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RESEARCH ARTICLE

The Effect of Broiler Breeder Maternal Age on Gene Expression of the Hen and the Embryo

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Abstract

Background and Objective: Broiler breeder hens begin laying at approximately 25 weeks of age and produce small eggs that are frequently non-settable or yield small chicks with reduced livability. The yolk sac (YS) and liver (LV) are the primary tissues supporting nutrient utilization during embryonic development, and hen age affects yolk size and hatchling weight. The objective of this study was to characterize age-related differences in gene expression and protein abundance in breeder hens and their embryos.

Materials and Methods: Cobb 500 breeder hens were artificially inseminated at 26 and 35 weeks of age, and hatching eggs were collected over a 7-d period at each age. Yolk sac and LV samples were collected from embryos on embryonic day 17 (E17), while abdominal fat (AF) and LV samples were collected from the hens that produced those eggs. Forty-eight genes related to fatty acid, glucose, and amino acid metabolism were profiled using a fold-regulation cutoff of 2.0 and $p<0.05$. Selected targets were subsequently confirmed by Western blotting.

Results: In the YS of embryos from 26-week-old hens, the most overexpressed genes were Pyruvate Carboxylase (PC), glutamic-pyruvate transaminase 2 (GPT2) and lipoprotein lipase (LPL), with fold increases of 75, 28 and 24, respectively. Three genes were underexpressed in the YS but overexpressed in the embryonic LV of the same embryos: fatty acid-binding protein 2 (FABP2), low-density lipoprotein receptor-related protein 2 (LRP2) and PKM2. In the LV of 26-wk-old hens, the most overexpressed genes were the fatty acid metabolism-related genes stearoyl-CoA desaturase (SCD), fatty acid elongase 6 (ELOVL6), and acetyl-CoA carboxylase alpha (ACACA). Protein abundance confirmed greater levels of GPT2, PC and LPL in the YS of embryos from young hens, whereas LRP2 protein abundance was greater in embryos from 35-wk-old hens.

Conclusion: Embryos from young breeder hens exhibit greater expression of genes involved in gluconeogenesis and carbohydrate metabolism, whereas embryos from prime-aged hens exhibit greater abundance of lipid transport proteins. These findings indicate an age-related shift in fuel utilization during late incubation rather than impaired fatty acid metabolism.

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INTRODUCTION

Eggs produced by young broiler breeder hens during early lay (approximately 25 to 29 week of age) are consistently smaller than those produced by hens at peak or later stages of production[1-3]. Reduced egg size during early lay is primarily attributed to lower yolk deposition, incomplete maturation of the reproductive tract, and lower overall nutrient allocation to the developing oocyte[4-6]. Because egg size is strongly and positively correlated with chick hatch weight, these smaller eggs result in lighter chicks at hatch, with chick body weight often reduced by 5-10% compared with chicks from eggs laid by older hens[7-9].

Lower hatch weight and reduced residual yolk mass have important downstream consequences for chick quality and post-hatch performance. Chicks hatched from young breeder flocks exhibit reduced early feed intake, slower intestinal development, poorer energy reserves and increased susceptibility to dehydration and metabolic stress during the first week post-hatch[10-13]. As a result, birds originating from young flocks frequently demonstrate reduced livability, inferior early growth rate and compromised feed efficiency compared with broilers derived from eggs produced by prime-aged or older breeders (>30 weeks of age)[3,14-16]. Collectively, these effects create a significant economic disadvantage for broiler producers by increasing early mortality and reducing uniformity and overall flock performance.

In addition to reduced egg size, the internal composition of eggs from young breeders differs substantially from that of eggs produced by older hens. Eggs laid by young flocks (29 weeks of age) contain a smaller yolk proportion, approximately 3.5% less, along with a proportionally greater amount of albumen compared with eggs from older flocks (59 week of age)[3]. Because the yolk supplies nearly all of the lipid-derived energy required for embryonic growth, reduced yolk allocation may limit energy availability during late embryogenesis and early post-hatch development[17,18]. Eggs from young breeders also differ in yolk fatty acid composition and in the efficiency with which yolk lipid is taken up by the late-term embryo[19,20]. Furthermore, eggs from younger hens have been associated with reduced fertility, by up to 18%, and reduced hatchability, by approximately 20%, compared with eggs from older breeders between 31 and 36 weeks of age[3,15].

According to performance objectives published by primary breeding companies, approximately 10-12% of total eggs produced over a 40-weeks production cycle are laid before hens reach 29 week of age[21,22]. Although only about 3% of these early-lay eggs meet standard hatching-egg weight recommendations (>50 g)[22], many commercial producers set lighter eggs to maintain chick supply, despite the known penalties in chick quality and broiler performance. Consequently, a meaningful proportion of broiler chickens originate from eggs produced by young breeders, approximately 26 weeks of age, underscoring the need to better understand the biological mechanisms underlying reduced embryonic efficiency and post-hatch performance in these birds.

The biological basis for the reduced performance of chicks hatched from first-laid eggs following sexual maturity remains incompletely understood. While egg size and yolk mass clearly contribute to these differences, it is unlikely that egg size alone fully explains the consistently lower performance observed in chicks from young breeders. Differences in the efficiency of nutrient transfer, yolk lipid mobilization, and embryonic metabolic programming may also play critical roles[23,24]. It is possible that embryos from young hens differ in the expression of genes involved in lipid metabolism, nutrient transport, or energy production, thereby limiting their ability to utilize available yolk nutrients efficiently during development[25,26].

