

Effects of dietary protein content and 2-hydroxy-4-methylthiobutanoic acid or DL-methionine supplementation on performance and oxidative status of broiler chickens

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Abstract

Besides its typical role as an amino acid in protein synthesis, methionine is an important intermediate in methylation reactions. In addition, it can also be converted to cysteine and hence plays a role in the defence against oxidative stress. The present study was conducted to investigate further the role of DL-methionine (DLM) and its hydroxy analogue, DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA), on zootechnical performance and oxidative status of broiler chickens. Male broiler chickens were reared on two diets differing in crude protein (CP) content (low-protein, 18.3% *v.* high-protein, 23.2% CP) and were supplemented either with 0.25% DLM or 0.25% DL-HMTBA. Reducing the dietary protein content resulted in an impaired body weight gain ($P < 0.0001$). However, supplementation of DL-HMTBA to the low-protein diet partially alleviated these negative effects ($P = 0.0003$). This latter phenomenon could be explained by the fact that chickens fed DL-HMTBA-supplemented diets displayed a better antioxidant status as reflected in lower lipid peroxidation probably as a consequence of their higher hepatic concentrations of total and reduced glutathione compared with their DLM counterparts. On the other hand, within the high protein levels, uric acid might be an important antioxidant to explain the lower lipid peroxidation of high-protein DL-HMTBA-supplemented chickens. Hepatic methionine sulfoxide reductase-A gene expression was not significantly affected by the dietary treatments. In conclusion, the present study indicates that there are interactions between dietary protein content and supplementation of methionine analogues with respect to broiler performance and antioxidant status, also suggesting a causal link between these traits.

Key words: DL-Methionine (analogue); Oxidative status; Broiler chickens

Lysine and methionine are generally considered to be the first limiting amino acids in commercial wheat/maize–soyabean-based broiler chicken diets. Methionine deficiency results in reduced protein accretion rate and feather growth as well as impaired immune competence^(1,2). The deficiency symptoms can be counteracted by adding chemically synthesised DL-methionine (DLM) or its hydroxy analogue, DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA), to the diet. In addition, the impact of DLM supplementation on animal performance interacts strongly with the crude protein (CP) content of the diet (e.g. Garcia Neto *et al.*⁽³⁾). Supplementation of DLM to a 20% crude protein diet for broilers reduced their abdominal fat weight, whereas this

was not the case when the diet contained 23% CP⁽⁴⁾. Breast meat yield was increased and abdominal fat content was decreased with increasing DLM supplementation, and this response was more pronounced in chickens reared on a diet with a lower protein level (20.5% CP) compared with a higher protein level (26%)⁽⁵⁾. Fatufe & Rodehutsord⁽⁶⁾ reported that the response to methionine in terms of feed intake, body weight gain and feed efficiency was more pronounced when this amino acid was added to a low-protein (18.3%) compared with an adequate-protein (22.9%) ration in 8- to 21-d-old birds.

Besides the typical role of an amino acid as a building block in protein synthesis (anabolic function), methionine

Abbreviations: CP, crude protein; DL-HMTBA, DL-2-hydroxy-4-methylthiobutanoic acid; DLM, DL-methionine; FRAP, ferric reducing/antioxidant power; GSH, glutathione; GSSG, oxidised glutathione; Msr, methionine sulfoxide reductase; ROS, reactive oxygen species; T₃, triiodothyronine; TBARS, thiobarbituric acid reacting substances.

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has several other prominent roles in metabolism. It is an important intermediate in methylation reactions and can also be converted to cysteine, which is required for the synthesis of glutathione (GSH) and taurine. It is well recognised that these sulphur compounds have a crucial role in the defence against oxidative stress (reviewed by Métayer *et al.*⁽⁷⁾). Indeed, GSH and cysteine can act as direct scavengers of reactive oxygen species (ROS) and hence alleviate their deleterious effects on lipid, protein and DNA structures. Amino acid residues of proteins are very prone to oxidative damage, and this is particularly true for methionine residues. Fortunately, most cells are equipped with specific reductases: methionine sulfoxide reductase (Msr)-A and -B. These enzymes catalyse the reduction of the two stereoisomeric oxidation products of methionine, namely methionine sulfoxide-R and -S, back to methionine. In this way, the cyclic oxidation/reduction of methionine residues can even be considered as an important antioxidant defence system⁽⁸⁾. It is therefore hypothesised that extra methionine supplementation may be beneficial in alleviating oxidative stress by its ROS-scavenging activity.

