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Abstract

The objective of this experiment was to investigate the effects of *in ovo* feeding of arginine (Arg), tryptophan (Trp), and threonine (Thr) on hatch performance, hatchability, and morphometry of newly hatched broiler chickens. A total of 400 fertile eggs from 28-week-old Arbor Acres broiler breeder flocks, with an average fertile egg weight of 52 ± 0.7 g, were collected for the experiment. The eggs were randomly assigned to five treatment groups (80 eggs per group, eight replicates of 10 eggs each): non-injected control (CON); phosphate-buffered saline (PBS) injection (100 μL of 1% PBS); Arg injection (100 μL/egg); Trp injection (100 μL/egg); and Thr injection (100 μL/egg). All eggs were incubated at the recommended temperature and humidity of 38.0 ± 0.2°C and 75.0 ± 3%, respectively. After hatching, one 1-day-old broiler chick per replicate with a body weight (BW) close to the average of each treatment was selected for hatchability and morphometry assessments. Results indicated that CON and PBS groups had significantly greater ($p < 0.05$) BW than Trp and Thr groups. The hatch window was greater ($p < 0.05$) in Thr group compared to CON, PBS, and Trp groups. However, no significant differences were observed among treatments for hatchability such as hatch of set and fertile, egg weight, survival rate, and chick yield. Morphometric analysis revealed that Arg, Trp, and Thr treatment groups exhibited significantly greater ($p < 0.05$) middle toe length compared to CON group. Conversely, small intestine length and chick body length were reduced ($p < 0.05$) in Arg, Trp, and Thr groups compared to CON group. No significant differences were observed in tibia and radius among groups. In conclusion, *in ovo* feeding Arg, Trp, and Thr influenced chick morphology and organ development, indicating that precise dosage optimization of amino acid injection during the embryonic stage is essential.

Keywords: arginine, broiler chicken, *in ovo* feeding, threonine, tryptophan

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INTRODUCTION

The broiler industry is a critical sector in global livestock production. With the steady increase in worldwide food demand, enhancing productivity has become increasingly vital to ensure a sustainable food supply [1,2]. The early growth stage of broiler chickens, particularly from the late embryonic stage to the first week post-hatch, is a critical phase that significantly influences future growth and overall health [3]. Nutritional status during this period profoundly affects adult body weight (BW), immune function, and organ development [4-7]. Broiler chickens often experience inadequate feed and water intake during the initial 24 to 72 hours post-hatch, which can adversely affect the development of the digestive system, reduce BW, and impair immune system maturation [8]. However, such nutritional deficiencies may lead to stunted growth and compromised immune function, ultimately decreasing broiler growth performance and survival rates in broiler chickens [9].

In ovo feeding is a technique that involves the direct administration of specific nutrients or vaccines into the yolk sac or amniotic fluid during embryonic development, promoting embryonic growth and providing essential nutrients required for optimal post-hatch development [10,11]. *In ovo* feeding has been reported to positively affect embryonic and post-hatch development, enhancing growth performance after hatch [12,13].

Amino acids are essential for embryonic growth and development, playing a vital role in supporting proper physiological processes in eggs [14]. Arginine (Arg) is an essential amino acid in poultry due to the absence of a functional urea cycle and limited endogenous synthesis capacity [15]. Additionally, Arg promotes skeletal muscle growth by activating the mechanistic target of rapamycin (mTOR) and nitric oxide (NO) signaling pathways [16]. Tryptophan (Trp), an essential amino acid for broiler chickens, enhances poultry growth performance by improving appetite, feed efficiency, protein synthesis, and immune response [17]. It also serves as a precursor for serotonin and melatonin, alleviating stress and behavioral issues [17]. Furthermore, Trp formed from niacin in embryo metabolism, allows niacin to reduce embryo mortality from early to late incubation stages [18]. Threonine (Thr), as the limiting essential amino acid for broiler chickens, facilitates intestinal development through mucin synthesis [19]. Thr modifies metabolic processes during the final stage of incubation to meet the elevated energy demands caused by limited oxygen availability [20]. Previous studies have documented improvements in hatchability and early growth when individual or combined amino acids including lysine, glutamine, glycine, proline, and Arg were administered via *in ovo* feeding. *In ovo* feeding of 1% Arg at 17.5 days of incubation has enhanced digestive organ development and duodenal morphology by stimulating gastrointestinal hormone release and mucosal enzyme activity in post-hatch chicks [21]. Additionally, *in ovo* feeding of 20 to 30 mg of Thr into

