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Influence of *in ovo* threonine on growth performance, immunocompetence and carcass characteristics in broiler chickens

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ABSTRACT

The experiment was conducted to investigate the effect of *in ovo* threonine (Thr) injection on 18th embryonation day at the broad end of the egg using 24G needle on growth performance, organs development, immunocompetence and carcass yield in broiler chickens. Fertile eggs (n=500) were randomly distributed to 5 groups, viz. (i) un-injected control, sham control (0.5 mL sterile water), Thr @15 mg/egg, Thr @30 mg/egg, and Thr @45 mg/egg. Hatchability was better in chicks treated with 45 mg Thr/egg compared to other Thr-injected groups. *In ovo* Thr injection had quadratic effect on weight gain (WG) and production index at the 2nd and 3rd weeks. Chicks fed 45 mg Thr/egg had better feed conversion ratio (FCR) at 2nd week compared to other groups while feed intake (FI) was not affected. Thr injected chicks had higher thymus weight (d0), bursa weight (d3), spleen weight (d3 and d7) whereas quadratic effect were observed on weights of bursa, thymus and spleen at d21. *In ovo* Thr administration improved (group-wise, linear or quadratic) the relative weights of gizzard, intestine, and liver at hatch, proventriculus at d7, as well as intestine and liver at d21. No significant difference was recorded for humoral and cell mediated immune response following *in ovo* Thr injection. Carcass traits of broiler chickens were not influenced by *in ovo* Thr administration, except breast meat which slightly differs, among the treatment groups. The results of this study suggested that *in ovo* Thr can improve growth performance, digestive and immune organs development at the early age.

Keywords: Amino acids, Immunity, Incubation, *In ovo* feeding, Lymphatic organs, Post-hatch growth

Survivability of newly-hatched chicks could be influenced by the amount of nutrient resources immediately available after hatching as well as how soon they gain access to exogenous feed (Bhanja *et al.* 2015). Due to long transit time between hatching and access to feed, chicks often mobilize the reserves meant for other functions i.e immune system function for body maintenance. More so, limited body reserves are noted to result in about 2–5% mortality in hatchlings (Bhanja *et al.* 2004, 2015). Besides, higher rate of nutrients depletion towards the later stage of embryonic development could be partly connected to the dynamism of morphological, cellular, and molecular changes of digestive organs which causes maximal relative size of these organs in preparation for digestion and utilization of exogenous dietary nutrients since chickens are precocial with the ability to feed almost immediately after hatch (Uni *et al.* 2012, Jha *et al.* 2019). Alabi *et al.* (2018) also observed that maximum intestine development (98% increase) occurred from 19th to 21st embryonated day (ED). Thus, the rationale for *in ovo* feeding (IOF) is premised on the need to supply adequate nutrients during the late term-embryonic stage to optimize embryonic development and to overcome the adverse effect of delayed access to feeds by poultry hatchlings during the early post-hatch periods (Bhanja *et al.* 2004, Kadam *et al.* 2008, Jha *et al.* 2019).

Threonine (Thr; 2-amino-3-hydroxybutyric acid, $C_4H_9NO_3$) is an essential amino acids in broilers which is known for its crucial role in gastro-intestinal tract (GIT) structure and function, mucin secretion and γ-globulin production (Corzo *et al.* 2007, Ayasan *et al.* 2009, Canogullari *et al.* 2009, Ayasan and Okan 2014). It is an important constituent of mucin, a major glycoprotein which protects the intestinal epithelium from injury and maintains intestinal immune function (Najafi *et al.* 2017, Rasheed *et al.* 2018). Because of its high demand, a larger proportion of the dietary Thr ingested is often catabolizes at the intestinal level for synthesis of intestinal-mucosal proteins

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and digestive secretions for optimum nutrient break-down, absorption and utilization (Corzo *et al.* 2007, Sigolo *et al.* 2017). In addition, Thr exerts an efficient immuno-modulatory effect through enhancement of antibody production (Najafi *et al.* 2017). In view of these benefits, therefore, IOF of Thr on may exert positive influence on enteric development and immunomodulatory effect in broilers through enhancement of antibody production.

Furthermore, Uni *et al.* (2005) had noted that as embryos prepare for hatching process, there would be a paradigm shift in energy metabolism by generating glucose from glycogenolysis and gluconeogenesis from amino acids in muscle protein to meet the metabolic glucose demands. Thus, it was hypothesized that *in ovo* injection of Thr (a glycogenic amino acid) into the embryonic yolk sac prior to hatch could minimize muscle protein depletion, enhance the hatch weight, improve enteric and immune organs development thus gives rise to better post-hatch growth performance and carcass yield. Meanwhile, previous studies on *in ovo* injection of amino acids (AA), including L-Thr, either singly or in combination, in poultry birds are available but with inconsistent results (Ohta *et al.* 1999, Kadam *et al.* 2008, Salmanzadeh *et al.* 2011, Gaafar *et al.* 2013, Shafey *et al.* 2014). The variation in reported findings might be partly connected to the difference in day of injection, deposition site, dosage (concentration) of substances injected, and strain of birds used. Therefore, the objective of this present study was to determine the impact of *in ovo* Thr injected on 18^{th} day of incubation on the growth performance, organ developments, immunocompetence and carcass yield in broiler chickens.