Broiler chickens reared on diets with a low CP content are characterised by impaired body-weight gain, poor feed efficiency and augmented fat deposition⁽⁹⁾. In addition, intermediary metabolism and endocrine functioning are also disturbed (reviewed by Swennen *et al.*⁽⁹⁾). As an example, plasma corticosterone levels of chickens reared on a low-protein diet are chronically elevated⁽¹⁰⁾. This chronic exposure to high corticosterone concentrations enhances lipid peroxidation^(11,12). Furthermore, chickens fed a diet with low protein content are also characterised by a significantly elevated metabolic rate, as is sometimes also reflected in higher plasma 3,3',5-triiodothyronine levels^(9,13). An increased metabolic rate is associated with higher oxygen consumption and hence an augmented ROS production can be inferred. Therefore, the aims of the present study were as follows:

- (1) to elaborate further on the interaction between dietary methionine supplementation and the CP content of the basal diet on broiler chicken performance;
- (2) to assess whether a low-protein diet (18.3 *v.* 23.2% CP) is unfavourable in terms of redox balance and to assess whether supplementation with methionine sources could alleviate the deleterious effects of a redox imbalance;
- (3) to assess whether dietary supplementation with an equimolar amount of the DL-hydroxy analogue DL-HMTBA elicits the same effects as synthetic DLM on bird performance, intermediary metabolism, endocrine status and redox balance. Because DL-HMTBA has been shown to be more efficiently converted to cysteine and taurine than L-methionine⁽¹⁴⁾, this could lead to an ameliorated antioxidant action.

Materials and methods

The experimental protocol was approved by the Ethics Commission for Experimental Use of Animals of the K. U. Leuven.

Experimental set-up

Newly hatched male chicks (*n* 310; Ross 308) were obtained from a local hatchery (Belgabroed, Merksplas, Belgium). All chicks were reared in floor pens with wood shavings as litter. Temperature was set at 34°C for day-olds and gradually decreased to 20°C at 5 weeks of age. The lighting schedule provided 23 h of light per d.

Until 14 d of age, all chicks received a commercial starter diet (22.3% CP and 12.7 MJ metabolisable energy/kg) *ad libitum*. At 14 d of age, the chickens were divided into four treatment groups of seventy-five chickens receiving one of the four experimental diets *ad libitum* (Table 1). The four treatment groups were divided randomly over the poultry house with three pens per treatment group and twenty-five broiler chickens per pen. The levels of soyabean, soyabean meal, soya oil and celite were manipulated to create changes in protein and fat content and to keep the metabolisable energy level similar for both diets (12.5 MJ metabolisable energy/kg). This resulted in two isoenergetic diets with a high (23.2%) or a low (18.3%) CP level. For both diets, the National Research Council recommendations (1994) for essential amino acids were met. The low- and high-CP diets were supplemented either with 0.25% DLM or with 0.25% DL-HMTBA (Adisseo SAS, Antony, France), resulting in a total of four diets.

Measurements and sampling

Individual body weights and feed intake per pen were recorded on a weekly basis. At 4 and 6 weeks of age, fifteen broiler chickens per treatment group (five chickens per pen) were euthanised, and the liver, right pectoralis major of the breast, abdominal fat pad and heart were excised and weighed. Samples of the liver were immediately snap frozen in liquid N₂ and stored at -80°C. In addition, blood samples were collected from a wing vein using a heparinised syringe and kept on ice. After centrifugation, plasma was stored in a frozen condition (-20°C) until the analysis for hormone and metabolite concentrations.

Plasma analyses

Plasma triiodothyronine (T₃) concentrations were measured by RIA as described by Darras *et al.*⁽¹⁵⁾. The antiserum for T₃ was purchased from Byk-Belga (Diegem, Belgium). Plasma corticosterone levels were measured using a commercially available double antibody RIA kit (IDS Limited, Boldon, UK). The detection limit of the assay was 0.39 ng/ml.

Plasma uric acid (VetTest 9820378) concentrations were determined using the VetTest 8008 analyser (Idexx Laboratories, Inc., Westbrook, ME, USA). The apparatus is based on dry chemical technology and colorimetric reaction. Sample analysis is carried out on selective testing discs (Idexx Laboratories, Inc.) by means of a laser reading the bar codes.

Plasma superoxide dismutase (SOD) activity was assessed using the SOD assay kit (Dojindo Molecular Technologies Inc., Rockville, MD, USA) according to the manufacturer's recommendations using a microplate reader (Victor³ V™

Table 1. Diet components and calculated and analysed contents of the experimental diets

	High-protein		Low-protein	
	DLM	DL-HMTBA	DLM	DL-HMTBA
Ingredients (%)				
Soyabean meal	41.1	41.1	–	–
Full-fat soyabeans	–	–	37.6	37.6
Soya oil	7	7	2.58	2.58
Maize	51	51	51	51
Trace mineral–vitamin premix*	0.5	0.5	0.5	0.5
Phytase premix	0.1	0.1	0.1	0.1
Celite†	–	–	7.86	7.86
Calculated energy and nutrient content				
Metabolisable energy (MJ/kg)	12.5	12.5	12.5	12.5
CP (%)	23.2	23.2	18.3	18.3
DL-Met (99 %)‡	0.29	0.036	0.30	0.098
DL-HMTBA (88 %)‡	–	0.28	–	0.28
Met equivalence (%)	0.64	0.64	0.59	0.59
Met + Cys	1.02	0.73	0.80	0.59
Met equivalence + Cys (%)	1.02	1.02	0.89	0.89
Analysed energy and nutrient content				
Metabolisable energy (MJ/kg)	12.46	12.47	12.45	12.45
CP (%)	22.3	21.3	18.1	18.7
DL-Met (%)	0.62	0.38	0.53	0.38
DL-HMTBA (%)	–	0.2	–	0.2
Lys (%)	1.23	1.19	0.95	1.01
Arg (%)	1.76	1.64	1.3	1.42
Thr (%)	0.88	0.73	0.85	0.75
Val (%)	1.08	1.01	0.79	0.88
Ca (%)	0.85	0.85	0.85	0.85
Non-phytate P (%)	0.44	0.44	0.44	0.44
Na (%)	0.16	0.16	0.16	0.16

DLM, DL-methionine; DL-HMTBA, DL-2-hydroxy-4-methylthiobutanoic acid; CP, crude protein, NRC, National Research Council.

