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Effect of excess dietary L-valine on laying hen performance, egg quality, serum free amino acids, immune function and antioxidant enzyme activity

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Abstract 1. The aim of this study was to evaluate the tolerance of laying hens for an excessive L-valine (L-val) supply on laying performance, egg quality, serum free amino acids, immune function and antioxidant enzyme activities of laying hens.

2. A total of 720 HyLine Brown hens were allocated to 5 dietary treatment groups, each of which included 6 replicates of 24 hens, from 40 to 47 weeks of age. Graded amounts of L-val were added to the basal diet

only consideration, but the tolerance for an excessive supply is also relevant. It has been reported in rats (Benton *et al.*, 1956; Rogers *et al.*, 1962) that antagonisms exist between the three branched chain amino acids.

The effect of an excessive supply of L-val in laying hens is not well known. Therefore, the aim of this study was to evaluate the tolerance of laying hens for an excessive L-val supply on laying performance, egg quality, serum free amino acids, immune function and antioxidant enzyme activities.

MATERIALS AND METHODS

The experiment was conducted in accordance with the Chinese guidelines for animal welfare and approved by the animal welfare committee of Animal Science College, Zhejiang University.

Birds and housing

A total of 720 HyLine Brown hens, 40 weeks of age and from a commercial layer farm, with similar performance were randomly allocated to 5 treatment groups, each of which included 6 replicates of 24 hens. The hens were housed at 4 birds per cage under the same managerial conditions in a windowed poultry house. They were divided into 5 experimental groups and assigned to the treatment replicates based on laying percentage and hen weight (on average 91.5% and 1900 g, respectively). Hens were kept in 3-layer complete ladder cages and fed *ad libitum* twice daily at 0600 and 1400 h; water nipples were available at all times. The photoperiod was 16L:8D throughout the experiment. The present study was carried out between May and June and the mean daily temperature was 23 ± 5°C.

Diets

Hens were fed diets based on corn, soya bean meal, peanut meal and crystalline amino acids. Graded amounts of L-val (98.5% purity, Specom Biochemical Co. Ltd, ZhangJiagang, China) were added to the basal diet at 0 (control), 1, 2, 3 and 4 g/kg, respectively. Dietary treatments were achieved by the addition of crystalline L-val at the expense of inert filler (kaolin) to derive dietary treatments. The study lasted 60 d, including a 7-d acclimation period and 53-d experimental period. Ingredient composition and analysed nutrients are presented in Table 1. Feed samples from each experimental diet were prepared in duplicate and analysed for amino acids (AOAC, 2000). To determine amino acids, samples were hydrolysed with 6 M HCl at 110°C for 24 h, and the major amino acid composition of hydrolysates was analysed by HPLC (Hitachi L-8900 Amino Acid Analyser, Tokyo, Japan).

Table 1. *Composition and nutrient content of the control diet*

Composition	g/kg
Maize	630
Soya bean meal, 44%	92
Peanut meal, 47.8%	140
Wheat bran	10
Soya bean oil	10
Limestone	85
CaHPO4	15
Sodium chloride	3
Premix[1]	5
DL-Meth	1.6
L-lysine HCl	1.8
L-threonine	0.3
L-tryptophan	0.3
L-isoleucine	2.0
L-valine	0.0
Filler[2]	4.0
Calculated concentration g/kg[3]	
Metabolisable energy, MJ/kg	11.31
Crude protein	161.6
Calcium	35.7
Available phosphorus	4.4
Amino acid concentration, g/kg[3] *(analysed)*	
Lysine	7.4 (7.2)
Methionine	3.6 (3.4)
Threonine	5.6 (5.3)
Isoleucine	6.5 (6.7)
Valine	7.0 (6.9)
Leucine	12.4 (13)
Tryptophan	1.83 (–)

[1]Premix provided the following per kg of diet: retinyl palmitate, 3.96 mg; cholecalciferol, 0.06 mg; DL-α-tocopheryl acetate, 20 mg; menadione sodium bisulphite, 4 mg; thiamine mononitrate, 1.63 mg; riboflavin, 5 mg; niacin, 30 mg; pantothenic acid, 10 mg; folic acid, 0.5 mg; biotin, 0.22 mg; choline chloride, 250 mg; cyanocobalamin, 0.012 mg; Mn, 48 mg; Zn, 40 mg; Fe, 24 mg; Cu, 16 mg; I, 0.6 mg; Se, 0.12 mg; moisture ≤ 10%.
[2]The dose titrations were achieved by the addition of L-valine at the expense of the inert filler (kaolin) to derive dietary treatments.
[3]Values were calculated from data provided by the Feed Database in China (2012).
[4]Chemical composition data are the results of a chemical analysis conducted in duplicate.

