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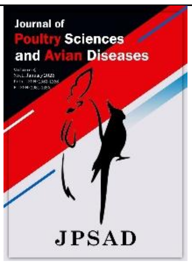

Dietary Digestible Methionine+Cystine Levels Influence Performance, Egg Quality, and Hepatic Gene Expression in Post-Molt Laying Hens

Mousa. Zarrin[1]*, Amir. Ahmadpour[1]*

[1] Department of Animal Science, Faculty of Agriculture and Natural Resources, Yasouj University, Yasouj, Kohgiluyeh and Boyer-ahmad, Iran

*** Corresponding authors' email address:** M.Zarrin@yu.ac.ir & ahmadpouramir@yu.ac.ir

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ABSTRACT

This study evaluated the effects of graded digestible methionine+cystine (dMet+Cys) levels (0.490–0.698%; dMet+Cys: lysine ratios of 75–107) on performance, egg quality, plasma metabolites, and hepatic gene expression in post-molt Hy-Line W-80 laying hens (79–95 weeks). One hundred eighty hens were assigned to five isonitrogenous (15.06% CP) and isoenergetic (~2,800 kcal/kg AME) treatments in a completely randomized design with six replicates of six hens each. Increasing dMet+Cys linearly improved hen-day laying rate (79.92% to 87.12%; $p<0.001$), egg mass (52.18 to 58.22 g/hen/day; $p<0.001$), and feed conversion efficiency ($p\leq 0.002$). Egg weight showed a quadratic response, peaking at 0.648% dMet+Cys (68.90 g; $p=0.003$). Haugh unit and albumen index increased ($p\leq 0.021$), while egg component proportions remained unchanged ($p>0.10$). Plasma methionine, cysteine, homocysteine, and glutathione rose linearly ($p\leq 0.003$), indicating enhanced SAA flux and antioxidant capacity. Hepatic qPCR revealed marked downregulation of sulfur amino acid metabolism genes (*MTR, CBS, CTH, BHMT, MAT1A, GPX1*) and egg quality-related genes (*OVAL, LYZ, TF, CA2, OC-17, KRT75*) at higher dMet+Cys levels relative to the deficient basal diet ($p<0.0001$), suggesting homeostatic feedback and systemic metabolic reprogramming under adequate SAA supply. These results indicate that 0.648% dMet+Cys (dMet+Cys: lysine ≈ 99) optimizes post-molt performance and egg quality in white-egg layers through balanced metabolic and nutrigenomic regulation.

Keywords: *Laying Hens, Post-Molt, Methionine+cCystine, Gene eExpression, Egg Quality*

1 Introduction

The global poultry industry relies heavily on laying hens for egg production, a staple protein source contributing significantly to human nutrition and food security. With annual worldwide egg output exceeding 80 million tons, optimizing hen performance through nutritional strategies is paramount, particularly in extended production cycles (Fao, 2022). Induced molting, a management practice involving controlled feed restriction or

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withdrawal, rejuvenates aging flocks by promoting feather renewal, reproductive tract regeneration, and improved post-molt productivity (Berry, 2003; Webster, 2003). This technique extends the economic lifespan of hens, enhancing egg yield by 20-30% in the second cycle while improving shell quality and albumen height (Gongruttananun et al., 2017; Mccowan et al., 2006). However, molting imposes metabolic stress, elevating requirements for key nutrients like sulfur amino acids (SAA), including methionine and cystine, which are critical for protein synthesis, antioxidant defense, and epigenetic regulation (Lebedev et al., 2025; Oliveira Filho et al., 2019). Under tropical heat stress, methionine supplementation in low-protein diets (14% CP) has been shown to mitigate negative effects, improving hen-day egg production, egg weight, and eggshell thickness while significantly reducing mortality rates compared to unsupplemented low-protein or standard-protein controls (Bunchasak & Silapasorn, 2005).

Methionine, an essential amino acid, serves as a precursor for cystine through the transsulfuration pathway, forming the SAA pool vital for avian physiology (Macelline et al., 2021). In laying hens, SAA supports feather keratinization comprising up to 90% cystine-rich proteins during post-molt recovery, where feather regrowth accounts for 5-10% of body protein turnover (Kim et al., 2021; Oliveira Filho et al., 2019). Deficiencies in digestible methionine+cystine (dMet+Cys) impair vitellogenesis and ovulation, reducing egg production by 15-25% and egg mass by 10-20% in molted flocks (Castro et al., 2019; Geng et al., 2021). Conversely, optimal SAA levels enhance feed efficiency and nitrogen retention, aligning with sustainable poultry practices by minimizing crude protein in diets (Hofmann et al., 2020; Liu et al., 2020).

Nutrigenomics reveals how SAA modulates gene expression in poultry, influencing metabolic pathways and egg quality. Methionine's role in S-adenosylmethionine (SAM) synthesis drives one-carbon metabolism, epigenetically regulating genes like *methionine synthase* (*MTR*) and *cystathionine beta-synthase* (*CBS*) for SAA homeostasis (Lee et al., 2021; Wu et al., 2024). In laying hens, SAA deficiency upregulates transsulfuration enzymes (e.g., CTH, BHMT) to conserve cysteine for glutathione peroxidase (GPX1), bolstering antioxidant capacity against oxidative stress post-molt (Lebedev et al., 2025; Wu et al., 2024). Recent studies show SAA supplementation downregulates these genes in the liver, preventing overload while supporting methylation for reproductive hormones (Gao et al., 2024; Xiao et al., 2016). For egg formation, SAA influences *ovalbumen* (*OVAL*) and *lysozyme* (*LYZ*) expression in the oviduct, enhancing albumen antimicrobial properties (Gamboa Gonzales et al., 2023; Ogbuewu et al., 2022). Furthermore, methionine in low-protein diets has been shown to enhance reproductive organ development, with linear increases in ovary and oviduct weight (as % of body weight) observed at higher Met levels (0.44%), though not fully matching standard-protein diets (Bunchasak & Silapasorn, 2005).

Egg quality, encompassing internal (Haugh unit, albumen index) and external (shell strength, thickness) traits, deteriorates in aging hens due to reduced SAA bioavailability, exacerbating post-molt challenges (Kakhki et al., 2023; Kim et al., 2021). SAA promotes disulfide bonds in ovomucin, improving albumen viscosity and Haugh units by 5-10% (Castro et al., 2019; Geng et al., 2021). Shell matrix genes like carbonic anhydrase 2 (*CA2*) and ovocleidin-17 (*OC-17*) rely on SAA for calcification, with deficiencies thinning shells by 15% (Liu et al., 2020; Ogbuewu et al., 2022). In molted hens, SAA requirements rise 10-20% due to heightened oxidative stress and protein demands, yet precise dMet+Cys levels and their mechanistic effects on hepatic gene expression in post-molt hens remain poorly defined. We hypothesized that graded increases in dMet+Cys would linearly improve performance and egg quality, concurrently downregulating hepatic genes involved in SAA metabolism and ectopic egg protein synthesis, thereby revealing a systemic response to SAA sufficiency. This study aimed to establish optimal dMet+Cys levels and elucidate the associated nutrigenomic mechanisms in post-molt Hy-Line W-80 hens.

Plasma metabolites reflect SAA status: elevated methionine and cysteine indicate adequate supply, while homocysteine accumulation signals imbalance, potentially inducing vascular issues (Gao et al., 2024; Wu et al., 2024). Glutathione surges with SAA fortification, mitigating lipid peroxidation in post-molt hens (Lebedev et al., 2025; Wu et al., 2024). Gut microbiota interactions further modulate SAA utilization; deficiencies alter Parabacteroides and Lactobacillus, correlating with reduced egg mass and increased feed conversion (Gamboa Gonzales et al., 2023; Geng et al., 2021). Similarly, supplementation of methionine in low-protein diets has been shown to increase Lactobacillus abundance and decrease Faecalibacterium proportions, which are closely associated with improvements in key performance parameters such as average daily feed intake, egg weight, and feed efficiency (Ma et al., 2021).

Despite advances, gaps persist in post-molt nutrigenomics: few studies integrate SAA levels with gene expression for egg quality optimization (Lee et al., 2021; Xiao et al., 2016). Recent meta-analyses suggest 0.60-0.70% dMet+Cys for molted hens, but breed-specific and age-related variations warrant investigation (Kakhki et al., 2023; Ma et al., 2021). In non-molted laying hens fed low-protein diets, an optimal methionine level of 0.38% has been reported to maximize production performance, including egg production and feed efficiency, while also enhancing nutrient metabolism and reproductive traits (Ma et al., 2021). This study addresses these by evaluating graded dMet+Cys on performance, metabolites, and genes in post-molt white-egg layers, hypothesizing that optimal levels enhance productivity and quality through metabolic gene modulation.