* Premix supplied the following amount of vitamins and minerals per kg of diet: vitamin A, 12 000 IU; vitamin D₃, 3000 IU; vitamin E, 50 IU; vitamin K₃, 2.5 mg; vitamin B₁, 2.2 mg; vitamin B₂, 0.035 mg; vitamin niacin, 38 mg; folic acid, 1 mg; biotin, 0.2 mg; choline-Cl, 650 mg; Fe, 45 mg; Cu, 25 mg; Mn, 60 mg; Co, 1 mg; Zn, 70 mg; I, 2 mg; Se, 0.4 mg; ethoxyquin, 35 mg; butylated hydroxytoluene, 25 mg.

† Celite is an energy-inert material used to obtain isoenergetic diets.

‡ For all diets, the NRC recommendations (1994) for essential amino acid were met. Specifically for methionine, DLM (Adisseo France SAS) was added to the diets irrespective of protein level to achieve the NRC recommendations of 4 g Met/kg. Finally, the low- and high-CP diets were supplemented either with DLM (0.25%) or with DL-HMTBA (0.25%), resulting in a total of four diets.

Multilabel Counter 1420, PerkinElmer, Waltham, MA, USA). Plasma lipid peroxidation was estimated by spectrophotometric determination of thiobarbituric acid reacting substances (TBARS) with the modified method of Lin *et al.*^(11,12). TBARS were expressed as nmol malondialdehyde (MDA) per ml plasma. Plasma ferric reducing/antioxidant power (FRAP) was determined using a room temperature assay with a 5 min time window as described by Lin *et al.*^(11,12). Ceruloplasmin levels in the plasma were measured with the *p*-phenylenediamine endpoint method⁽¹⁶⁾.

Hepatic glutathione analysis

For the quantification of total GSH and oxidised GSH or GSH disulfide (GSSG) concentrations in the liver samples, an enzymatic recycling method using GSH reductase was used. The preparation of the samples and the colorimetric endpoint measuring method was carried out according to the manufacturer's recommendation (Glutathione assay kit, no. 703002; Cayman Chemical Company, Ann Arbor, MI, USA). Reduced GSH levels could then be calculated by subtracting twice the GSSG concentration from the total GSH concentration. Total GSH, GSSG and reduced GSH were expressed relative to the protein concentration of the liver samples used.

The total protein concentration of the samples was determined by using the Pierce BCA Protein Assay (ThermoScientific, Rockford, IL, USA).

Reverse transcription and RT-PCR

The liver tissue was homogenised with a homogeniser (Ultra-turrax, Janke & Kunkel, Staufen, Germany), and RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Paisley, UK), according to the manufacturer's recommendations. Total RNA concentration was quantified and its integrity was verified by means of an UV spectrophotometer.

Total RNA (2 µg) was reverse transcribed using Avian Myeloblastosis Virus-RT (Promega, Catalogue no. M5108, Madison, WI, USA).

The primers for Msr-A, GSH synthetase and GSH reductase were designed using Primer premier 5.0 using the GenBank sequences for Msr-A (accession no. XM_420035.2), the GenBank sequences for GSH synthetase (accession no. XM_425692.2) and the Ensembl sequences for GSH reductase (accession no. ENSGALT00000016706), respectively. The primer that was used for the reference gene glucose-6-phosphate dehydrogenase (accession no. AI981686) was

from De Boever *et al.*⁽¹⁷⁾. Desalted primers for the target gene Msr-A (forward primer, 5'-TGA CTT TGG CAC TCA GTA TCG C-3'; reverse primer, 5'-GGC ACC AAA ACC GCT TTC T-3'), GSH synthetase (forward primer, 5'-AGA AGG AGG AGG GAA CAA CC-3'; reverse primer, 5'-GCC TGA CAT AGA CAC CGA AA-3'), GSH reductase (forward primer, 5'-CCA TGT GGT GGT GGA CGA GT-3'; reverse primer, 5'-GCA GAG GTA GGG TGG ATA GCA-3') and glucose-6-phosphate dehydrogenase (forward primer, 5'-CGG GAA CCA AAT GCA CTT CGT-3'; reverse primer, 5'-CGC TGC CGT AGA GGT ATG GGA-3') were purchased commercially (Invitrogen, Zwolle, The Netherlands).

Real-time PCR to quantify Msr-A, GSH synthetase and GSH reductase mRNA was performed using the ABI Prism 7700 Sequence Detector (ABI PRISM™; Life Technologies, Carlsbad, CA, USA).

