Theses of Doctoral (PhD) Dissertation

Physiological responses to DL-methionine and L-methionine supplementation in the early life of TETRA-SL LL hybrid and Hungarian Partridge Colored Hen genotypes

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1. INTRODUCTION AND AIM OF THE THESIS

The main challenge for the coming century in livestock farming systems includes meeting the high predicted demand for meat due to the human population, which is increasing exponentially. To overcome that challenge, farmers must precisely control animal performance by enhancing the quantification of animal requirements (EL-TARRAS *et al.* 2019). Methionine supplementation maximises the bird's performance (MAJDEDDIN *et al.* 2019). Methionine is considered the first limiting amino acid for birds, primarily due to amino acid demand for muscle and feather metabolism and the composition of feed used (KIM *et al.* 2019; REHMAN *et al.* 2019; CHEN *et al.* 2020). Protein supplementation in the bird's diet traditionally involved groundnut and fish meals. Nevertheless, despite the limited availability of the quality of fish meal and poor quality protein, and the mycotoxins problem of groundnut meal, soybean meal has been used as the primary source of supplementation in the poultry diet along with cereal maize (corn) (ZHANG 2016). However, the amino acid methionine (Met) is the first limiting in the commercial soybean-corn meal-based diet of poultry. Therefore, supplementation with limiting amino acids in the poultry diet is required to reduce protein-rich feedstuffs while maintaining performance and reducing nitrogen excretion through better utilization for protein synthesis (MANDAL *et al.* 2004; SHEN *et al.* 2015)**.** Met is the only amino acid obtained by chemical synthesis, resulting in a ceramic mixture of D and L forms (DL–Met). Met can be supplemented in the feed (ULLRICH *et al.* 2019) in drinking water (CADIRCI & KONCAGUL 2014) as well as *in-ovo* feeding (CHEN *et al.* 2020) as L- methionine (L-Met), a mixture of the D and L forms (DL-Met), and the methionine analogue, DL-2hydroxy-4-(methylthio) butanoic acid (DL-HMTBA). DL–Met is the most common form of sulfur amino acid supplementation for birds (AGOSTINI *et al.* 2016; ZHANG 2016)**.** However, recently, L-Met became widely available and registered as an additive feed for poultry (MILLECAM *et al.* 2020).

Few studies have investigated the effect of DL-Met and L-Met sources on growth performance, intestinal development and health and antioxidant status of poultry via dietary supplementation but not during embryonic development. The dietary supplementation revealed that either DL-Met or L-Met affected the growth performance of ducks in the starter phase and enhanced the small intestinal morphology and feather development (ZHANG *et al.* 2019). However, they reported that L-Met was more effective than DL-Met, with a range of 12% to 140% for the growth performance at the starter phase and 153% for the feather traits at the finisher phase. The same trend was reported in broiler chicks, where L-Met improved the redox status and intestinal development compared with DL-Met (SHEN *et al.* 2015). Furthermore, L-Met showed

a positive effect on the intestinal development, immune responses and antioxidant system of broilers challenged with *Eimeria spp*. (TENG *et al.* 2023). Contrarily, another current study has shown that both Met sources (DL-Met, L-Met and MHA-FA) had a similar effect on intestinal barrier function and microbiota in broilers (BAREKATAIN & KLUENEMANN 2023).

The *in-ovo* injection strategy overcomes these issues, bridges the gap between hatching, and provides a tool to overcome the imbalances between antioxidants and pro-oxidants (KADAM *et al.* 2013). Studies have shown that the amnion is an effective site for implying the *in-ovo* injection method, and the embryo ingests the amniotic fluids before pipping. Therefore, this procedure is called *in-ovo* feeding (DANG *et al.* 2022b). *In-ovo* feeding provides nutrients to chicks at the critical stage, facilitating embryo development and post-hatch growth performance (JHA *et al.* 2019). The high demand for protein for growth and to reduce the negative effect of oxidative stress during late embryonic development are shown to be achieved by the in-feeding of specific nutrients (DANG *et al.* 2022a). *In-ovo* feeding of methionine (Met) has been shown not only to alleviate oxidative stress (BHANJA *et al.* 2012; ELWAN *et al.* 2019; DANG *et al.* 2022a) but also to be used as a source of amino acids for protein synthesis and, hence, decrease protein-based gluconeogenesis in hatchlings (COSKUN *et al.* 2018).

Therefore, the present research program aims to study the physiological responses of laying birds TETRA-SL and Hungarian partridge colored hen (HPC) to DL-Met and L-Met supplementation. Therefore, the outcomes from this study are expected to give significant results not only on the physiological responses of layers and chicks (embryo) but also on direct applicability for industrial use and to poultry farmers.

In general, this Ph.D. programme aimed to evaluate the physiological responses of newly hatched and young chicks to *in-ovo* and dietary supplementation of DL-Met and L-Met in **TETRA-SL LL hybrid (TSL)** and Hungarian partridge colored hen breed (**HPC)** layer genotypes. The specific objectives of the thesis are as follows:

- ✓ To determine the effect of *in-ovo* injection of DL and L-Met on blood biochemical parameters (AST, ALT, uric acid, FRAP) and jejunum morphology of TSL and HPC chicks.

- ✓ To examine the influence of *in-ovo* injection of methionine sources on antioxidant status (GSH, TAC) in the liver, intestine, and pectoral muscles of the newly hatched chicks of the two genotypes.
- ✓ To evaluate the effect of *in-ovo* feeding of methionine sources on the gene expression related to growth, antioxidant status, and intestinal tight-junction proteins of the newly hatched chicks of TSL and HPC.
- ✓ To assess the biological efficacy of DL and L-Met supplementation on growth performance, feathers development and hematological parameters of the TSL chicks from day 1 to 28 days of life.

2. MATERIALS AND METHODS

Experiments were conducted at the Kismacs Experimental Station of Animal Husbandry University of Debrecen under ethical guidelines and national authority approval (6/2021/DEMÁB). Two experiments were conducted to meet the dissertation objectives: in-ovo feeding and dietary supplementation of Met experiments.

2.1. Experiment I: Effect of *in-ovo* application of different Met sources and dosages on the physiological parameters of layer chicks

2.1.1. Hatching eggs

A total of 570 hatching eggs with correct shape were obtained, 360 eggs for TSL genotypes were procured from the TETRA Bábolna Ltd. (Bábolna, Hungary), and 210 eggs for HPC genotypes were collected from the gene reserve flock of the University of Debrecen. All the eggs were marked with T for TSL and H for HPC, respectively, and eggs were fumigated before incubation.

2.1.2. Incubation and experimental setup

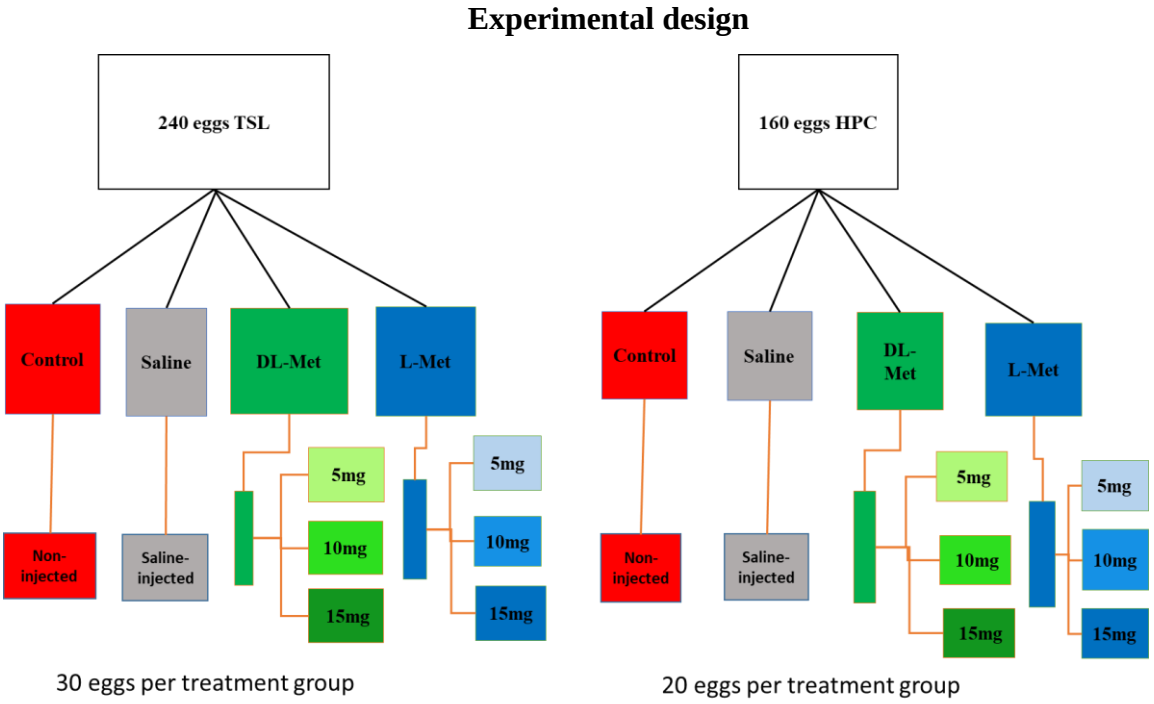
All eggs were initially stored at 21°C for 24 hours. They were then incubated at a temperature of 37.8°C with a relative humidity of 60% until day 17.5, as per the protocol described by Fasenko in 2007. From 17.5 to 21 d of incubation, the relative humidity was raised to 65-70%. The eggs were placed and incubated in an automatic egg-turning incubator (PLM 3600, PL Maschine KFT Budapest-Hungary), which turned them automatically every hour. On the 10^{th} day, the eggs were candled, and non-fertile eggs and dead embryos were removed. In total, 34 infertile eggs and 11 early dead embryos were removed from the incubator for HPC, while 21

infertile eggs and 7 early dead embryos were removed for TSL. The unfertilised and dead embryos were sorted based on illumination, as per the protocol described by CHEN *et al.* (2020).