Laying performance parameters and egg quality

Hen-d egg production and egg weight were recorded daily, and feed consumption was recorded weekly on a replicate basis. Egg mass was calculated (egg weight × egg production). Feed conversion ratio was calculated as g of feed intake per g of egg mass produced. Mortalities and health status were visually observed and recorded daily throughout the entire experimental period. At the end of the experiment, 24 eggs from each treatment were randomly collected to assess egg quality parameters. The eggs were weighed, opened and albumen

height, Haugh units, yolk colour and eggshell strength were measured with the digital egg tester (DET-6000, NABEL, Kyoto, Japan). Eggshell breaking strength was measured by applying increased pressure to the broad pole of the shell.

Blood sampling and laboratory analyses

At the end of the experiment (week 47), 6 hens per treatment were slaughtered by cervical dislocation and blood samples were collected. Immediately following slaughter, the livers of each bird were collected and snap-frozen with liquid nitrogen, and stored at −80°C until analysis. The serum was centrifuged for 10 min (958 × *g*) at room temperature. Serum samples were aspirated by pipette and stored in 1.5-ml Eppendorf tubes at −70ºC until analysed. The samples were then thawed at 4°C before analysis. Serum concentrations of total protein, albumin, total amino acids, blood urea nitrogen (BUN), uric acid, lactate dehydrogenase (LDH), alkaline phosphatase (AKP), Ca and P were measured spectrophotometrically (UV-2000, Unico Instruments Co. Ltd., Shanghai, China) using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum glucose (GLU) was measured using a commercial kit (Shanghai Rongsheng Biotech Co. Ltd., Shanghai, China). Concentrations of serum IgA, IgG, IgM and complements (C3 and C4) were measured using a commercial kit (Shanghai Fosun Long March Medical Science Co., Ltd). Standards of thyroxine (T4) and 3,5,3′-triiodothyronine (T3) were from Sigma (St. Louis, MO). A stable deuterium-labelled internal standard, l-thyroxine-d2, was synthesised as previously described (Burman *et al.*, 1981; Tai *et al.*, 2002)

Serum free amino acids

To determine free amino acids, serum was deproteinised by mixing equal volumes of serum and trichloroacetic acid (7.5% w/v) in a 1.5-ml microcentrifuge tube. The samples were vortexed (30 s) and centrifuged for 15 min at 15 000 × *g*. A 20-µl aliquot of the supernatant was injected into the HPLC column (Hitachi L-8900 Amino Acid Analyser, Tokyo, Japan). Amino acids were separated by cation exchange using lithium buffers with ultraviolet light detection (570 nm) of individual amino acids (440 nm for proline) achieved by postcolumn ninhydrin derivatisation.

Serum oxidant and antioxidant status

Lipid peroxidation was estimated by measuring serum malondialdehyde (MDA) content and antioxidant enzyme concentrations in serum were assessed on the basis of superoxide dismutase (SOD) and total antioxidative capability (T-AOC). The analyses were performed spectrophotometrically (UV-2000, Unico Instruments Co. Ltd., Shanghai, China) using MDA, SOD and total antioxidative capability (T-AOC) assay kits, which were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Serum MDA content was measured using the thiobarbituric acid method (Ohkawa *et al.*, 1979), reading the absorbance at 532 nm with the spectrometer. Serum SOD activity was assayed by the xanthine oxidase method (Winterbourn *et al.*, 1975), which monitors the degree of inhibition of nitroblue tetrazolium reduction by O_2 generated from xanthine and xanthine oxidase, and the change in the absorbance of the solution was recorded at 550 nm using a spectrophotometer. Total antioxidative capability (T-AOC) was evaluated by the method of ferric reducing/antioxidant power assay (Benzie and Strain, 1996). In the reaction mixture, ferric ion was reduced by antioxidant reducing agents and blue complex Fe(2+)–TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) was produced. Then this blue complex reacted with phenanthroline to generate a stable complex which could be monitored by the absorbance at 520 nm. Data were expressed as U/ml serum.