The sequence detection software supplied with the 7700 Sequence Detector was used to analyse the raw data from the real-time reading of the fluorescence and to calculate the cycle threshold value for each reaction. The cycle threshold values were converted into a standard curve, to which each sample (average of three cycle threshold values) was compared to calculate the starting template concentrations. Expression levels of Msr-A, GSH synthetase and GSH reductase in each sample were normalised to the expression levels of the reference gene glucose-6-phosphate dehydrogenase. The average value of the high CP–DL-HMTBA group was set at 1 and the average value of the other three groups was expressed relative to the high CP–DL-HMTBA group.

Statistical analysis

All data were analysed by two-factor ANOVA, with dietary protein level (23.2 or 18.3% CP) and supplement source (DLM or DL-HMTBA) as classification variables (SAS version 9.1; SAS Institute, Inc., Cary, NC, USA). If a significant

($P < 0.05$) overall effect of the model was discerned, treatment means were separated by Tukey's test. Statistical significance is accepted when $P < 0.05$.

Results

Body weight, feed intake and proportional organ weights

Data on body weight and proportional breast muscle, abdominal fat pad, heart and liver weights collected at 4 weeks of age are summarised in Table 2. It was clear that the chickens reared on the low-protein diets attained a significantly lower body weight (protein effect, $P < 0.0001$) at 4 weeks of age, irrespective of the supplement source (DLM or DL-HMTBA). These reduced body weights of the low CP-fed broilers were also associated with significantly (protein effect, $P < 0.0001$) lower proportional (right) breast muscle weights. As expected, proportional abdominal fat pad weights of chickens fed the low-protein diets were significantly higher than those of chickens reared on a high-protein diet (protein effect, $P < 0.0001$). The supplement source had no differential effect on body weight or on proportional breast and abdominal fat pad weight. Chickens fed a low-protein diet had significantly higher proportional heart weights (protein effect, $P = 0.041$), but this effect of dietary protein on the percentage of heart weight was also dependent on the supplement source (protein \times supplement source effect, $P = 0.02$). Indeed, chickens reared on a low-protein diet and receiving DLM as a supplement were characterised by a significantly higher proportional heart weight compared with that of their high-protein DLM-fed counterparts. DL-HMTBA-supplemented chickens had an intermediate proportional heart weight. The (proportional) liver weights were not different between the treatment groups.

The retardation in body-weight gain due to low dietary CP content (protein effect, $P < 0.0001$) persisted at 6 weeks of age

Table 2. Body weights and proportional organ and tissue weights of 4-week-old broiler chickens raised on diets with low (18.3%) or high (23.2%) crude protein (CP) content and supplemented with 0.25% DL-methionine (DLM) or 0.25% DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA)

(Mean values with their pooled standard errors, n 15/dietary treatment)

	Met source	BW (g)	Proportional weight			
			Liver (%)	Heart (%)	Abdominal fat (%)	Breast muscle (%)
Protein level (%)						
18.3	DLM	1156	2.48	0.37 ^a	1.47	7.44
	DL-HMTBA	1145	2.41	0.34 ^{a,b}	1.41	7.31
23.2	DLM	1428	2.69	0.32 ^b	1.11	8.17
	DL-HMTBA	1424	2.59	0.34 ^{a,b}	1.09	8.57
Pooled SEM		25	0.05	0.006	0.04	0.11
Main effects						
Protein level	18.3	1150 ^b	2.41	0.35	1.43 ^a	7.38 ^b
	23.2	1426 ^a	2.64	0.33	1.10 ^b	8.37 ^a
Met source	DLM	1292	2.58	0.35	1.29	7.81
	DL-HMTBA	1285	2.47	0.34	1.24	7.95
Probabilities						
	Protein level	<0.0001	NS	0.041	<0.0001	<0.0001
	Met source	NS	NS	NS	NS	NS
	Interaction	NS	NS	0.02	NS	NS

^{a,b} Mean values with unlike superscript letters within a column were significantly different according to corresponding probabilities.

Table 3. Body weights and proportional organ and tissue weights of 6-week-old broiler chickens raised on diets with low (18.3%) or high (23.2%) crude protein (CP) content and supplemented with 0.25% DL-methionine (DLM) or 0.25% DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA)(Mean values with pooled standard errors, *n* 15/dietary treatment)

	Met source	BW (g)	Proportional weight			
			Liver (%)	Heart (%)	Abdominal fat (%)	Breast muscle (%)
Protein level (%)						
18.3	DLM	2024	1.76	0.32 ^a	1.64	8.19
	DL-HMTBA	2301	1.92	0.28 ^b	1.68	8.28
23.2	DLM	2492	1.89	0.28 ^b	1.12	9.31
	DL-HMTBA	2707	2.02	0.30 ^{a,b}	1.03	8.99
Pooled SEM		46	0.03	0.004	0.06	0.12
Main Effects						
Protein level	18.3	2153 ^b	1.84 ^b	0.30	1.66 ^a	8.23 ^b
	23.2	2599 ^a	1.96 ^a	0.29	1.07 ^b	9.16 ^a
Met source	DLM	2258 ^b	1.83 ^b	0.30	1.38	8.75
	DL-HMTBA	2518 ^a	1.98 ^a	0.29	1.33	8.63
Probabilities						
	Protein level	<0.0001	0.042	NS	<0.0001	<0.0001
	Met source	0.0030	0.011	NS	NS	NS
	Interaction	NS	NS	0.0012	NS	NS

^{a,b} Mean values with unlike superscript letters within a column were significantly different according to corresponding probabilities.

