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L-Leucine *In Ovo* Administration Causes Growth Retardation and Modifies Specific Amino Acid Metabolism in Broiler Embryos

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L-Leucine (L-Leu) *in ovo* administration was demonstrated to afford thermotolerance and modified amino acids metabolism in post-hatched broiler chicks under heat stress. This study aimed to investigate the changes in embryonic growth and amino acid metabolism after *in ovo* injection of L-Leu. Fertilized broiler eggs were subjected to *in ovo* injection of sterile water or L-Leu on embryonic day (ED) 7. The weight of embryos and yolk sacs were measured on ED 12, 14, 16, and 18. Plasma and livers were collected on ED 14 and 18 for free amino acid analysis. The weight and relative weight of embryos were significantly lowered by *in ovo* administration of L-Leu, but those of yolk sacs were not altered. Moreover, L-Leu *in ovo* injection significantly reduced the plasma proline concentration during embryogenesis and increased the plasma concentrations of tyrosine (Tyr) and lysine (Lys) in ED 18. Hepatic Lys concentration was also significantly increased by L-Leu *in ovo* injection. Interestingly, Leu concentrations in the plasma and liver were not affected by L-Leu administration. These results indicated that *in ovo* administered L-Leu was metabolized before ED 14 and affected embryonic growth and amino acid metabolism during embryogenesis.

Key words: amino acid, chick, embryo, growth, L-leucine

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Introduction

Heat stress is a severe problem that restricts the efficient and healthy production of broiler chickens for a long time (Zaboli *et al*., 2019). In order to reduce the negative impacts of heat stress on poultry production, many studies have been conducted to propose suitable and efficient strategies using broiler chicks and chickens (Lin *et al*., 2006; Renaudeau *et al*., 2012; Chowdhury, 2019). The utilization of feed additives and controlling the environment are the main strategies currently used for overcoming heat stress in the poultry industry (Lin *et al*., 2006). These are passive methods used to alleviate heat-related damage or to promote a lower room temperature; however, they have a low efficiency and a high cost associated (Wasti *et al*., 2020).

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It has been demonstrated that thermal manipulation (TM), a technique that involves increasing the incubation temperature before hatching, affords thermotolerance in post-hatched broiler chicks and chickens (Piestun *et al*., 2008; Loyau *et al*., 2014). In our previous studies, we found that TM altered amino acid concentrations, including that of leucine (Leu), in the brain and liver of embryos (Han *et al*., 2017). Then, L-Leu was administered to the embryo, and it was found to improve thermotolerance and growth performance under heat stress in broiler chicks and chickens, respectively (Han *et al*., 2019a; 2020).

Amino acid metabolism was affected even after a short exposure to heat stress (Ito *et al*., 2014). Amino acids also play critical roles in the regulation of body temperature. For instance, L-citruline (Chowdhury *et al*., 2017) and D-aspartic acid (D-Asp) (Erwan *et al*., 2014) have hypothermic functions that can afford thermotolerance in neonatal chicks. Recent studies showed that L-Leu *in ovo* injection influenced amino acid metabolism under acute or chronic heat stress in broilers without changes in Leu concentration in chicks or chickens (Han *et al*., 2019b, 2020). These results suggest that L-Leu may be a trigger, rather than a long-term regulator of L-Leu-mediated thermotolerance in broiler chicks. Thus, it is necessary to clarify the change in amino acid concen-

trations after L-Leu *in ovo* injection during embryogenesis. Therefore, the first aim of the current study was to investigate the effects of L-Leu *in ovo* injection on amino acid metabolism in broiler embryos.