2 Material and Methods

2.1 Experimental Design and Dietary Treatments

A completely randomized experimental design was employed, consisting of five dietary treatments with six replicates per treatment and six Hy-Line W-80 commercial white-egg laying hens per replicate (total n=180 birds). The trial encompassed the period from 79 to 95 weeks of age (16 weeks in total), divided into four consecutive 28-day subperiods, following induced molting initiated at 72 weeks of age when hen-day egg production had declined to approximately 75%. Experimental diets were formulated to be isonitrogenous (15.06% crude protein) and isoenergetic (approximately 2,800 kcal/kg apparent metabolizable energy; AME), incorporating graded levels of digestible methionine+cystine (dMet+Cys) designed to span deficient to supra-adequate concentrations relative to established requirements for second-cycle laying hens (National Research & Subcommittee on Poultry, 1994). These levels were achieved by supplementing a basal diet deficient in dMet+Cys with graded inclusions of DL-methionine (98% purity; Evonik Industries AG, Essen, Germany) in substitution for L-glutamic acid (Ajinomoto Co., Inc., Tokyo, Japan). Digestible lysine was fixed at 0.653%, yielding dMet+Cys: lysine ratios of 75, 83, 91, 99, and 107, respectively. All other essential amino acids were balanced relative to lysine, and diet formulations met or exceeded established nutritional recommendations for second-cycle laying hens (National Research & Subcommittee on Poultry, 1994). Diets were mixed in a horizontal mixer and provided as mash twice daily (at 07:00 and 17:00 h) to ensure ad libitum access. The ingredient composition and calculated nutrient profiles of the experimental diets are presented in Table 1.

Table 1. Ingredient composition and calculated nutrient composition of experimental diets with graded levels of digestible methionine+cystine for Hy-Line W-80 laying hens during the second production cycle.

Ingredient / Nutrient	Treatments				
	dMC75	dMC83	dMC91	dMC99	dMC107
Ingredients (%)					
Corn	40.412	40.412	40.412	40.412	40.412
Sorghum	17.9	17.9	17.9	17.9	17.9
Soybean meal	19.8	19.8	19.8	19.8	19.8
Wheat middlings	7.8	7.8	7.8	7.8	7.8
Soybean oil	2.99	2.99	2.99	2.99	2.99
Dicalcium phosphate	1.255	1.255	1.255	1.255	1.255
Limestone	8.89	8.89	8.89	8.89	8.89
Choline chloride (60%)	0.02	0.02	0.02	0.02	0.02
Common salt	0.491	0.491	0.491	0.491	0.491
Vitamin premix[1]	0.1	0.1	0.1	0.1	0.1
Mineral premix[2]	0.05	0.05	0.05	0.05	0.05
Butylated hydroxytoluene (BHT)	0.01	0.01	0.01	0.01	0.01
L-Glutamic acid	0.224	0.171	0.116	0.063	0.01
DL-Methionine (98%)	0.058	0.111	0.166	0.219	0.272
Calculated nutritional composition					
Crude protein (%)	15.06	15.06	15.06	15.06	15.06
Metabolizable energy (kcal/kg)	2802	2803	2804	2805	2806
Calcium (%)	3.818	3.818	3.818	3.818	3.818
Available phosphorus (%)	0.341	0.341	0.341	0.341	0.341
Sodium (%)	0.227	0.227	0.227	0.227	0.227

Potassium (%)	0.609	0.609	0.609	0.609	0.609
Digestible lysine (%)	0.653	0.653	0.653	0.653	0.653
Digestible methionine (%)	0.275	0.327	0.379	0.431	0.483
Digestible methionine + cystine (%)	0.49	0.542	0.594	0.648	0.698
Digestible threonine (%)	0.498	0.498	0.498	0.498	0.498
Digestible tryptophan (%)	0.172	0.172	0.172	0.172	0.172
Digestible valine (%)	0.636	0.636	0.636	0.636	0.636
Digestible arginine (%)	0.96	0.96	0.96	0.96	0.96
Digestible leucine (%)	1.309	1.309	1.309	1.309	1.309
Digestible isoleucine (%)	0.584	0.584	0.584	0.584	0.584
Digestible histidine (%)	0.372	0.372	0.372	0.372	0.372
Digestible phenylalanine (%)	0.686	0.686	0.686	0.686	0.686

[1] Vitamin premix (levels per kg diet): vitamin A 12,000,000 IU; vitamin D_3 3,600,000 IU; vitamin E 3,500 IU; vitamin B_1 2,500 mg; vitamin B_2 8,000 mg; vitamin B_6 3,000 mg; pantothenic acid 12,000 mg; biotin 200 mg; vitamin K 3,000 mg; folic acid 3,500 mg; nicotinic acid 40,000 mg; vitamin B_{12} 20,000 µg; Se 130 mg; carrier to 1,000 g.

[2] Mineral premix (levels per kg diet): manganese 160 g; iron 100 g; zinc 100 g; copper 20 g; cobalt 2 g; iodine 2 g; excipient to 1,000 g.

2.2 *Animals, Housing, and Management*

Hens were procured from a commercial flock and managed in accordance with breed-specific guidelines throughout the rearing, growing, and initial production phases. Induced molting was executed via a modified protocol involving a 10-day fasting interval (with ad libitum water access) followed by provision of a pullet developer diet until egg production resumed at 50% by 79 weeks of age. The study was conducted at the commercial laying facility of SabzBavaran-e-NouAndish Co., located in Qir and Karzin County, southern Fars Province, Iran. Birds were housed in pairs within galvanized wire cages (dimensions: 25×40×45 cm) in an open-sided poultry house featuring clay tile roofing and lateral mesh for natural ventilation. A photoperiod of 16 h light:8 h dark was upheld throughout the trial. Feed and water were provided ad libitum via trough feeders and nipple drinkers, respectively. Ambient temperature was recorded twice daily at bird height using maximum-minimum thermometers. All experimental procedures received approval from the Institutional Animal Care and Use Committee, in alignment with Iranian animal welfare regulations and guidelines of the Veterinary Organization.

2.3 *Production Performance Evaluation*

Egg production was monitored daily by collecting and counting eggs per replicate to calculate hen-day laying percentage (%). Eggs from the final four days of each 28-day subperiod were individually weighed using a digital analytical balance (Mettler-Toledo, Columbus, Ohio; precision: 0.01 g; calibrated to international standards) to determine mean egg weight (g) and egg mass (g/hen/day). Feed intake was measured at the end of each subperiod by subtracting residual feed from the amount provided, allowing calculation of daily digestible methionine+cystine (dMet+Cys) intake (mg/hen/day). Feed conversion efficiency was calculated as kg feed per dozen eggs and g feed per g egg mass. Body weights of the hens were recorded at 79 and 95 weeks of age using a calibrated platform scale (Ohaus, Parsippany, New Jersey; precision: 1 g).

2.4 *Egg Quality Assessment*

Four eggs per replicate were collected from the final three days of each 28-day subperiod: two for the determination of component proportions and two for the assessment of internal quality attributes. Eggs were individually weighed, manually broken onto a flat surface, and the yolks, together with air-dried shells (dried for 24 h at ambient temperature), were separately weighed; albumen weight was calculated by difference. Component proportions (% shell, % yolk, % albumen) were expressed relative to total egg weight. Internal quality parameters included the Haugh unit, calculated as $100\times\log(H+7.57-1.7\times W^{0.37})$, where H is albumen height (mm), and W is egg weight (g) (Roberts, 2004); yolk index (yolk height divided by average yolk diameter); and albumen index (albumen height divided by average albumen diameter). Heights were measured using a digital micrometer (Mitutoyo, Kawasaki, Japan; precision: 0.001 mm), and diameters were determined with a digital caliper (Mitutoyo, Kawasaki, Japan; precision: 0.01 mm).

2.5 *Blood Metabolite Quantification*

At 95 weeks of age, blood samples (5 mL) were collected from the brachial vein of three hens per replicate (n=90 total) into heparinized vacutainer tubes. Plasma was isolated via centrifugation (3,000×g for 10 min at 4°C) and stored at −80°C until analysis. Concentrations of plasma methionine, cysteine, homocysteine, and glutathione were quantified using high-performance liquid chromatography (HPLC) with fluorescence detection post-derivatization with o-phthalaldehyde on a reversed-phase C18 column (Agilent 1260 Infinity II system, Agilent Technologies, Santa Clara, CA, USA). Intra-assay and inter-assay coefficients of variation (CV) for these analytes were 1.5–6.8% and 2.8–9.2%, respectively. Total protein and albumin levels were determined employing commercial colorimetric assay kits (total protein: Biuret method kit; albumin: Bromocresol Green method kit; both from Erba Lachema, Brno, Czech Republic) on an automated biochemistry analyzer (Ellipse, AMS Analyzers, Rome, Italy). Intra-assay and inter-assay CV for total protein and albumin assays were 1.2–3.1% and 2.4–4.6%, respectively. All assays were performed in triplicate, with overall coefficients of variation below 5%.