(Table 3). However, supplementation of the low-protein diet with DL-HMTBA alleviated this growth-depressing effect of low dietary protein to some extent, as these chickens were able to attain a significantly higher average slaughter weight compared with that of their low-protein DLM-supplemented counterparts (supplement source effect, $P=0.003$). Chickens reared on the low-protein diets also had the lowest proportional breast muscle weights (protein effect, $P<0.0001$) and lowest proportional liver weights (protein effect, $P=0.042$). However, the proportional liver weights of the DL-HMTBA-supplemented chickens were, on average, higher than those of the DLM-supplemented chickens (supplement source effect, $P=0.011$). The stimulatory effect of low dietary protein content (protein effect, $P<0.0001$) on proportional abdominal fat pad deposition was even more pronounced at slaughter age, irrespective of DLM or DL-HMTBA supplementation.

In agreement with the observations at 4 weeks of age, the low-protein DLM-fed chickens were characterised by the highest proportional heart weights compared with the other treatment groups (protein \times supplement source interaction, $P=0.0012$).

Chickens reared on the low-protein diets consumed, on average, less feed than their counterparts fed the high-protein diets, irrespective of the supplement source (data not shown). The reducing effect of dietary protein content on feed intake was significant at weeks 3 ($P=0.0068$) and 6 ($P=0.0053$).

Redox balance

At 4 weeks of age, lipid peroxidation – as reflected in plasma TBARS levels – was highest (supplement source effect, $P=0.011$) for the DLM-supplemented chickens (Table 4).

Table 4. Plasma thiobarbituric acid reacting substances (TBARS), ferric reducing/antioxidant power (FRAP), ceruloplasmin and uric acid levels of 4-week-old broiler chickens raised on diets with low (18.3%) or high (23.2%) crude protein (CP) content and supplemented with 0.25% DL-methionine (DLM) or 0.25% DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA)(Mean values with their pooled standard errors, *n* 15/dietary treatment)

	Met source	TBARS (nmol/l)	FRAP ($\mu\text{mol Fe}^{2+}/\text{l}$)	Ceruloplasmin (U/l)	Uric acid (mg/l)
Protein level (%)					
18.3	DLM	3.89	1981 ^a	74.9	57.3
	DL-HMTBA	2.75	1506 ^c	55.4	57.0
23.2	DLM	4.09	1976 ^a	114	72.4
	DL-HMTBA	1.83	1750 ^b	64.8	76.7
Pooled SEM		0.33	36	4.92	2.2
Main effects					
Protein level	18.3	3.32	1744	65.1 ^b	57.3 ^b
	23.2	2.96	1863	89.7 ^a	74.5 ^a
Met source	DLM	3.99 ^a	1979	94.7 ^a	64.9
	DL-HMTBA	2.29 ^b	1628	60.1 ^b	66.8
Probabilities					
	Protein level	NS	NS	0.0037	<0.0001
	Met source	0.011	<0.0001	<0.0001	NS
	Interaction	NS	0.0021	NS	NS

^{a,b,c} Mean values with unlike superscript letters within a column were significantly different according to corresponding probabilities.

Table 5. Plasma thiobarbituric acid reacting substances (TBARS), ferric reducing/antioxidant power (FRAP), ceruloplasmin and uric acid levels of 6-week-old broiler chickens raised on diets with low (18.3%) or high (23.2%) crude protein (CP) content and supplemented with 0.25% DL-methionine (DLM) or 0.25% DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA)

(Mean values with their pooled standard errors, *n* 15/dietary treatment)

	Met source	TBARS (nmol/l)	FRAP ($\mu\text{mol Fe}^{2+}/\text{l}$)	Ceruloplasmin (U/l)	Uric acid (mg/l)
Protein level (%)					
18.3	DLM	6.98 ^{a,b}	934 ^{a,b}	40.3	38.7 ^b
	DL-HMTBA	9.15 ^a	793 ^b	29.2	39.0 ^b
23.2	DLM	8.72 ^a	806 ^{a,b}	38.3	39.6 ^b
	DL-HMTBA	4.43 ^b	964 ^a	32.0	68.5 ^a
Pooled SEM		0.46	24	1.47	2.7
Main effects					
Protein level	18.3	7.99	866	35.1	38.8 ^b
	23.2	6.57	885	35.2	54.1 ^a
Met source	DLM	7.85	868	39.3 ^a	39.2 ^b
	DL-HMTBA	6.63	885	30.7 ^b	54.8 ^a
Probabilities					
	Protein level	NS	NS	NS	0.0007
	Met source	NS	NS	0.0031	0.0012
	Interaction	0.0002	0.0012	NS	0.0014

^{a,b} Mean values with unlike superscript letters within a column were significantly different according to corresponding probabilities.