Amino acids not only serve as building blocks of proteins, but also play important roles in growth (Wu, 2009). It has been demonstrated that *in ovo* administration of amino acids has the potential to improve post-hatch growth (Ohta *et al*., 1999) and the feed conversion ratio (Kadam *et al*., 2008) in neonatal chicks. L-Leu and its metabolites have been reported to suppress protein degradation and promote muscle growth (Duan *et al*., 2016) by activating the mechanistic target of rapamycin (mTOR) signaling pathway in skeletal muscle and adipose tissues (Shimomura *et al*., 2015). *In ovo* administration of L-Leu, but not L-isoleucine (L-Ile) and L-valine, on embryonic day (ED) 7 improved embryo weight on ED 14 without affecting the body weight on the hatching day (Kita *et al*., 2015). In our previous reports, L-Leu *in ovo* administration on ED 7 had no effect on the body weight at hatching (Han *et al*., 2017). However, the effects of L-Leu *in ovo* feeding on embryonic growth performance have not yet been fully elucidated. Thus, the second aim of this study was to investigate the changes in embryonic weight after *in ovo* administration of L-Leu in broiler embryos.

Materials and Methods

Experiment Design

One hundred and twenty fertilized broiler eggs (Ross 308 strain; 80-weeks old) were purchased from a local hatchery in Nanjing, China. Eggs were divided into two groups (control and L-Leu; $n=60$/ group) based on the egg weight, to form groups as uniform as possible. The average egg weights of the control and L-Leu groups were 66.9 g. Eggs were placed into an incubator (Hongde 2112 type incubator, Hongde Comp., Shandong, China) at 37.6℃ with 60−70% relative humidity and auto-turning every 1.5 h. On ED 7, thirteen unfertilized eggs (4 eggs from the control group and 9 eggs from the L-Leu group) were detected via candling and discarded properly. The remaining eggs were subjected to *in ovo* injection of sterile water (0.5 mL/egg) for the control, and L-Leu solution (34.5 μmol/0.5 mL sterile water/egg) for the L-Leu group, as described elsewhere (Han *et al*., 2017, 2018). Briefly, a small hole was made at the blunt end of the egg after disinfecting the eggshell. The sterile water or L-Leu solution was injected into the yolk sac using a 1-mL disposable syringe with a 25-gauge needle. After injection, the small holes were immediately sealed with scotch tape, which was sterilized with 70% ethanol, and the eggs were returned to the incubator.

On ED 12, 14, 16, and 18, developing embryos ($n=10$−12) were randomly selected from each group to measure the weight of the egg, yolk-free embryo, and yolk sac. The sampling of the embryo and yolk sac was performed by the same person without knowing the grouping information. The relative embryo weight (%) was calculated as the ratio between yolk-free embryo weight and the initial egg weight. The relative yolk weight (%) was calculated as the ratio between the yolk sac weight and the initial egg weight. On ED 14 and 18, blood was collected from the umbilical vein using a heparinized 1-mL syringe with a 27-gauge needle, as described in our previous study (Han *et al*., 2018). The blood was placed into heparinized tubes immediately after sampling and centrifuged at 10,000×*g* and 4℃ for 4 min to collect the plasma. After blood collection and measurement, the liver was collected, snap frozen using liquid nitrogen, and then stored in plasma at −80℃ until further analysis.

This study was performed according to the Guidelines for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (permit number SYXK (Su) 2011-0036).

Analysis of Free Amino Acids in the Plasma and Liver

The free amino acid concentrations in the liver and plasma were analyzed using a fully automatic amino acid analyzer (L-8080 type, Hitachi, Japan), according to the method described by Zhang *et al*. (2017). The liver samples were weighed and homogenized in a 5% sulfonic acid solution and left for deproteinization on ice. The plasma was only well mixed with a 5% sulfonic acid solution for deproteinization. After 30 min, the liver or plasma samples were centrifuged at 4℃ and 20,000×*g* for 20 min. The supernatant was collected and filtered using a 0.22-μm filter (Biosharp, Guangzhou saiguo biotech Co., LTD, Guangzhou, China). The filtrate and standard solution were incorporated into the amino acid analyzer. The amino acid concentrations were expressed as pmol/mg of wet tissue in the liver and as pmol/μL in the plasma. Since the system applied here could not separate the L- and D-forms of amino acids, the results of the determined amino acids only used the names of the amino acids.