On the 17.5th day of embryonic development, the eggs were candled again and randomly allocated to *in-ovo* treatments. A total of 400 embryonated eggs were divided into16 groups (8 groups for each genotype), with 30 eggs for TSL and 20 eggs for HPC genotype. The treatment groups consisted of non-injected (Control), saline solution injected (0.5 ml), and 5, 10 and 15 mg of either DL-Met or L-Met injected (in 0.5 ml saline solution) for both HPC and TSL breeds (Figure 1). The treatment was set up as a 2x2x3 factorial arrangement based on two sources of Met (DL and L-Met), 2 genotypes of layers (TETRA–SL and HS breeds), and 3 doses of Met for each source.

Met sources, L-Met and DL-Met were purchased from Evonik Nutrition and Care GmbH or Sigma Aldrich with a purity greater than 99.5% from Germany. The solution of Met sources was prepared by dissolving them in normal saline (0.75% NaCl) of the desired concentration. The procedure involved disinfecting the surface of every egg with a 70% ethanol-soaked cotton ball before injection. A small hole with a 2mm diameter was then made at the big end of the egg using a drill. The egg was injected with 0.5 mL solution containing Met a dose of 5, 10, or 15 mg either L or DL-Met into the amniotic sac using a 21-gauge needle. After injection, the holes were immediately sealed with hot paraffin and returned to the incubator. All eggs were maintained outside the incubator during the injection for less than 30 minutes.

Figure 1



TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed

2.1.3. Sample collection

The weight of the chicks was recorded on the day of hatching and the day of sampling, along with their liver and heart weight. They were then euthanized by cervical dislocation for blood and tissue sampling. Blood samples (about 1 mL) were collected in EDTA-coated tubes from the jugular vein on the day of hatching and centrifuged at 3,000x g for 10 minutes at room temperature to separate the plasma. The plasma samples were stored at -80°C for further analysis. The liver, small intestine, and pectoral muscle tissues were snap-frozen in liquid nitrogen and stored at -80°C for further analysis. Additionally, about 1 cm of the jejunum was taken, washed with buffer, and fixed in 10% formalin for histology examination.

2.1.4. Plasma biochemical analysis

In this study, blood samples were collected by spinning at 3000 g for 10 minutes and stored at -80°C. A kit protocol was used to determine the Ferric Reducing Ability of Plasma (FRAP). The assay relies on the reduction of oxidized Fe III to Fe II in acidic conditions, forming a blue-colored complex. To prepare the 1X assay buffer, 7 mL of buffer 10X was diluted with 63 mL of deionized water. Plasma samples were diluted by combining 20 µL of plasma with 40 µL of 1X assay buffer. Meanwhile, standards were prepared by adding 20 µL of 10mM ferrous

Chloride to a tube containing 180 µL 1X assay buffer. A serial dilution of the standards was made. 75 µL of FRAP color solution was added to each well for the FRAP assay. After that, 20 µL of standard or diluted samples were introduced. The mixture was then incubated for 30 minutes at room temperature, and the absorbance was read at 560 nm. The unknown sample concentration was determined from the standard curve and expressed as µM FeCl2 equivalent. In addition, the blood samples were analyzed for AST, ALT, and uric acid using an auto-analyzer (Lab-Analyse 10261, OrvosTechnika Kft Budapest) and its corresponding kits. Three replicates were performed for each parameter, with uric acid measured via the endpoint method and AST and ALT measured via the Kinetic method. The units used were µmol/L for uric acid, U/L for AST and ALT.

2.1.5. Histology analysis

To analyze the histomorphology of the jejunum, we collected five samples for each treatment before Merkel's diverticulum. These samples were washed in a buffer solution and fixed in 10% buffered formalin. Following standard histological laboratory procedures, we dehydrated the samples in ethanol and embedded them in paraffin. Hematoxylin-eosin stain was used to stain tissue sections of four micrometres, which were then observed under a light microscope with a magnification of 200x. To determine the villus height and crypt depth, we randomly selected thirty (30) villi per chick from the cross-section of each individual. The villus height (VH) was measured from the villus tip to the villus-crypt junction, while the crypt depth (CD) was calculated as the depth between two villi invaginations. We determined the villus height, width, area, and crypt depth. Villous width (VW) was measured at three points: the base (VBW), middle (VMW), and the tip of the villous height (VAW). The villus surface area (VA) was calculated using the villous height and width at half-height by UNI *et al*. (2003). The villus surface area was determined using the formula $2\pi \times VH \times (VW/2)$. All measurements were taken using an Olympus light microscope and Olympus CellSens software.

2.1.6. Tissues Antioxidants analysis

2.1.7. Gene expression analysis

2.1.7.1. Sample Preparation and Total RNA Isolation

Samples of the liver and intestine were homogenized using an ultraturax homogenizer, and total RNA was isolated using TRIzol reagents as per the Direct-zol RNA Miniprep, R2052 kit protocol. RNA quality was checked using the Qubit RNA IQ assay kit (# Q33222, Thermo

Fisher Scientific) and Qubit 4 fluorometer (Invitrogen by Thermo Fisher Scientific). The total RNA was either used directly to synthesize cDNA or stored at -80 ˚C for less than four days before cDNA was made.

2.1.7.2. cDNA synthesis and RT-qPCR

1 µg of total RNA was used to create cDNA using the LunaScript RT SuperMix Kit. The cDNA was diluted in a 1:5 ratio for qPCR analysis. The 5x HOT FIREPol EvaGreen qPCR Mix plus kit was used to amplify cDNA samples, which were run in duplicate using an AriaMx Real-Time PCR system. The 18sRNA was the reference gene for liver and intestine tissue. The relative mRNA expression was calculated using the $2^{-\Delta\Delta}$CT model (LIVAK & SCHMITTGEN 2001).

2.2. Experiment II: Early development of TSL chicks as affected by dietary methionine supplementation

2.2.1. Experimental animals, housing and dietary treatments

The Tetra SL LL (TSL) breeder eggs were obtained from Bábolna Tetra Ltd. (Bábolna, Hungary) and incubated at standard temperature and humidity (37.8 ˚C and 60 % Rh, PLM 3600, PL Machine KFT Budapest-Hungary). After hatching, a total of 96 one-day-old chicks were weighed and distributed to six treatments, with four replicates having four chicks (125 cm2/bird) per pen. The experimental design was a complete randomized block with a 2 x 3 factorial arrangement (2 Met sources x 3 Met levels). The chicks had similar initial body weight for each treatment, and pen, feed and water were provided ad libitum. The pens were bedded with wood shaving. Infrared lamps (optima plus II 175 W) provided extra heating in the pens, and the temperature in the pen was maintained according to the breeder's recommendation (BÁBLONA 2020). The chicks were fed with ad-libitum of a standard mash diet that was formulated according to the manual guideline of TSL, except for the Met levels. The diets were formulated to include 90, 100, and 110% of either DL (MetAmino, feed grade 99%, Evonik Degussa GmbH, Wesseling, Germany) or L- Met (L-Met 100, feed grade 99%, CJ Europe GmbH., Schwalbach/Taunus, Germany) of the nutrient requirements of the breeders.

2.2.2. Measurements

All chicks in each pen were included in measuring the length of the fourth primary feather and body weight weekly for four weeks (ZENG *et al.* 2015). The length of the feather was measured in millimetres using a Vernier-calliper with 0.01 mm accuracy. Due to the immeasurable

amount of feed waste and the fact that the experiment utilized layer genotype, the average feed intake and conversion ratio were not calculated in this study. No mortalities were observed for the entire experimental period.