2.6 Gene Expression Analysis

At the termination of the trial (95 weeks of age), three hens per replicate (n=90 total) were humanely euthanized by cervical dislocation. Tissue samples were immediately collected from the liver (~100 mg), magnum and uterine segments (~50 mg each), and skin, including feather follicles (~50 mg), for potential future analyses. For the present study, only liver tissue was used for all qPCR analyses, including genes involved in sulfur amino acid metabolism (*MTR*, *CBS*, *CTH*, *BHMT*, *MAT1A*, *GPX1*), as well as genes traditionally associated with egg formation (*OVAL*, *LYZ*, *TF*, *CA2*, *OC-17*) and feather keratin (*KRT75*) to assess ectopic expression in the liver. All samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) with on-column DNase I digestion (RNase-Free DNase Set; Qiagen GmbH, Hilden, Germany). RNA purity (A260/A280 ratio>1.8) was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and integrity was confirmed by RNA integrity number (RIN>7) on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). First-strand cDNA was synthesized from 1 µg of total RNA using SuperScript IV Reverse Transcriptase (catalog no. 18091050; Thermo Fisher Scientific Inc., Waltham, MA, USA) with a combination of random hexamers and oligo(dT) primers. Quantitative real-time PCR (qPCR) was performed on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA) using PowerUp SYBR Green Master Mix (catalog no. A25742; Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantitative PCR procedures were performed in accordance with MIQE guidelines. No-template controls and no-reverse-transcription controls were included for each primer set, and single-product amplification was confirmed via melt curve analysis.

The analysis targeted 12 genes of interest: *methionine synthase* (*MTR*), *cystathionine beta-synthase* (*CBS*), *cystathionine gamma-lyase* (*CTH*), *betaine-homocysteine methyltransferase* (*BHMT*), *methionine adenosyltransferase* 1A (*MAT1A*), *ovalbumen* (*OVAL*), *lysozyme* (*LYZ*), *ovotransferrin* (*TF*), *carbonic anhydrase* 2 (*CA2*), *ovocleidin*-17 (OC-17), *keratin* 75 (*KRT75*), and *glutathione peroxidase* 1 (*GPX1*). Two housekeeping genes were used for normalization: *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *beta-actin* (*ACTB*).

Primers were *Gallus gallus*-specific, designed to produce amplicons of 100–200 bp with amplification efficiencies of 90–110% (synthesized by Integrated DNA Technologies Inc., Coralville, IA, USA). Each reaction (20 µL) contained cDNA equivalent to 20 ng RNA, 200 nM forward and reverse primers, and master mix. Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a subsequent melt curve analysis to verify specificity.

In the present study, gene expression levels are presented on a linear scale using the formula: $\Delta Ct = 20–(Ct_{target}–Ct_{reference})$, where Ct_{target} is the threshold cycle of the target gene, and $Ct_{reference}$ is the geometric mean of the two housekeeping genes (*GAPDH* and *ACTB*). This transformation ensures that higher index values correspond to higher expression levels. Table 6 reports these expression indices as ΔCt (using this definition).

For comparison between treatments, the difference in expression index is calculated as $\Delta\Delta Ct = \Delta Ct_{treatment}–\Delta Ct_{control}$ (control=dMC75). Thus, positive ΔΔCt indicates upregulation and negative ΔΔCt indicates downregulation relative to the control. Figures 3 and 4 present these ΔΔCt values.

For conventional biological interpretation, fold change was calculated as $2^{−\Delta\Delta Ct}$. These yields fold change>1 for upregulation and <1 for downregulation. Figures 1 and 2 and the corresponding Results text report fold change.

Amplification efficiency for each primer pair was determined using a five-point serial dilution standard curve. Only primers with efficiencies between 90% and 110% and

$R^2>0.99$ were accepted. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method adjusted for individual amplification efficiency where appropriate. All samples were analyzed in triplicate. The sequences of primers used for qPCR, amplicon sizes, NCBI accession numbers, and annealing temperatures are presented in Table 2.

Table 2. qPCR primer sequences, amplicon sizes, accession numbers, and annealing temperatures for target and housekeeping genes in Gallus gallus

Gene Symbol*	Primers (5' → 3')		NCBI Accession (mRNA)	Amplicon Size (bp)	Annealing Temp (°C)
	Forward	Reverse			
Methionine metabolism and the methylation pathway					
MTR	CCGGCAGATACCCAGGAAAG	CGTGCTGCTGGATCATGGTA	NM_001031104.2	103	60
CBS	GAA AGC CCT GTC CCA CAT CA	GCC TCC TCC AAG TCA CCA TC	XM_040652613.2	81	60
CTH	AGG GCT GTG CTG ATG TGA TC	AAG GAC GCT GGA AAT ACG CA	XM_422542.8	87	60
BHMT	TGT CTG GGC AGT TGA AGT CC	ACA GCA CAT TGT CCA GGA GG	XM_414685.7	108	60
MAT1A	ACG AGT TGT CCG AGA TGC AA	ACT GCT GTT CCA ATG CCA CT	NM_001199519.2	101	60
GPX1	CTT CCA AAG CCT CGA AGT GC	ACA ACG TTA CCC AGA CTC GC	NM_001277853.3	101	60
Egg formation and structural integrity					
LYZ	TGC AAC ATC CCG TGC TCA G	CGT TCA TGC CGT TTC CAT CG	NM_205281.2	94	60
TF	AGC TTT ACG GTG TCT GGT CG	CTT GGC CCA ATC AGC TTT GT	NM_205304.2	100	60
CA2	GCC AAA GCC ATC GTC AAC AA	CCT CCT TGC AGC ACT GAC TT	NM_205317.2	80	60
OC-17	CGC AAT GCC TTC GTC TGC	GCG TTG TTG CGT TGT TGC	XM_040702883.2	87	60
KRT75	ATC TCC TGT GGG TTT GGT GG	AGG CTT TGG TTG ACG GAG AC	NM_001001314.3	110	60
OVAL	ACC AAC GCC GTT CTC TTC TT	GTG CTC TGG GTC TTG TTG GA	NM_205152.3	91	60
Reference (Housekeeping) genes					
GAPDH	TCT TCA CCA CCG CTC AGT TC	TAT CAG CCT CTC CCA CCT CC	NM_204305.2	114	60
ACTB	TAC AGC TTC ACC ACC ACA GC	GGC CAT CTC CTG CTC GAA AT	NM_205518.2	93	60

*The genes are as follows: *MTR – methionine synthase; CBS – cystathionine beta-synthase; CTH – cystathionine gamma-lyase; BHMT – betaine-homocysteine methyltransferase; MAT1A – methionine adenosyltransferase 1A; OVAL – ovalbumin; LYZ – lysozyme; TF – ovotransferrin; CA2 – carbonic anhydrase 2; OC-17 – ovocleidin-17; KRT75 – keratin 75; GPX1 – glutathione peroxidase 1; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; ACTB – beta-actin.*

2.7 *Statistical Analysis*

Normality of residuals and homogeneity of variances were verified using the Shapiro–Wilk and Levene's tests, respectively. For repeated-measures variables (e.g., performance traits and egg quality parameters), data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The statistical model included treatment and period as fixed effects and replicate (pen) as a random effect. When appropriate, the period was specified as a repeated factor, and the covariance structure was selected based on the lowest Akaike information criterion.

Variables measured once at the end of the experiment (terminal data) were analyzed by one-way ANOVA using the GLM procedure of SAS, with treatment as the fixed effect. When treatment effects were significant, orthogonal polynomial contrasts were applied to evaluate linear and quadratic dose–response trends. Differences among least squares means were separated using Tukey's multiple comparison test. For gene expression analyses, RNA samples from three hens within each replicate cage were pooled before cDNA synthesis. Therefore, the experimental unit for gene expression was the replicate cage (n=6 per treatment).

Statistical significance was declared at $p \leq 0.05$, and tendencies were discussed at $0.05 < p \leq 0.10$. Results are presented as least squares Means±Standard Error of the Mean (SEM).

3 Results

3.1 *Production Performance*

The provision of graded dietary levels of digestible methionine+cystine (dMet+Cys) exerted dose-dependent effects on production performance parameters in Hy-Line W-80 laying hens during the second production cycle (79 to 95 weeks of age; Table 3). Hen-day laying percentage increased linearly as dMet+Cys concentration rose from dMC-75 to dMC-107 (linear $p<0.001$; quadratic $p=0.458$). The lowest laying rate was recorded at dMC-75 (79.92±4.05%), whereas the highest value occurred at dMC-107 (87.12±4.05%), representing an absolute improvement of 7.2 percentage points across the treatment range. This linear trend was consistent across the four 28-day subperiods, with no evidence of a plateau within the tested range.