Statistical Analysis

The data were statistically analyzed using a two-way analysis of variance (ANOVA) to determine the main effects of *in ovo* administration and embryo development age, as well as of their interaction. When a significant interaction was detected, the *post hoc* analysis of Holm–Sidak's multiple comparisons test was applied to examine the interaction effect of *in ovo* L-Leu feeding and age on the dependent variables. Statistical analysis was conducted using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). A $P<0.05$ was used to denote significant differences. All data in each group were first subjected to a Thompson's rejection test, as described by Kobayashi and Pillai (2013), to eliminate the outliers ($P<0.01$), and the remaining data were subjected to analysis among groups. All results are expressed as mean±standard error of mean (SEM). The number of embryos used for statistical analysis in each group is shown in the figure legends and table notes.

Results

Changes of Embryonic Weight and Yolk Weight During Incubation

Egg, embryo, and yolk weight changes, as well as the relative weights of the embryo and yolk are shown in Figs. 1 and 2. During embryonic development, egg weight did not

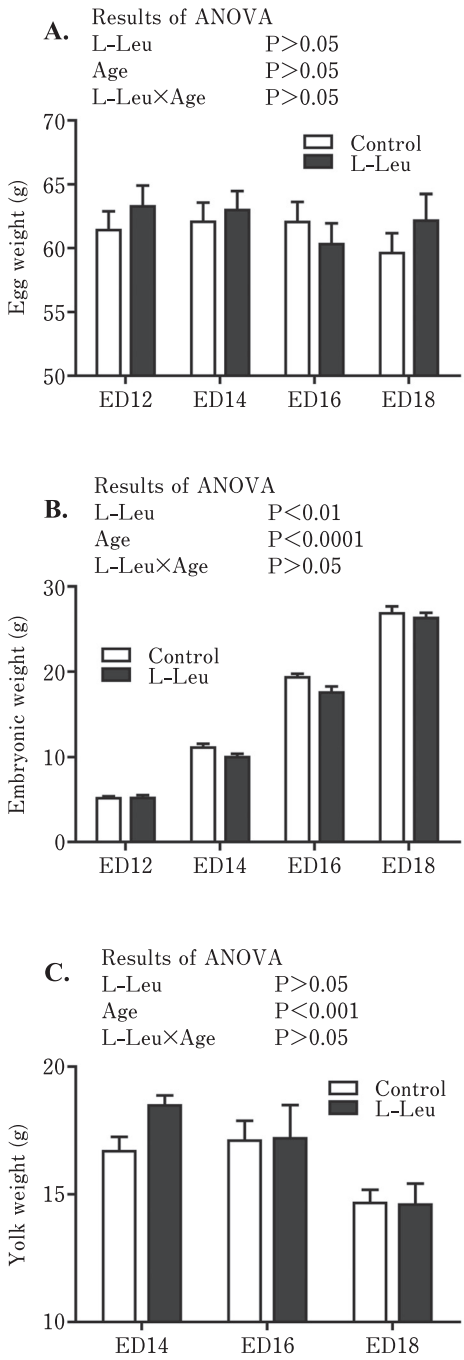

Fig. 1. **Effects of L-Leu *in ovo* injection on weight changes in egg (A), embryo (B), and yolk sac (C) during incubation.** The number of chick embryos in each group was n =9 to 12. Values are represented as mean±SEM. L-Leu, L-leucine; ED, embryonic day.