2.2.3. Blood sampling and haematological analysis

At the end of the 28-day trial, 2 chicks were randomly chosen from each pen, making a total of 8 birds per treatment group. Blood samples were collected from the cutaneous ulnar vein, also known as the brachial wing vein, and put into EDTA-coated tubes following the procedures outlined in (KELLY & ALWORTH 2013). The samples were kept on ice and transferred to the laboratory, where they were allowed to reach room temperature and gently mixed before analysis. As per the Urit-3000Vet Plus automated Haematology analyzer operation manual, a pre-diluent method was used, where 20 µL of blood was pipetted and diluted to 1 mL dilution buffer. The following haematological parameters were analyzed: the number of red blood cells (RBC, 1012/L), haemoglobin (Hb, g/dL) concentration, haematocrit (Ht; %), the number of white blood cells (WBC, 109/L), and platelet count. Additionally, the following parameters were obtained: mean corpuscular volume of red blood cells (MCV, fL), mean corpuscular haemoglobin (MCH, pg), mean corpuscular haemoglobin concentration (MCHC, g/dL), lymphocyte percentage and count (LYM% and LYM#), mid-range (eosinophil + basophil) percentage and number (MID% and MID#), granulocyte percentage and number (GRAN% and GRAN#). The haematological parameters were analyzed using an automated haematology analyzer (URIT-3000 Vet Plus, Orvostechnika Ltd., Budapest), with triplicate readings and the average of the runs taken.

2.3. Statistical analysis

The statistical analyses were performed using R-version 4.2.2. The data from the *in-ovo* feeding experiment, the individual bird (chick), was considered an experimental unit for all parameters except for the histology data. For early development of the TSL experiment, the experimental unit for ADG was the pen (n = 4/treatment). In contrast, for the BW, FL (n = 16/treatment) and hematological parameters (n = 8/treatments), the individual birds were considered as the experimental unit. The data were checked for normality by using the Shapiro-Wilrk test. Data were analyzed using variance ANOVA analysis and a general linear model appropriate for evaluating the main effects (the genotypes, methionine levels, and dietary methionine sources). Data were presented as means, and the significance level for differences was set at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of *in-ovo* injection of methionine on TSL and HPC genotypes

3.1.1. Hatchability

In-ovo feeding of methionine sources decreased the hatching percentage in TSL and HPC genotypes (Table 1). The highest hatchability of 100% was observed in the control group, and the lowest was in the group injected with 5 mg of DL-Met (Table 1). Unlike arginine, methionine *in-ovo* feeding does not support embryonic development. The reduced hatchability of methionine-injected eggs could be due to factors such as high concentration of the amino acid solution and temperature instability during *in-ovo* operations. The hatchability of the eggs injected with 5 mg of methionine sources ranged from 72% to 90%, which is consistent with what has been reported in the literature. (Coşkun et al. 2014; Coskun et al. 2018; Elwan et al. 2021). The lowest hatchability (10%) was recorded in 15 mg DL-Met for TSL genotypes and 11% hatchability in 10 mg L-Met for HPC genotype (Table 1)

Table 1

Effect of *in-ovo* injection of different Met source and levels at 17.5th day of embryo development on hatchability of the TSL and HPC (%)[1]

Hatchability	**Control**	**Saline**	**DL-Met**			**L-Met**		
Genotype			5 mg	10 mg	15 mg	5 mg	10 mg	15 mg
TSL	100.0	88.9	90.0	30.0	10.0	75.9	44.8	23.1
HPC	89.1	84.2	72.2	31.6	22.2	72.2	11.8	17.6

[1] Hatchability (%) is calculated as the percentage of eggs hatched over the number of fertile eggs subjected to the respective treatment. TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed, Control: non-injected group, Saline: saline injected group, DLMet: DL-Met injected group, L-Met: L-Met injected group

3.1.2. Hatching body weight and relative liver and heart weight

The study found significant differences in chick-hatching weight and relative heart weight among treatments and genotypes (Table 2). DL-Met improved the hatching weight of the L-Met group. Inoculating solutions containing a variety of amino acids *in-ovo* enhanced hatching rates and birth weight. Feeding late-term embryos *in-ovo* enhanced hatching weight compared to controls. The liver relative weight was positively influenced by the *in-ovo* injection of methionine. L-Met *in-ovo* feeding at 5 mg/egg might be toxic in layers genotypes compared to DL-Met *in-ovo* feeding.

Table 2

Effect of *in-ovo* injection of 5 mg DL and L-Met in eggsof TSL and HPC genotypes at 17.5 days of incubation on hatching performance

Parameter		HBW (g)	ALW (g)	AHW (g)	RLW (%)	RHW (%)
Pooled effects						
Genotype	TSL	41.2 [a]	1.27	0.37 [a]	2.84 [b]	0.90
	HPC	35.7 [b]	1.15	0.33 [b]	2.97 [a]	0.92
Treatment	Control	39.7 [a]	1.17	0.36 [a]	2.99	0.92
	Saline	38.2 [ab]	1.17	0.34 [ab]	3.05	0.90
	DL-Met	39.5 [a]	1.21	0.37 [a]	3.08	0.95
	L-Met	36.5 [b]	1.10	0.32 [b]	3.02	0.88
P - values	Genotype	**0.0001**	0.6365	**0.0289**	**0.0321**	0.1641
	Treatment	**0.0350**	0.5025	**0.0380**	0.9011	0.5653
	Interaction	0.2186	**0.0279**	0.3487	0.0631	0.3736
RMSE		3.291	0.229	0.015	0.578	0.132
Treatment effect by genotype						
TSL	Control	43.6 [c]	1.20	0.38	2.78	0.88
	Saline	40.7 [c]	1.29	0.37	3.15	0.92
	DL-Met	41.6 [c]	1.12	0.37	2.71	0.89
	L-Met	38.3 [d]	1.08	0.34	2.81	0.88
	P-value	**0.0061**	0.2209	0.2898	0.3348	0.8756
	RMSE	2.7041	0.2113	0.0465	0.5145	0.1144
HPC	Control	35.69	1.11	0.35	3.16	0.98
	Saline	35.48	1.00	0.31	2.84	0.89
	DL-Met	37.71	1.33	0.37	3.51	0.99
	L-Met	34.81	1.12	0.31	3.22	0.88
	P-value	0.4673	0.0909	0.0732	0.2323	0.3059
	RMSE	3.7875	0.2452	0.0557	0.6355	0.1467

[a,b] Means within the column of the main effect with similar superscript letters are not significantly different and [c,d] means with similar superscript letters are not signicant different within column of TSL genotype ($P > 0.05$). HBW: Hatching body weight, ALW: absolute liver weight, AHW: absolute heart weight, RHW: relative heart weight, RLW: liver relative weight. TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed, control: non-injected, saline: saline injected, DL-Met: DL-Met injected, L-Met: L-Met injected (5 mg of Met /0.5 mL of NaCl).

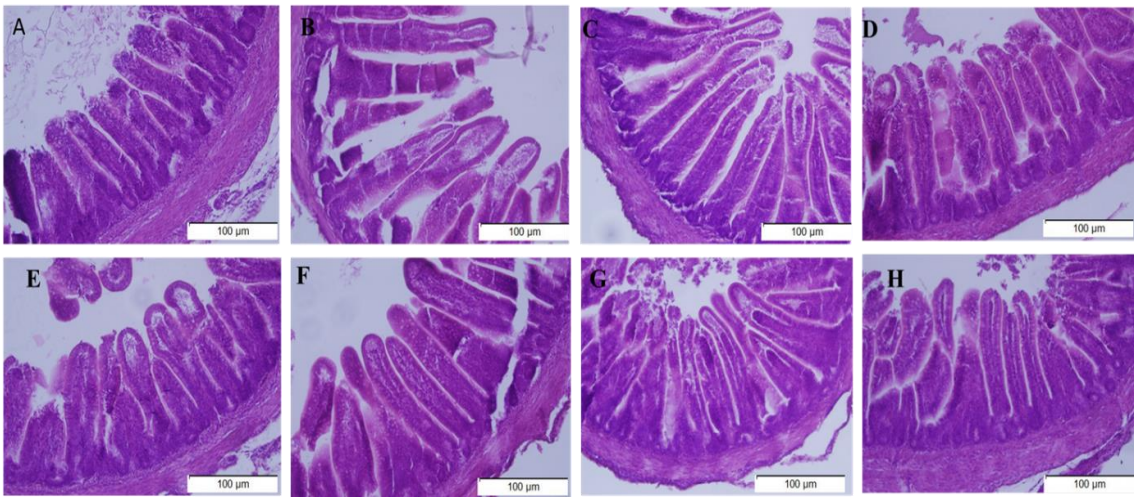
3.1.3. Jejunum histomorphomentry

Upon examination with H&E staining (see representative images in Picture 1), it was revealed that the jejunum villi height was increased in the DL-Met group compared to both the L-Met group and the control treatment (Table 3). The *in-ovo* injection significantly increased (except for crypt depth) the jejunum morphology parameters measured: villi height, middle villi width, and the villi height crypt ratio (Table 3, $P < 0.001$). In addition, genotype influence on jejunum morphology parameters was observed in apical villi width, middle villi width and villi area only. The interaction effect of genotype and *in-ovo* injection treatment was significant on all measured parameters except villi crypt depth and villi height crypt ratio (Table 3, $P < 0.001$).