Table 3. Performance parameters of HylineW80 laying hens fed graded levels of digestible methionine+cysteine during the second production cycle (79 to 95 weeks of age)

Variable	Treatments					SEM	*p*-value	
	dMC75	dMC83	dMC91	dMC99	dMC107			
							Linear	Quadratic
Feed intake (g/hen/day)	111.55^{e}	113.78^{d}	113.8^{c}	113.54^{b}	113.78^{a}	0.68	0.412	0.587
Digestible Met+Cys intake (mg/hen/day)	546.6^{a}	616.7^{ab}	675.95^{ab}	735.76^{ab}	794.22^{b}	3.92	<0.001	0.921
Feed conversion per dozen eggs (kg/dozen)	1.71^{a}	1.68^{ab}	1.63^{ab}	1.63^{ab}	1.59^{b}	0.09	0.002	0.684
Feed conversion per egg mass (g feed/g egg mass)	2.15^{a}	2.05^{ab}	2.01^{ab}	1.97^{b}	1.97^{b}	0.08	0.001	0.312
Laying rate (%)	79.92^{c}	82.28^{bc}	84.11^{b}	84.13^{b}	87.12^{a}	4.05	<0.001	0.458
Egg weight (g)	65.32^{c}	67.75^{ab}	67.82^{ab}	68.9^{a}	66.82^{b}	0.99	0.034	0.003
Egg mass (g/hen/day)	52.18^{b}	55.71^{ab}	57.03^{a}	57.93^{a}	58.22^{a}	2.39	<0.001	0.219

Egg mass (g/hen/day) followed a highly significant linear response to increasing dMet+Cys inclusion ($p<0.001$; quadratic $p=0.219$). Values progressed from 52.18±2.39 g/hen/day at the lowest level to 58.22±2.39 g/hen/day at the highest level, corresponding to a 11.6% relative increase.

Mean egg weight displayed a significant quadratic pattern (linear $p=0.034$; quadratic $p=0.003$). Egg weight increased progressively from 65.32±0.99 g at dMC-75 to a maximum of 68.90±0.99 g at dMC-99, followed by a modest decline to 66.82±0.99 g at dMC-107. Egg weight peaked at dMC-99 (68.90±0.99 g) and declined at dMC-107 (66.82±0.99 g).

Feed conversion efficiency improved markedly with increasing dMet+Cys. When expressed as kilograms of feed per dozen eggs, the feed conversion ratio decreased linearly from 1.71±0.09 kg/dozen at dMC-75 to 1.59±0.09 kg/dozen at dMC-107 (linear $p=0.002$; quadratic $p=0.684$). Similarly, feed conversion per gram of egg mass declined linearly from 2.15±0.08 g feed/g egg mass to 1.97±0.08 g feed/g egg mass (linear $p=0.001$; quadratic $p=0.312$).

Daily feed intake did not differ significantly among treatments (range: 111.55±0.68 to 113.80±0.68 g/hen/day; linear $p=0.412$; quadratic $p=0.587$). Consequently, calculated daily digestible methionine+cystine intake increased linearly and proportionally with dietary concentration, rising from 546.6±3.92 mg/hen/day at dMC-75 to 794.2±3.92 mg/hen/day at dMC-107 ($p<0.001$).

3.2 *Egg Quality*

Haugh unit exhibited both linear ($p=0.021$) and quadratic ($p=0.004$) responses (Table 4). Values ranged from 88.44±1.94 at dMC-75 to a peak of 92.20±1.94 at dMC-107, with intermediate levels clustering around 87–89. The quadratic component was driven by a particularly high value at the highest inclusion level.

Table 4. Internal egg quality parameters of HylineW80 laying hens fed graded levels of digestible methionine+cysteine during the second production cycle (79 to 95 weeks of age)

Variable	Treatments					SEM	*p*-value	
	dMC75	dMC83	dMC91	dMC99	dMC107			
							Linear	Quadratic
Haugh unit	88.44^{ab}	88.86^{ab}	87.05^{b}	89.15^{a}	92.2^{a}	1.94	0.021	0.004
Albumen index	0.109^{c}	0.111^{bc}	0.113^{bc}	0.119^{b}	0.121^{a}	0.007	0.002	0.712
Yolk index	0.498	0.502	0.493	0.49	0.487	0.013	0.189	0.456

Shell (%)	9.93	9.84	9.57	9.82	9.77	0.21	0.567	0.312
Yolk (%)	25.86	25.22	25.79	25.81	26.05	0.76	0.834	0.621
Albumen (%)	64.21	64.94	64.65	64.39	64.17	0.87	0.412	0.789

Albumen index increased linearly with dMet+Cys concentration ($p=0.002$; quadratic $p=0.712$), progressing from 0.109±0.007 at dMC-75 to 0.121±0.007 at dMC-107.

Yolk index remained statistically unaffected by treatment (linear $p=0.189$; quadratic $p=0.456$), with values fluctuating narrowly between 0.487 and 0.502±0.013. Similarly, the relative proportions of shell (9.57–9.93±0.21%), yolk (25.22–26.05±0.76%), and albumen (64.17–64.94±0.87%) showed no significant linear or quadratic responses (all $p>0.30$).

3.3 Plasma Metabolites

Circulating concentrations of key sulfur-containing metabolites and related proteins responded strongly to dietary dMet+Cys supply (Table 5). Plasma methionine concentration increased linearly from 48.2±3.1 µmol/L at dMC-75 to 71.3±3.1 µmol/L at dMC-107 ($p<0.001$; quadratic $p=0.842$). A similar linear elevation was observed for cysteine (12.4±1.4 to 21.6±1.4 µmol/L; $p<0.001$; quadratic $p=0.567$), homocysteine (8.9±0.7 to 12.1±0.7 µmol/L; $p=0.003$; quadratic $p=0.914$), and total glutathione (18.7±1.8 to 30.2±1.8 µmol/L; $p<0.001$; quadratic $p=0.312$).

Table 5. Plasma metabolite concentrations in HylineW80 laying hens fed graded levels of digestible methionine+cysteine at 95 weeks of age

Variable	Treatments					SEM	p-value	
	dMC75	dMC83	dMC91	dMC99	dMC107		Linear	Quadratic
Methionine (µmol/L)	48.18^{c}	52.67^{bc}	58.08^{b}	64.54^{ab}	71.33^{a}	3.1	<0.001	0.842
Cysteine (µmol/L)	12.41^{b}	14.84^{b}	16.92^{ab}	19.17^{ab}	21.56^{a}	1.4	<0.001	0.567
Homocysteine (µmol/L)	8.88^{b}	9.59^{ab}	10.44^{ab}	11.22^{a}	12.11^{a}	0.7	0.003	0.914
Total glutathione (µmol/L)	18.73^{c}	21.28^{bc}	24.46^{b}	27.83^{ab}	30.22^{a}	1.8	<0.001	0.312
Total protein (g/L)	48.52	49.19	49.76	50.08	50.38	1.2	0.078	0.456
Albumin (g/L)	18.21	18.63	18.88	19.12	19.26	0.5	0.145	0.678

Total plasma protein concentration showed a weak linear tendency to increase with dMet+Cys level (48.5±1.2 to 50.4±1.2 g/L; $p=0.078$; quadratic $p=0.456$), whereas plasma albumin remained statistically unchanged across treatments (18.2±0.5 to 19.3±0.5 g/L; linear $p=0.145$; quadratic $p=0.678$).

3.4 Tissue Gene Expression

Quantitative real-time PCR analysis of liver tissue at 95 weeks of age showed highly significant treatment effects on the relative expression of all twelve target genes (Table 6; Figures 1-4). Expression was quantified as fold change ($2^{-\Delta\Delta Ct}$) relative to the lowest dietary level (dMC-75; T1=1.00), with normalization to the geometric mean of *GAPDH* and *ACTB*.

Table 6. Cycle threshold (ΔCt*) values for genes related to sulfur amino acid metabolism, egg formation, and antioxidant defense in tissues of Hy-Line W-80 laying hens fed graded levels of digestible methionine+cystine at 95 weeks of age.