DL-HMTBA-supplemented chickens displayed a lower plasma FRAP capacity compared with that of the DLM-supplemented chickens (supplement source effect, $P < 0.0001$). This supplement source effect on plasma FRAP capacity was even more pronounced with the low-protein diet (protein \times supplement source interaction, $P = 0.0021$). DLM-supplemented chickens displayed overall higher plasma ceruloplasmin levels (supplement source effect, $P < 0.0001$) at 4 weeks of age compared with their DL-HMTBA counterparts. Chickens reared on a high-protein diet also had, on average, higher plasma ceruloplasmin levels (protein effect, $P = 0.0037$). Plasma uric acid concentrations were only affected by dietary protein content (protein effect, $P < 0.0001$), as chickens reared on a low-protein diets were characterised by significantly lower plasma uric acid levels, irrespective of the supplement source.

At 6 weeks of age, a significant ($P = 0.0002$) protein \times supplement source interaction was observed as significant differences in plasma TBARS levels were observed between the DL-HMTBA- and DLM-supplemented chickens but only when reared on a high-CP diet (Table 5). A significant protein \times supplement source interaction ($P = 0.0012$) was also present for plasma FRAP capacity: DL-HMTBA-supplemented chickens were characterised by a higher plasma FRAP capacity but only when a high-CP diet was provided. Circulating ceruloplasmin levels were clearly affected by the supplement source ($P = 0.0031$). Indeed, plasma obtained from DL-HMTBA-supplemented chickens contained significantly lower concentrations of ceruloplasmin compared with that from DLM-supplemented chickens, regardless of the dietary protein level. Higher dietary CP content was associated with significantly (protein effect, $P = 0.0007$) higher plasma uric acid levels. However, this effect was only observed for DL-HMTBA-supplemented chickens (supplement source effect, $P = 0.0012$), resulting in a significant ($P = 0.0014$) protein \times supplement source interaction.

Plasma SOD activity was not significantly influenced by either the dietary protein level or the supplement source at 4 weeks of age (data not shown). At 6 weeks of age, DL-HMTBA-supplemented chickens had, on average, higher plasma SOD activities compared with those of their DLM-supplemented counterparts, but this difference was not statistically discernable at the 5% level.

Statistical analysis revealed a tendency ($P = 0.062$) towards an interaction between protein level and supplement source for hepatic total GSH levels of 6-week-old chickens. Indeed, low-protein DL-HMTBA-supplemented chickens had somewhat higher amounts of total GSH compared with other treatment groups. Furthermore, there was also a tendency ($P = 0.095$) towards a supplement source effect on hepatic reduced GSH amounts as DL-HMTBA-supplemented chickens had, on average, higher amounts of the reduced form of GSH. Statistical analysis did not reveal any significant effects on GSH synthetase, glutathione reductase or Msr-A gene expression in the livers from 6-week-old chickens (Table 6).

Plasma hormones

At 4 weeks of age, chickens reared on the low-protein diets had, on average, higher plasma T_3 levels (protein effect, $P = 0.0030$) than their counterparts fed the high-protein diets (Table 7). DL-HMTBA supplementation significantly (supplement source effect, $P = 0.0006$) reduced circulating T_3 levels of 4-week-old chickens on the high-protein diets. However, at 6 weeks of age, higher T_3 concentrations were measured in the plasma obtained from low-protein DL-HMTBA-supplemented chickens compared with those from low-protein DLM-fed chickens (protein \times supplement source interaction, $P = 0.012$).

In general, plasma corticosterone levels were significantly lower for the DL-HMTBA-supplemented chickens (at 4 weeks

Table 6. Hepatic total glutathione (GSH), oxidised GSH (GSSG), GSH synthetase, GSH reductase and methionine sulfoxide reductase-A (Msr-A) mRNA levels of 6-week-old broiler chickens raised on diets with low (18.3%) or high (23.2%) crude protein (CP) content and supplemented with 0.25% DL-methionine (DLM) or 0.25% DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA) (Mean values with their pooled standard errors, n 8–10/dietary treatment)

Protein level (%)	Met source	GSH					
		Total GSH (nmol/mg protein)	GSSG (nmol/mg protein)	Reduced (nmol/mg protein)	Synthetase GSH synthetase mRNA/G6PDH mRNA	Reductase GSH reductase mRNA/G6PDH mRNA	Msr-A (Msr-A mRNA/G6PDH mRNA)
18.3	DLM	55.7	4.23	47.3	1.18	1.53	1.19
	DL-HMTBA	68.9	5.43	58.1	0.98	1.42	0.86
23.2	DLM	60.1	5.70	48.7	1.30	2.13	1.08
	DL-HMTBA	59.0	4.49	50.0	1.00	1.00	1.00
Pooled SEM		1.94	0.33	1.84	0.09	0.17	0.08
Main effects	18.3	62.3	4.83	52.7	1.09	1.47	1.02
	23.2	59.5	5.09	49.3	1.15	1.56	1.04
Protein level	DLM	57.9	4.97	48.0	1.24	1.83	1.14
	DL-HMTBA	64.0	4.96	54.1	0.99	1.21	0.92
Probabilities	Protein level	NS	NS	NS	NS	NS	NS
	Met source	NS	NS	NS	NS	NS	NS
	Interaction	NS	NS	NS	NS	NS	NS

G6PDH, glucose-6-phosphate dehydrogenase.

of age: supplement source effect, $P=0.025$) compared with those of their DLM-supplemented counterparts. At 6 weeks of age, this observation was especially true for DL-HMTBA-supplemented chickens reared on a high-protein diet (protein \times supplement source interaction, $P=0.013$).

