

Protein expression of *pectoralis major* muscle in chickens in response to dietary methionine status

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The present study evaluated the effect of dietary methionine on breast-meat accretion and protein expression in skeletal muscle of broiler chickens *in vivo*. All broilers received a common pre-test diet up to 21 d of age, and were subsequently fed either a methionine-deficient (MD) or -adequate (MA) diet (3.1 v. 4.5 g/kg diet) from age 21 to 42 d. Dietary cystine levels were 3.7 v. 3.6 g/kg diet for the MD and MA diet, respectively. Detrimental effects on carcass yield ($P=0.004$), abdominal fat percentage ($P=0.001$), and breast-meat weight ($P=0.001$), yield ($P=0.001$), and uniformity ($P=0.002$) were observed and validated in birds fed MD diets. Via tandem MS, a total of 190 individual proteins were identified from *pectoralis major* (PM) muscle tissue. From the former composite, peptides from three proteins were observed to be present exclusively in breast muscle from those chickens fed the MD diet (pyruvate kinase, myosin alkali light chain-1, ribosomal-protein-L-29). No proteins were observed to be uniquely expressed in chickens fed MA diets. Research is warranted to further explore the possibility of the proteins pyruvate kinase, myosin alkali light chain-1, or ribosomal protein L-29, as potential biological indicators of differences in protein expression of PM of chickens in response to a dietary methionine deficiency.

Mass spectrometry: Methionine: Protein expression: Proteomics

Animal nutritionists face constant challenges to balance dietary amino acids for optimal production at minimal nutrient expense. Because of the composition of feedstuffs used in poultry feed manufacturing, some amino acids become more limiting than others. In practical diets fed to poultry worldwide, methionine is the first limiting amino acid. Apart from being a component of bodily proteins of all living organisms, methionine is known for its role as a precursor of sarcosine, betaine, and choline via transmethylation, and for the conversion to cystine or cysteine (via homocysteine).

The muscles located in breast meat of poultry are the *pectoralis major* (PM) and *minor*, commonly referred to as fillets and tenders, respectively. The main genetic selection traits used to improve commercial broiler chickens are increased food intake, and improved PM conformation and mass. Hence, dietary amino acid composition and balance considers PM accretion rates, in addition to nutrient utilisation. Methionine composition of PM is among the lowest of all essential amino acids (Murphy, 1994), but its dietary inadequacy, based on the order of amino acid limitation, compromises PM accretion and nutrient utilisation efficiency (Hickling *et al.* 1990; Jeroch & Pack, 1995; Schutte & Pack, 1995*a,b*; Huyghebaert & Pack, 1996). As such, methionine needs, interactive effects with other amino acids, and ingredient source availability and efficacy are well researched in poultry (National Research Council, 1994).

The influence of dietary amino acids in gene and protein expression has become an important area of research. It has been shown how under conditions of amino acid undernutrition certain genes are consequently expressed aiming to regulate amino acid homeostasis (Andrulis *et al.* 1987; Nicholson & McGivan, 1996; Blommaert *et al.* 1997; Fafournoux *et al.* 2000; van Sluijters *et al.* 2000; Nedergaard *et al.* 2002; Jousse *et al.* 2004; Chen *et al.* 2005; Corzo *et al.* 2005). In some instances, regulatory effects in protein metabolism have been observed at the skeletal muscle level in response to amino acid signalling (Buse & Reid, 1975; Hong & Layman, 1984; Garlick & Grant, 1988; Long *et al.* 2000; Tremblay & Marette, 2001). Therefore, it is reasonable to assume that methionine may have a multitude of functions related to gene expression in the chicken. For that purpose, a study was conducted to create a scenario where dietary methionine would be supplied in deficient or adequate concentrations and, upon validation, compare the proteomes derived from the PM muscle and the influence that dietary methionine may have on the expression of those proteins.

Materials and methods

Chickens and diets

Male broiler chicks (1 d old; n 72; Ross × Ross 708) were obtained (Aviagen North America, Huntsville, AL, USA)

Abbreviations: MA, methionine-adequate; MD, methionine-deficient; PM, *pectoralis major*.