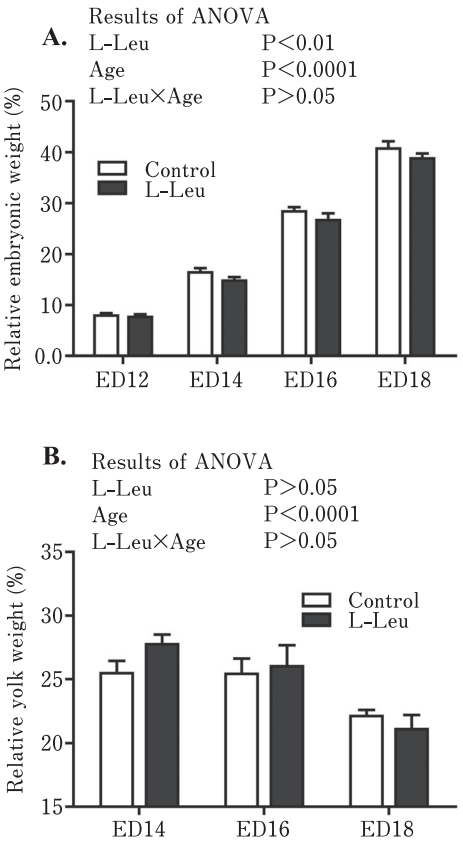

Fig. 2. **Effects of L-Leu *in ovo* injection on the relative weight changes of embryo (A) and yolk sac (B) during incubation.** The number of chick embryos in each group was n=9 to 12. Values are represented as mean±SEM. L-Leu, L-leucine; ED, embryonic day.

change. The embryonic weight was significantly increased by the age of the embryo, with a reduction in the yolk weight. However, *in ovo* administration of L-Leu significantly reduced embryonic weight but had no effect on yolk weight (Fig. 1). The results of relative embryo and yolk weights showed a similar tendency as those of their weight, and L-Leu *in ovo* injection also significantly lowered the relative embryonic weight but showed no effect on the relative yolk weight (Fig. 2).

Free Amino Acid Concentrations in the Plasma and Liver

The changes in free amino acid concentrations in the plasma and liver of broiler embryos are shown in Figs. 3 and 4 and Tables 1 and 2. Plasma tyrosine (Tyr) significantly (P <0.0001) decreased toward ED 18, but a significant interaction between L-Leu and age indicated that the decline did not occur so sharply in L-Leu in the *in ovo*-administered group compared to the control group (Fig. 3A). *In ovo* L-Leu administration caused a significant (P<0.01) increase in plasma lysine (Lys) concentration (Fig. 3B). The significant interaction between L-Leu and age that was observed in plasma Lys suggested that plasma Lys decreased with age in the control group; however, it increased with age in the L-Leu *in ovo* administration group (Fig. 3B). Embryonic development caused a significant (P<0.0001) increase in plasma proline (Pro) concentration during incubation (Fig. 3C). However, this increase was significantly (P<0.05) suppressed by L-Leu *in ovo* administration. In the liver, the