Statistical analyses

Table 2. *Effect of dietary L-valine supplementation on the laying performance of laying hens (mean of 6 replications, with 24 hens per replicate)*

	L-val, g/kg							Contrast *P*-value[1]		
Trait	0.0	1.0	2.0	3.0	4.0	SEM	Val	L	Q	C
Hen-day egg production, %	91.56	91.91	94.42	91.48	91.16	2.29	0.62	0.81	0.28	0.93
Egg weight, g	63.44	63.19	61.81	63.84	63.35	0.87	0.23	0.81	0.22	0.48
Egg mass, g/hen/d	58.07	58.05	58.35	58.39	57.72	1.25	0.98	0.89	0.64	0.71
Feed intake, g/h/d[2]	113.48^{ab}	113.52^{ab}	115.37^{a}	113.56^{ab}	109.72^{b}	1.65	0.04	0.06	0.01	0.31
FCR, g of feed/g of egg mass	1.95	1.95	1.97	1.94	1.90	0.06	0.98	0.89	0.65	0.71

[1]L = Linear. Q = Quadratic. C = Cubic.
[2]Values with different superscripts are significant at $P < 0.05$

mass by 5 to 10%. In the current study, the highest concentration of total dietary valine was 1.19%. Therefore, we did not find depression in egg mass. Also, Zhang *et al.* (2007) reported that an increase in dietary leucine reduced food intake in rats and mice. In addition, Peganova and Eder (2002*b*) studied the response of laying hens to an excess of isoleucine and reported that feed consumption decreased significantly at higher concentrations of L-isoleucine. In general, animals decrease their feed consumption when fed diets in which the protein content is very low, very high or deficient in an indispensable amino acid, or in which the amino acid proportions in the protein are grossly imbalanced from the amino acid needs of the animal (Anderson, 1977).

L-val supplementation at 2 g/kg increased significantly the concentration of serum glucose (Table 3). There is a link between blood glucose and appetite that led to the glucostatic theory of appetite control. Van Der Wal *et al.* (1999) reported that blood glucose concentrations declined in broilers experiencing food deprivation. Corzo *et al.* (2005) reported that supplementation of dietary tryptophan increased feed intake and blood plasma glucose.

Serum albumin concentration was significantly affected by supplemental L-val, and the response was maximised at 2 g/kg (Table 4). Total protein and albumin concentrations are indicators for the protein status of the blood. Laborde *et al.* (1995) suggested that the albumin fraction of total protein is more of an indicator of the long-term protein status. The non-albumin part of total protein includes globulin, fibrinogen, peptide hormones, enzymes and amino acids (Kaneko, 1989). This means that dietary L-val up to 2 g/kg can be successfully supplemented into

Table 3. *Effect of dietary L-valine supplementation on serum blood parameters and antioxidant enzyme activity (n = 6 hens per group)*

	L-valine, g/kg							Contrast *P*-value[1]		
Trait[2]	0.0	1.0	2.0	3.0	4.0	SEM	Val	L	Q	C
Glucose, mM	8.37^{b}	8.81^{ab}	12.06^{a}	8.36^{b}	8.75^{ab}	1.22	0.03	0.90	0.04	0.64
LDH, U/l	4897.03	4971.26	4987.06	4757.98	5059.54	153.98	0.37	0.75	0.61	0.09
AKP, King units/100 ml	48.55	57.06	59.64	61.65	61.86	11.00	0.60	0.73	0.98	0.20
Ca, mM	2.06	2.16	2.94	2.87	2.67	0.23	0.37	0.14	0.21	0.41
P, mM	1.71	1.79	2.11	1.72	1.78	0.21	0.32	0.89	0.17	0.66
T-SOD, U/ml	184.52	169.84	175.84	170.86	180.96	6.89	0.19	0.69	0.04	0.72
T-AOC,U/ml	6.63	5.98	6.53	6.78	6.35	0.68	0.80	0.87	0.95	0.23
MDA,U/ml	7.17	4.37	4.14	4.20	3.58	0.97	0.94	0.54	0.61	0.91

[1]L = Linear. Q = Quadratic. C = Cubic.
[2]LDH = lactate dehydrogenase; AKP = alkaline phosphatase; T-SOD = superoxide dismutase; T-AOC = total antioxidative capability; MDA = malondialdehyde.