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and placed in six floor pens (0.9 × 1.2 m/pen) after equalisation of body weight across pens (± 1.5 g/chick). Water and feed were supplied *ad libitum*. The lighting programme was 23 h light and 1 h of darkness. The temperature was set for the birds to achieve thermoneutrality throughout the study (Aviagen North America, 2005). All animal procedures were approved by the Universities Institutional Animal Care and Use Committee.

Ingredients used in the experimental diets were analysed for amino acids by HPLC after acid hydrolysis (Llames & Fontaine, 1994). Upon analyses, the nutrient matrix of the feed ingredients used was updated and all the test diets were then formulated using linear programming that solved for energy, amino acids, mineral and vitamin needs that satisfied optimal growth. A common pre-test diet in crumbled form, formulated to satisfy all nutrient recommendations (National Research Council, 1994), was fed to all the broilers in the study from placement until 21 d of age (Table 1). Subsequently, the test diets were fed from age 21 to 42 d (Table 1), composed primarily of maize, soyabean meal and pet-food-grade poultry meal. It was formulated to satisfy all nutrient recommendations for the 21–42 d period with the exception of methionine, which was considerably deficient (National Research Council, 1994; Kalinowski *et al.* 2003). Dietary cystine, cysteine and choline were above recommended levels (National Research Council, 1994; Kalinowski *et al.* 2003). Because poultry can efficiently utilise dietary methionine in both D- and L- isoforms, DL-methionine (98 %) was supplemented at the expense of an inert filler to achieve a dietary adequate level (methionine-adequate; MA).

This was fed to half of the pens while the other half received the methionine-deficient (MD) test diet (calculated methionine 3.2 v. 4.7 g/kg diet). Composite samples of both dietary treatments were obtained and analysed for amino acid composition by HPLC after brief cold acid washing (Llames & Fontaine, 1994). To prevent sulfur amino acid degradation during hydrolysis of the raw feed ingredients (before formulation of the diets) and the test diets, oxidation using performic acid was used to derive stable products of methionine and cystine. The formed products, methionine sulfone and cysteic acid, were then determined by chromatography and calculated back to the methionine and cystine content in the experimental diets in accordance with AOAC 994.12 (Gaithersburg, MD, USA) as described by Llames & Fontaine (1994).

The mean bird weight of all pens was recorded at the initiation (21 d) and termination (42 d) of the experimental phase. At 42 d of age, eleven birds from each pen were randomly removed and processed. Carcass and abdominal fat weights were obtained and recorded. Carcasses were chilled for 4 h, followed by a manual deboning and weighing of the breast muscles.

Skeletal muscle dissection, preparation and analyses

A bird from each pen was randomly selected at 42 d of age and killed via cervical dislocation. Following *post mortem*, 1 cm³ from each bird was aseptically dissected from the cranial region of the left PM, and immediately frozen at -80°C . The blocks were then removed from the -80°C storage and allowed to warm to -21°C in a cryotome (Minotome

Table 1. Diet composition (g/kg)

Ingredients	Common diet (0–21 d)	Test diets (21–42 d)	
		Methionine deficient	Methionine adequate
Maize	608.9	642.3	642.3
Soyabean meal	282.2	246.2	246.2
Pet-food-grade poultry meal	60.0	60.0	60.0
Poultry fat	18.5	25.6	25.6
Dicalcium phosphate	9.2	7.8	7.8
Calcium carbonate	9.1	8.4	8.4
Sodium chloride	4.7	4.0	4.0
Vitamin and mineral premix*	2.5	2.5	2.5
DL-Methionine†	2.5	–	1.5
Filler‡	–	1.6	0.1
L-Lysine	1.5	0.8	0.8
Salinomycin§	0.5	0.5	0.5
Analysed composition (g/kg)			
Methionine	5.7	3.1	4.5
Cystine	4.0	3.7	3.6
Lysine	13.6	10.8	11.0
Threonine	8.5	7.2	7.3
Isoleucine	9.3	7.7	7.7
Valine	10.2	9.1	9.0
Leucine	17.4	15.5	15.7
Histidine	5.9	5.3	5.2

* The vitamin and mineral premix contained (per kg diet): retinyl acetate, 2654 µg; cholecalciferol, 110 µg; dl- α -tocopheryl acetate, 9.9 mg; menadione, 0.9 mg; vitamin B₁₂, 0.01 mg; folic acid, 0.6 µg; choline, 379 mg; d-pantothenic acid, 8.8 mg; riboflavin, 5.0 mg; niacin, 33 mg; thiamin, 1.0 mg; d-biotin, 0.1 mg; pyridoxine, 0.9 mg; ethoxyquin, 28 mg; Mn, 55 mg; Zn, 50 mg; Fe, 28 mg; Cu, 4 mg; I, 0.5 mg; Se, 0.1 mg.