MATERIALS AND METHODS

Ethical protocol: The study protocols in this experiment were in accordance with the Institutional Animal Ethics Committee guidelines of ICAR-Central Avian Research Institute, Izatnagar, India as approved vide number CARI/CPCSEA/2017/2 dated 05-08-2017.

Incubation and in ovo injection procedure: Fertile eggs (n=635) were obtained from broiler breeder flock reared under good management husbandry practices at the Poultry Housing and Management Section, ICAR-Central Avian Research Institute, Izatnagar, Bareilly, India. Eggs were weighed before setting. The eggs were set in a vertical force draft incubator (AP Poultry Equipments, India) under optimal conditions (Temp.=37.5°C and RH=60%). On 18 embryonated day (ED), all the unfertile or nonviable eggs were removed after candling. Thereafter, a total of 500 viable eggs in the range of mean±SD (62.5±2 g) were randomly distributed into 5 groups (n=100 each) and were returned back in the incubator. The treatment groups were un-injected control (T1), sham control (T2; 0.5 mL sterile water), *in ovo* Thr supplement @15 mg/egg (T3), 30 mg/egg (T4) and 45 mg/egg (T5). The required amount of L-Thr (Pure 99%; Sisco Research Laboratories Pvt Ltd, Mumbai, India) were weighed and dissolved in sterile water (0.5 ml).

On 18^{th} ED, 0.5 ml of Thr solution and sham (sterile water) were injected into the amniotic cavity through a pinhole made at the broad end of the egg, using a 24-gauge hypodermic needle (25 mm long) as described by Bhanja *et al.* (2004). Prior to injection, the broad end of the egg was suitably sterilized with 70% ethanol. The whole injection procedure was carried out within 20 min under laminar flow system and the pinhole site was sealed with sterile paraffin wax immediately after injection. In parallel, the un-injected control was taken out of the incubator for 20 min to equalize the conditions for all treatment groups. Then, the eggs were transferred to the hatcher and placed in pedigree-hatching boxes until hatch.

Post-hatch rearing and feeding management: On the day of hatch, each chick was weighed, wing banded, and transferred to 4-tier electrically heated battery cages (8 chicks per cage) that measured 80 × 75 × 65 cm (length, width, and height, respectively) which were situated at the PHM brooder shed of the Institute. All chicks received a corn-soybean meal basal diet formulated per all the nutrients based on NRC (1994) recommendations (Table 1) up to d42 of age. Feed (mash form) and water were supplied *ad lib.* The chicks were vaccinated against Newcastle disease (Ranikhet) and infectious bursal disease as per the recommendation of the Institute.

Growth performance: Birds were weighed individually while FI per replicate cage was recorded on a weekly basis in order to determine average daily weight gain (ADWG) and average daily feed intake (ADFI), respectively. Feed conversion ratio (FCR) was estimated as the ratio of feed consumed to weight gain and was adjusted for mortality, if any. Production index (PI) was computed as the ratio of the square of weight gain to the feed intake.

$$\text{Production index (PI)} = \frac{(\text{Weight gain})^2}{\text{Feed intake}}$$

Measurements of organs development: Six random chicks (equal male and female) from each of the *in ovo* and control treatments were sampled for analysis on the day of hatch (d 0), d 3, 7, 21 and 42 post-hatch. Body weight, lymphatic organs (bursa, thymus and spleen) as well as digestive and immune organs (empty gizzard, heart, liver, intestine, proventriculus, and pancreas) weights were recorded for each bird sampled using a sensitive microbalance (KERN/ABT 220–5DM; Kern & Sohn GmbH, Balingen, Germany) and the obtained values were expressed as percentage of respective live weights.

In vivo cellular immune response: The *in vivo* cutaneous basophilic hypersensitivity response to the lectin Phytohaemagglutinin from *Phaseolus vulgaris* (PHA-P; HiMedia Laboratories Pvt. Ltd., Mumbai, India) was studied at d 28 post-hatch as a measure of cell-mediated immune response using the method of Corrier and Deloach (1990). The toe web thicknesses between the 3^{rd} and 4^{th} inter-digital space of both left and right feet (n = 8 birds per group) were measured using a micrometer caliper (Mitutoyo, Japan). Thereafter, 0.1 ml of PHA-P [100 µg PHA-P dissolved in 0.1 ml phosphate-buffered saline (PBS)] was

Table 1. Diets composition and nutrient contents of the basal diets (as fed basis)

Ingredient (g/kg)	Starter (1–21 d)	Finisher (22–42 d)
Maize	541.5	572.0
Soybean meal (46% CP)	322.5	281.5
Guar meal (42% CP)	51.7	51.1
Fish meal (45% CP)	21.0	0.0
Deoiled rice bran	0.0	40.0
Soybean oil	27.5	20.0
Dicalcium phosphate	16.0	15.0
Limestone	11.5	12.0
Salt (NaCl)	3.0	3.0
L-Lysine HCL	1.0	1.0
DL-Methionine	1.3	1.4
Vitamins and minerals premix*	2.5	2.5
Choline chloride	0.5	0.5
Total	1000.0	1000.0
Nutrient composition (*g/kg, except ME*)		
Metabolizable energy (Kcal/kg)	3028.41	3000.11
Crude protein	230.00	210.20
Ether extract	54.30	47.00
Crude fibre	29.30	33.30
Calcium	10.50	9.20
Available phosphorus	4.50	3.80
Lysine	11.50	10.20
Methionine	4.60	4.40
Threonine	7.10	6.40
Threonine/lysine	6.20	6.30
Methionine + cysteine	7.50	7.10
Tryptophan	2.40	2.20
Arginine	15.20	14.00
Valine	9.40	8.80
Histidine	5.40	5.00
Leucine	17.30	16.10
Isoleucine	8.30	7.50
Glycine	7.70	6.90
Phenylalanine	9.90	9.00