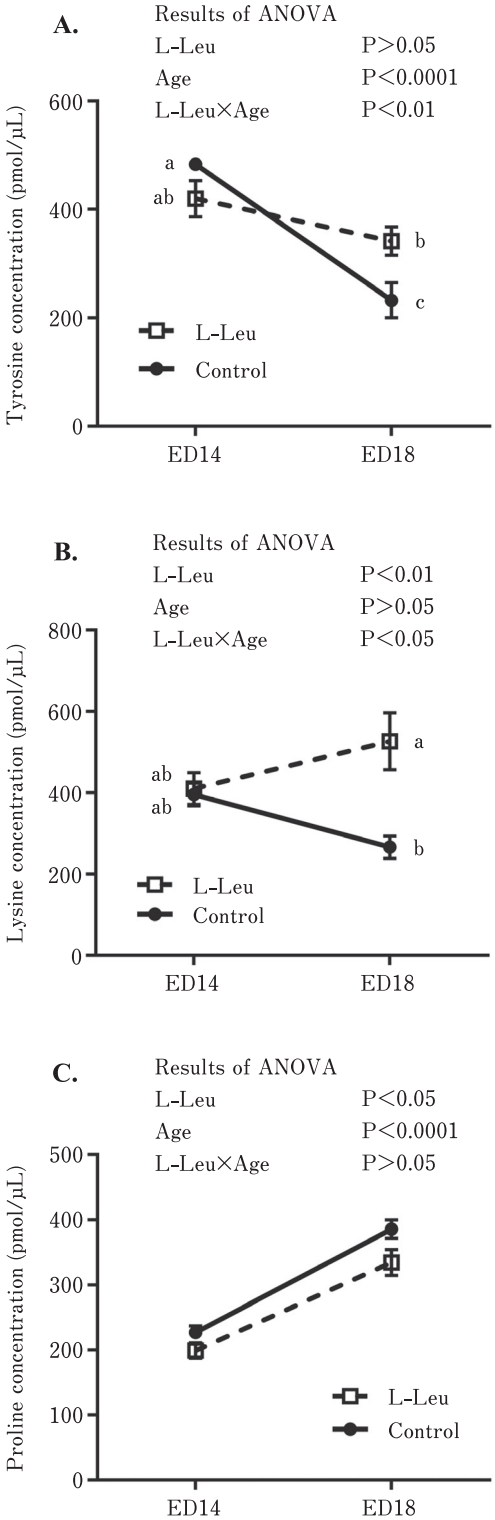

Fig. 3. **Effects of L-Leu *in ovo* injection on tyrosine (A), lysine (B), and proline (C) concentration changes in the plasma of chick embryos.** The number of chick embryos in each group was $n=6$. Values are represented as mean±SEM. Different letters beside the symbols indicate significant ($P<0.05$) differences. L-Leu, L-leucine; ED, embryonic day.

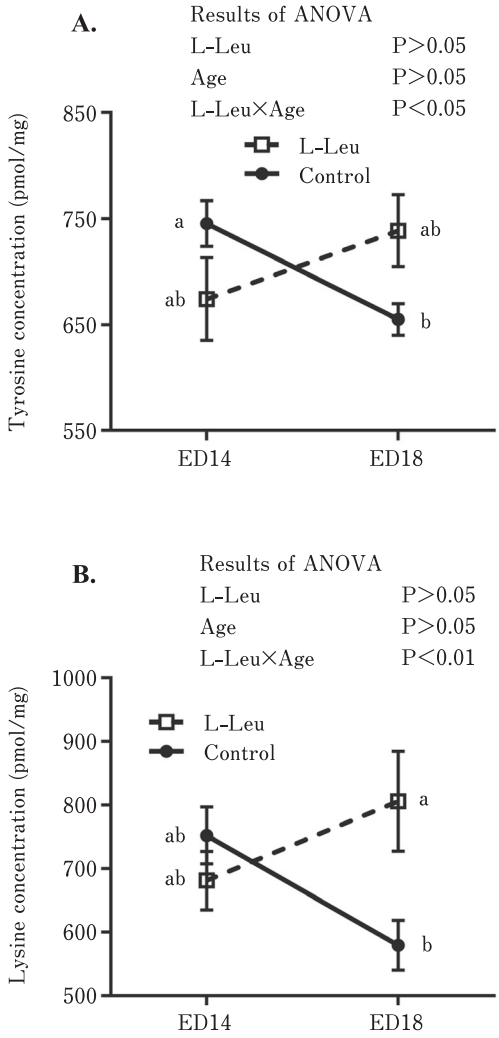

Fig. 4. **Effects of L-Leu *in ovo* injection on tyrosine (A) and lysine (B) concentration changes in the liver of chick embryos.** The number of chick embryos in each group was $n=6$. Values are represented as mean±SEM. Different letters beside the symbols indicate significant ($P<0.05$) differences. L-Leu, L-leucine; ED, embryonic day.

significant interactions between L-Leu and age observed in Tyr ($P<0.05$) and Lys ($P<0.01$) concentrations, indicated that both amino acids decreased with the progression of age in the control group, but increased with age in the L-Leu *in ovo* administration group (Fig. 4). As shown in Tables 1 and 2, the concentrations of many amino acids in the plasma and liver were significantly increased by the embryonic age. However, only glycine (Gly) and Asp in the liver decreased with age. Notably, no effects of L-Leu *in ovo* administration were detected in these amino acids.