† DL-Methionine, 98 % pure (Degussa Corporation, Kennesaw, GA, USA).

‡ Filler represents inert space (sand) in the diet to which DL-methionine was added at its expense.

§ Provided 60 g salinomycin Na/907.2 kg diet to prevent intestinal coccidia from developing.

|| Calculated composition of other nutrients common to both methionine-adequate and -deficient diets were: apparent metabolisable energy, 13.18 MJ/kg; crude protein, 212 g/kg; Ca, 8.4 g/kg; available P, 4.2 g/kg.

For details of diets and procedures, see p. 703.

Plus; Triangle Biomedical Sciences Inc., Durham, NC, USA). Sections were then made ($5 \times 10 \mu\text{m}$) from each 1 cm^3 of PM. Each section was placed into an individual pre-weighed $500 \mu\text{l}$ Eppendorf tube that had been prepared by being placed in a vacuum chamber overnight and moisture removed. These tubes were then pre-cooled to -21°C in the cryotome before use. Each tube and its tissue section were weighed on dry ice and the tissue section weights determined. One section was chosen from each bird that was closest to the population mean weight. The sample with the lowest weight was used to normalise all others after solubilisation. The tissue samples were then solubilised in lysis buffer (7 M-urea, 2 M-thiourea, 4% [(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate, 8 mM-phenylmethylsulfonyl fluoride) with repetitive pulsed sonication on ice and centrifugation (18 000 g; 5 min; 4°C) and volumes normalised to the lowest weight tissue section.

Proteins were subsequently precipitated in a half-volume of ice-cold 50% TCA ($280 \mu\text{l}$) and acetone ($1000 \mu\text{l}$) and re-suspended in 100 mM-ammonium bicarbonate, 5% acetonitrile solution ($100 \mu\text{l}$). Proteins were then reduced by adding 50 mM-dithiothreitol ($50 \mu\text{l}$; 5 min; 65°C), alkylated with 0.1 M-iodoacetamide ($50 \mu\text{l}$; 30 min, 30°C) and digested with $100 \mu\text{l}$ trypsin ($0.2 \mu\text{g}/\mu\text{l}$; overnight at 37°C), followed by centrifugation (11 340 g; 5 min). The peptides were desalted using a $20 \mu\text{g}$ peptide microtrap (Michrom BioResources, Inc., Auburn, CA, USA) and eluted using 0.1% trifluoroacetic acid, 95% acetonitrile solution ($20 \mu\text{l}$) following the manufacturer's instructions. Desalted peptides were dried in a vacuum centrifuge and re-suspended in 0.1% formic acid ($20 \mu\text{l}$). Liquid chromatography was done directly inline with a micro electrospray-ionisation ion trap mass spectrometer (LCQ Deca XP Plus; ThermoElectron Corporation, San Jose, CA, USA) that was used for the ionisation and tandem mass spectral analysis. The liquid chromatography was first done by strong cation exchange followed by reverse-phase liquid chromatography. Samples were loaded into the liquid chromatography gradient ion exchange system (Thermo Separations P4000 quaternary gradient pump; ThermoElectron Corporation) coupled with a $0.32 \times 100 \text{ mm}$ BioBasic strong cation exchange column. A salt gradient was applied in steps of 0, 5, 10, 20, 25, 30, 35, 40, 45, 50, 57, 64, 71, 79, 90, 110, 300 and 700 mM-ammonium acetate in 5% acetonitrile and 0.1% formic acid, and the resultant peptides loaded directly into the sample loop of a $0.18 \times 100 \text{ mm}$ BioBasic C18 reverse-phase liquid chromatography column of a Proteome X workstation (ThermoElectron Corporation). The reverse-phase gradient used 0.1% formic acid and increased the acetonitrile concentration in a linear gradient from 5% to 30% in 30 min, 30% to 65% in 9 min, followed by 95% for 5 min and 5% for 15 min. Each reverse-phase gradient was directly eluted to the electrospray-ionisation source. The spectrum collection time was 59 min for every strong cation exchange step. The mass spectrometer was configured to optimise the duty cycle length with the quality of the data acquired by alternating between a single full MS scan followed by three tandem MS scans on the three most intense precursor masses (as determined by Caliber mass spectrometer software in real time) from the full scan. Dynamic mass exclusion windows were set at 2 min and all spectra were measured with an overall mass:charge (m/z) ratio range of 300–1700.