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the yolk sac has been shown to improve post-hatch growth and positively affect humoral immune responses in broiler chicks [22]. Moreover, *in ovo* feeding of 0.5% Trp has been found to enhance digestive capacity by promoting feed intake and improving nutrient utilization [23]. Previous studies have reported that *in ovo* feeding of amino acids such as Arg, Trp, and Thr at concentrations of approximately 0.5 to 1% improved post-hatch growth and intestinal development without adverse effects [21-23]. However, studies examining the effects of *in ovo* feeding of amino acids remain limited, particularly those directly comparing the distinct effects of individual amino acids.

Based on these findings, we selected a 1% concentration for Arg, Trp, and Thr in the present study to evaluate their effects on embryonic development and early growth performance. Therefore, the objective of this study was to investigate the effects of *in ovo* feeding of Arg, Trp, and Thr on hatch performance, hatchability, and morphometry of newly hatched broiler chickens.

MATERIALS AND METHODS

The protocol for this experiment was approved by the Institutional Animal Care and the Use Committee (IACUC) at Chungbuk National University (IACUC approval No. CBNUA-2126-23-01).

Egg and incubation

A total of 400 fertile eggs from 28-week-old Arbor Acres broiler breeder flocks, averaging a fertile egg weight of 52 ± 0.7 g, were collected from the hatchery. All fertile eggs were incubated at the recommended temperature and humidity of 38.0 ± 0.2°C and 75.0 ± 3%, respectively. Fertile eggs, sourced from Isu farm in Dangjin-si, Republic of Korea, were randomly placed in an automatic incubator (Rcom MARU Deluxe max 200, Autoelex Co., Ltd., Gimhae-si, Republic of Korea) under optimal conditions for temperature, humidity, and ventilation. The eggs were turned automatically every hour up to day 18.

Experimental design

Four hundred fertile eggs were divided into five treatment groups, each consisting of 80 eggs divided into eight replicates (10 eggs per replicate). The groups included: CON, control (no injection); PBS, injected with phosphate-buffered saline (100 μL of 1% PBS); Arg, injected with arginine (100 μL/egg); Trp, injected with tryptophan (100 μL/egg); Thr, injected with threonine (100 μL/egg). Following hatching, one 1-day-old chick per replicate, with a BW close to the average of each treatment, was selected for measuring morphometry, hatchability, and hatch performance.

Solution and injection procedure

The injection solution was prepared as a 1% (w/v) solution by dissolving 0.2 g of each L-Arg, L-Trp, and L-Thr (≥ 98%, Sigma-Aldrich Inc., St. Louis, MO, USA) in 20 mL of phosphate-buffered saline (PBS, LB004-01, Welgene Inc., Gyeongsan-si, Republic of Korea). The solution preparation and injection procedure were conducted according to the method described by Yu et al. [24] with minor modifications. After complete dissolution, the solution was autoclaved at 120℃ for 15 minutes and then incubated at 37℃ before use. On day 18 of incubation, each egg was removed from the incubator, candled with an electric torch, and marked at the site corresponding to the yolk sac. The injection sites were sterilized with 70% alcohol, punctured with a 21-gauge needle, and injected using a 21-gauge syringe (Korea Vaccine Co., Ltd., Ansan-si, Republic of Korea). A volume of 100 μL of the prepared solution was carefully injected into the yolk sac to a depth of approximately 1 - 2 cm. After the injection, the injection site was immediately sealed with parafilm to prevent contamination. In the present study, injections were conducted on day 18 of incubation into the yolk sac. Roto et al. [25] reported that the administration of amino acids into the yolk sac during the late stage of incubation, particularly on days 17 to 18, was the most effective in enhancing embryonic development and improving growth performance.