Discussion

In the current study, we administered L-Leu *in ovo* and examined the growth of embryos at different stages during

Table 1. **Plasma free amino acid concentration in chick embryos**

	Control		L-Leu		*P* value		
Amino acids	ED 14	ED 18	ED 14	ED 18	L-Leu	Age	L-Leu×Age
Histidine	90±8	133±21	97±8	137±14	NS	$P<0.01$	NS
Threonine	483±90	682±34	345±27	746±150	NS	$P<0.01$	NS
Valine	449±16	611±37	450±39	528±27	NS	$P<0.001$	NS
Isoleucine	219±10	218±24	235±16	237±5	NS	NS	NS
Leucine	175±9	199±24	199±12	216±14	NS	NS	NS
Phenylalanine	101±5	156±21	105±6	176±12	NS	$P<0.001$	NS
Methionine	55±4	107±12	56±5	102±9	NS	$P<0.001$	NS
Lysine	396±28^{ab}	266±27^{b}	410±39^{ab}	527±70^{a}	$P<0.01$	NS	$P<0.05$
Tyrosine	483±9^{a}	232±33^{c}	420±33^{ab}	341±26^{b}	NS	$P<0.0001$	$P<0.01$
Proline	227±10	386±14	199±12	335±20	$P<0.05$	$P<0.0001$	NS
Arginine	265±15	308±39	268±6	369±20	NS	$P<0.01$	NS
Glycine	186±18	180±22	165±16	187±6	NS	NS	NS
Aspartic acid	13±2	16±3	13±4	15±2	NS	NS	NS
Alanine	273±17	318±37	245±18	328±7	NS	$P<0.05$	NS
Glutamic acid	37±3	118±22	41±2	125±11	NS	$P<0.001$	NS
Cysteine	43±4	102±12	38±3	94±9	NS	$P<0.001$	NS
Serine	345±34	637±28	343±32	593±33	NS	$P<0.001$	NS

Different superscripts in the same row indicate significant differences ($P<0.05$). The number of embryos used in each group was $n=6$. Values are represented as mean±SEM in pmol/μL. Control, sterile water injection; L-Leu, L-Leucine injection; ED, embryonic day; NS, not significant.

Table 2. **Hepatic free amino acid concentration in chick embryos**

	Control		L-Leu		*P* value		
Amino acids	ED 14	ED 18	ED 14	ED 18	L-Leu	Age	L-Leu×Age
Histidine	350±27	498±13	372±23	487±16	NS	$P<0.0001$	NS
Threonine	2749±93	3149±417	2598±148	3514±56	NS	$P<0.05$	NS
Valine	563±26	772±39	540±28	726±22	NS	$P<0.0001$	NS
Isoleucine	218±12	244±13	213±5	258±11	NS	$P<0.01$	NS
Leucine	559±34	681±22	539±28	719±30	NS	$P<0.0001$	NS
Phenylalanine	320±15	381±13	309±10	385±14	NS	$P<0.0001$	NS
Methionine	102±13	139±8	98±12	160±10	NS	$P<0.001$	NS
Lysine	752±45^{ab}	579±39^{b}	681±46^{ab}	806±79^{a}	NS	NS	$P<0.01$
Tyrosine	746±22^{a}	655±15^{b}	674±39^{ab}	739±34^{ab}	NS	NS	$P<0.05$
Proline	234±25	367±14	205±1	305±5	NS	$P<0.001$	NS
Arginine	380±21	506±28	367±26	547±34	NS	$P<0.0001$	NS
Glycine	1228±55	951±10	1185±51	1000±29	NS	$P<0.0001$	NS
Aspartic acid	2097±39	1310±91	1899±60	1476±114	NS	$P<0.0001$	$P<0.05$
Alanine	1094±25	1072±42	960±40	1175±74	NS	NS	$P<0.05$
Glutamic acid	3071±133	2841±240	2960±106	3153±138	NS	NS	NS
Serine	1510±58	1637±96	1479±34	1650±73	NS	$P<0.05$	NS

Different superscripts in the same row indicate significant differences ($P<0.05$). The number of embryos used in each group was $n=6$. Values are represented as mean±SEM in pmol/mg wet tissue. Control, sterile water injection; L-Leu, L-Leucine injection; ED, embryonic day; NS, not significant.