The *in-ovo* feeding of Met significantly enhanced all the jejunum parameters studied in the HPC genotype compared to the control group. However, in the TSL genotype, the villus area decreased by *in-ovo* feeding of Met. The comparison of the two Met sources showed that DL-Met *in-ovo* feeding significantly increased the villus basal and middle width, crypt depth and villus crypt depth ratio than L-Met (Picture 1). Our results indicated that the injection of methionine on day 17.5 of embryonic development increased the villus surface area, suggesting an improved jejunum absorption rate (NAZEM *et al.* 2019). DL-met injected improved intestinal development compared with the control in all measure parameters (Table 3).

Picture 1

Cross sections of the jejunum from newly hatched TSL and HPC chicks as responses to *in-ovo* injected with methionine sources (representative images: Scale bar = 100 µm, 200x magnification)



A to D representative images of jejunum villi of HPC chicks hatched from A) non-injected. B) saline-injected group. C) Injected with 5mg of DL-Met group. D) Injected with 5 mg of L-Met group. E to H representative images of jejunum villi of TSL chicks hatched from E) non-injected group, F) saline-injected group, G) DL-Met-injected group, H) L-Met injected group. TSL= TETRA SL layer hybrid, HPC= Hungarian Partridge colored hen breed.

Table 3

Effect of *in-ovo* injection of 5 mg DL-Met and L-Met on intestine histology parameters of newly hatched chicks from TSL and HPC genotypes

Parameter		Villus basal width µm	Villus apical width µm	Villus middle width µm	Villi height µm	Crypt depth µm	Villus Height/ Crypt depth ratio	Villus area (x10^3 µm^2)
Pooled effects								
Genotype	TSL	74.72 ^{b}	61.63	72.75 ^{b}	349.9	61.89	5.89	80.09 ^{b}
	HPC	82.47 ^{a}	61.06	79.79 ^{a}	345.2	61.45	5.73	87.05 ^{a}
Treatment	Control	72.79 ^{b}	54.72 ^{c}	73.35	321.9 ^{c}	60.43 ^{b}	5.60 ^{b}	74.69 ^{b}
	Saline	77.25 ^{ab}	63.76 ^{ab}	75.26	378.9 ^{a}	69.25 ^{a}	5.59 ^{b}	89.92 ^{a}
	DL-Met	80.85 ^{a}	61.52 ^{b}	80.62	349.3 ^{b}	53.64 ^{c}	6.62 ^{a}	89.10 ^{a}
	L-Met	77.50 ^{ab}	66.68 ^{a}	73.52	339.3 ^{bc}	64.96 ^{a}	5.31 ^{b}	77.89 ^{b}
***P*-values**	Genotype	0.0001	0.861	0.001	0.577	0.522	0.714	0.013
	Treatment	0.0001	0.001	0.065	0.001	0.001	0.001	0.001
	Interaction	0.0001	0.001	0.001	0.001	0.001	0.145	0.001
	RMSE	15.48	10.66	13.66	48.4	9.30	1.32	21.27
Treatment effects by genotypes								
TSL	Treatment							
	Control	80.14 ^{d}	57.07 ^{e}	79.65 ^{d}	332.0 ^{e}	62.38 ^{d}	5.59 ^{e}	83.05^{d}
	Saline	71.23 ^{de}	58.04 ^{de}	71.71 ^{d}	359.8 ^{d}	67.43 ^{d}	5.46 ^{e}	81.35^{d}
	DL-Met	80.82 ^{d}	64.50 ^{d}	76.75 ^{d}	347.9 ^{de}	52.59 ^{e}	6.71 ^{d}	84.31^{d}
	L-Met	66.10 ^{e}	66.40 ^{d}	63.26 ^{e}	354.0 ^{d}	66.94 ^{d}	5.43 ^{e}	70.52^{e}
	P-value	0.0001	0.0024	0.0001	0.0009	0.0001	0.0001	0.0002
	RMSE	14.51	10.62	12.73	42.6	9.61	1.43	20.90
HPC	Treatment							
	Control	65.03 ^{e}	52.33 ^{e}	64.04 ^{e}	303.5 ^{f}	59.70 ^{ef}	5.14 ^{e}	61.22 ^{f}
	Saline	85.10 ^{d}	66.15 ^{d}	81.25 ^{d}	411.1 ^{d}	72.34 ^{d}	5.81 ^{de}	104.3 ^{d}
	DL-Met	84.99 ^{d}	58.35 ^{e}	84.49 ^{d}	350.7 ^{e}	54.68 ^{f}	6.54 ^{d}	93.89 ^{de}
	L-Met	88.65 ^{d}	68.21 ^{d}	83.39 ^{d}	325.1 ^{f}	63.06 ^{de}	5.20 ^{e}	84.98 ^{e}
	P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
	RMSE	14.62	10.71	14.62	54.1	8.94	1.17	21.69

^{a,b,c} Means with similar superscript letters are not significantly different within the column of main effect and ^{d,e,f} means with similar superscript letters are not signicant different within column of TSL and HPC genotype respectively ($P > 0.05$), TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed. Control: non-injected group, Saline: Saline injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group, RMSE - the root of the Mean Square Error.

3.1.4. Liver enzymes and kidney status indicators (AST, ALT and Uric acid) and Ferric reducing ability of the plasma (FRAP)

The genotypes did not significantly influence plasma parameters, except for AST: ALT ratio, uric acid and FRAP (*P < 0.05*, Table 4). The TSL genotype had significantly increased uric acid and FRAP content and a lower AST:ALT ratio than the HPC genotype (*P < 0.05)*.

Treatment with *in-ovo* feeding of Met sources significantly influenced the plasma uric acid, AST, ASL/ALT ratio and FRAP (*P < 0.05)*. I*n ovo* feeding with L-Met significantly lowered the levels of uric acid and FRAP (*P < 0.05)* in the plasma compared with the other groups. In addition, the L-Met group had a significantly lower FRAP content than the DL-Met group (*P < 0.05)* but not significantly different from DL-Met and saline groups. The saline group had a significantly higher AST level than DL-Met and a higher ASL/ALT ratio than all other groups (*P < 0.05,* Table 4*).*

In the HPC genotype, the treatment significantly influenced all the measured plasma parameters. Comparable effects of the control, DL-Met, and L-Met were observed in all parameters except for FRAP. The DL-Met group exhibited significantly higher plasma FRAP levels ($P < 0.05$, Table 4) than L-Met but were comparable with the other experimental groups in the HPC genotype. Saline in-ovo injection significantly increased AST levels and the AST:ALT ratio more than all other treatment groups ($P < 0.05$) but significantly reduced ALT levels compared to the control group in the HPC genotype ($P < 0.05$). However, in the TSL genotype, significant effect of treatment was observed in uric acid and FRAP levels ($P < 0.05$). DL-Met significantly increased uric acid content in TSL chicks compared to L-Met ($P < 0.05$) but was not better than the other groups (Table 4).

Table 4

The effect of *in-ovo* injection of 5 mg DL-Met and L-Met on the plasma biochemical parameters of one-day-old TSL and HPC chicks

Plasma parameter		AST (U/L)	ALT (U/L)	AST: ALT	Uric acid (µmol/l)	FRAP (µM $FeCl_2$ equivalent)
Pooled effects						
Genotype n = 32						
	TSL	93.63	57.00	1.64 [b]	301.73[a]	78.96 [a]
	HPC	98.08	59.50	2.02 [a]	267.67[b]	70.27 [b]
Treatment n = 8						
	Control	93.20 [b]	63.63	1.42 [b]	323.1 [a]	81.36 [a]
	Saline	109.13 [a]	49.90	2.81 [a]	283.99 [ab]	73.01 [a]
	DL-Met	88.11 [b]	63.59	1.43 [b]	284.22 [ab]	84.80 [a]
	L-Met	93.12 [b]	55.89	1.66 [b]	247.49 [b]	59.30 [b]
P- Values	Genotype	0.2750	0.6820	0.0342	0.0391	0.0171
	Treatment	0.0017	0.0563	0.0001	0.0110	0.0002
	Interaction	0.0007	0.0772	0.2331	0.0010	0.0021
RMSE		15.17	16.35	0.82	59.74	14.88
Treatment effect by genotype n = 8						
TSL	Control	102.22	57.23	1.71	329.44 [de]	90.12 [d]
	Saline	94.09	57.56	1.56	365.36 [d]	87.39 [d]
	DL-Met	87.54	63.47	1.42	276.59 [e]	78.21 [d]
	L-Met	91.19	49.73	1.87	235.54 [f]	60.12 [e]
	P-value	0.3200	0.3584	0.2361	0.0075	0.0069
	RMSE	15.97	15.024	0.4422	61.93	16.89
HPC	Control	84.18 [e]	70.02 [d]	1.12 [e]	316.78 [d]	72.60 [e]
	Saline	124.16 [d]	42.23 [e]	4.06 [d]	202.61 [e]	58.62 [e]
	DL-Met	88.67 [e]	63.71 [de]	1.44 [e]	291.85 [d]	91.38 [d]
	L-Met	95.06 [e]	62.03 [de]	1.45 [e]	259.43 [de]	58.48 [e]
	P-value	0.0001	0.0267	0.0001	0.0035	0.0001
	RMSE	14.26	17.58	1.0761	57.81	12.34

[a,b] Means with the same superscript letters are not significantly different within the main effect column and [d,e,f] means with similar superscript letters are not signicant different within column of TSL and HPC genotype respectively (*P > 0.05)*. TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed, Control: non-injected, Saline: saline-injected: DL-Met: DL-Met injected, L-Met: L-Met injected. AST: aspartate aminotransferase, ALT: alanine aminotransferase, AST/ALT: aspartate aminotransferase to alanine aminotransferase ratio, FRAP: ferric reducing ability of the plasma.