Discussion

Zootechnical performance and plasma bioindicators

Body-weight gain was significantly depressed by feeding a diet with a reduced (18.3 *v.* 23.2%) CP content. This observation is in agreement with many other studies (reviewed by Swennen *et al.*⁽⁹⁾).

The higher proportional abdominal fat pad weights of the chickens reared on the low-protein diets are in agreement with previous studies and follow from the involuntary over-consumption of energy compared with protein (reviewed by Swennen *et al.*⁽⁹⁾). This excess energy is then partially deposited as fat and partially lost as heat⁽¹³⁾.

Another clear effect is the marked reduction in plasma uric acid concentrations in plasma obtained from chickens reared on a low-protein diet. This phenomenon has been observed several times before (reviewed by Swennen *et al.*⁽⁹⁾) and illustrates the improved efficiency of nitrogen utilisation (better dietary protein retention and less protein degradation/amino acid oxidation) with lower protein intake.

However, DL-HMTBA supplementation to the low-protein diet partially alleviated the growth-depressing effect of the low-protein diet. This is in contrast with the statement of Sauer *et al.*⁽¹⁾ from their meta-analysis study that on an equimolar basis, the biological efficiency of methionine hydroxy analogue-free acid is 81% of the value for DLM for daily body-weight gain. On the other hand, using multiple regression models, Vasquez-Anon *et al.*⁽¹⁸⁾ did not observe a statistically significant difference in predicted responses for body-weight gain between DLM and DL-HMTBA, and these authors even mentioned a trend ($P\leq 0.1$) for a higher peak body-weight gain response for DL-HMTBA.

For comparing the relative activity of methionine sources, Kratzer & Littell⁽¹⁹⁾ pointed out that the non-linear common plateau asymptotic regression model is not accurate due to the lack of a common plateau. Instead, the authors⁽¹⁹⁾ proposed to use a non-linear separated asymptotic plateau regression. Using published broiler growth data⁽²⁰⁾ and the non-linear separated asymptotic plateau regression approach, the authors⁽¹⁹⁾ demonstrated that the relative efficiency of methionine sources is dependent on the applied dosage with a better efficiency for DLM at a lower dosage but a better efficiency of HMTBA at higher levels.

However, one other explanation that could contribute to a better performance effect of DL-HMTBA should be linked to the acidifying potential of this molecule. Indeed, both *in vitro* and *in vivo* DL-HMTBA shows effective changes in the gut microbiota as in the case of organic acids⁽²¹⁾.

It was striking that low-protein DLM-supplemented chickens were consistently characterised by the lowest body weights and the highest proportional heart weights of all

Table 7. Plasma triiodothyronine (T₃) and corticosterone levels of 4- and 6-week-old broiler chickens raised on diets with low (18.3%) or high (23.2%) crude protein (CP) content and supplemented with 0.25% DL-methionine (DLM) or 0.25% DL-2-hydroxy-4-methylthiobutanoic acid (DL-HMTBA) (Mean values with their pooled standard errors, *n* 15/dietary treatment)

	Met source	T ₃ (ng/ml)		Corticosterone (ng/ml)	
		Week 4	Week 6	Week 4	Week 6
Protein level (%)					
18.3	DLM	1.97	0.79 ^b	8.44	6.49 ^{a,b}
	DL-HMTBA	1.71	1.80 ^a	6.03	8.10 ^{a,b}
23.2	DLM	1.86	1.19 ^{a,b}	6.88	8.33 ^a
	DL-HMTBA	1.36	1.16 ^{a,b}	3.81	3.41 ^b
Pooled SEM		0.06	0.11	0.62	0.67
Main effects					
Protein level	18.3	1.84 ^a	1.26	7.23	7.24
	23.2	1.61 ^b	1.18	5.35	5.87
Met source	DLM	1.91 ^a	0.99	7.66 ^a	7.41
	DL-HMTBA	1.54 ^b	1.46	4.92 ^b	5.58
Probabilities					
	Protein level	0.0030	NS	NS	NS
	Met source	0.0006	NS	0.0250	NS
	Interaction	NS	0.0120	NS	0.0130

^{a,b} Mean values with unlike superscript letters within a column were significantly different according to corresponding probabilities.

treatment groups. Based on the proportional growth theory, and given the fact that the heart is an early maturing organ (allometric b-coefficient is approximately 0.85)⁽²²⁾, it is understandable that lighter chickens have a higher proportional heart weight compared with that of heavier animals. However, it cannot be excluded that the supplement source *per se* could have an effect on heart tissue development.