Genes**	Treatments					SEM	p-Value
	dMC75	dMC83	dMC91	dMC99	dMC107		
Reference genes mean	16.48	16.38	16.34	16.35	16.36	0.04	0.212
Methionine metabolism and the methylation pathway							
MTR	6.09^{a}	8.49^{b}	8.42^{b}	11.47^{c}	14.77^{d}	0.18	<.0001
CBS	6.09^{a}	8.48^{b}	10.10^{c}	13.35^{d}	18.15^{e}	0.15	<.0001
CTH	5.78^{a}	9.66^{b}	9.89^{b}	14.27^{c}	16.12^{d}	0.19	<.0001

BHMT	5.98^{a}	7.19^{b}	10.03^{c}	12.29^{d}	14.93^{e}	0.25	<.0001
MAT1A	6.19^{a}	5.39^{b}	8.30^{c}	10.37^{d}	12.02^{e}	0.20	<.0001
GPX1	6.12^{a}	7.86^{b}	9.25^{c}	13.19^{d}	14.15^{e}	0.23	<.0001
Egg formation and structural integrity							
OVAL	5.97^{a}	9.49^{b}	12.62^{c}	11.70^{c}	17.45^{d}	0.24	<.0001
LYZ	5.94^{a}	6.95^{a}	8.96^{b}	10.77^{c}	13.91^{d}	0.26	<.0001
TF	6.20^{a}	6.21^{a}	8.02^{b}	9.43^{c}	12.05^{d}	0.21	<.0001
CA2	6.01^{a}	7.83^{b}	9.34^{c}	7.36^{b}	9.09^{c}	0.22	<.0001
OC17	5.83^{a}	6.87^{b}	8.37^{c}	8.29^{c}	10.62^{d}	0.26	<.0001
KRT75	6.00^{a}	8.35^{b}	11.06^{c}	11.90^{cd}	12.73^{d}	0.23	<.0001

[*] ΔCt = 20 – (Ct_target – Ct_reference). Higher values indicate higher expression. Statistical comparisons were performed on these index values. For fold change ($2^{-\Delta\Delta Ct}$) relative to dMC75, see Figures 1–4 and the Results text.

[**] The genes are as follows: MTR – methionine synthase; CBS – cystathionine beta-synthase; CTH – cystathionine gamma-lyase; BHMT – betaine-homocysteine methyltransferase; MAT1A – methionine adenosyltransferase 1A; OVAL – ovalbumin; LYZ – lysozyme; TF – ovotransferrin; CA2 – carbonic anhydrase 2; OC-17 – ovocleidin-17; KRT75 – keratin 75; GPX1 – glutathione peroxidase 1

3.5 Egg quality and albumen/shell matrix-related genes (Figures 1 and 3)

The six genes associated with egg formation and quality (*OVAL*, *LYZ*, *TF*, *CA2*, *OC-17*, *KRT75*) also exhibited highly significant treatment effects ($p<0.0001$). Expression was downregulated at elevated dMet+Cys levels relative to T1. The greatest magnitude of downregulation occurred for *OVAL* (1429-fold at T5), *TF* (71-fold), and *KRT75* (107-fold), followed by *LYZ* (555-fold), *OC-17* (27-fold), and *CA2* (8.5-fold). Pairwise comparisons consistently separated T1 from all higher treatment levels ($p<0.05$), with progressive increases in fold change from T2 through T5 in most cases. No gene in this group showed upregulation at any tested level.

All data are presented as least squares Mean±Standard Error of the mean (SEM) unless otherwise indicated. Statistical significance was declared at $p\leq0.05$, with tendencies noted at $0.05<p\leq0.10$. Orthogonal polynomial contrasts were used to evaluate linear and quadratic dose–response patterns, while Tukey's test separated treatment means where overall ANOVA effects were significant.

3.6 Sulfur amino acid metabolism and methylation pathway genes (Figures 2 and 4)

All genes in this functional group (*MTR*, *CBS*, *CTH*, *BHMT*, *MAT1A*, *GPX1*) displayed highly significant overall treatment effects ($p<0.0001$). Relative to T1, expression was progressively downregulated with increasing dMet+Cys. The most pronounced downregulation at T5 occurred for *CBS* (>3700-fold), *CTH* (>1000-fold), and *BHMT* (>340-fold). *MAT1A* showed a biphasic response: upregulation at T2 (1.74-fold) followed by downregulation at higher levels (0.236-fold at T3, 0.056-fold at T4, 0.018-fold at T5). Within each gene, Tukey-adjusted pairwise comparisons separated most treatment means ($p<0.05$), with the clearest distinctions occurring between T1 and T3–T5.

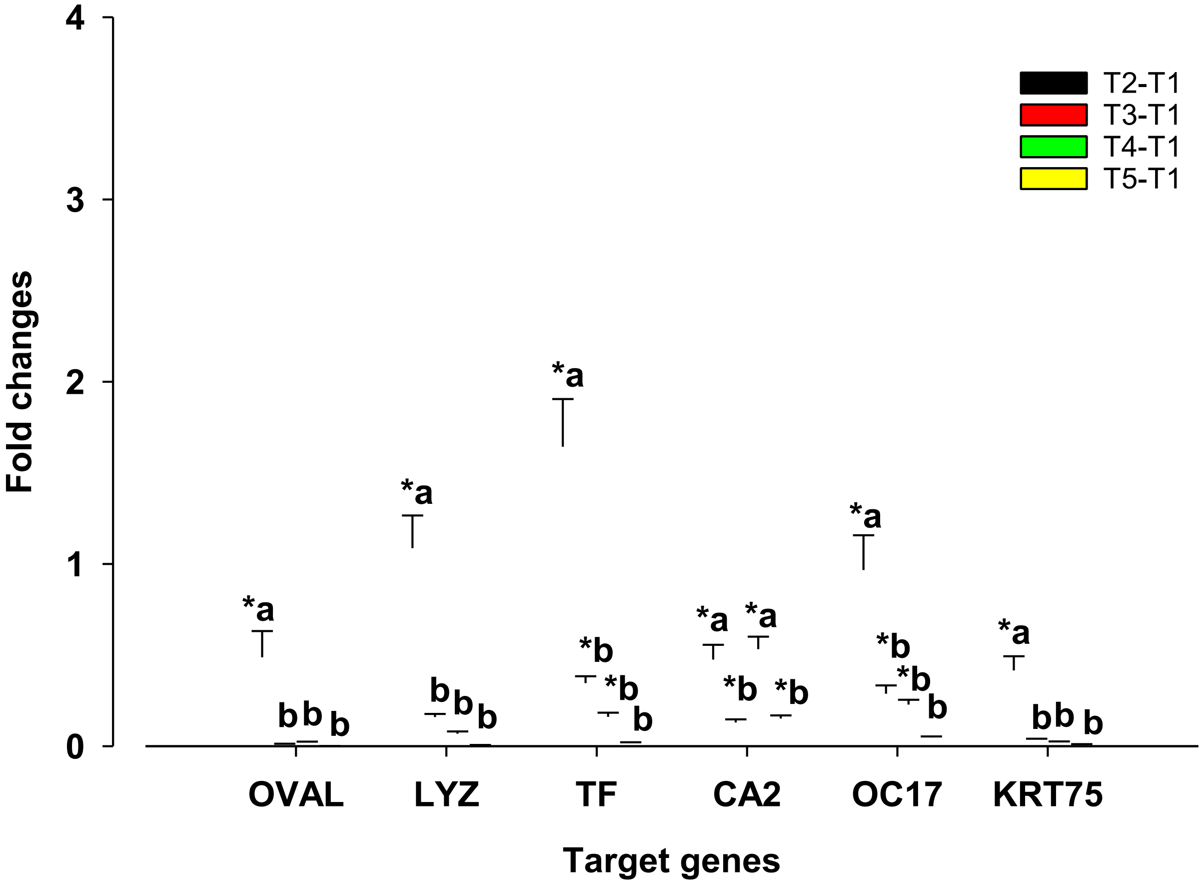

Figure 1. Fold changes in relative gene expression of sulfur amino acid metabolism and methylation pathway genes in the liver of Hy-Line W-80 laying hens at 95 weeks of age fed graded levels of digestible methionine + cystine (dMet+Cys). Bars represent mean fold changes (±SEM, n=6 replicates/treatment) relative to the lowest dietary level (T1 = dMC-75; 0.490% dMet+Cys, dMet+Cys: lysine ratio=75). Treatments were: T1=dMC-75, T2=dMC-83, T3=dMC-91, T4=dMC-99, T5=dMC-107. Expression was normalized to the geometric mean of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (beta-actin) housekeeping genes using the 2-ΔΔCt method. Genes shown are: MTR (methionine synthase), CBS (cystathionine beta-synthase), CTH (cystathionine gamma-lyase), BHMT (betaine-homocysteine methyltransferase), MAT1A (methionine adenosyltransferase 1A), and GPX1 (glutathione peroxidase 1). Different lowercase letters (a–b) above bars within each gene indicate significant differences among treatments (p≤0.05; Tukey's test). Asterisks (*) denote significant upregulation relative to T1 (p≤0.05).