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Hatch performance

In this study, hatch performance measurements were conducted using the method described by Muyyarikkandy et al. [26] with minor modifications. On day 14 of embryonic development, eggs were candled using an electric torch to identify and mark unfertilized and nonviable eggs. These eggs were recorded but not removed from the incubator. The hatch of set was calculated as the percentage of 400 eggs that hatched. Conversely, hatch of fertile was calculated as the percentage of fertilized eggs that hatched out of 400 eggs. Survival rates were measured for all hatched eggs. Survival rate was calculated as the number of live chicks divided by the number of fertilized eggs, excluding chicks that died post-hatching. Egg weight was measured prior to incubation using an electronic scale (HS-1000A, Hansung Co., Ltd., Gwangmyeong-si, Republic of Korea). The hatch window was determined by the time interval between the earliest and latest hatching chicks and was related to the total number of fertilized eggs. After hatching, the number of hatched chicks and the BW of each chick were recorded. Chick yield is defined as the ratio of the average weight of hatched chicks to the total number of fertilized eggs.

Hatchability

Hatchability measurements were conducted using the method described by Muyyarikkandy et al. [25] with slight modifications. The fertility of set eggs was calculated by determining the percentage of fertilization among all set eggs per replicate. All unhatched eggs were opened to identify the cause of non-hatching. The standard for contaminated eggs involved a deep discoloration of the egg contents accompanied by the emission of rotten odors (Investigating Hatchery Practice, Ross tech, Scotland, UK). Embryo mortality was measured during early (0 - 7 days), middle (8 - 14 days), and late (15 - 19 days) stages of embryonic development. Eggs in which the embryo's beak had broken through the shell but subsequently died were classified as pipps. Culls are defined as chicks presenting with unhealed navels, skin lesions, deformed beaks, or abnormal leg conformations.

Morphometry

At day 10 post-hatch, chicks were euthanized using CO_2 to assess various morphometric parameters and the weights of the breast, leg meat, and liver. These tissues were carefully excised and individually weighed using a digital scale (HS1000A, Hansung, Seoul, Republic of Korea). Morphometric measurements included middle toe, tibia, radius, small intestine, and chick body lengths. The middle toe was measured from the base to the tip of the third toe using a 30-cm ruler. The tibia and radius were measured after carefully removing the surrounding muscles from the leg and wing, respectively, using a 30-cm ruler. The small intestine was gently extended without excessive tension and measured with a 30-cm flexible ruler to minimize tissue damage. Chick body length was measured from the tip of the beak to the base of the middle toe using a 15-cm ruler. All measurements were performed by the same trained individual to ensure consistency and minimize inter-observer variability.

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Relative organ weight

The relative organ weights of the heart, liver, gizzard, glandular stomach, kidney, yolk, and small intestine in broiler chicks were measured using a digital scale (HS1000A, Hansung, Seoul, Republic of Korea) and expressed relative to BW. The egg yolk that was not absorbed by all newborn chicks was measured using a scale.

Breast meat, leg meat, and liver color

Lightness (L*), redness (a*), and yellowness (b*) values of the breast meat, leg meat, and surface of the liver were measured precisely using a colorimeter (model CR-10, Konica Minolta, Tokyo, Japan).

Statistical analysis

Statistical analysis was conducted using a completely randomized design by using the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). Each replicate was considered an experimental unit. All data were checked for normal distribution and outliers were checked with the UNIVARIATE procedure of SAS. The least significant difference test was conducted to calculate treatment means and the PDIFF option of SAS was used to separate means if the difference was significant. Significance for statistical tests was set at $p < 0.05$.

RESULTS

No significant differences were observed in the hatch of set, hatch of fertile, egg weight, survival rate, and chick yield. However, the 1-day-old BW was significantly less ($p < 0.05$) in Trp and Thr groups than in CON and PBS groups. *In ovo* feeding in Thr group resulted in significantly greater ($p < 0.05$) hatch window than in CON, PBS, and Trp groups. Hatch performance, including fertility, contamination of set eggs, embryo mortality, pipps, and culls was not affected by any group. Furthermore, middle toe length in Arg, Trp, and Thr groups was significantly greater ($p < 0.05$) than in CON group. The lengths of the small intestine and chick body were significantly less ($p < 0.05$) in Arg, Trp, and Thr groups than in CON group. However, tibia and radius measurements remained unchanged across all groups. Abdominal yolk sac absorption was significantly less ($p < 0.05$) in Arg, Trp, and Thr groups than in CON and PBS groups. There were no significant differences in the relative weights of the heart, liver, gizzard, proventriculus, and small intestine. In the kidneys, *in ovo* feeding of amino acids showed a significant increase ($p < 0.05$) compared to PBS group. *In ovo* feeding of amino acids increased ($p < 0.05$) L* value of leg meat, but decreased ($p < 0.05$) a* value. The b* values of Liver in Trp and Thr groups were less ($p < 0.05$) than in PBS group. However, breast meat color was not significantly affected by *in ovo* feeding of amino acids.