*Supplied per kg of diet: Vit A, 12000 IU; Vit D3, 5000 IU; Vit E, 75 IU; Vit K3, 3 mg; Vit B_1, 3 mg; Vit B_2, 8 mg; Vit B_6, 5 mg; Vit B_{12}, 0.016 mg; Pantothenic acid, 13 mg; Niacin, 55 mg; Folic acid, 2 mg; Biotin, 0.2 mg; Cu, 16 mg; Ý, 1.2 mg; Se, 0.3 mg; Mn, 120 mg; Fe, 40 mg; Zn, 100 mg.

injected at the right foot whereas the left foot which served as control was injected with 0.1 ml PBS. The inflammatory response was determined after 24 h of injection by measuring the thickness of the respective toe webs. The foot web index was calculated as a difference between the swelling in the right and left feet before and after 24 h of injection. The foot web/pad index was calculated as follows:

$$\text{Foot web index (mm)} = (R2–R1) – (L2–L1)$$

where R2, thickness of right foot web after 24 h of injection; R1, thickness of the right foot web before injection; L2, thickness of left foot web after 24 h of injection; and L1, thickness of the left foot web before injection.

In vivo humoral immune response: The *in vivo* humoral immune response to the Sheep Red Blood Cell (SRBC) antigen was studied at 28th day post-hatch according to the standard procedures (Siegel and Gross 1980, van der Zijpp 1983). Fresh sheep blood was collected and was suspended in Alsevar's solution. The blood was washed three times in isotonic PBS (*p*H 7.2) using centrifugation (3,000 rpm; 10 mins), and 1% SRBC suspension was prepared. Thereafter, 8 chicks from each treatment group were injected intravenously with 1 ml of 1% SRBC suspension. After 5 days post-injection, blood samples (2 ml) were obtained from the jugular vein of each chick and allowed to clot for serum collection. The antibody response to SRBC was determined using a standard haemagglutination assay. The reciprocal of highest dilution in which there were complete haemagglutination was the end point of titre and the values were expressed as $\log_2$.

Carcass yield: At d 42, 6 birds per treatment (equal male and female) whose weights are nearest to the average weight per replicate group were selected, slaughtered, defeathered, and eviscerated following standard commercial procedures (Debnath *et al.* 2019). The body weights and dressed weights were measured while the dressed yields were calculated. Primal-cut parts, such as breast, drumstick, thigh, and back were weighed using an electronic weighing scale (Goldline®-GLF3; Goldline, Delhi, India). The weights were expressed as percentage of respective live weights.

Statistical analysis: Data generated from this study were subjected to One-Way Analysis of Variance technique in a completely randomized design using General Linear Model procedure of SAS (2007) (SAS for Windows, 9.1.3 portable version, Cary, NC, USA) according to the following model:

$$Y_{ij} = \mu + A_i + \varepsilon_{ij}$$

where Y_{ij}, observation; μ, overall mean; A_i, effect of effect of *in ovo* Thr injection and ε_{ij}, random error term. Means of significant results were compared by Tukey's HSD Test where necessary at P<0.05 or otherwise stated. Orthogonal polynomials were used to assess the linear and quadratic effects of varying concentration of *in ovo* Thr.

RESULTS AND DISCUSSION

Hatch index: Varying concentration of *in ovo* injected Thr had no significant effect on egg weight, chicks weight, and chick to egg weight ratio across all the treatment groups (P>0.05) (Table 2). Meanwhile, providing 15 and 30 mg L-Thr to late-term embryos increased hatching weights by 3.0 and 5.9%, respectively over sham control. The observed non-significance difference in chicks' weight, and chick to egg weight ratio suggested the adequacy of endogenous nutrients supplied through maternal nutritional plane to support optimum development of hatched chicks. Previous reports by Awachat *et al.* (2017) showed similar chick weight and chick to egg weight ratio with *in ovo* supplementation of Arg + Gln + Thr and control group (P>0.05). Also, IOF of Thr at the 14th embryonated day (ED) produced no significant difference in the chick weight at hatch, but chick to egg weight ratio was significantly higher compared to the control groups (Kadam *et al.* 2008,

Table 2. Effect of *in ovo* L-Thr administration on hatch traits of broiler chicks

Item	Control		Thr (mg/egg)			Pooled SEM	P value
	Un-injected (T1)	Sham (T2)	15 (T3)	30 (T4)	45 (T5)		
Egg weight (g)	60.10	59.81	59.96	59.95	59.93	1.39	0.999
Chick weight (g)	45.23	44.39	45.73	47.01	44.51	1.44	0.703
Ratio of chick weight to egg weight (%)	75.41	74.23	76.36	78.49	74.47	3.27	0.882
Hatchability (%)[#]	89.36[a]	82.50[a]	73.42[b]	75.00[ab]	85.00[a]	3.01	0.001

[ab]Means with different superscripts within the same row differ significantly (P<0.05). [#]Percentage of fertile eggs used for *in ovo* injection; SEM, Standard error of means.