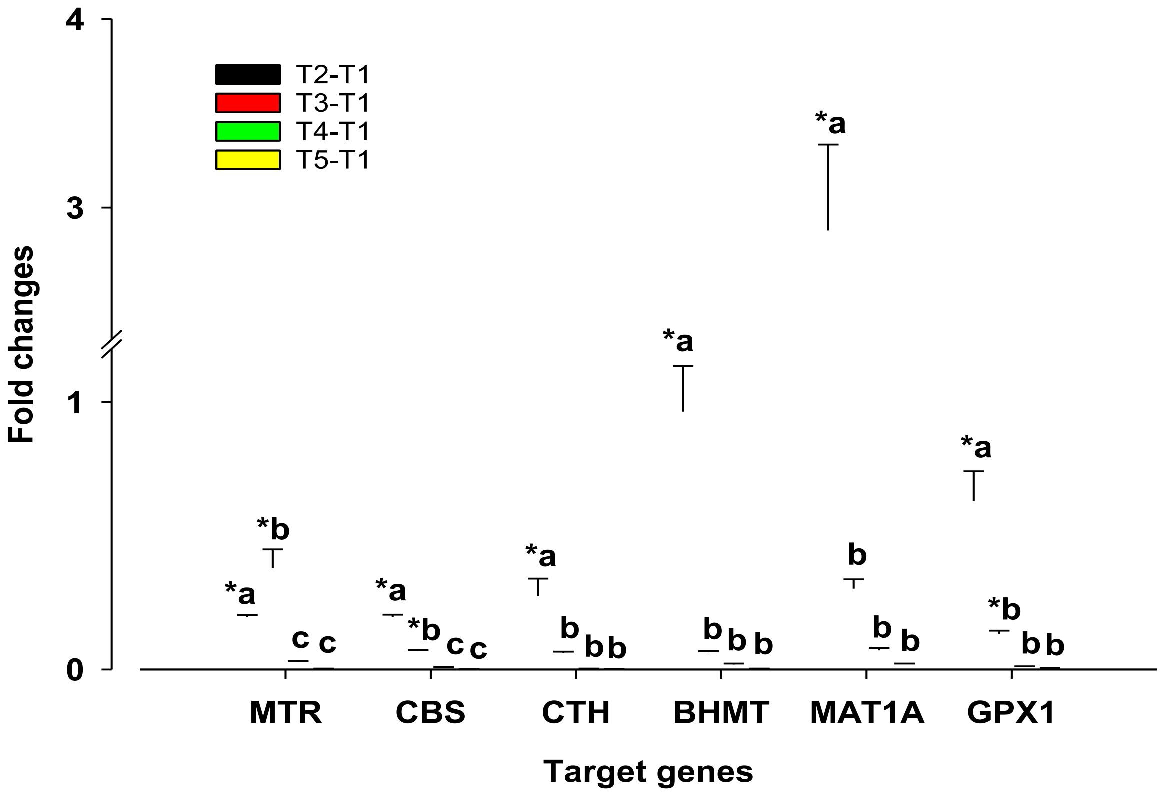

Figure 2. Fold changes in relative gene expression of sulfur amino acid metabolism and methylation pathway genes in the liver of Hy-Line W-80 laying hens at 95 weeks of age fed graded levels of digestible methionine + cystine (dMet + Cys). Bars represent mean fold changes (± SEM, n = 6 replicates/treatment) relative to the lowest dietary level (T1 = dMC-75; 0.490% dMet + Cys, dMet + Cys: lysine ratio = 75). Treatments were: T1 = dMC-75, T2 = dMC-83, T3 = dMC-91, T4 = dMC-99, T5 = dMC-107. Expression was normalized to the geometric mean of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (beta-actin) housekeeping genes using the 2-ΔΔCt method. Genes shown are: MTR (methionine synthase), CBS (cystathionine beta-synthase), CTH (cystathionine gamma-lyase), BHMT (betaine-homocysteine methyltransferase), MAT1A (methionine adenosyltransferase 1A), and GPX1 (glutathione peroxidase 1). Asterisks (*) indicate significant upregulation relative to T1 (p≤0.05). Different lowercase letters (a–c) above bars within each gene indicate significant differences among treatments (p≤0.05; Tukey's test).

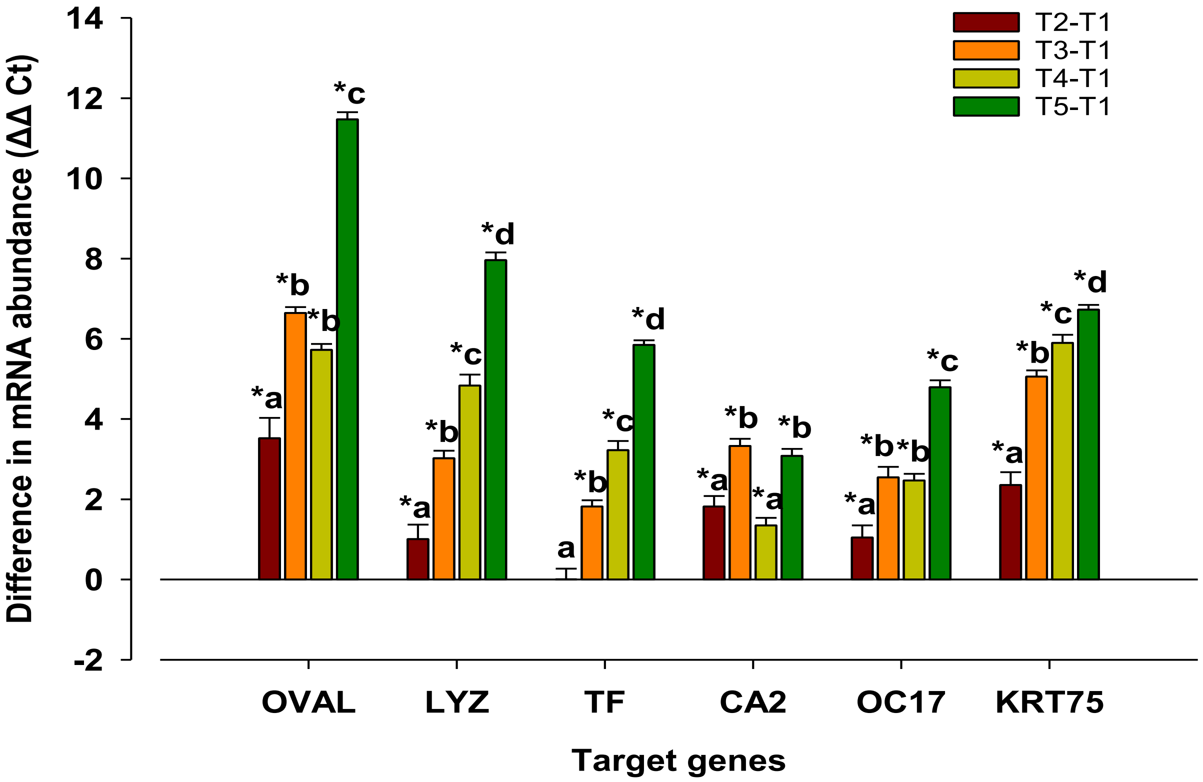

Figure 3. Difference in relative gene expression (ΔΔCt values) of egg quality and albumen/shell matrix-related genes in the liver of Hy-Line W-80 laying hens at 95 weeks of age fed graded levels of digestible methionine + cystine (dMet + Cys). Bars represent mean ΔΔCt values (±SEM, n=6 replicates/treatment) relative to the lowest dietary level (T1 = dMC-75; 0.490% dMet + Cys, dMet + Cys: lysine ratio = 75). Treatments were: T1 = dMC-75, T2 = dMC-83, T3 = dMC-91, T4 = dMC-99, T5 = dMC-107. Expression was normalized to the geometric mean of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (beta-actin) housekeeping genes. Genes shown are: OVAL (ovalbumin), LYZ (lysozyme), TF (ovotransferrin), CA2 (carbonic anhydrase 2), OC-17 (ovocleidin-17), and KRT75 (keratin 75). ΔΔCt = ΔCt_treatment – ΔCt_control, where ΔCt = 20 – (Ct_target – Ct_reference). Positive values indicate upregulation; negative values indicate downregulation relative to dMC75. This linear scale is used for visual clarity. Fold change (2–ΔΔCt) is reported in the text and in Figures 1–2. Asterisks (*) indicate a significant difference from T1 (p≤0.05). Different lowercase letters (a–d) above bars within each gene indicate significant differences among treatments (p≤0.05; Tukey's test).