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DISCUSSION

Chicks utilize nutrients stored in the yolk during embryonic development to establish a foundation for growth [27]. However, the nutrient composition of the yolk varies depending on factors such as the breeder hen's age, genetics, and environmental conditions, subsequently influencing embryonic development and post-hatch growth performance of broiler chicks [28]. Enhanced nutrient availability during the early developmental stages facilitates optimal growth and is a key determinant of market value, such as meat yield and final BW in the poultry industry [29]. Shafey et al. [30] reported that *in ovo* feeding of an amino acid mixture including lysine, glutamine, glycine, proline, and Arg on day 21 of incubation increased post-hatch growth performance including BW and BW gain. Therefore, amino acids may contribute to supplementing deficient nutrients during embryonic development, which is expected to enhance post-hatch growth performance of broiler chickens. In poultry, Arg is an essential amino acid critical for growth, immunity, metabolism, and muscle development [31]. It serves as a substrate for the synthesis of NO, polyamines, and glutamate [32]. Additionally, Arg promotes gut health, enhances mucosal recovery, and modulates immune responses through various physiological roles in broiler chickens [33]. Similarly, Thr is an essential amino acid for broiler chickens, as it plays a key role in protein synthesis, mucin production, and maintaining intestinal health [34]. Moreover, Trp contributes to protein synthesis and acts as a precursor to serotonin and melatonin, influencing growth, feed intake, immunity, and reducing oxidative stress in broiler chickens [35]. In our study, therefore, amino acids such as Arg, Trp, and Thr were injected to supplement nutrients that may be lacking during embryonic development.

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In the present study, *in ovo* feeding of Arg did not significantly affect the BW of 1-day-old chicks, which is consistent with the findings of Gao et al. [36]. In contrast, Trp and Thr significantly reduced BW. The observed reduction may be attributed to elevated kynurenine metabolite levels induced by excessive Trp, which may promote free radical generation and subsequently lead to oxidative stress in embryos [37]. Similarly, a previous study reported that high-dose Thr injection (e.g., 40 mg) reduced hatch weight, likely due to amino acid imbalance [22]. These findings suggest that moderate *in ovo* supplementation supports growth, excessive supplementation of Trp and Thr may impair embryonic development.

The hatch window refers to the interval between the first and last chick hatching [38]. In the present study, the hatch window was significantly extended by *in ovo* feeding of Thr. An extended hatch window implies that first-hatched chicks experience delays in accessing external nutrients compared to later-hatched chicks [39]. This delay may adversely affect chick quality, growth, and small intestinal development post-hatching [40]. The increase in hatch window caused by *in ovo* feeding of Thr may be linked to altered developmental rates among embryos. Our results indicate that the typical hatch window ranges from 24 to 48 hours [39]. In this study, there were no significant differences in chick yield across all treatment groups, suggesting that *in ovo* feeding of amino acids exerts minimal impact on chick yield.

In the present study, *in ovo* feeding of Arg, Trp, and Thr resulted in significantly longer middle toe lengths than CON. This effect can be attributed to Arg's role as an essential precursor for NO production, which serves as a vital signaling molecule in bone cell formation [41]. Additionally, Arg promotes skeletal muscle development via mTOR pathway [33]. Trp acts as a precursor for serotonin, enhancing bone formation by influencing bone density and growth [42]. The hydroxyl group in Thr helps stabilize collagen structure, thus supporting the integrity and function of connective tissues, including bone [43]. In avian species, toes are essential for maintaining balance and stability on various surfaces such as branches, fence posts, and nests, supporting activities such as digging, nesting, and food selection [44]. A longer middle toe in poultry might aid in distributing BW and maintaining balance, especially in heavier birds, contributing to the prevention of leg disorders by evenly distributing pressure on the legs during locomotion and rest [45]. Therefore, the elongation of the middle toe induced by *in ovo* feeding of amino acids is anticipated to promote bone formation in the later developmental stages.