Mass spectra and tandem mass spectra were used to search subsets of the non-redundant protein database downloaded from the National Center for Biotechnology Information (2005) Entrez Protein web page. From this, an avian subset was produced (AvDb; search term: gallus; 23 525 proteins) of the non-redundant protein database, which includes 'predicted' proteins. Searches were done using TurboSEQUEST (Bioworks Browser 3.2; ThermoElectron Corporation). Trypsin digestion, including mass changes due to cysteine carbamidomethylation and methionine oxidation (+16 Da), was applied in silico to each database search. The peptide mass tolerance was set to 1.5 Da and the fragment ion mass tolerance was set to 1.0 Da. Peptide matches were considered genuine if they were equal or larger than six amino acids and consistent with accepted high-stringency Sequest cross-correlation and ΔCn values (Washburn *et al.* 2001). Protein databases contain all known protein sequences and are redundant at the level of protein identity. The method employed in the present study, although well accepted and commonly used, often does not identify subtle sequence variants; it identifies proteins by peptide tags, and the derived protein lists contain protein identification redundancies. Therefore, the redundancies were later searched for and manually removed from the dataset obtained.

Statistical analyses

Data derived from the live production phase were evaluated by one-way ANOVA in a completely randomised design. Pen was used as the experimental unit for analysis. Uniformity data (CV) were calculated after determining the weight and proportion of all PM values within a pen. All live production data were analysed using the general linear models procedure (SAS/STAT version 7.12 for Windows; SAS Institute, Cary, NC, USA). Treatment effects ($P \leq 0.05$) were separated using a *t* test. Peptides generated by MS were evaluated for accuracy using the following biostatistical parameters: cross-correlation > 1.7 for a +1 charge, > 2.5 for a +2 charge, or > 3.0 for a +3 charge; delta correlation value > 0.1 ; preliminary score > 200 ; rank preliminary score < 11 . A χ^2 test compared categorical data (present *v.* absent proteins) obtained from MS.

Results and discussion

Growth and pectoralis muscle accretion

All broiler chicks being raised with a common diet from placement until 21 d of age grew at an adequate growth rate ($805.9 \pm 53.2 \text{ g/bird}$) before being fed the experimental diets. Calculated and analysed dietary methionine levels of the experimental diets were in close agreement (Table 1). It can be observed in Table 2 how a deficient level of dietary methionine resulted in birds with lower ($P=0.08$) body-weight gain, and carcass absolute and relative weights ($P=0.004$). Abdominal fat percentage was increased ($P=0.001$) in carcasses of broilers fed the MD diet. In confirmation of the planned methionine deficiency, the yield of boneless-skinless breast meat was significantly lower ($P=0.001$). Total breast meat as well as its PM muscle was also adversely affected when fed MD diets ($P=0.001$). Furthermore, after calculating the CV from each pen for total

Table 2. Effect of dietary methionine on productive traits of commercial broiler chickens (Observed means of three pens, each providing about eleven carcasses, and pooled standard errors of the mean)

Methionine group	Body-weight gain (kg)†	Abdominal fat (%)	Carcass*		<i>Pectoralis major</i>		Total breast meat		
			Weight (kg)	Relative (%)‡	Weight (g)	Relative (%)§	Weight (g)	Relative (%)	CV (%)¶
3.1 g/kg (MD)	1.22	2.63	1.37	71.5	255	18.8	320	23.6	7.9
4.5 g/kg (MA)	1.55	2.11	1.72	72.9	371	21.6	468	27.3	5.6
Pooled SEM	0.10	0.095	0.036	0.33	9.4	0.30	11.7	0.32	0.23
<i>P</i>	0.08	0.001	0.001	0.004	0.001	0.001	0.001	0.001	0.002

MA, methionine-adequate; MD, methionine-deficient.

* Represents eviscerated carcass without feathers, head, neck, feet and shanks.