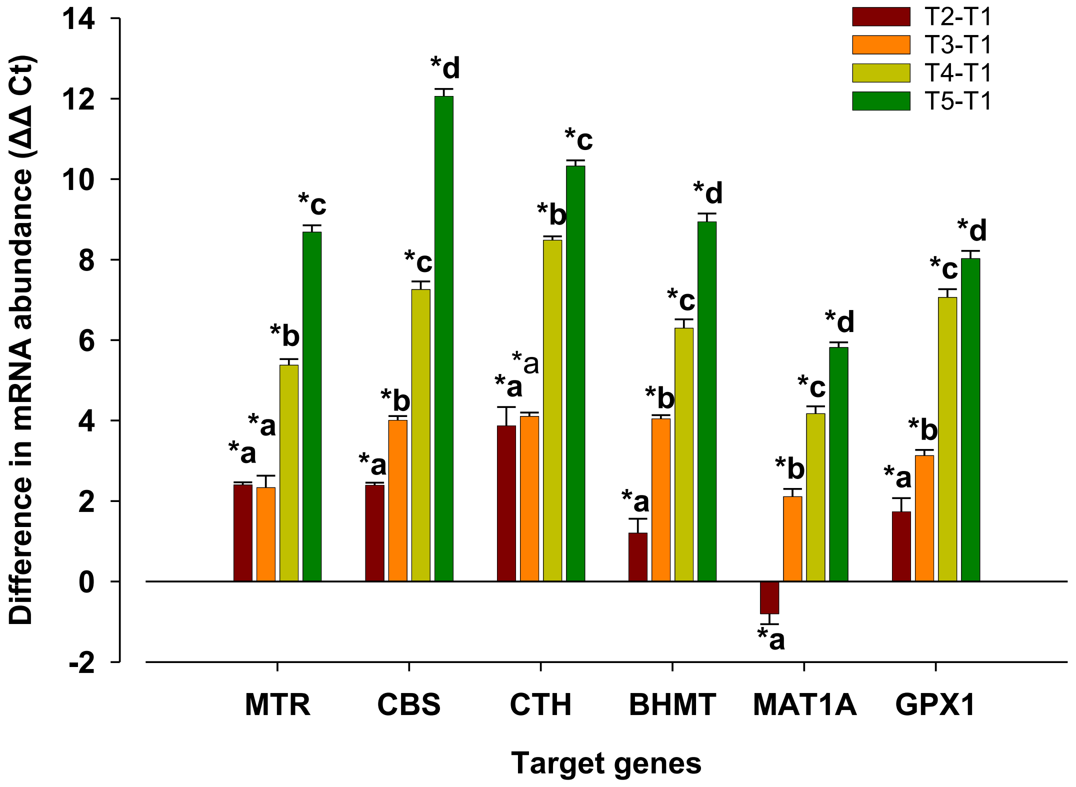

Figure 4. Difference in relative gene expression (ΔΔCt values) of sulfur amino acid metabolism and methylation pathway genes in the liver of Hy-Line W-80 laying hens at 95 weeks of age fed graded levels of digestible methionine + cystine (dMet + Cys). Bars represent mean ΔΔCt values (± SEM, n = 6 replicates/treatment) relative to the lowest dietary level (T1=dMC-75; 0.490% dMet+Cys, dMet+Cys: lysine ratio=75). Treatments were: T1=dMC-75, T2=dMC-83, T3=dMC-91, T4=dMC-99, T5 =dMC-107. Expression was normalized to the geometric mean of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (beta-actin) housekeeping genes. Genes shown are: MTR (methionine synthase), CBS (cystathionine beta-synthase), CTH (cystathionine gamma-lyase), BHMT (betaine-homocysteine methyltransferase), MAT1A (methionine adenosyltransferase 1A), and GPX1 (glutathione peroxidase 1). ΔΔCt=ΔCt_treatment–ΔCt_control, where ΔCt= 20–(Ct_target–Ct_reference). Positive values indicate upregulation; negative values indicate downregulation relative to dMC75. This linear scale is used for visual clarity. Fold change (2–ΔΔCt) is reported in the text and in Figures 1–2. Asterisks (*) indicate significant difference from T1 ($p \leq 0.05$). Different lowercase letters (a–d) above bars within each gene indicate a significant difference among treatments ($p \leq 0.05$; Tukey's test).

4 Discussion

The present study demonstrates coordinated, dose-dependent adaptations in productive performance, egg quality, circulating sulfur metabolites, and hepatic transcriptional activity in response to graded dMet+Cys supply. The linear improvements in laying rate and egg mass indicate that the basal diet imposed a functional SAA constraint during the metabolically demanding post-molt phase. One interpretation is that when sulfur amino acids are scarce, hens prioritize maintenance over reproduction, and only once the immediate demands of feather regrowth and antioxidant defense are met does the extra SAA become available for egg production. The linear increase in laying rate with rising SAA levels is consistent with the view that reproduction is a lower priority for limiting resources (Macelline et al., 2021; Oliveira Filho et al., 2019). Incremental supplementation may have shifted this allocation toward reproductive output once maintenance and recovery demands were satisfied, thereby restoring productive efficiency without evidence of supra-physiological stimulation. This finding aligns with the work of Domingues et al. (2012), who demonstrated that increasing digestible Met+Cys levels during the post-molt period of Isa Brown hens significantly influenced performance in the subsequent production cycle, with higher levels promoting greater egg weight (Domingues et al., 2012). The present results extend these observations by quantifying the linear response in a modern white-egg strain and establishing a higher optimal intake, which may reflect genetic progress over the intervening decade.

Although methionine participates in one-carbon metabolism via SAM, direct epigenetic activation of vitellogenin or hypothalamic signaling was not measured in the present study. Thus, the observed 7.2-percentage-point increase in laying rate should be interpreted primarily as a consequence of improved amino acid adequacy supporting hepatic yolk precursor synthesis and ovarian recrudescence, rather than as definitive evidence of altered neuroendocrine regulation.

Egg weight followed a quadratic trajectory with maximal response at 0.648% dMet+Cys. This pattern may reflect a transition from SAA-limited deposition to a multi-factorial ceiling in egg component synthesis. Once sulfur amino acid adequacy was achieved, additional supply may have encountered constraints imposed by magnum secretory capacity, energy availability, lysine balance, or endocrine regulation, thereby attenuating further increases in egg size. The linear rise in plasma homocysteine across treatments, coupled with the absence of adverse performance or protein alterations, suggests increased methyl-cycle throughput rather than metabolic dysregulation. Therefore, the modest decline in egg weight at the highest inclusion level is best interpreted as a biological plateau reflecting saturation of reproductive synthetic capacity rather than evidence of SAA toxicity.

Improvements in feed conversion efficiency may reflect enhanced nitrogen utilization efficiency as methionine limitation was alleviated. Adequate SAA supply is known to improve amino acid balance, reduce amino acid oxidation, and support more efficient protein accretion (Hofmann et al., 2020). However, extrapolation to crude protein reduction strategies should be approached cautiously, as the present experiment did not evaluate graded protein dilution.

Feed intake was not significantly affected by dietary treatment, indicating that the observed performance responses were driven primarily by changes in nutrient utilization efficiency rather than voluntary intake adjustments. This stability strengthens inference regarding requirement estimation, as differences in egg output can be attributed directly to SAA supply rather than being confounded by intake variability.

Based on observed consumption levels, estimated daily dMet+Cys intake at the performance plateau ranged from approximately 735 to 794 mg/hen. These values exceed historical NRC (1994) recommendations, which were derived from earlier genetic strains and shorter production cycles (National Research & Subcommittee on Poultry, 1994), but align with more recent evaluations in high-producing second-cycle hens (Oliveira Filho et al., 2019). This discrepancy may reflect ongoing genetic progress and increased egg mass output in modern commercial lines.

Beyond simple performance metrics, the gains in Haugh unit and albumen index point to improvements in albumen structure. These changes may stem from enhanced disulfide bonding, as cysteine residues are essential for ovomucin complexes that contribute to albumen viscosity and stability. Improved sulfur amino acid adequacy may therefore have supported more efficient albumen protein synthesis and structural stabilization. However, direct measurements of albumen protein composition or disulfide bond density were not performed, and mechanistic interpretation should remain conservative. These findings resonate with reports in heat-stressed layers, where SAA supplementation mitigates protein denaturation and preserves internal quality indices (Kachungwa Lugata et al., 2022; Sarsour & Persia, 2022). The concept is reinforced by the work of Martínez-Aguilar et al. (2015), who demonstrated that supplementing hypoproteic diets with DL-methionine and L-lysine in laying hens significantly improved albumen height and Haugh units (Martínez-Aguilar et al., 2015). The present results show that the qualitative improvements in albumen are associated not only with dietary SAA levels but also with systemic metabolic and transcriptional adaptations, providing a possible mechanistic link between nutrition and egg internal quality.

Although SAA supplementation did not alter the basic proportions of shell, yolk, or albumen, it did improve certain quality attributes. This distinction suggests that SAA may be involved in fine-tuning glycoprotein structure rather than driving bulk synthesis, potentially via selective enhancement of matrix glycoproteins such as ovocleidin-17 (Castro et al., 2019; Kachungwa Lugata et al., 2022). The tendency for thicker shells at higher inclusions might implicate SAA in carbonic anhydrase II catalysis, facilitating bicarbonate availability for calcite nucleation in the uterus (Hofmann et al., 2020; Yang et al., 2024). While the present study did not measure bone parameters directly, the observed shifts in sulfur metabolism raise the possibility that adequate SAA might also support skeletal health through collagen cross-linking. However, such interpretations remain speculative based on the current data (Castro et al., 2019; Xiao et al., 2016).