3.1.4.1. Tissues Total GSH content

The content of liver GSH in one-day-old chicks was affected by the *in-ovo* treatment and genotypes ($P < 0.001$), but their interaction did not show any effect. Unexpectedly, the GSH content was higher in chicks hatched from non-injected eggs than those from injected/treated groups (Table 5). The TSL genotype showed higher GSH content as compared to its counterpart genotype in all treatment groups. The liver GSH concentration in the TSL genotype follows the order: Non-injected > Saline injected > DL-Met=L-Met. However, the *in-ovo* treatments showed a different response in the HPC genotype in the following order: Non-injected > DL-Met > L-Met = saline injected (Table 5).

In the muscles and intestines, only the content of GSH was influenced by the genotype ($P< 0.05$). However, the treatment also tended to control the muscle's GSH content ($P = 0.075$). The treatment showed a significant effect on the GSH content of the HPC genotype, where DL-Met significantly reduced the muscle's GSH content than the control group ($P < 0.05$). The muscle content of the TSL genotype was not affected by the treatment. In the intestine, only the genotype effect on GSH content was observed ($P = 0.001$). In addition to the intestine, in the muscles, only genotype influenced the concentration of GSH ($P = 0.048$) (Table 5).

3.1.4.2. Total antioxidant capacity (TAC) in tissues

The total antioxidant capacity of the liver tissue was affected only by the genotype and the interaction of the genotype and treatment ($P < 0.05$). The interaction of genotype and treatment was more evident between the control and Met group. In the HPC genotype, DL-Met and L-Met groups significantly reduced the hepatic TAC level than the control and saline-injected groups (Table 5, $P < 0.05$), but not in the TSL genotype. No significant effect of the treatment and genotype was observed in the muscles TAC, but the interaction effect was noted. The DL-Met significantly increased the muscle TAC content compared to the saline-injected group in the HPC genotype but not in the TSL genotype (Table 5, $P < 0.05$). In the intestine, the *in-ovo* injection of Met did not influence the TAC significantly in both genotypes ($P > 0.05$). However, in the HPC, the saline injection slightly increased the TAC compared to other treatment groups (Table 5, $P < 0.05$).

Table 5

The effect of *in-ovo* injection of 5 mg DL-Met and L-Met on tissues GSH and TAC of 1d post-hatch chick of the TSL and HPC genotypes

Parameter		***GSH content***			***TAC content***		
	Tissue	Liver	Intestine	Muscles	Liver	Intestine	Muscles
Pooled effects							
Genotype n = 32							
	TSL	4.13 [a]	2.78 [a]	0.75 [b]	13.1 [a]	4.14	2.67
	HPC	2.62 [b]	1.70 [b]	0.92 [a]	10.2 [b]	4.00	2.74
Treatment n= 8 per treatment							
	Control	4.52 [a]	2.39	1.021	12.1	3.95	2.73
	Saline	3.11 [b]	2.21	0.815	11.8	4.21	2.64
	DL-Met	3.03 [b]	2.21	0.792	11.4	4.00	2.79
	L-Met	2.86 [b]	2.16	0.712	11.3	4.13	2.66
***P*-values**	Genotype	0.0001	0.0004	0.0488	0.0001	0.2873	0.3586
	Treatment	0.0001	0.9179	0.0751	0.1985	0.4880	0.5718
	Interaction	0.0926	0.8208	0.2173	0.0069	0.1281	0.0224
	RMSE	0.9829	1.1313	0.3374	1.1900	0.5342	0.3295
Treatment effects by genotypes							
TSL	Treatment						
	Control	5.55 [d]	2.73	0.859	12.80	4.24	2.58
	Saline	4.17 [e]	2.54	0.812	12.97	4.05	2.78
	DL-Met	3.32 [e]	2.92	0.701	13.28	4.12	2.61
	L-Met	3.47 [e]	2.94	0.628	13.39	4.17	2.70
	P-value	0.0021	0.9466	0.3931	0.7831	0.9199	0.6563
	RMSE	1.1460	1.5031	0.2920	1.2942	0.5413	0.3508
HPC	Treatment						
	Control	3.48 [d]	1.59	1.182 [d]	10.87 [de]	3.66 [e]	2.89 [de]
	Saline	2.04 [e]	1.87	0.772 [de]	11.16 [d]	4.38 [d]	2.50 [e]
	DL-Met	2.73 [de]	1.49	0.724 [e]	9.51 [ef]	3.88 [de]	2.96 [d]
	L-Met	2.24 [de]	1.84	1.002 [de]	9.11 [f]	4.09 [de]	2.62 [de]
	P-value	0.0048	0.3944	0.0765	0.0012	0.0659	0.0165
	RMSE	0.7868	0.5138	0.3773	1.0761	0.5268	0.3068

[a,b] Means with the same superscript letters are not significantly different within the main effect column and [d,e,f] means with similar superscript letters are not signicant different within column of TSL and HPC genotype respectively (*P > 0.05)*. TSL: TETRA –SL layer hybrid, HPC: Hungarian Partridge colored hen breed, GSH: glutathione, TAC: total antioxidant capacity. Control: non-injected, Saline: saline-injected, DL-Met: DL-Met injected and L-Met: L-Met injected.

3.1.5. Hepatic gene expression

The genotype significantly influenced the expression of *IGF1* and *IGF1R* genes (*P < 0.05*) and no significant influence on the *TLR4* (*P* = 0.0579) and *GHR* gene expression (Table 6, *P* = 0.1505). There is no interaction effect of genotypes and *in-ovo* feeding on the gene expression. The study found that *in-ovo* feeding of methionine did not significantly affect the expression of

hepatic genes in one-day-old chicks (Table 6, $P > 0.05$). However, the effect of *in-ovo* feeding of Met sources varied with the genotypes. In the HPC genotype, *in-ovo* feeding of L-Met tended to increase the expression of *IGF1R* ($P = 0.16$), while DL-Met tended to decrease the expression of TLR4 (Table 6) compared to the control. No significant effects or tendencies in the expressions of the genes (*IGF1, IGF1R, GHR, and TLR4*) were noted for TSL genotype chicks (Table 6).

Table 6

Effect of *in-ovo* feeding of 5mg DL-Met and L-Met on the liver gene expression of TSL and HPC chicks

Parameter		***IGF1***	***IGF1R***	***GHR***	***TLR4***
Pooled effects					
Genotype n= 32					
	TSL	1.92[b]	1.11[b]	2.20	1.66
	HPC	2.29[a]	2.17[a]	2.44	2.07
Treatment n = 8 per treatment					
	Control	2.28	1.52	2.36	1.95
	Saline	2.09	1.62	2.51	2.10
	DL-Met	2.03	1.63	2.24	1.51
	L-Met	2.01	1.78	2.16	1.92
***P*-values**	Genotype	0.0165	0.0001	0.1505	0.0579
	Treatment	0.5414	0.3860	0.4230	0.2862
	Interaction	0.7978	0.3492	0.5624	0.2194
	RMSE	0.5647	0.4133	0.6160	0.8734
Treatment effects by genotypes					
TSL	Treatment				
	Control	2.11	1.107	2.31	1.37
	Saline	1.78	0.978	2.26	2.14
	DL-Met	1.94	1.186	2.28	1.46
	L-Met	2.19	1.186	1.96	1.67
	P-value	0.6965	0.6264	0.5836	0.3664
	RMSE	0.5615	0.3516	0.5378	0.8817
HPC	Treatment				
	Control	2.45	1.940	2.42	2.53
	Saline	2.39	2.263	2.76	2.06
	DL-Met	2.13	2.083	2.21	1.55
	L-Met	2.19	2.376	2.36	2.16
	P-value	0.6333	0.2789	0.4426	0.1851
	RMSE	0.5678	0.4669	0.6828	0.8650

[a,b,c] Means with similar superscript letters are not significantly different within the effect. TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed. Control: non-injected group, Saline: saline injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group. *IGF1*: insulin-like growth factor 1, *IGF1R*: insulin-like growth factor 1 receptor, *GHR*: growth hormone receptor, *TLR4*: toll-like receptor 4.