The yolk serves as the primary nutrient source for embryonic growth and development in poultry [46]. Consequently, the size and composition of the residual yolk at hatching are critical factors influencing the energy reserves available to chicks during the early rearing period [47]. In the present study, *in ovo* feeding of Arg, Trp, and Thr significantly reduced small intestine and body lengths compared to the CON. These findings suggest that alterations in nutrient absorption during embryogenesis may have contributed to the observed reductions in intestinal and overall body growth.

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The small intestine plays a crucial role in nutrient digestion and absorption, and its underdevelopment can severely impair nutrient uptake efficiency during the early post-hatch period [48]. Previous studies [32] have shown that amino acid imbalances or excessive supplementation during the embryonic stage can disrupt growth and even cause death. *In ovo* feeding of a single amino acid is less effective than *in ovo* feeding of multiple amino acids simultaneously, as the excess of a single amino acid can disrupt the balance of other amino acids [49].

The body length of chicks serves as a critical indicator of growth performance, survivability, and overall health status in broiler chickens, reflecting the efficient conversion of yolk nutrients into body growth with increased yolk absorption at hatch [50,51]. Therefore, the observed decreases in small intestine and body lengths in this study are likely attributable to impaired yolk nutrient absorption following *in ovo* feeding of Arg, Trp, and Thr.

Consumers often evaluate chicken freshness based on its appearance, which influences purchasing decisions. Chicken thighs and drumstick meat are typically dark red. Generally, lower L* and a higher a* values are generally associated with superior meat quality [52]. However, this may differ from the meat color at marketing weight in broiler chickens because our analysis focused on the leg meat color in young chicks. Earlier studies have indicated that Arg enhances muscle protein synthesis and regulates muscle development, thus improving blood circulation in skeletal muscle [16]. In the current study, *in ovo* feeding of Arg decreased the L* value of the leg. However, *in* *ovo* feeding of Trp and Thr decreased the a* value of the leg. These findings suggest that kynurenine constitutes about 94% of the Trp metabolism pathway, stimulating hepcidin expression, which limits iron bioavailability by regulating its absorption and metabolism [53]. Similarly, Thr is crucial for collagen formation, which enhances water holding capacity (WHC) and increases muscle pH, potentially reducing meat redness [54,55]. In the present study, *in ovo* feeding of Trp reduced the redness value of the leg. It was anticipated that *in ovo* feeding of Trp could cause iron deficiency in the embryo due to kynurenine [53]. Iron deficiency diminishes the oxygen-carrying capacity of myoglobin, leading to a decrease in muscle pH, protein denaturation, and a reduction in WHC of muscle. Consequently, the meat becomes paler and exhibits a brighter color [56,57]. Therefore, this study suggests that *in ovo* feeding of Arg, Trp, and Thr may influence meat pigmentation by modifying muscle metabolism.

The liver, which is the largest gland in broiler chickens, detoxifies substances that enter the body, aids in digestion, and supports metabolic processes [58]. Previous studies have shown that adult chickens usually have livers ranging from dark red to red-brown, whereas chicks typically have yellow-colored livers due to yolk absorption, which is the primary source of carotenoids giving the liver its yellow hue in newly hatched chicks [59,60]. In the present study, *in ovo* feeding of Trp and Thr significantly decreased b* values in the liver compared to those of PBS. As approximately 94% of Trp metabolism occurs through the kynurenine pathway, it can be deduced that activation of this pathway contributes to reduced yolk absorption [53]. Similarly, excessive Thr can disrupt metabolic process distribution, causing competition with other amino acids required for various metabolic pathways [61]. Excessive availability of Thr could redirect amino acids towards intestinal development and mucin synthesis, thus limiting the metabolic resources available for yolk utilization. Therefore, the decrease in b* value observed in this study may have also resulted from reduced yolk absorption induced by Trp and Thr.

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CONCLUSION

In conclusion, *in ovo* feeding of amino acids significantly increases middle toe length and kidney weight, accompanied by reducing abdominal yolk sac absorption and small intestine length. *In ovo* feeding of Trp and Thr adversely affects BW and yolk absorption in hatched chicks. In addition, it reduces meat pigmentation, as indicated by decreased redness and yellowness values in the leg and liver tissues. These findings suggest that *in ovo* feeding of amino acids may enhance certain developmental and quality traits; however, precise optimization of their dosage is essential to minimize potential adverse effects and to improve the overall performance of broiler chickens.