incubation. The weight and relative weight of embryos were suppressed by L-Leu *in ovo* administration, especially during ED 14–18. Our previous results showed that the relative embryonic weight was significantly reduced by L-Leu *in ovo* administration on ED 18 and ED 19 (unpublished data). However, the body weight of neonatal chicks at hatching was not affected by L-Leu administration (Han *et al*., 2017), even with an increased dose of L-Leu (Han *et al*., 2019a). L-Leu *in ovo* administration stimulated oxygen consumption and heat production of embryos at ED 14. It was hypothesized that the *in ovo* administration of L-Leu might have stimulated the metabolic rate during the middle stages (Han *et al*., 2018) with a decline in the embryonic growth and metabolic rate at later stages of incubation. In the current study, a clearly lower level of embryonic weight was found at ED 14, 16, and 18, which suggested that the stimulation of L-Leu on

metabolic activity might also have occurred on ED 12. Future studies will clarify the suppressed embryo growth by measuring the metabolic activity and growth-related hormones at different days after *in ovo* administration of L-Leu.

L-Leu is a branched chain amino acid (BCAA), and it has been demonstrated to improve muscle protein metabolism through the mTOR signaling pathway (Shimomura *et al*., 2015). Kita *et al*. (2015) reported that Leu *in ovo* injection caused an increase in the weight of whole embryos at ED 14 in layer chicks (Single Comb White Leghorn strain), in contrast with our current results in broiler chicks (Ross 308 strain). Similar results in turkey (hybrid converter strain) showed that *in ovo* injection of BCAAs on ED 22 reduced the (relative) weight of the embryo, but did not affect the yolk weight on ED 24 (Kop-Bozbay and Ocak, 2019). This difference could be explained by several decades of an intensive selection in chickens, which resulted in significant differences in growth, metabolite rate, and other physiological functions in embryos and chickens between layer and broiler strains (Havenstein *et al*., 2003; Sato *et al*., 2006; Druyan, 2010).

During embryogenesis, fatty acid oxidation contributes with more than 90% to the total energy requirements (Noble and Cocchi, 1990). The yolk is mainly the lipid source and is consumed by embryos during incubation. Leu was reported to increase the mitochondrial biogenesis (Duan *et al*., 2016) and hepatic mitochondrial function, and to be involved in fatty acid oxidation and lipid metabolism (Lindquist *et al*., 2017). L-Leu *in ovo* injection stimulated lipid metabolism and improved the metabolic activity on ED 14, as reported in a previous study (Han *et al*., 2018). However, in the current study, the yolk weight was not affected by L-Leu *in ovo* injection during embryogenesis, and the average relative yolk weight in the L-Leu group was higher than that in the control group on ED 14 ($P=0.0495$ using unpaired t-test between control and L-Leu groups, $n=9$/group). Future studies on the changes in L-Leu and its metabolites between ED 7 and ED 14 will be investigated to clarify this matter.

The free amino acid pool plays a critical role in embryonic growth. The different patterns of amino acid changes and lipid metabolism were related to the growth differences in broiler and layer embryos (Sato *et al*., 2006, 2009). *In ovo* injection of amino acids caused an increase in the growth of neonatal chicks by improving amino acid utilization and the development of digestive organs in embryos or chicks (Ohta *et al*., 1999; Gao *et al*., 2018). Most of the amino acid concentrations were increased and the free amino acid pool was enlarged during incubation to match the amino acid requirements for embryonic growth. This is in agreement with previous reports on broiler embryos (Ohta *et al*., 2004; Han *et al*., 2017). However, the levels of Ile, Leu, Gly, and Asp in the plasma did not significantly change with embryo development in the current study. Ohta *et al*. (2004) reported that the plasma Ile, Leu, Gly, and Asp concentrations increased with an increasing age in layer and broiler embryos. *In ovo* injection of an amino acid solution on ED 7 altered the plasma amino acid metabolism in neonatal chicks (Ohta *et al*., 2004), which suggested that the metabolism profiles of Ile, Leu, Gly, and Asp were altered by *in ovo* injected L-Leu, in this study. On the other hand, only Asp and Gly levels decreased, and that of glutamic acid did not change in the liver with the progress of embryonic age in the present study. This observation can be explained by uric acid synthesis, since uric acid synthesis in chicken embryos increases with the advancement of age (Fiske and Boyden, 1926) and these three amino acids are involved in uric acid synthesis.