3.1.6. Intestinal gene expression related to growth, antioxidants, and tight junction

3.1.6.1. Jejunum growth-related genes

The injection of Met *in-ovo* significantly affected the expression of *IGF1* ($P = 0.0108$), and on *IGF1R* mRNA expression ($P = 0.0454$). The results suggested that the L-Met injection tends to decrease the expression of the *IGF1* gene in the HPC genotype compared to the control (Table 7, $P = 0.1521$). The genotype significantly affected *GHR, IGF1,* and *IGF1R* mRNA expression ($P < 0.0001$). The TSL genotype had higher expression levels of *IGF1R* (Table 7, $P < 0.01$) and *GHR* ($P < 0.01$) than the counterpart genotype. In contrast, the HPC genotype had higher expression levels of *IGF1* when compared to the TSL genotype (Table 7, $P < 0.01$).

3.1.6.2. Jejunum antioxidants-related genes

Based on the data presented in Table 7, it appears that injecting DL-Met had a significant impact on the expression of *NRF2* in the HPC genotype but did not noticeably affect other genes related to antioxidants. The genotype significantly influenceed the expression of antioxidant genes, except *GPX1* and *NRF2*. Additionally, compared to the TSL genotype, the HPC genotype showed increased mRNA expression of *SOD1* and *GST3* genes (Table 7) and decreased mRNA expression of *TLR4* ($P < 0.05$). The interaction effect observed on the expression of *NRF2* mRNA only. The L-Met *in-ovo* feeding significantly increased the expression of *NRF2* in the HPC genotypes but not TSL genotypes repectively when compared to the treatments (Table 7, $P = 0.0375$).

Table 7

Effects of *in-ovo* injection of 5 mg DL-Met and L-Met on jejunum relative mRNA expression levels of growth-related genes in TSL and HPC chicks at one day of age

Parameter		*IGF1*	*IGF1R*	*GHR*	*TLR4*	*GPx*	*GST3*	*NRF2*	*SOD*	*MD2*	*OCLN*	*TJP2*
Pooled effects												
Genotype	TSL	0.913^{b}	0.904^{b}	0.617^{b}	1.64^{a}	0.690	1.69^{b}	0.842	0.875^{b}	0.91^{b}	0.828^{b}	2.35^{a}
	HPC	1.484^{a}	2.092^{a}	1.276^{a}	1.07^{b}	0.853	4.76^{a}	0.828	1.539^{a}	2.05^{a}	2.485^{a}	1.55^{b}
Treatment	Control	1.368	1.47^{ab}	0.895	1.13	0.697	3.48	0.956	1.23	1.53	1.93	1.93
	Saline	1.066	1.30^{b}	0.924	1.23	0.705	3.38	0.988	1.22	1.44	1.63	1.88
	DL-Met	1.367	1.48^{ab}	0.857	1.58	0.817	2.93	0.924	1.14	1.53	1.52	2.02
	L-Met	0.994	1.74^{a}	1.108	1.49	0.867	3.12	1.099	1.24	1.42	1.54	1.99
***P*-values**	Genotype	0.0001	0.0001	0.0001	0.0010	0.1790	0.0001	0.7990	0.0001	0.0001	0.0001	0.0001
	Treatment	0.0108	0.0454	0.3949	0.1776	0.7184	0.2488	0.5248	0.9319	0.7463	0.0778	0.8314
	Interaction	0.6222	0.9910	0.5445	0.6574	0.6222	0.2419	0.0375	0.2554	0.9030	0.4156	0.3474
	RMSE	0.3980	0.4686	0.4613	0.6503	0.4611	0.8815	0.3097	0.4315	0.4324	0.5167	0.5255
Treatment effects by genotypes												
TSL	Treatment											
	Control	1.01	2.067	1.180	1.549	0.778	1.94	0.841	0.772	0.915	1.268	2.13
	Saline	0.89	1.921	1.382	1.383	0.671	1.60	0.808	1.059	0.852	0.700	2.33
	DL-Met	1.06	2.063	1.206	1.913	0.676	1.25	0.939	0.747	0.951	0.593	2.43
	L-Met	0.70	2.318	1.335	1.721	0.634	1.97	0.780	0.923	0.923	0.749	2.52
	P-value	0.3461	0.5094	0.7624	0.3678	0.9468	0.0350	0.778	0.3375	0.9344	0.0269	0.5791
	RMSE	0.4175	0.5245	0.4451	0.6147	0.5039	0.5226	0.3244	0.3789	0.3163	0.4514	0.5781
HPC	Treatment											
	Control	1.729	0.879	0.611	0.714	0.616	5.02	0.759	1.683	2.142	2.595	1.72
	Saline	1.245	0.671	0.467	1.084	0.739	5.15	0.858	1.381	2.117	2.557	1.43
	DL-Met	1.676	0.894	0.508	1.244	0.958	4.60	0.598	1.533	2.021	2.451	1.61
	L-Met	1.287	1.171	0.881	1.253	1.100	4.28	1.096	1.559	1.926	2.337	1.45
	P-value	0.0281	0.1493	0.3601	0.3836	0.1243	0.4475	0.0228	0.6617	0.8534	0.8240	0.5518
	RMSE	0.3766	0.4026	0.4774	0.6853	0.4121	1.1404	0.2938	0.4801	0.5264	0.5770	0.4646

[a, b] Means that having similar superscript letters within the effect are not significantly different within the factor ($P < 0.05$). TSL = TETRA-SL layer hybrid, HPC= Hungarian Partridge colored hen breed, Control = non-injected group, Saline = saline-injected group, DL-Met = DL-Met injected group, L-Met = L-Met infiltrated group. *GHR* = growth hormone receptor, *IGF1* = insulin-like growth factor 1, *IGF1R* = insulin-like growth factor 1 receptor. Data are presented as estimated marginal means and RMSE

3.1.6.3. Jejunum tight junction-related genes

The study revealed significant genotype effects on the expression of *OCLN*, *TJP2*, and *MD2* ($P < 0.01$, Table 7). No significant treatment or interaction effects were found on tight junction genes in both genotypes ($P > 0.0.5$, Table 7) except for the *OCLN*, where a significant effect and a tendency of reduction was noted in the TSL genotype due to *in-ovo* injection of DL-Met ($P = 0.02$), Saline ($P = 0.07$) and L-Met ($P = 0.12$) when compared to control (Table 7). *OCLN and MD2* mRNA expression were higher in the HPC genotype compared to the TSL genotype ($P < 0.01$, Table 7), while the TSL genotype exhibited increased expression of *TJP2* ($P < 0.01$).

3.2. Effect of methionine source and levels supplementation on TETRA-SL chicks

3.2.1. Growth performance

Generally, neither Met source nor Met levels influenced the body weight of the chicks for the whole period of the experiment except for the interaction effect on week four (Table 8). Moreover, the dietary Met source and Met level did not significantly influence the ADG except for the fourth week of age, where the interaction was noted. In week 4, the highest ADG value (14.6 g/day) was obtained in the DL-Met group with a supplementation level of 110%, which increased by 7.6% from the control (DL-Met at 100%) (Table 8). Growth performance has been used to determine the bioavailability (digestion, absorption, and utilisation) of amino acids, particularly methionine, the first limiting amino acid in maize and soybean meal-based poultry diets (SHEN *et al.* 2014).

Table 8

Effects of different Met sources and levels on growth performance in TETRA –SL layer hybrid chicks

Traits		BW on day 1, g	BW on day 7, g	ADG, g/day	BW on day 14, g	ADG, g/day	BW on day 21, g	ADG, g/day	BW on day 28, g	ADG, g/day	ADG, g/day
Pooled effects				Week 1		Week 2		Week 3		Week 4	Overall
Met source	DL-Met	42.39	78.22	5.11	124.05	6.50	205.01	11.57	271.05	10.34	8.39
	L-Met	42.38	76.77	5.04	117.86	6.03	197.37	11.41	265.85	9.48	8.02
Met level	90%	42.36	77.58	5.02	121.88	6.75	203.69	11.69	260.83	8.38	7.99
	100%	42.40	78.27	5.21	120.59	5.97	198.27	10.67	263.15	9.50	8.04
	110%	42.40	76.71	4.98	120.55	6.16	202.04	12.15	281.30	11.74	8.59
***P*-values**	Met sources	0.9835	0.4031	0.7826	0.1198	0.2599	0.3035	0.8669	0.6377	0.4046	0.5035
	Met levels	0.9981	0.6758	0.6921	0.9476	0.2170	0.8386	0.3880	0.2398	0.0643	0.6065
	Interaction	0.9993	0.2813	0.3508	0.5582	0.4419	0.4851	0.8128	0.0130	0.0174	0.0667
	RMSE	2.96	7.81	0.58	18.84	0.87	34.85	2.05	51.07	2.62	1.27
Effect among all treatment combinations											
DL-Met	90%	42.35	77.86	5.04	125.63	6.69	205.59	11.42	250.46 [a]	8.22 [a]	7.88
	100%	42.41	77.68	5.04	120.76	6.16	197.85	11.02	255.49 [ab]	8.23 [a]	7.61
	110%	42.41	79.06	5.24	125.78	6.67	211.61	12.26	306.87 [b]	14.56 [b]	9.68
L-Met	90%	42.37	77.36	5.00	118.13	6.84	201.67	12.04	270.5 [ab]	8.52 [ab]	7.90
	100%	42.37	79.00	5.39	120.40	5.71	198.78	10.20	271.33 [ab]	10.76 [ab]	8.47
	110%	42.38	74.03	4.72	115.19	5.65	191.84	12.00	255.72 [ab]	8.91 [ab]	7.49
	P-value	1.000	0.5631	0.6905	0.5803	0.3178	0.7042	0.7922	0.0400	0.0218	0.2136
	RMSE	2.95	7.81	0.58	18.84	0.87	34.85	2.05	51.07	2.62	1.27