Table 4. *Effect of dietary L-val supplementation on serum total protein, albumin, total amino acids, blood urea nitrogen and uric acid (n = 6 hens per group)*

	L-valine, g/kg							Contrast *P*-value[1]		
Trait	0.0	1.0	2.0	3.0	4.0	SEM	Val	L	Q	C
Total protein, g/l	52.15	52.02	59.49	59.69	58.30	5.26	0.37	0.09	0.48	0.44
Albumin, g/l[2]	23.27^{ab}	22.01^{ab}	24.20^{a}	22.92^{ab}	20.82^{b}	0.95	0.01	0.07	0.05	0.05
Total amino acids, μmole	16.24	14.43	18.34	16.00	13.23	2.50	0.25	0.41	0.20	0.25
Blood urea nitrogen, mM	4.27	5.06	4.35	5.71	6.55	1.11	0.23	0.05	0.47	0.70
Uric acid, μM	269.65	254.29	309.86	266.59	269.59	21.84	0.14	0.80	0.29	0.61

[1]L = Linear. Q = Quadratic. C = Cubic.
[2]Values with different superscripts are significant at $P < 0.05$.

diets without detrimental effects on serum protein concentration which is required for egg formation. Serum uric acid or BUN (Table 4) did not change due to L-val supplementation. This result is in agreement with that of Corzo *et al.* (2003, 2005) and Azzam *et al.* (2011). These authors found no changes in blood uric acid concentrations when dietary lysine, tryptophan or threonine, respectively, were increased in the diet. The current results showed that serum free valine increased as L-val concentration increased and the response was maximised between 1 and 2 g and then decreased linearly at 4 g supplemental L-val (Table 5). The maximum concentrations of serum free valine occurred at 1 and 2 g L-val, suggesting a requirement for L-val is within these two levels of supplementation.

Serum concentration of triiodothyronine (T3) increased significantly at 2 g L-val/kg supplementation compared to the control group, but serum T4 concentrations were not significantly altered by graded levels of L-val (Table 6). This result is in agreement with that of Carew *et al.* (1998) reporting that chicks fed an excess of valine had plasma T3 concentrations that were statistically higher than control treatment concentrations. Thyroid hormones are recognised as the key metabolic hormones of the body, with T3 being the most functionally active form. The serum concentration of thyroid hormones is associated with protein synthesis and energy production (Hornick *et al.*, 2000; Smith *et al.*, 2002).

Adding L-val did not affect egg quality (Table 7). Huyghebaert *et al.* (1991) also found that Haugh units were unaffected by isoleucine concentration.

From a nutritional viewpoint, amino acids affect the synthesis of effector molecules (immunoglobulins, nitric oxide, lysozyme and complement). The addition of L-val did not affect concentrations of immunoglobulins IgG, IgA, IgM, C3 or C4 (Table 8). Also, in broilers, Thornton *et al.* (2006) reported that there were no effects of valine on innate or adaptive immunity. In contrast, Bhargava *et al.* (1971) evaluated valine responses when birds were inoculated with Newcastle disease virus and reported that valine is required for antibody production. It has been reported that other supplemental AAs like L-threonine or L-tryptophan increased serum IgG and IgM concentration in laying hens

Table 5. *Effect of dietary L-valine supplementation on serum free amino acids (n = 6 hens per group)*