† Values correspond to pen mean body-weight gain for the experimental period (21 to 42 d of age).

‡ Values correspond to the proportion of the carcass to the live weight.

§ Values correspond to the *pectoralis major* as a proportion of the carcass weight.

|| Values correspond to the total breast meat as a proportion of the carcass weight.

¶ Values correspond to the CV for total breast meat relative weight (breast-meat yield).

For details of diets and procedures, see p. 703.

breast-meat yield, it was observed how MA-fed broilers resulted in more uniform relative weights than those fed MD diets ($P=0.002$). Perhaps this increased variability in breast meat relative to weight is the result of a small proportion of the MD-fed population being able to compensate for the methionine deficiency by increasing their feed consumption and therefore meeting their cumulative methionine needs, but at the same time increasing the variability within the experimental unit. However, the majority of the population was unable to compensate for the deficiency, resulting in reduced lean tissue accretion and increased lipogenesis in confirmation of many earlier studies of methionine deficiency (Jensen *et al.* 1989; Baker *et al.* 1996; Kalinowski *et al.* 2003).

Pectoralis major muscle proteome

Tissue samples from all six birds yielded a combined 190 unique proteins. No difference between the numbers of proteins or peptides ($P>0.05$) detected from PM of broilers fed either MA or MD diets was observed. An increase ($P=0.005$) in the numbers of myosin-related proteins was observed in tissue samples corresponding to MD-fed birds (4.7 and 7.3 (SEM 0.33) proteins/50 μ^2 , for MA- and MD-fed birds respectively). On a cumulative muscle-mass basis, it is likely that the PM muscle from broilers fed MA diets may have higher myosin- and actin-related protein.

However, the observed higher concentration of myosin-related proteins in a specific unit area in PM from broilers fed MD diets could be an indication of a structural adaptation of broilers fed MD diets, or perhaps the presence of a more diverse or concentrated PM proteome considering that it has reduced mass or space to coexist.

When comparing treatment proteomes, most of the detected peptides were present in muscle tissue obtained from birds that were fed both dietary treatments. However, three proteins were present in the three PM muscle samples corresponding to the MD diet, but were absent in the three samples from birds fed the MA diet (Table 3). A χ^2 test analysed the presence of these three proteins and resulted in a probability value of $0.02 > P > 0.01$ (χ^2 6.0) giving a reasonable significance to the presence of these proteins considering the low number of observations per treatment. The first protein found to be present solely in muscle from MD-fed chickens was the enzyme pyruvate kinase. This enzyme was first annotated in the chicken PM more than two decades ago (Lonberg & Gilbert, 1983), and is known for its role in the irreversible conversion to pyruvate from phosphoenolpyruvate. However, this glycolytic enzyme is ubiquitous in nature; the fact that it is present in all cells probably discards it as being a metabolic biomarker for methionine inadequacy in broiler chickens.

Table 3. Proteins originating exclusively from *pectoralis* muscles in methionine-deficient (MD)-fed broilers*

Protein name	Accession number	Sequence of detected peptides
Muscle pyruvate kinase	45382651	VIDVGSK GSGTAEVELK DIQDLK
Myosin alkali light chain-1	2144836	ALGQNPTNAEINK EPAIDLK
Ribosomal protein L-29†	50754217	KM#QANNAK

M#, oxidized methionine.

* Listed proteins were observed solely in all three *pectoralis major* muscle samples from MD-fed broilers and absent in broilers fed methionine-adequate diets. Four proteins were found in two muscle samples solely from MD-fed broilers: connectin; aldolase C; glycogen phosphorylase; sarcoplasmic/endoplasmic reticulum calcium ATPase-2. One protein was found in two samples solely from MA-fed broilers: BM-011.

† A predicted protein from an annotated genomic sequence.