In the current study, the amino acid profile was proposed to find some clues for L-Leu-mediated improvement of metabolism in the embryos of broiler chicks, as demonstrated in our previous studies (Han *et al*., 2018, 2019a, 2020). Tyr and Lys concentrations in the blood and liver of control embryos declined with embryonic development after ED 14 in the current study and in previous reports (Huether and Lajtha, 1991; Han *et al*., 2017). However, the changes in the concentration of Tyr and Lys were modified by L-Leu *in ovo* injection, resulting in an increase in Tyr and Lys levels in the blood and liver (unpaired t-test, $P=0.0388$ for Tyr in the liver) at ED 18. The developmental profiles of Tyr and Lys are almost identical in the blood and brain during embryogenesis (Huether and Lajtha, 1991). It could be predicted that the Tyr and Lys levels in the brain of the embryo might also be improved by L-Leu *in ovo* feeding at later stages of embryogenesis.

Tyr serves as a precursor of dopamine, epinephrine, and norepinephrine. Dopaminergic activity and norepinephrinergic metabolism were stimulated, and the Tyr concentration in the brain (mesencephalon) was also improved under fasting stress in neonatal chicks (Hamasu *et al*., 2012; Tran *et al*., 2015). The central administration of neuropeptide Y regulated monoamine metabolism and corticosterone response under heat stress, and reduced the body temperature without heat stress in chicks (Bahry *et al*., 2017). TM during incubation increased the Tyr level on ED 14 and reduced that of Tyr on ED 19 in broiler embryos (Han *et al*., 2017), affording thermotolerance in post-hatch chicks (Piestun *et al*., 2008). Thus, it could be hypothesized that the improved Tyr concentration may promote monoamine metabolism and influence brain function. The plasma Pro level was increased with embryo development (Ohta *et al*., 2004); however, the increase in plasma Pro was attenuated by L-Leu *in ovo* injection in the current study. Pro was reported to suppress dopamine and serotonin metabolism under stress conditions (Hamasu *et al*., 2009). The improved Tyr might be related to the reduced Pro via the alteration of monoamine metabolism. Future studies on monoamine analysis will be needed to clarify this matter.

In previous studies, Lys concentration was reduced via TM in the liver and brain of the embryo and decreased by an acute heat stress in the blood of broiler chicks (Han *et al*., 2017, 2019b). Moreover, *in ovo* administration of L-Leu caused an increase in hepatic and diencephalic Lys concentrations under acute heat stress in young broiler chicks (Han *et al*., 2019b), and improved the Lys concentration in the blood of broiler chickens after chronic heat stress (Han *et al*.,

2020). Lys is an essential amino acid and the second limiting amino acid in broiler chicken diets based on corn and soybean. Dietary supplementation of Lys reduced the feed conversion ratio by increasing the plasma serotonin level in broiler chickens (Ishii *et al*., 2019). Both Leu and Lys are exclusively ketogenic amino acids, and their carbon skeletons can be broken down into acetyl-CoA and acetoacetate, a ketone body (Voet and Voet, 1995). L-Leu-mediated ketogenesis is expected to stimulate lipid metabolism during the middle stage of embryogenesis (Han *et al*., 2018). In the current study, the supplied L-Leu was totally catabolized before ED 14. Thus, the catabolism of supernumerary L-Leu might cause a reduction in Lys consumption during the middle stage of embryogenesis and increase Lys concentration at later stages. However, the reports about Lys supplementation *in ovo* are limited, and future studies on *in ovo* injection of Lys should be conducted to explore new functions of Lys.

In conclusion, L-Leu *in ovo* injection caused embryonic growth retardation and altered the metabolism of some amino acids in broiler embryos. Tyr and Lys may play potential roles in embryonic development. Unaffected Leu concentration in the blood and liver suggested that the injected L-Leu was totally metabolized before ED 14 and did not enhance the free amino acid pool.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

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