is because eggshell formation need about 20 hours and mainly take place during night, resulting in the Ca deficiency without diet supply and therefore Ca mobilization from bone. The decreased serum Ca observed at D4 after the second egg oviposition might be resulted from the Ca consumption of bone reabsorption in pigeon treated with the 12-h fasting, suggesting supplementation of dietary Ca was still necessary for pigeon bone recovery immediately after laying egg.

Amino acids transporters are responsible for shuttling AAs in and out of cells or cellular organelles and these AAs transporters are grouped into neutral, cationic, anionic and others (Kandasamy, et al., 2018). Based on the different side chains, 20 types of AAs are divided into 2 anionic AAs (Asp and Glu), 3 cationic AAs (Arg, Lys and His) and the other 15 neutral AAs. In this study, 2 cationic AAs (Arg and Lys) were transported into cells from outside by *CAT1*, whereas 2 anionic AAs by *EAAT3*, respectively. The other 11 neutral AAs were moved into cells from extracellular fluid by the 3 selected neutral AA transporters as follows: *SNAT2*, *LAT4*, and B^0AT1 (Bodoy, et al. 2005; Oparija, et al., 2019). Moreover, B^0AT1 can mediate transport of all neutral AAs, but *LAT4* specially transported part of neutral AAs, such as the large neutral AAs (Phe) and the neutral hydrophobic AAs (Ala, Ile, Leu and Val). However, y^+LAT2 worked as an antiport by coupling the efflux of cationic AAs to the influx of neutral AAs (Bröer, et al., 2004). In pigeons, gene expression of various AA transporters had been analyzed in parent crop tissue (Xie, et al., 2020a) and in small intestine of chick or parent during chick-rearing period (Gao, et al., 2016; Zhang, et al., 2017; Xie, et al., 2020b). In the present study, the B^0AT1 expression increased and the expression of *LAT4*, *EAAT3*, *CAT1* and y^+LAT2 decreased in the ovary at D10 and D13, suggesting the neutral AAs transport was mainly carried out by B^0AT1 and the transport of anionic and cationic AAs exhibited a less active status during the period of yolk formation. In the liver, the expression of B^0AT1 and y^+LAT2 increased and the *LAT4* expression decreased at D10 and D13, suggesting the hepatic cells might mainly transport AAs by B^0AT1 to synthesize proteins for yolk formation. In addition, 6 of NPRS were observed in the y^+LAT2 band, indicating the neutral AAs transport was enhanced by coupling the efflux of cationic AAs. In the oviduct, the *SNAT2* expression increased and the *LAT4* expression decreased at D2, indicating *SNAT2* played an important role in the neutral AAs transport for egg albumen formation.

Biosynthesis of AAs occurred to support protein production if the supply from dietary AAs did not meet tissue AA utilization. For example, Cys, a semiessential AA, can be synthesized endogenously from Met via the transulfuration pathway mainly in liver and the addition of dietary cysteine reduced the requirement for Met

(Baker, 2009; Silva, et al., 2020). In this study, the increased serum Cys observed across the 7 DELCs during fasting period might come from the body methionine. Glutamate dehydrogenase 1 is a key enzyme for glutamine production (Spanaki, et al., 2015) and *ASL* is responsible for de novo synthesis of Arg (Nagamani, et al., 2012). Serine hydroxymethyltransferase 2 is the enzyme in serine catabolism that converts serine to glycine (Hebbring, et al., 2012), whereas *PDH* is the first and rate-limiting enzyme in the de novo serine biosynthetic pathway (Mullarky, et al., 2019). During crop milk production in pigeon, these genes encoding *GDH1*, *SHMT2*, *PDH* and *ASL* expressed and showed dynamic variations in the mRNA expression during the 25-d chick-rearing period (Xie, et al., 2020a). In this study, there were sharp increases in the *GDH1* expression and decreases in the *SHMT2* expression at D10 and D13 in the liver, indicating hepatic cells might increase the utilization of Glu and Ser for the raw material synthesis of yolk. However, the *ASL* expression obviously increased and the other three genes stayed in a lower level at D10 and D13 in the ovary, suggesting there might be a limited capacity of AA synthesis in ovary cells and Arg participated the protein synthesis of pigeon yolk. In the oviduct, the expression of *SHMT2* and *PDH* significantly increased at D2, indicating Ser might provide important precursors for protein synthesis for egg albumen formation. Considering the increased Glu content and the undetected *GDH1* expression at D2, the oviduct might only utilize blood Glu for protein synthesis. Meanwhile, dynamic variations of serum AAs during one ELC were positively corelated with the mRNA expression of the AA synthesis genes (*SHMT2, PDH* and *ASL*) and the AA transporter genes in oviduct magnum of laying pigeons in this study, suggesting the AA utilized for egg albumen formation probably not only coming from blood AA, but also obtained from biosynthesis of AAs in oviduct magnum.

CONCLUSION

Although pigeons were treated with the 12-h fasting, the dynamic variations in serum AAs were still detected during one ELC, characterized by high levels of serum TAA and most of the detected AAs around the egg-laying period, suggesting endogenous AAs were mobilized to support egg white formation. Heat map showed the percentage of positive r values with significant difference in oviduct magnum were much higher than those in liver or ovary, and dynamic variations of serum AAs during one ELC were positively corelated with the mRNA expression of the AA transport genes and AA synthesis genes in oviduct magnum of pigeon, implying the up-regulated serum AAs might be necessary to meet the AAs requirement for egg white formation and an additional AAs in diet should be considered for pigeon egg production around the egg-laying period.

Oparija et al., 2019

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

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