[ab] Means with similar letters superscript do not differ ($p > 0.05$) within the effect. The result was presented as mean and RMSE (n = 4/treatment for ADG and n = 16/treatment for BW). 90%, 100%, and 110% supplementation levels of the recommendation of methionine. BW-Body weight, ADG- average daily gain, RMSE –root mean square error.

3.2.2. Feathers development

The results showed that neither Met source nor Met levels affected the feather length development in the chicks from the first week to the third week of rearing. However, in the fourth week, the interaction of source and level was observed (Table 9). These findings agree with the result reported by CHEN et al.(2020), where no effect of DL-Met and L-Met supplementation was observed on the featherweights of the broiler chicks. Similarly, ZHAO et al. (2018) and ZENG et al. (2015) reported that DL-Met and DL-HMTBA showed the same efficiency for feather growth in Cherry Valley ducks and Pekin ducks, respectively. Our findings indicated that as in BW, dietary DL and L-Met sources have similar efficacy on the feather development of TSL chicks till 28 days of age.

Table 9

Effects of different dietary Met sources and levels of supplementation on feathers development of TSL chicks from 1-28 days of age

		Feather length on day 7 (mm)	Feather length on day 14 (mm)	Feather length on day 21 (mm)	Feather length on day 28 (mm)
Pooled effects					
Met source	DL-Met	38.58	57.50	73.80	84.94
	L-Met	36.67	55.99	72.74	84.24
Met level	90%	36.32	56.46	72.98	84.98
	100%	39.71	57.88	73.80	83.87
	110%	36.85	55.92	73.08	84.96
P-values	Source	0.1898	0.1609	0.1709	0.4310
	Level	0.1250	0.3108	0.6245	0.5215
	Interaction	0.0720	0.0325	0.0265	0.0625
RMSE		7.07	5.24	3.83	4.34
Effect among all treatment combinations					
DL-Met	90%	35.37	55.76	73.37	84.96
	100%	40.38	58.13	73.09	83.14
	110%	39.99	58.62	74.96	86.72
L-Met	90%	37.26	57.20	72.54	85.01
	100%	39.04	57.62	74.51	84.59
	110%	33.71	53.23	71.07	83.09
	P-value	0.0528	0.0516	0.0797	0.1865
	RMSE	7.07	5.24	03.83	4.34

TSL: TETRA-SL layer hybrid. The result presented as mean and pooled SEM (n = 16/treatment)

3.2.3. Hematological parameters

The blood parameters of chicks were influenced by Met source and levels both separately and interactively (Table 10). Met source had a significant effect on the number of erythrocytes (RBC) in the blood of chicks ($p = 0.004$), as did Met concentrations ($p = 0.016$), but there was no significant interaction between Met source and levels and the number of RBC (Table 10). Hb concentration was significantly different between Met sources ($p = 0.001$) and among Met levels ($p = 0.013$), but there was no significant interaction effect of Met source and levels on the Hb concentration of chicks' blood. The Ht value was significantly affected by both Met source ($p = 0.005$) and levels ($p = 0.007$), with a demonstration of the interaction of Met source and Met levels ($p = 0.024$). However, there were no significant differences in MCV, MCH, and MCHC values exerted by either dietary Met sources, levels, or their interaction (Table 10). The dietary Met source significantly affected the platelet count in blood liters ($p = 0.001$), with higher values observed in L-Met as compared to DL-Met. No significant effects of Met levels or interactions of Met sources and Met levels were revealed. The highest value of platelet count (10.44×10^9/L) was obtained in the L-Met group with 110% inclusion, while the lowest (2.33×10^9/L) was in the DL-Met group at the same dose (Table 10).

WBC count in the blood liter was affected by both Met sources ($p = 0.003$), Met levels ($p = 0.009$), and their interaction ($p = 0.001$). The percentage of lymphocytes was also significantly influenced by both dietary Met source and dietary Met levels with their interaction, the same as for WBC count. The number of LYM cells was only affected by Met sources ($p = 0.044$) and levels ($p = 0.023$) with no interaction effect observed. While the MID percentage was not influenced by the source or the levels, the MID number was significantly affected by both factors but not by their interactions (Table 10). Similarly, the percentage of GRAN % was statistically affected by both Met source ($p = 0.014$) and levels ($p = 0.012$) with no interaction effect. However, the GRAN cell count was significantly affected by Met-source ($p = 0.005$) and Met levels ($p = 0.001$), as well as having a significant interaction ($p = 0.002$). The highest dietary inclusion levels in the L-Met group resulted in the lowest number and percentage (26.18×109/L and 37.63%, respectively) of GRAN compared to other nutritional inclusion levels in both sources (Table 10). It should be noted that no similar study was found on the effect of the Met sources and levels on the TSL genotype.

Table 10

Effect of different dietary Met-source and levels of supplementationon the hematological parameters of TSL chicks from 1 to 28 d of age

		RBC (10^{12}/L)	Hb (g/dL)	Ht (%)	MCV (fL)	MCH (pg)	MCHC g/dL	Platelet (10^9/L)	WBC (10^9/L)	LYM (%)	MID (%)	GRAN (%)	LYM (10^9 cells/L)	MID (10^9 cells/L)	GRAN (10^9 cells/L)
Pooled effects															
Met source	DL Met	2.99 ^{b}	11.91 ^{b}	38.42 ^{b}	127.66	39.5	30.76	2.70 ^{a}	84.49 ^{b}	45.09 ^{a}	13.04	41.77 ^{b}	38.35 ^{b}	11.06 ^{b}	36.15 ^{b}
	L Met	2.77 ^{a}	10.99 ^{a}	35.67 ^{a}	127.70	39.11	30.84	8.00 ^{b}	77.39 ^{a}	47.45 ^{b}	12.97	40.05 ^{a}	36.06 ^{a}	9.92 ^{a}	31.85 ^{a}
Met level															
	90%	2.94 ^{ab}	11.4 ^{ab}	36.56 ^{ab}	126.79	38.62	30.70	5.54	78.82 ^{a}	46.50 ^{b}	13.17	40.47 ^{ab}	35.41 ^{a}	10.02 ^{a}	32.52 ^{a}
	100%	2.97 ^{b}	11.96^{b}	38.77 ^{b}	129.21	39.69	30.68	3.86	86.67 ^{b}	44.57 ^{a}	12.85	42.36 ^{b}	39.21 ^{b}	11.27 ^{b}	38.01 ^{b}
	110%	2.76 ^{a}	11.05 ^{a}	35.62 ^{a}	126.55	39.56	31.01	6.76	78.94 ^{a}	47.61 ^{b}	13.02	39.97 ^{a}	37.04 ^{ab}	10.22 ^{ab}	31.69 ^{a}
P-values	Source	0.0039	0.0006	0.0045	0.9806	0.3649	0.7146	0.0005	0.0030	0.0006	0.512	0.0140	0.0442	0.0039	0.0045
	Level	0.0156	0.0125	0.0068	0.1699	0.0487	0.4701	0.3101	0.0093	0.0010	0.124	0.0116	0.0234	0.0150	0.0006
	Inter-action	0.2395	0.0864	0.0244	0.2585	0.8934	0.0727	0.1241	0.0014	0.0204	0.586	0.0904	0.1464	0.0650	0.0017
RMSE		0.21	0.77	2.55	3.68	1.05	0.73	4.09	6.69	1.95	0.39	2.13	3.27	1.36	4.08
Effect among all treatment combinations															
DL Met	90%	2.97 ^{a}	11.49^{a}	36.14 ^{ab}	126.53	38.85	30.69	2.52	76.54 ^{ab}	46.30 ^{a}	13.18	40.59 ^{ab}	35.32	10.05 ^{a}	31.17 ^{ab}
	100%	3.09 ^{a}	12.51^{a}	40.17 ^{b}	128.03	39.73	30.98	3.22	92.34 ^{c}	43.56 ^{a}	12.97	42.90 ^{b}	40.13	11.75 ^{b}	40.15 ^{c}
	110%	2.91 ^{ab}	11.68^{a}	37.99 ^{b}	128.14	39.77	30.60	2.33	86.83 ^{bc}	45.36 ^{a}	13.00	41.65 ^{b}	39.59	11.22 ^{b}	36.42 ^{bc}
L Met	90%	2.91 ^{ab}	11.32^{ab}	36.81 ^{ab}	126.95	38.42	30.71	9.06	81.11^{abc}	46.68 ^{a}	13.15	40.36$^{a\,b}$	35.48	9.99 ^{ab}	33.87 ^{bc}
	100%	2.85 ^{ab}	11.31^{ab}	37.14 ^{b}	130.58	39.64	30.39	4.50	82.13 ^{bc}	45.57 ^{a}	12.71	41.72 ^{b}	38.29	10.70 ^{ab}	35.52 ^{bc}
	110%	2.54 ^{b}	10.15 ^{b}	32.31 ^{a}	125.28	39.31	31.43	10.44	69.73 ^{a}	50.24 ^{b}	13.04	37.63 ^{a}	34.50	9.06 ^{a}	26.18 ^{a}
P-value		0.0059	0.008	0.0007	0.2816	0.1820	0.2254	0.0039	0.0002	0.0001	0.357	0.0057	0.0156	0.0015	0.0001
RMSE		0.21	0.77	2.55	3.68	1.05	0.73	4.09	6.69	1.95	0.39	2.13	3.27	1.09	4.08