Bhanja *et al.* 2012). Early feeding of AA mixture at 15th ED in a study by Shafey *et al.* (2014) also increased the relative chick weight at hatch. Gaafar *et al.* (2013) reported heavier weights of ducklings at hatch due to IOF of amino acids mixture at the 12th ED compared to the un-injected control. Meanwhile, Salmanzadeh *et al.* (2011) reported higher chick weight in eggs injected with 30 and 35 mg L-Thr at 8th ED when compared with control group. This could probably suggests that *in ovo* L-Thr supplementation at early stage could augment bio-available amino acids which are required for formation of internal organs, limbs and muscle tissue synthesis which occur massively at this stage thereby having positive influence on the chick weight at hatch (Alabi *et al.* 2018). Being a precursor of Gly, supplemental L-Thr could have increased Gly synthesis which usually has higher demand during early stage of embryonic development (Kadam *et al.* 2008). To support this assertion, Ohta *et al.* (1999) observed improvement in ratio of body weight of hatched chicks to egg weight when 53 mg AA/egg were injected into broiler breeder eggs at 7th ED.

Hatchability on a fertile egg set basis was better in chicks treated with 45 mg Thr/egg compared to other Thr-injected groups. This might suggests that higher dose of *in ovo* Thr solution could enhance hatchability. Reduced hatchability (P<0.001) observed in chicks that received 15 mg Thr/egg compared to other groups could be attributed to the pH of the substrate at the time of injection. Because L-Thr used in this study was extra pure (99% purity), and not monohydrochloride, the pH of Thr solution reduces as Thr concentration increases. Reijrink *et al.* (2008) had noted that pH of fertile eggs plays an important role in embryo development and hatchability due to its influence on embryo micro-environment within the egg, gases exchange, as well as transport of nutrients and electrolytes (Na^+ and K^+). Previous work by Alabi *et al.* (2018) reported that the yolk pH maintained slight fluctuation from 11th day of incubation until day of hatching due to the nature of its buffer system (non-bicarbonate based), movements of water from albumen to yolk (Reijrink *et al.* 2008), and nutrients from yolk to embryo (Uni *et al.* 2012). Therefore, pH of the *in ovo* supplement should be adjusted close to that of embryo yolk pH prior to *in ovo* injection.

Growth performance: Growth performance measurements as affected by *in ovo* Thr are presented in Table 3. Chicks that received an *in ovo* injection of Thr 45 mg had highest (P<0.001) ADWG (22.61 g) at 2nd week than other groups. Also, there was quadratic effect on ADWG at the 2nd (P<0.001) and 3rd (P=0.004) weeks due to dietary treatment. This suggests that *in ovo* Thr could exert its growth-enhancing effects during starter phase which is characterized by massive cell differentiation, muscle tissue synthesis and maintenance of body protein turnover, and that chicks could require higher dietary Thr level for body gain at this stage. Earlier studies by Kadam *et al.* (2008) have shown that broiler chicks given *in ovo* Thr injections had heavier BWs from 14 to 28 d than untreated controls. Rasheed *et al.* (2018) attributed higher weight gain and better FCR with increasing levels of Thr in the diets of Japanese quails to improved nutrient digestion and nitrogen retention due to higher amylase secretion in the digestive tract. In support, Canogullarý *et al.* (2009) observed that increasing Thr levels in laying quails improved FCR. However, in the present study, higher (P=0.001) ADWG was observed in T2 at the 5th week. Along with our findings, Najafi *et al.* (2017) found that increase in dietary Thr level from 0.65 to 0.89 g/kg diet improved BWG and FCR of broilers in the 2nd week and 1 to 14 days period without significant influence on feed intake at this period. Baylan *et al.* (2006) observed significant increase in BWG and improved FCR in growing quails fed 0.96% dietary Thr in 1–21 and 1–28 days period with no significant influence on feed intake. Debnath *et al.* (2019) also reported both linear and quadratic response in average daily gain in 21 d broilers with increase in dietary threonine levels while Ayasan *et al.* (2009) observed linear increase in BWG and threonine intake in finishing broilers (22–42 d) as dietary threonine level increases. In a study by Bhanja *et al.* (2012), BWG in broilers did not vary at the starter phase, but in finisher phase, due to IOF of amino acids among the treatment groups. Toghyani *et al.* (2019) observed higher BW in 42 d broiler chickens fed IOF of 25 mg Thr than broilers who received 35 mg Arg or combination of Arg + Thr.

Feed intake (FI) is an essential key to producing heavy broilers due to interactions between nutrients intake, BWG and livability of broilers. Voluntary FI is often influenced by the energy density, levels of other nutrients as well as diet's quality (digestibility and bioavailability of ingested feeds). Average daily feed intake (ADFI) did not differ

Table 3. Effect of *in ovo* L-Threonine on growth performance of broiler chickens