	L-val, g/kg							Contrast *P*-value[1]		
Amino acid, μg/ml	0.0	1.0	2.0	3.0	4.0	SEM	Val	L	Q	C
Essential AA										
Arginine	83.83	80.12	79.28	89.87	84.29	10.09	0.84	0.64	0.77	0.41
Histidine	26.12	32.11	25.22	27.58	29.42	3.43	0.30	0.79	0.92	0.12
Isoleucine	17.23	17.79	17.10	19.23	17.48	1.78	0.76	0.63	0.70	0.51
Leucine	31.41	32.87	30.65	34.87	30.05	2.72	0.42	0.90	0.40	0.38
Lysine	97.00	103.00	95.50	125.31	92.57	17.13	0.34	0.72	0.38	0.21
Methionine	15.81	14.26	16.25	16.67	16.80	1.56	0.50	0.22	0.66	0.28
Phenylalanine	20.45	20.60	21.65	22.83	22.70	1.78	0.54	0.10	0.92	0.58
Threonine	35.81	35.77	36.61	43.98	34.287	4.67	0.28	0.62	0.31	0.09
Valine	24.27[a]	25.01[a]	24.27[a]	20.78[ab]	15.45[b]	3.85	0.10	0.01	0.15	0.96
Nonessential AAs										
Alanine	46.13	50.58	52.67	48.32	54.86	3.32	0.10	0.05	0.79	0.08
Aspartic Acid	13.83	17.44	14.30	18.00	16.45	1.81	0.11	0.16	0.47	0.71
Cystine	10.18	9.97	11.66	10.77	12.68	1.84	0.57	0.17	0.73	0.83
Glutamic acid	30.35	34.21	31.85	30.74	31.50	3.53	0.83	0.88	0.60	0.31
Glycine	50.45	49.64	51.16	54.35	53.06	3.53	0.66	0.22	0.94	0.39
Proline	18.51	18.41	18.34	19.24	17.10	1.76	0.82	0.62	0.51	0.44
Serine	71.81	88.61	77.75	87.47	75.61	7.88	0.16	0.71	0.09	0.73
Tyrosine	24.10	26.99	28.14	24.70	31.08	3.50	0.30	0.15	0.79	0.15

[1]L = Linear. Q = Quadratic. C = Cubic.

Table 6. *Effect of dietary L-valine supplementation on serum concentration of thyroid hormones in laying hens (n = 6 hens per group)*

	L-val, g/kg							Contrast *P*-value[1]		
Thyroid hormone, nmole/ml	0.0	1.0	2.0	3.0	4.0	SEM	Val	L	Q	C
T3	0.71[b]	0.82[ab]	0.89[a]	0.80[ab]	0.70[b]	0.06	0.04	0.64	0.13	0.04
T4	21.99	22.25	23.93	22.83	21.37	2.00	0.81	0.88	0.27	0.68

[1]L = Linear. Q = Quadratic. C = Cubic.

without infection-challenge (Azzam *et al.,* 2011; Dong *et al.,* 2012). On the other hand, the current findings showed that adding L-val did not affect immune function negatively, all laying hens appeared healthy and no mortality occurred throughout the entire experimental period. Other excess AAs suppressed immune function; for example, excess methionine (Gershoff *et al.,* 1968) and leucine (Aschkenasy, 1979) suppressed humoral immune function in the rat.

Recently, it has been reported that amino acid supplementation affects antioxidant enzyme activity. For example, Dong *et al.* (2012) reported that serum T-SOD increased significantly at 0.4 g L-tryptophan/kg diet. Also, Azzam *et al.* (2012) concluded that L-threonine supplementation at 0.2% maximised the concentration of T-SOD in both serum and liver of laying hens. Moreover, in juvenile Jian carp (*Cyprinus carpio* var. Jian), Ile supplementation improved the activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase ($P < 0.05$). In the present study, supplementation of L-val did not affect serum concentrations of total antioxidative capability (T-AOC), SOD and MDA (Table 3) or the liver concentrations of these antioxidant enzymes (data not shown).

In conclusion, these results suggest that high concentrations of L-val are tolerated and can be successfully fed to laying hens without detrimental effects on laying performance or immunity. Further studies are required to investigate whether the inclusion of L-val in combination with lower protein concentrations can maintain production performance and reduce nitrogen excretion.

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Azzam, M.M.M., Djournal

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