^{abc} Means with similar superscript letters in a column are not significantly different ($P > 0.05$). The result was presented as mean (n = 8/treatment). RBC: red blood cells, Hb: haemoglobin, Ht: hematocrit, MCV: mean corpuscular volume of red blood cell, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, WBC: white blood cells, LYM: lymphocyte, MID: mid-range, GRAN: granulocyte, TSL: TETRA-SL layer hybrid.

4. CONCLUSIONS

In conclusion, *in-ovo* feeding of DL-methionine has advantages over L-methionine in promoting higher hatching weight, heart weight, and antioxidant capacity (FRAP) in the plasma. However, these advantages are not superior to those observed in the control and saline groups. Both DL-methionine and L-methionine show similar effects on liver enzyme levels (AST, ALT), glutathione content, and plasma uric acid levels, which are relatively less or equal to the saline group. This suggests that both Met sources at 5 mg can effectively support liver health and metabolism, and the benefits of methionine on GSH content may not be justified. The commercial genotype TSL appears to have improved the antioxidant defense system and enhanced hatching body weight, liver health and function compared with the indigenous HPC genotype. This result demonstrates the difference in growth performance and metabolism between the two genotypes. The practical implications of these findings are significant for poultry management and nutrition and could lead to enhanced breeding and production practices.

Furthermore, *in-ovo* feeding of DL-Met and L-Met sources has been shown to enhance intestinal development in the late-term embryonic development of chicks. This study found that DL-Met injection exhibited higher efficacy in promoting intestinal development by increasing the villus height, villus height crypt ratio and villus surface area compared to L-Met injection, but not when compared to saline in-ovo injection. Therefore, the positive effect of in-ovo injection on intestinal morphology may be attributed to moisturization rather than Met supply. Nevertheless, more extensive research is needed to evaluate the benefits of saline in-ovo injection on the gut morphology of layers.

Moreover, it is noteworthy that the effectiveness of *in-ova* injection of L-Met and DL-Met on the expression of tight junction genes (*OCCLN, MD2, and TJP2*), antioxidant defense (*GST3, GPX1, SOD1, and NRF2*), immune response (*TLR4)* and growth-related genes (*IGF1R, GHR, and IGF1*) was comparable. However, in comparison with the control group, neither treatment demonstrated significant effectiveness. Our results also suggest that the HPC genotype displays beneficial characteristics regarding the antioxidant defense system and tight junction barrier function compared to the TSL genotype. On the other hand, the TSL genotype demonstrates better hatching body weight, *TLR4, TJP2, GHR, and IGF1R* gene expression than the HPC genotype. These findings suggest potential impacts on intestinal physiology and function and highlight the importance of genotype-specific gene expression patterns for optimizing breeding strategies and enhancing poultry productivity and health.

Lastly, dietary supplementation of the pre-starter diet with 90% of the recommended Met level

can maintain the growth performance and support feather growth the same as 100% and 110% Met levels of either DL or L Met from day 1 to 28 days of TSL chicks age. Generally, DL-Met improved the hematological parameters of TSL chicks reared for 28 days compared to L-Met supplementation. This research suggests that DL-Met and L-Met have similar effects on early TSL chicks' development and feather growth. This implies they may have the same biological efficacy for average daily gain and body weight, but different utilization for the response of hematological parameters.

5. NEW SCIENTIFIC RESULTS

1. *In-ovo* feeding of 5 mg DL-methionine injection facilitates intestinal development ($P < 0.05$) by increasing the villus height (7.8%), villus apical width (11.1%), villus surface area (16.0%) and villus height/crypt ratio (15.4%) while decreasing the crypt depth (11.2%) in the jejunum of newly hatched (1d) chicks of TETRA-SL and Hungarian partridge colored hen when compared to the non-injected group.
2. *In-ovo* feeding with 5 mg of methionine sources improves the liver and renal functions of the newly hatched TETRA –SL and Hungarian partridge colored hen chicks compared with the control but is not better than saline injection.
3. *In-ovo* injection of 5 mg DL-methionine improves ($P < 0.05$) the ferric-reducing ability of the plasma as well as the intestinal antioxidant defence system and tight barrier function of the Hungarian Partridge-Colored layer hen, but not the TETRA-SL layer hybrid genotype.
4. In layers, 90% of the recommended dietary sidMet level either in the form of DL or L-methionine could effectively support the feather's growth and growth performance ($P > 0.05$) as 100% (0.40% Met of TSL recommended) or more of the Met supplementation in early life.
5. Our results suggest that dietary DL-methionine supplementation in pre-starters is more beneficial in maintaining the physiological status by improving the hematological parameters ($P < 0.05$) of TETRA-SL LL hybrid layer chicks than L-methionine.

6. PRACTICAL USABILITY OF RESULTS

I. *In-ovo* feeding of Methionine source affects hatchability and antioxidant status. Poultry farmers should weigh the pros and cons of DL-methionine vs L-methionine for their flocks. Understanding specific needs optimizes supplementation.

II. The study shed light on the potential of 5 mg DL-Met to improve the antioxidant status of chicks and how both Met sources enhanced intestinal development and support liver

health, which are critical for nutrient absorption and overall health, respectively. In practice, this information may be used to design the *in-ovo* feeding programme to promote intestinal development and maximize nutrient absorption and overall production performance in later life.

III. The study revealed the significance of genotype-specific responses; hatcheries should consider the genotype-specific variation if they consider *in-ovo* feeding programs to improve their flocks' growth performance and overall health.

IV. The 90% dietary supplementation of methionine, particularly DL-Met, is enough to support the feather's development and growth performance and maintain the health status of the mid-heavy layers at a young age. This implies that 0.36% methionine in the diet is as effective as the 0.40% methionine required in the early life of the layers. The findings of this study can be a useful resource for poultry farmers and nutritionists to improve the nutritional content of diets for TSL chicks. By considering the quality and quantity of methionine, they can enhance healthy growth, efficient utilization of resources, and the birds' overall well-being. The study also emphasizes the significance of monitoring haematological parameters in poultry health management.

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8. PUBLICATIONS IN THE FIELD OF RESEARCH

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Subject: PhD Publication List

Candidate: James Kachungwa Lugata
Doctoral School: Doctoral School of Animal Husbandry
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List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (1)

1. **Lugata, J. K.**, Ozsváth, X. E., Ndunguru, S. F., Kidane, G. R., Knop, R., Angyal, E., Oláh, J., Szabó, C.: Effect of genotype on the hematological parameter of TETRA-SL and Hungarian Partridge coloured chickens at young age.
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Foreign language conference proceedings (1)

11. Ortega, A. D. S. V., Xayalath, S., **Lugata, J. K.**, Szabó, C.: Effects of heat stress-induced oxidative stress on the reproduction of sows and its alleviation by dietary antioxidants: a review.
In: XXV. Tavaszi Szél Konferencia 2022 : Tanulmánykötet I.. Szerk.: Molnár Dániel, Molnár Dóra, Nagy Adrián Szilárd, Doktoranduszok Országos Szövetsége, Budapest, 13-25, 2022. ISBN: 9786156457134

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In: XXV. Tavaszi Szél Konferencia 2022 : Absztraktkötet. Szerk.: Molnár Dániel, Molnár Dóra, Doktoranduszok Országos Szövetsége, Budapest, 72-73, 2022. ISBN: 9786158205481

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