Item	Control		Thr (mg/egg)			Pooled	Significance#		
	Un-injected (T1)	Sham (T)	15 (T3)	30 (T4)	45 (T5)	SEM	T	L	Q
Average daily weight gain (g)									
1st week	8.79	9.26	9.09	8.97	9.32	0.13	0.699	0.568	0.809
2nd week	21.03ab	20.13b	20.67b	18.24c	22.61a	0.31	<0.001	0.375	<0.001
3rd week	34.74	36.89	37.80	37.98	34.98	0.45	0.045	0.554	0.004
4th week	41.60	43.79	42.09	42.73	43.26	0.50	0.668	0.618	0.820
5th week	42.77b	49.66a	42.95b	42.39b	45.11ab	0.70	0.001	0.424	0.790
6th week	46.29	48.02	51.60	48.73	48.06	0.74	0.243	0.481	0.061
1–6 week	32.53b	34.63a	34.03ab	33.17ab	33.89ab	0.49	0.044	0.419	0.106
Average daily feed intake (g)									
1st week	12.46	11.91	12.45	12.43	12.69	0.14	0.489	0.514	0.441
2nd week	35.04	34.93	35.32	35.17	34.87	0.18	0.940	0.924	0.576
3rd week	59.88	60.59	64.23	62.97	61.14	0.67	0.224	0.259	0.088
4th week	88.79	90.92	88.90	91.55	94.27	1.39	0.742	0.251	0.616
5th week	107.48	114.02	109.51	112.71	110.23	0.86	0.108	0.459	0.128
6th week	120.33	117.86	123.35	123.48	124.73	1.24	0.406	0.116	0.837
1–6 week	70.66	71.71	72.29	73.05	72.99	1.13	0.553	0.104	0.634
FCR (g/g)									
1st week	1.42	1.29	1.37	1.40	1.37	0.02	0.163	0.686	0.732
2nd week	1.68bc	1.74b	1.71b	1.93a	1.54c	0.03	<0.001	0.991	0.7021
3rd week	1.73	1.65	1.70	1.66	1.75	0.02	0.428	0.485	0.555
4th week	2.14	2.09	2.11	2.14	2.18	0.02	0.722	0.405	0.353
5th week	2.52ab	2.31b	2.58ab	2.66a	2.45ab	0.04	0.046	0.270	0.161
6th week	2.61	2.47	2.40	2.57	2.60	0.03	0.228	0.791	0.002
1–6 week	2.04ab	1.96b	2.01ab	2.08a	2.02ab	0.02	0.011	0.337	0.365
Production Index									
1st week	43.43	50.90	46.51	45.92	48.00	1.18	0.374	0.586	0.527
2nd week	89.12b	81.68b	84.88b	66.37c	102.63a	2.47	<0.001	0.300	<0.001
3rd week	141.37	157.99	156.95	160.91	140.71	3.29	0.129	0.949	0.014
4th week	136.62	149.00	140.00	140.00	139.20	2.18	0.468	0.791	0.353
5th week	119.77b	152.51a	119.16b	112.19b	129.67ab	3.78	0.003	0.364	0.900
6th week	124.85	138.73	152.03	135.74	129.85	3.69	0.178	0.782	0.027
1–6 week	109.19b	121.8a	116.59ab	110.19b	115.01ab	3.71	0.002	0.948	0.072

abcMeans with different superscripts within the same row differ significantly (P<0.05).

within experimental birds during the entire experimental period irrespective of the Thr levels (P>0.05). This is an indication that nutrient profile in feeds offered to the chicks was adequate to supply the needed dietary nutrients for birds' maintenance and productive functions. This response is in line with the studies of Ayasan *et al.* (2009) where broiler's feed intake was not affected by dietary threonine supplementation. However, the present findings did not agree with those of Kadam *et al.* (2008) and Toghyani *et al.* (2019) who observed higher FI in broilers injected different levels of *in ovo* Thr. Furthermore, studies by Estalkhzir *et al.* (2013) and Debnath *et al.* (2019) found that average FI increased significantly in broilers as dietary Thr levels increases. Rasheed *et al.* (2018) indicate higher FI in Japanese quails fed control diet compared with dietary Thr supplemented diets.

FCR measures the efficient utilization of dietary nutrients for BWG. Better FCR value often indicates reduced FI and/ or higher BWG on the same amount of feed. In this present study, IOF of 45 mg Thr resulted in better (P<0.05) FCR at 2nd week among the injected groups while FCR was significantly poorer (P<0.05) in group T4 at 2nd and 5th weeks. FCR also exhibited quadratic effect (P=0.002) at the 6th week. This finding is in congruence with the reports of Kidd *et al.* (1996), Ayasan and Okan (2006) and Estalkhzir *et al.* (2013) who found significant improvements in FCR values as a result of dietary Thr supplementation in broilers. However, this finding contradicts the studies done by Bhanja *et al.* (2012), Shafey *et al.* (2014) and Toghyani *et al.* (2019) who reported no significant difference in the FCR of birds injected with individual or combined AAs throughout the entire experimental period. Also, Azzam and El-Gogary (2015) and Debnath *et al.* (2019) observed no variation in FCR with the increasing level of dietary threonine. Production index (PI) was significantly higher in groups T5 and T2 at the 2nd and 5th week, respectively. An increasing concentration of Thr had quadratic effect on PI at the 2nd (P<0.001), 3rd (P=0.014) and 6th (P=0.027) week. Significantly higher PI in broilers with *in ovo* Thr was a reflection of higher body gain and better feed utilization as feed consumption was similar among the treatment groups. This might be attributed to the crucial role of Thr in secretion of mucin, digestive enzymes, and enhanced development of digestive system for optimum nutrient absorption and assimilation (Kadam *et al.* 2013, Toghyani *et al.* 2019).