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The second protein found exclusively in PM from MD-fed chickens was myosin alkali light chain-1. This protein, first described and sequenced by Matsuda *et al.* (1981), is a structural component of skeletal muscle in the chicken, along with two heavy chains and three other light chains. This structural protein differentiates from all other myosin light chains in that it has the lowest anionic mobility. Considering that PM in chickens requires all six myosin proteins for its assembly and continual accretion, it is unlikely that this protein is present as a sign of myosin synthesis. Hence, its exclusive presence in PM obtained from chickens fed MD diets could be the indication of being residual as a result of proteolysis for gluconeogenic purposes due to methionine malnutrition and/or physiological stress. In the event of stress-induced proteolysis, it has been previously described how muscle depletion is coupled with an increase in fat deposition in the chicken (Nagra & Meyer, 1963; Bartov *et al.* 1980), as observed with the effect on abdominal fat percentage of MD-fed chickens (Table 2). Nevertheless, this protein's presence in MD-fed broilers is open to interpretation and warrants more research.

The last of the MD exclusively expressed breast muscle proteins was ribosomal protein L-29. This protein has been predicted for the avian species as a result of computational biology from an annotated genomic sequence; thus its occurrence in nature was previously hypothetical, and in the present paper we are documenting its existence. It has been previously shown how amino acid deprivation has enhanced mRNA synthesis for ribosomal proteins L-17 and S-25 (Hitomi *et al.* 1993; Laine *et al.* 1994). In the case of L29, little is known about this protein apart from being a structural unit of the protein synthesis apparatus of the cell, the ribosome. Its existence has been documented for the rat and other species (Nika & Hultin, 1984), but still remains as one of the ribosomal proteins whose function is yet to be fully understood (Wool *et al.* 1995; Brodersen & Nissen, 2005). Therefore, little can be concluded at this point from the presence of this protein in muscle from broiler chickens fed an MD diet.

Although there may be some detected proteins among the observed proteome from MA muscle that may serve as potential indicators of maximisation in breast-meat development, or that may have regulatory-type roles in decrease or increase in breast-meat accretion in response to dietary methionine, none can be reported by the present paper. In Table 3, a list of five proteins found to be present in two samples from a treatment but absent in all three samples from the other treatment is given (χ^2 3; $0.10 < P < 0.05$). Of the five proteins, four were observed in two samples from MD-fed broilers while absent in all three samples from MA-fed broilers. The remaining fifth protein was observed in two samples from MA-fed broilers while absent in all three samples from MD-fed broilers. Those four proteins from MD-derived muscle are mostly common enzymes, with the exception of connectin, which is a cytoskeletal elastic protein known for its role within the sarcomere during motoneuronal activities (Soeno *et al.* 1999). The single protein observed to be expressed exclusively in muscle obtained from MA-fed broilers is known as BM-011. Its abbreviation refers to the fact that it is expressed in bone marrow. Apart from being predicted from computational analysis, little is known about its function. More research is needed to elucidate the reason behind the observed expression in muscle and not just bone marrow of broiler chickens fed MA diets.

It has been proposed that dietary methionine deficiency regulates the expression of asparagine-synthetase and C/EBP-homologous-protein (Fafournoux *et al.* 2000). In the present study, three proteins were noted to differ distinctly between the treatment proteomes and could be considered as potential biomarkers regulated by a methionine deficiency in broiler chickens. However, it may be premature to label any of these proteins as biomarkers for decreased PM tissue deposition in response to methionine dietary inadequacy. It should be noted that we have reported considerably fewer proteins than possible due to the stringent biostatistical parameters employed. By decreasing MS biostatistical evaluation criteria the number of detected proteins would have increased by several-fold. The inability to detect a higher number of proteins becomes more complex if we consider that higher abundance proteins in any specific proteome may decrease the detection of lower abundance proteins (Adkins *et al.* 2002). In this particular case, the high presence of proteins in PM of broilers such as the heavy and/or light chains of myosin, actin, connectin, tinin, troponin, or tropomyosin, among many others, may have interfered with the detection of lower abundance proteins that could have provided a better indication of protein expression in muscle as influenced by dietary methionine. Furthermore, it is possible that the interference of high abundance proteins may have prevented the detection in MA-fed broilers of those three proteins exclusively observed in samples originated from MD-fed broilers. It is recommended that a higher number of observations than those used in the present study (three per treatment) be used when comparing protein expression in muscle tissue response to nutritional regimens, in an attempt to reduce any current equipment or sampling limitations, and experimental variability. Nevertheless, the importance of dietary methionine was demonstrated once again with the vast effects on production traits, particularly those associated with breast-meat absolute and relative mass accretion. Furthermore, a model for future protein expression comparisons in broiler chicken muscles in response to methionine or other amino acid starvation is proposed.

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