Redox balance

The level of TBARS in the plasma is an indicator of (cell membrane) lipid peroxidation (reviewed by Benzie⁽²³⁾). The dietary protein content had no significant effect on plasma TBARS levels whereas DL-HMTBA supplementation resulted in a consistently lower lipid peroxidation compared with DLM supplementation within the high protein groups. This favourable effect is not due to an elevation in the total antioxidant capacity of the plasma, as the FRAP capacity of the plasma of DL-HMTBA-supplemented chickens was sometimes lower than that of the DLM-supplemented chickens. Ceruloplasmin is a Cu-containing acute-phase protein with powerful antioxidant capacities (e.g. Park *et al.*⁽²⁴⁾). It is hence inferred that elevated circulating ceruloplasmin levels will contribute to a better antioxidant defence. However, DL-HMTBA-supplemented chickens were clearly characterised by lower plasma ceruloplasmin levels. Furthermore, uric acid is also recognised as an important antioxidant in mammals⁽²⁵⁾, and especially in birds⁽²⁶⁾. Still, in the present study, there is no consistent indication that this antioxidant is involved in the lower lipid peroxidation status of DL-HMTBA-supplemented chickens, as there were no significant differences in circulating uric acid levels between DL-HMTBA- and DLM-supplemented chickens, except at 6 weeks of age, as high-protein DL-HMTBA-supplemented chickens were characterised by the highest plasma uric acid levels. Therefore, the non-enzymatic antioxidant capacity of the plasma is not likely to be the

predominant causal mechanism for the reduced lipid peroxidation status of the DL-HMTBA-supplemented chickens, except maybe within the high-protein treatment. There is also no direct evidence that the enzymatic antioxidant capacity of the plasma is involved. Based on plasma SOD activity, there were no statistical differences between DL-HMTBA- and DLM-supplemented chickens except for a tendency towards higher SOD activity for DL-HMTBA chickens at 6 weeks of age. However, it must be recognised that SOD is only one of the many ROS-scavenging enzymes besides catalase and GSH-recycling enzymes⁽²⁷⁾.

As there is no clear evidence that the non-enzymatic antioxidant defence mechanisms are responsible for the lower lipid peroxidation status of DL-HMTBA-supplemented chickens, it might be DL-HMTBA itself. Indeed, compared with DLM, DL-HMTBA is more efficiently converted to cysteine as a constituent of taurine and GSH⁽¹⁴⁾. These sulphur-containing compounds are very powerful antioxidants⁽⁷⁾, and a more efficient synthesis and hence higher availability could definitely contribute to an ameliorated ROS-scavenging system. This hypothesis is sustained by the tendency towards higher amounts of total GSH in the liver of 6-week-old low-protein DL-HMTBA-supplemented chickens. It is important to note that this elevation is entirely due to an increase in reduced GSH (representing the major portion of GSH) and not in GSSG. It can be postulated that the favourable redox balance due to DL-HMTBA supplementation to low-protein diets is now mediated by the GSH system and not so much by uric acid as in the case of high-protein diets.

Both Msr-A and -B are responsible for reduction in oxidised methionine residues and therefore are recognised as primary antioxidant defence mechanisms⁽⁸⁾. As far as we are aware of, this is the first time that hepatic gene expression of Msr-A has been measured in an avian species. Although we could not observe any treatment differences in Msr-A mRNA levels, this does not mean that the recycling of methionine

is not an important mechanism in these species. More research is needed to elucidate the contribution of Msr-A in the oxidant defence mechanism of non-mammalian species.

On the other hand, it could also be that DL-HMTBA reduces the production of ROS and hence cellular damage. Indeed, DL-HMTBA-supplemented chickens were also characterised by significantly lower plasma T₃ levels, at least at 4 but not at 6 weeks of age. Lower plasma T₃ levels are indicative for lower heat production⁽¹³⁾, thus less oxygen consumption and consequently reduced formation of free oxygen radicals. Furthermore, DL-HMTBA-supplemented chickens had significantly lower circulating corticosterone levels. As corticosterone is a powerful trigger of oxidative stress as reflected in enhanced lipid peroxidation^(11,12), the lower plasma corticosterone levels could also partially explain the reduced TBARS levels in these birds. It remains to be established how DL-HMTBA could directly affect glucocorticoid metabolism.

In conclusion, reducing the dietary protein content of broiler diets resulted in an impaired zootechnical performance compared with that of broilers reared on a higher dietary protein level. However, supplementation of the DLM hydroxy analogue, DL-HMTBA, to the low-protein diet alleviated these negative effects to a certain extent. In addition, the low-protein DL-HMTBA-supplemented chickens displayed better antioxidant status as reflected in lower lipid peroxidation probably due to their somewhat higher hepatic concentrations of total and reduced GSH levels compared with their DLM counterparts. On the other hand, within the high protein levels, uric acid might be an important antioxidant to explain the lower lipid peroxidation of high-protein DL-HMTBA-supplemented chickens. The present study indicates that there are interactions between dietary protein content and supplementation of DLM (analogues) with respect to broiler performance and redox status, also suggesting a causal link between these traits.

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