Relative weight of lymphatic organs: The effects of *in ovo* Thr administration on lymphatic organs of broiler chicks at d 0, 3, 7, 21, and 42 are shown in Table 4. *In ovo* supplementation with 45 mg of Thr resulted in higher thymus weight (P=0.024; linear, P=0.036; quadratic, P=0.022) at day of hatch when compared with other groups. Chicks that received *in ovo* Thr 30 mg had higher bursa weight (P=0.012) among the injected groups at d 3. More so, *in ovo* Thr injection produced quadratic effect on spleen weight on d 3 (P=0.028) and d 7 (P=0.048). At 21 d, there were quadratic increase in the weights of bursa (P=0.038), thymus (P=0.013) and spleen (P=0.020). Meanwhile, no variation was observed in the lymphoid organs weight at 42 d. Lymphatic organs are important component of adaptive or acquired immune system which consists of T lymphocytes, B lymphocytes and humoral factors (Erf 1997), and increased in their relative weights has been considered to be positively correlated with the improvement in immunity of broiler chickens (Toghyani *et al.* 2019). Bursa of Fabricius plays active role in antibody production through the amplification and differentiation of B lymphoid progenitors within its follicular micro environment which ensure functional maturation of the B cell, while thymus provides a unique microenvironment for differentiation and functional maturation of T lymphocyte precursors into immunocompetent lymphocytes (Erf 1997). The spleen provides a site for proliferation of immune cells (lymphocyte) within its tissue because poultry birds lack lymph nodes (Toghyani *et al.* 2019). It also exerts its immune surveillance by developing immune response against antigens circulating in the bloodstream and, by its blood-cleansing functions, removes defective blood cells, debris and foreign matter from blood flowing through its sinuses (Erf 1997).

Increased in lymphatic organs weight of broiler chicks following *in ovo* Thr administration (either among the treatment groups, linearly or quadratically) up to d 21 in this trial is an indication of positive influence of Thr on enhancement of immunity status. Previously, Thr was well-known to be an immunostimulant which promotes the growth of thymus gland, and can probably promote cell immune defense function. These results are in agreement with previous studies by Bhanja *et al.* (2004) who reported that thymus and spleen weights were comparatively higher in AA injected group than control birds. In another study, Bhanja *et al.* (2012) showed that weight of bursa of Fabricius and spleen were higher in *in ovo* injected groups compared to sham and un-injected controls, respectively.

Table 4. Effect of *in ovo* L-Thr on lymphatic organs weights of broiler chicks

Item (g/kg BW)	Control		Thr (mg/egg)			Pooled	Significance#		
	Un-injected (T1)	Sham (T2)	15 (T3)	30 (T4)	45 (T5)	SEM	T	L	Q
d 0 (*day of hatch*)									
Bursa	0.91	1.20	1.10	0.83	1.17	0.01	0.255	0.72	0.901
Thymus	0.83^{b}	0.88^{b}	0.74^{b}	0.76^{b}	1.37^{a}	0.01	0.024	0.036	0.022
Spleen	0.44	0.42	0.80	0.70	0.61	0.01	0.065	0.062	0.119
d 3									
Bursa	1.52^{b}	1.47^{c}	1.36^{c}	1.86^{a}	1.60^{b}	0.02	0.012	0.255	0.740
Thymus	1.00	0.95	1.14	0.99	1.23	0.02	0.638	0.308	0.670
Spleen	0.76	0.81	0.76	0.64	0.55	0.01	0.168	0.275	0.028
d 7									
Bursa	2.32	2.38	1.95	2.01	2.52	0.02	0.342	0.964	0.112
Thymus	1.25	1.14	1.04	0.78	1.15	0.02	0.337	0.300	0.202
Spleen	1.18	0.92	0.93	1.00	1.33	0.02	0.306	0.466	0.048
d 21									
Bursa	4.10	3.17	3.24	3.23	3.35	0.04	0.436	0.259	0.038
Thymus	1.60	1.43	1.36	1.35	1.87	0.01	0.058	0.289	0.013
Spleen	1.74	1.50	1.96	1.25	1.58	0.02	0.194	0.391	0.020
d 42									
Bursa	2.75	2.53	2.73	2.65	2.23	0.03	0.716	0.33	0.544
Thymus	1.19	1.23	1.23	1.29	1.15	0.01	0.962	0.983	0.562
Spleen	2.83	2.58	2.27	2.33	1.97	0.04	0.600	0.122	0.918

[abc]Means with different superscripts within the same row differ significantly (P<0.05). SEM, Standard error of means. #Probability of a significant effect of treatment (T), or linear (L) and quadratic (Q) orthogonal contrasts for *in ovo* Thr levels.

Gaafar *et al.* (2013) observed higher relative weights of bursa, thymus and spleen in male and female Muscovy ducks injected with 0.50 ml AA, while 0.75 ml AA increased lymphoid organ weights in male ducks only as compared to the control group. Debnath *et al.* (2019) recorded linear increase in weights of Bursa of Fabricius and thymus with the increased dietary L threonine supplementation. On the contrary, Bhanja and Mandal (2005) and Kadam *et al.* (2008) found no significant changes in the lymphoid organs of 21 day-old broiler chicks following *in ovo* AA and Thr supplementation, respectively. No variation in lymphatic organs at d 42 post-hatch, as observed in this current study, have been reported following *in ovo* AA injection (Toghyani *et al.* 2019) or in Thr supplemented diets (Azzam and El-Gogary 2015, Sigolo *et al.* 2017). This is probably because lymphoid organs become fully mature at 21 d in modern day broiler strains and that the effect of *in ovo* or dietary Thr might not exert any significant effect on organs development after the starting phase.