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ACCEPTED

Table 1. Effects of *in ovo* feeding of arginine (Arg), tryptophan (Trp), and threonine (Thr) on hatch performance of broiler chickens[1)]

	In ovo feeding[2)]						
Items	CON	PBS	Arg	Trp	Thr	SEM	*p*-value
Hatch of set[3)], %	63.75	70.00	47.50	56.25	65.00	6.416	0.137
Hatch of fertile[4)], %	70.35	79.13	60.66	68.06	75.03	6.802	0.387
Egg weight, g	51.69	52.26	52.66	52.34	52.55	0.375	0.480
Body weight, g	39.57^{a}	39.50^{a}	37.65^{ab}	36.83^{b}	37.38^{b}	0.692	0.021
Survival rate[5)], %	75.11	84.45	70.64	74.03	81.56	6.790	0.598
Hatch window[6)], h	11.48^{b}	13.87^{b}	12.97^{b}	12.01^{b}	26.79^{a}	2.882	0.028
Chick yield[7)], %	76.10	75.96	73.82	74.32	77.41	1.464	0.430

^{a,b}Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Each value represents the mean of 8 replicates per each treatment.

[2)]*In ovo* feeding = CON, control (non-*in ovo* feeding); PBS, *in ovo* feeding of phosphate buffer saline; Arg, *in ovo* feeding of arginine; Trp, *in ovo* feeding of tryptophan; Thr, *in ovo* feeding of threonine.

[3)]Hatch of set = (number of eggs hatched / number of eggs set) x 100.

[4)]Hatch of fertile = (number of eggs hatched / number of fertile eggs) x 100.

ACCEPTED

[5)]Survival rate = (number of live chick / number of fertile eggs) x 100.

[6)]Hatch window = (time that last hatched chick – time that first hatched chick) / number of fertile eggs x 100.

[7)]Chick yield = (average hatched chick weight / average fertile egg weight) x 100.

ACCEPTED

Table 2. Effects of *in ovo* feeding of arginine (Arg), tryptophan (Trp), and threonine (Thr) on hatchability of broiler chickens[1)]

Items	*In ovo* feeding[2)]					SEM	*p*-value
	CON	PBS	Arg	Trp	Thr		
Fertility of set eggs[3)], %	91.25	87.50	76.25	83.75	87.50	3.856	0.092
Contaminated eggs[4)], %	0.58	0.35	1.21	1.18	0.00	0.509	0.388
Embryo mortality[5)], %							
Early	0.42	0.52	0.59	1.38	0.19	0.495	0.518
Middle	0.31	0.00	0.62	0.75	0.16	0.276	0.300
Late	2.13	1.38	1.98	2.46	1.59	0.716	0.835
Pipps[6)], %	0.41	0.39	1.03	0.79	0.17	0.324	0.355
Culls[7)], %	0.00	0.63	0.00	0.42	0.14	0.297	0.497

[1)]Each value represents the mean of 8 replicates per each treatment.

[2)]*In ovo* feeding = CON, control (non-*in ovo* feeding); PBS, *in ovo* feeding of phosphate buffer saline; Arg, *in ovo* feeding of arginine; Trp, *in ovo* feeding of tryptophan; Thr, *in ovo* feeding of threonine.

[3)]Fertility of set eggs = (number of fertile eggs / number of eggs set) x 100.

[4)]Contaminated eggs = (number of contaminated eggs / number of fertile eggs) x 100.

ACCEPTED

[5)]Early (1 - 7 days), middle (8 - 14 days) or late mortality (15 - 21 days) calculated based upon the number of fertility eggs.

[6)]Pipps = (number of chicks that died after pipping / number of fertile eggs) x 100.

[7)]Culled chicks (unhealed navel, skin lesions, deformed beak or abnormal conformation of legs) calculated based upon the number of fertile eggs.

ACCEPTED

Table 3. Effects of *in ovo* feeding of arginine (Arg), tryptophan (Trp), and threonine (Thr) on morphometry of broiler chickens[1)]

Items	*In ovo* feeding[2)]					SEM	*p*-value
	CON	PBS	Arg	Trp	Thr		
Middle toe[3)], %	11.00^{c}	11.20^{bc}	11.67^{ab}	12.19^{a}	11.77^{ab}	0.205	0.002
Tibia[4)], %	20.73	20.04	20.74	20.10	21.45	0.551	0.375
Radiu[5)], %	28.13	28.45	27.72	27.27	28.71	0.882	0.791
Small intestine, cm	41.68^{a}	37.14^{bc}	34.80^{c}	38.48^{b}	37.56^{b}	0.942	<0.001
Chick length[6)], cm	16.14^{a}	15.66^{ab}	15.20^{bc}	15.18^{bc}	14.99^{c}	0.188	<0.001

[a-c]Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Each value represents the mean of 8 replicates per each treatment.