Plasma methionine, cysteine, homocysteine, and glutathione increased proportionally with dietary inclusion, suggesting that systemic SAA pools expanded in parallel with intake and that absorptive mechanisms were not limiting within the tested range. The concomitant downregulation of *CBS*, *CTH*, *BHMT*, and *MTR* is consistent with reduced reliance on endogenous remethylation and transsulfuration pathways once dietary supply became sufficient. Rather than implying transsulfuration overload, the elevation in homocysteine may reflect increased methyl-cycle flux under higher methionine availability. Notably, the rise in circulating glutathione despite suppression of *GPX1* transcription suggests substrate-driven enhancement of antioxidant buffering capacity rather than compensatory oxidative stress signaling. Although direct oxidative biomarkers were not measured, this coordinated metabolite–transcript response is consistent with improved redox equilibrium during post-molt recovery (Lebedev et al., 2025; Lee et al., 2021). The functional significance of this enhanced glutathione status is supported by research in broilers, where supplementation of DL-methionine above NRC recommendations during heat stress increased glutathione concentrations in the liver and breast muscle and reduced markers of lipid oxidation in muscle tissue (Zeitz et al., 2020). While the present study did not measure tissue oxidative markers, the linear increase in plasma glutathione suggests that elevated SAA supply similarly bolstered systemic antioxidant capacity in post-molt hens.

The marginal rise in total protein, without changes in albumin, implies selective hepatic export prioritization, possibly related to acute-phase responses (Wan et al., 2017; Wu et al., 2024). Co-fortification with betaine or choline could theoretically potentiate homocysteine remethylation via BHMT, but such speculation requires direct testing (Ma et al., 2024; Wu et al., 2024).

The coordinated suppression of *MTR*, *CBS*, *CTH*, and *BHMT* at higher dMet+Cys levels is consistent with substrate-mediated feedback regulation within the methionine cycle and transsulfuration pathway. This transcriptional attenuation may reflect decreased necessity for homocysteine recycling and cysteine generation once dietary intake satisfies metabolic demand. This pattern aligns with models in which elevated intracellular methionine and SAM concentrations signal reduced need for endogenous synthesis and recycling pathways (Wan et al., 2017). The transient modulation of *MAT1A* further supports a dynamic adjustment of methyl-donor flux rather than linear activation. Collectively, these findings are consistent with metabolic elasticity, whereby hepatic SAA metabolism contracts under sufficiency to prevent unnecessary energy expenditure and futile cycling while preserving methylation capacity. The downregulation of *MTR*, *CBS*, *CTH*, and *BHMT* observed in this study provides an in vivo example of this regulatory principle in post-molt layers. This is consistent with the work of Wan et al. (2017), who reported that increasing dietary methionine levels in broiler breeders quadratically altered the expression of hepatic genes involved in methyl group metabolism (Wan et al., 2017), including *GNMT* and *SAHH*, demonstrating a sensitive transcriptional response to methionine supply. The present findings corroborate this sensitivity and show that the response extends to core enzymes of the transsulfuration and remethylation pathways.

The transient *MAT1A* upregulation at intermediate levels may represent a biphasic response, initially boosting SAM for DNA methylation before feedback normalization (Ma et al., 2021; Sarsour & Persia, 2022). *GPX1* suppression is consistent with attainment of redox equilibrium, as excess SAA may obviate the need for glutathione peroxidase overexpression (Kachungwa Lugata et al., 2022).

One unexpected observation was that as dietary SAA increased, the liver downregulated genes traditionally considered oviduct-specific (*OVAL*, *LYZ*, *TF*, *CA2*, *OC-17*, *KRT75*). Ectopic expression of tissue-specific genes in the liver has been reported previously (Li et al., 2025). In the present study, when SAA was lowest (dMC75), the liver showed higher expression of these genes; when SAA became plentiful, expression was reduced. One possible interpretation is that the liver may act as a metabolic sensor, increasing ectopic transcription under amino acid scarcity and reducing it when supply is adequate. However, this hypothesis requires direct experimental validation.

A recent comprehensive transcriptomic analysis of the liver in growing laying hens by Wu et al. (2024) identified a wide array of differentially expressed genes related to amino acid transport and metabolism (Wu et al., 2024), highlighting the liver's central and dynamic role in systemic nutrient sensing. The present study adds that the liver's transcriptional landscape is not only responsive to amino acid levels but also shows suppression of non-hepatic protein genes (*OVAL*, *LYZ*, *TF*) under conditions of SAA sufficiency. This observation raises the possibility that the liver may prevent wasteful synthesis of unneeded proteins in a non-target tissue, though this remains speculative.

The consistent suppression of these transcripts under SAA sufficiency is consistent with a systemic transcriptional program that downregulates protein synthesis machinery in non-target tissues when amino acid supply is no longer limiting. One hypothesis is that under deficiency (T1), the liver may act as a non-specific amino acid sink, ectopically expressing these genes. Upon adequate SAA supply, this potential futile cycle is repressed, possibly reallocating resources toward the oviduct for egg protein synthesis, which could explain the improvements in albumen quality (Castro et al., 2019; Yang et al., 2024). This proposed inter-organ nutrient partitioning model, wherein SAA sufficiency signals the liver to downregulate non-essential ectopic transcription, thereby conserving amino acids for prioritized synthesis in the oviduct, is intriguing but remains hypothetical. Further studies are needed to test whether such reallocation actually occurs and whether it underlies the enhanced albumen quality observed in this study (Castro et al., 2019; Hofmann et al., 2020).

The optimal response observed near 0.648% dMet+Cys (dMC99) aligns with requirement estimates reported for the second-cycle or aged layers under high production pressure (Liu et al., 2020; Xiao et al., 2016). Differences between the present findings and earlier recommendations for non-molted hens may reflect altered physiological priorities following induced molt, including feather regeneration and accelerated ovarian redevelopment, both of which increase sulfur amino acid demand (Lebedev et al., 2025; Oliveira Filho et al., 2019).

Although genetic selection may influence amino acid efficiency over time (Lee et al., 2021), the present data do not permit inference regarding future requirement shifts. Similarly, potential interactions between SAA supply and gut-derived methyl donors remain hypothetical, as neither microbiota composition nor folate metabolism was assessed. These considerations warrant targeted investigation but should not be inferred directly from the current dataset (Khazab et al., 2026; Wu et al., 2024).

From a practical perspective, the findings support the precise formulation of sulfur amino acids in post-molt diets to maximize productive efficiency while avoiding unnecessary oversupply. Improved albumen quality and enhanced antioxidant-related metabolites suggest that adequate SAA nutrition contributes to physiological stability during second-cycle production. While oxidative status has been linked to immune competence in layers (Oliveira Filho et al., 2019; Wan et al., 2017), direct effects on pathogen resistance were not evaluated in this study and therefore cannot be concluded.

The integration of nutrigenomic markers into requirement modeling represents a promising direction for precision nutrition. However, application to microbiota modulation or digital feed optimization systems will require mechanistic validation and longitudinal performance trials (Ma et al., 2021; Wan et al., 2017). The present study provides foundational physiological and transcriptional data but does not independently substantiate broader systems-level interventions.

5 Conclusion

This study demonstrates that optimizing dietary digestible methionine+cystine at 0.648% (dMet+Cys: Lys ≈ 99) maximizes post-molt performance and egg quality in white-egg layers. Hens fed this level showed superior laying rates, egg mass, and feed efficiency, along with improved albumen quality and elevated plasma glutathione, indicating enhanced antioxidant capacity. These benefits stem from both adequate SAA supply for reproductive demands and a distinct metabolic shift: sufficient SAA downregulated hepatic expression of genes involved in sulfur metabolism (*MTR*, *CBS*, *CTH*) and, notably, suppressed ectopic transcription of egg-protein genes (*OVAL*, *LYZ*, *TF*) in the liver. This coordinated response suggests the liver prioritizes resource allocation toward the oviduct when SAA is no longer limiting.

For commercial practice, these findings support precision formulation of post-molt diets at 0.648% dMet+Cys to improve flock productivity and longevity while minimizing nitrogen excretion. From a mechanistic standpoint, the observed nutrigenomic adaptations reveal how the status of sulfur amino acids orchestrates inter-organ nutrient partitioning. This concept opens new avenues for research into targeted nutritional strategies to extend laying cycles.

Acknowledgements

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Use of Artificial Intelligence

During the preparation of this manuscript, the authors used AI-assisted tools (ChatGPT-4) for language editing and refinement of specific sections to improve clarity and readability. Following the use of these tools, the authors thoroughly reviewed and revised the content and take full responsibility for the final version of the manuscript.

Conflict of Interest

We declare that no conflict of interest.

Author Contributions

M.Z.: Conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing—review and editing, visualization, supervision.

A.A.: Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing—original draft, writing—review and editing, visualization, supervision, project administration, funding acquisition.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Considerations

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Yasouj University (approval number: 1403-4231923949-A) and conducted in compliance with Iranian animal welfare regulations and guidelines of the Veterinary Organization of Iran.

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