Relative weight of digestive organs: The gastrointestinal tract (GIT) of broiler chickens plays crucial role in nutrient digestibility and FCR. In addition, the GIT tissue acts as a physical and immunological barrier to the harmful chemicals and infectious agents that enter the host (Svihus 2014, Jha *et al.* 2019). In the current study, *in ovo* Thr injection enhanced the relative weights of gizzard (P=0.003; quadratic, P=0.008) and intestine (P=0.018; quadratic, P=0.011) while liver tended to increase both linearly (P=0.056) and quadratically (P=0.041) at day of hatch (Table 5). No significant variation was seen in the relative organs weight at d 3. At d 7, proventriculus weight was significantly higher (P<0.05) in chicks that received 15 mg Thr (T3) when compared with the sham control (Group T2). An increasing concentration of Thr increased the

Table 5. Effect of *in ovo* L-Thr on digestive and immune organs weight of broiler chicks

Item (g/kg BW)	Control		Thr (mg/egg)			Pooled	Significance#		
	Un-injected (T1)	Sham (T2)	15 (T3)	30 (T4)	45 (T5)	SEM	T	L	Q
day of hatch									
Gizzard	38.64^{b}	54.83^{a}	53.97^{a}	40.65^{b}	46.50^{ab}	0.29	0.003	0.867	0.008
Heart	8.77	8.93	9.36	9.39	9.28	0.07	0.956	0.514	0.723
Liver	20.78	26.59	26.95	27.31	26.67	0.19	0.136	0.056	0.041
Intestine	36.87^{c}	41.64^{b}	50.86^{a}	39.87^{b}	39.15^{b}	0.48	0.018	0.856	0.011
Proventriculus	7.99	9.19	10.11	8.31	8.58	0.10	0.559	0.924	0.218
d 3									
Gizzard	108.30	114.35	94.46	94.82	93.95	0.52	0.093	0.011	0.756
Heart	8.59	8.58	9.50	8.52	8.65	0.06	0.798	0.976	0.508
Liver	46.86	45.32	45.37	39.36	45.32	0.31	0.503	0.375	0.456
Intestine	111.85	116.86	110.16	118.97	113.58	0.79	0.930	0.827	0.861
Proventriculus	13.36	14.42	12.58	12.86	13.16	0.11	0.819	0.596	0.888
d 7									
Gizzard	87.18	77.29	82.10	95.88	82.32	0.44	0.077	0.529	0.922
Heart	7.63	8.57	7.86	8.75	8.29	0.07	0.768	0.510	0.653
Liver	38.33	41.45	36.80	38.52	39.42	0.21	0.617	0.909	0.807
Intestine	144.75	130.34	137.67	149.89	151.89	0.73	0.256	0.164	0.188
Proventriculus	13.85^{ab}	11.60^{b}	15.01^{a}	14.00^{ab}	14.56^{ab}	0.07	0.041	0.117	0.664
d 21									
Gizzard	32.57	32.81	34.26	29.71	33.79	0.22	0.511	0.708	0.219
Heart	6.75	7.09	7.09	6.02	7.57	0.05	0.247	0.712	0.047
Liver	26.51	29.12	31.74	31.36	32.17	0.17	0.171	0.026	0.17
Intestine	92.51^{b}	82.32^{b}	102.84^{ab}	97.08^{ab}	107.70^{a}	0.57	0.045	0.024	0.568
Proventriculus	8.10	8.94	9.09	8.51	8.83	0.06	0.793	0.602	0.061
Pancreas	4.46	4.94	4.91	4.57	4.94	0.03	0.764	0.589	0.034
d 42									
Gizzard	25.19	22.59	25.71	23.18	23.90	0.13	0.401	0.627	0.837
Heart	5.30	4.92	5.27	5.93	5.63	0.03	0.207	0.093	0.685
Liver	27.42	23.28	24.18	24.43	23.35	0.13	0.191	0.105	0.276
Intestine	61.23^{a}	55.94^{ab}	52.24^{bc}	49.35^{bc}	46.33^{c}	0.30	0.014	0.001	0.632
Proventriculus	6.29	5.43	5.39	5.23	5.81	0.04	0.254	0.311	0.065
Pancreas	2.82	3.00	3.06	2.83	3.18	0.02	0.478	0.295	0.925

[abc]Means with different superscripts within the same row differ significantly (P<0.05). SEM = Standard error of means. [#]Probability of a significant effect of treatment (T), or linear (L) and quadratic (Q) orthogonal contrasts for *in ovo* Thr levels.

weights of intestine (P=0.045; linear, P=0.024) and liver (quadratic, P=0.026) at d 21. However, at d 42, weight of intestine decreased (P=0.014, linear, P=0.001) as the Thr concentration increased. Previous investigations showed that digestive organs of avian embryos undergo series of morphological, cellular, and molecular changes during late-term stage to few days post-hatch which causes maximal relative size of these organs in preparation for digestion and utilization of exogenous dietary nutrients since chickens are precocial with the ability to feed almost immediately after hatch (Alabi *et al.* 2018, Jha *et al.* 2019). As stated by Svihus (2014), the direct relationship between size of the gizzard and weight of gizzard contents has been recognized, and increase in its size connotes increased grinding activity which would stimulate an upsurge in pancreas and liver secretions thereby leading to improvement in birds' ability to digest and absorb nutrients.