[2)]*In ovo* feeding = CON, control (non-*in ovo* feeding); PBS, *in ovo* feeding of phosphate buffer saline; Arg, *in ovo* feeding of arginine; Trp, *in ovo* feeding of tryptophan; Thr, *in ovo* feeding of threonine.

[3)]Middle toe = (middle toe length / chick length) x 100.

[4)]Tibia = (tibia length / chick length) x 100.

[5)]Radius = (radius length / chick length) x 100.

[6)]Chick length = measured from the tip of the middle toe to the tip of the beak.

ACCEPTED

Table 4. Effects of *in ovo* feeding of arginine (Arg), tryptophan (Trp), and threonine (Thr) on relative organ weight of broiler chickens[1)]

Items[3)]	*In ovo* feeding[2)]					SEM	*p*-value
	CON	PBS	Arg	Trp	Thr		
Heart, %	1.17	0.92	1.12	1.06	1.03	0.065	0.082
Liver, %	2.58	2.30	2.57	2.70	2.68	0.122	0.176
Gizzard, %	4.95	4.72	4.42	5.13	4.82	0.237	0.307
Proventriculus, %	1.01	0.86	0.81	0.90	0.93	0.059	0.169
Kidney, %	0.54^{a}	0.25^{b}	0.38^{ab}	0.43^{a}	0.42^{a}	0.056	0.020
Residual yolk sac[4)], %	6.47^{bc}	5.68^{c}	9.88^{a}	8.94^{a}	8.79^{ab}	0.824	0.004
Small intestine, %	4.82	4.81	4.22	4.97	4.90	0.242	0.212

$^{a\text{-}c}$Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Each value represents the mean of 8 replicates per each treatment.

[2)]*In ovo* feeding = CON, control (non-*in ovo* feeding); PBS, *in ovo* feeding of phosphate buffer saline; Arg, *in ovo* feeding of arginine; Trp, *in ovo* feeding of tryptophan; Thr, *in ovo* feeding of threonine.

[3)]The relative organ weight was expressed as a percentage of BW.

[4)]Young chicks that have hatched recently still have yolk sac remaining within the abdominal cavity.

ACCEPTED

Table 5. Effects of *in ovo* feeding of arginine (Arg), tryptophan (Trp), and threonine (Thr) on breast, leg, and liver color of broiler chickens[1)]

Items[3)]		*In ovo* feeding[2)]					SEM	*p*-value
		CON	PBS	Arg	Trp	Thr		
Breast	L*	51.60	51.55	61.54	52.13	51.24	1.435	0.995
	a*	11.28	12.53	11.39	9.98	11.44	0.630	0.106
	b*	23.10	24.10	20.89	22.36	22.13	1.287	0.499
Leg	L*	48.54^{ab}	45.55^{bc}	43.58^{c}	49.99^{a}	48.93^{ab}	1.506	0.027
	a*	15.33^{a}	14.70^{ab}	14.50^{ab}	12.75^{b}	12.49^{b}	0.775	0.050
	b*	20.75	18.94	17.39	18.31	20.33	1.040	0.187
Liver	L*	33.26	35.93	33.74	30.00	30.23	1.662	0.080
	a*	20.78	21.34	19.91	20.53	19.01	0.705	0.199
	b*	17.16^{ab}	22.61^{a}	18.13^{ab}	13.95^{b}	14.48^{b}	1.948	0.025

$^{a-c}$Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Each value represents the mean of 8 replicates per each treatment.

[2)]*In ovo* feeding = CON, control (non-*in ovo* feeding); PBS, *in ovo* feeding of phosphate buffer saline; Arg, *in ovo* feeding of arginine; Trp, *in ovo* feeding of tryptophan; Thr, *in ovo* feeding of threonine.

ACCEPTED

[3)] L*, lightness; a*, redness; b*, yellowness.

ACCEPTED