Higher relative weights of digestive organs observed in this study could be connected to higher daily weight gain and improved growth performance during starter phase. The results of this study further confirm the role of Thr in functional development of digestive and immune organs, synthesis of mucosal and digestive enzymes, as well as enhancement of gut health (Kadam *et al.* 2008, Jha *et al.* 2019). In agreement with the results of the current work, Awachat *et al.* (2017) found that IOF of AA improved the relative weights of gizzard, proventriculus and small intestine during the immediate post-hatch period, but not during post hatch growing periods. However, these results are in contrasts with previous studies which reported no significant difference in the weight of digestive organs of chicks following *in ovo* injection of AA, either individually or in combination, at day old age (Bhanja *et al.* 2012, Toghyani *et al.* 2019) or at 21 d post hatch (Bhanja and Mandal 2005).

In vivo *humoral and cell-mediated immune response:* As an important structural component of mucin and immunoglobulins, Thr is known to modulate immune functions. The results of *in ovo* Thr supplementation on immune response revealed no significant difference (P>0.05) in cell-mediated immune response (*in vivo* PHA-P response as footpad index) and humoral response to SRBC inoculation among the different *in ovo* injected groups. This could be adduced to the non-significant variation in the weights of lymphatic and immune organs after 21 d post hatch. The reason might be that the effects of *in ovo* Thr injected in chicks had worn out prior to inoculation of the antigens since the birds were fed a common diet containing similar basal Thr level. This correlates with previous studies of Bakyaraj *et al.* (2012) and Gaafar *et al.* (2013) which demonstrated that IOF of AA blend containing Thr did not affect the antibody titer against SRBC in broilers and Avian Influenza disease virus in Muscovy ducks, respectively as compared to control group. In addition, Kadam *et al.* (2007) did not find difference in humoral and cellular immune responses in broiler chickens fed AA supplemented low-CP diets. Conversely, Kadam *et al.* (2008) demonstrated that IOF of Thr improved the humoral response, but not inflammatory response to PHA-P antigen, in broiler chickens. Meanwhile, Bhanja and Mandal (2005) found significant difference in the cell mediated and humoral immune response in *in ovo* AA injected broilers. Therefore, further investigations deserve to be carried out in order to ascertain the effect of *in-ovo* injection of Thr on the humoral and cell mediated immunity in broiler chicks during early post-hatch period.

Carcass yield: Research evaluating the effect of *in ovo* Thr on carcass yield of broilers is sparse. The carcass traits of broiler chickens as influenced by in ovo Thr supplementation are shown in Table 6. No significant (P>0.05) variation was seen in the dressed yield and primal cuts among the treatment groups, except breast meat which was significantly higher (P<0.05) in sham control. This is in congruence with the observed results of growth performance and organs weight at the finisher phase. Breast meat is most valuable and preferred part in the carcass of broiler chickens (Baylan *et al.* 2006). Hence, optimum concentration of *in ovo* L-Thr required to achieve higher breast meat yield in broiler chickens needs to be established. Results of the current experiment are in line with the findings of Bhanja *et al.* (2004) and Gaafar *et al.* (2013) where carcass and cut-up parts yield did not vary between AA injected and control birds in broiler chickens and Muscovy ducks, respectively. Previous studies also reported no significant variation in breast and thigh yields with dietary L-threonine inclusion in Japanese quails (Baylan *et al.* 2006) and broilers

Table 6. Effect of *in ovo* L-Thr on carcass traits of broiler chickens

Item (% BW)	Control		Thr (mg/egg)			Pooled	Significance[#]		
	Un-injected (T1)	Sham (T2)	15 (T3)	30 (T4)	45 (T5)	SEM[$]	T	L	Q
Dressed yield	63.91	62.69	63.72	61.62	62.47	0.56	0.720	0.346	0.838
Breast	17.30[ab]	17.69[a]	16.70[ab]	15.31[b]	16.80[ab]	0.15	0.046	0.057	0.382
Back	15.44	14.15	15.47	15.74	15.15	0.22	0.178	0.521	0.841
Drumstick	8.82	8.66	9.10	8.82	9.06	0.11	0.628	0.415	0.912
Thigh	10.34	10.31	10.29	10.39	10.26	0.15	0.999	0.941	0.952
Wings	7.82	8.02	8.04	8.11	8.63	0.13	0.379	0.074	0.517

[ab]Means with different superscripts within the same row differ significantly (P < 0.05). [$]SEM, Standard error of means. [#]Probability of a significant effect of treatment (T), or linear (L) and quadratic (Q) orthogonal contrasts for *in ovo* Thr levels.

(Rezaeipour and Gazani 2014). Similarly, Debnath *et al.* (2019) observed no difference in carcass yield, except breast meat, of broiler with increase in dietary L threonine supplementation. However, Kheiri and Alibeyghi (2017) observed higher carcass, breast, and thigh yields in chicks that received higher Thr concentration. Earlier studies reported improved carcass weight and breast meat yield in broiler chickens (Hosseinpour *et al.* 2012, Estalkhzir *et al.* 2013, Ayasan and Okan 2014) and Japanese quails (Rasheed *et al.* 2018) fed dietary Thr-supplemented diets.

Summarizing, the results of the present study suggested that *in ovo* supplementation of Thr can improve growth performance, digestive and immune organs development at the early age. However, humoral and cellular immunity as well as carcass yield were not influenced following *in ovo* Thr injection. Further studies are needed to authenticate the role of *in ovo* injected Thr on immunity status of broiler chicks at early post-hatch period.

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