Supporting this concept, the embryonic yolk sac has been shown to be a highly active metabolic organ expressing numerous genes involved in lipid metabolism, lipoprotein receptors, fatty acid transport, and energy homeostasis[27]. These genes function to compensate for the immaturity of embryonic digestive and metabolic systems by facilitating nutrient absorption and transfer to developing tissues. However, to the authors' knowledge, differences in embryonic yolk sac gene expression or protein abundance between embryos derived from eggs laid by young versus older breeder hens have not previously been reported.

Previous studies have clearly demonstrated that breeder age significantly affects hatchability, chick quality, and offspring growth performance[2,8,28,29]. Given that nearly 90% of the energy required by the developing embryo is derived from the oxidation of yolk fatty acids, impairments in yolk lipid composition, uptake, or oxidation could have profound consequences for embryonic development and post-hatch performance[17]. In support of this mechanism, breeder hen age has been reported to alter yolk fatty acid profiles and yolk utilization during embryogenesis, with embryos from 26-week-old hens exhibiting reduced fatty acid absorption compared with those from older hens[30].

Therefore, the objective of the present study was to evaluate differences in gene expression and protein abundance in breeder hens and their embryos to better understand age-related differences in nutrient utilization between embryos from young (26-week-old) and prime-aged (35-week-old) broiler breeder hens.

MATERIALS AND METHODS

Breeders and management: A total of 80 Cobb 500 Fast Feathering (FF) breeder pullets and 10 Cobb MV cockerels, all 25 weeks of age at housing, were obtained from a commercial rearing facility and transferred to the University research unit. Birds were individually housed in battery cages constructed of galvanized wire. Female cages measured 47×30.5×47 cm (height×width×depth), and male cages measured 47×45.7 × 63.5 cm, with each cage equipped with an individual feed trough and nipple drinker.

Pullets were photostimulated at 21 weeks of age following the primary breeder lighting program for Cobb 500 breeders[31]. Throughout the experimental period, hens were fed a common breeder diet formulated to contain 15.5% crude protein and 2,860 kcal/kg ME (Table 1) and water was provided *ad libitum*.

As birds were maintained under cage-breeder management, feed allocation was controlled, and no post-peak feed withdrawal program was implemented, in contrast to floor-pen breeders. All hens received 140 g feed/bird/d, which represents a standard cage-breeder intake and is below the peak feed intake typically used in floor-managed breeders[32]. Consequently, hens at 26 weeks of age received the same feed allocation as those at 35 weeks of age, although the younger hens had not yet reached peak egg mass relative to nutrient intake at egg collection.

Artificial insemination and egg collection: All hens were artificially inseminated at 26 and 35 weeks of age to synchronize fertility status between age groups. Each hen received 50 µL of pooled semen containing approximately 1×10^6 sperm cells per insemination, following established procedures for broiler breeders[33,34]. Semen was pooled across cockerels to minimize male-to-male variation.

Following insemination, hatching eggs were collected daily from each hen for a consecutive 7-d collection period at each breeder age (26 and 35 weeks). Eggs were identified by hen, weighed at lay, and stored large-end up at 18°C and 75% relative humidity until incubation[35].

Experimental design and tissue collection: To evaluate age-related differences in embryonic and maternal nutrient utilization, tissues were collected from both embryos and hens. For embryonic sampling, three embryos per breeder age (26 or 35 weeks) were randomly selected on embryonic day 17 of incubation (E17).

At E17, eggs were opened and the yolk sac (YS) was carefully separated from the yolk contents. The YS, defined as the vascularized epithelial tissue surrounding the yolk mass, was gently rinsed with sterile saline to remove residual yolk lipids. A standardized section of the YS was excised using sterile instruments. The remaining yolk mass was discarded. Yolk sac and embryonic liver (LV) tissues were weighed, flash-frozen in liquid nitrogen, and stored at -80°C until analysis.

Table 1: Breeder 1 diet

Ingredients	Percentage	Nutrient	Percentage
Corn	66.30	**Calculated**	
Soybean Meal	23.10	AME[3] (kcal/kg)	2860.00
Poultry Oil	1.30	Crude Protein	15.50
Calcium Carbonate	6.78	Dig Lysine	0.76
Dicalcium Phosphate	1.81	Dig Met+Cys	0.67
Salt	0.17	Dig. Methionine	0.42
D-L Methionine	0.19	Dig. Threonine	0.52
Choline	0.10	Dig. tryptophan	0.16
Vitamin premix[1]	0.07[1]	Dig. Arginine	0.95
Sodium bicarbonate	0.10	Crude fat	3.38
Mineral premix[2]	0.08[2]	Calcium	3.25
		Available P	0.41
		Sodium	0.2
		Analyzed	
		Crude protein	15.30
		Crude fat	4.87
		Ash	10.31
Total	100.0	Calcium (ppm)	33604.00

[1]The vitamin premix contained per kilogram of diet: vitamin A from retinyl acetate, 60,000 IU; vitamin D_3, 2,167 IU; vitamin E from dl-α-tocopherol acetate, 33 IU; vitamin B_{12}, 0.01 mg; vitamin B_6 from pyridoxine mononitrate, 3 mg; niacin, 27 mg; d-pantothenic acid, 10 mg; menadione, 2 mg; folic acid, 0.87 mg; thiamine from thiamine mononitrate, 3 mg; ethoxyquin, 67 mg; d-biotin, 0.3 mg; and riboflavin, 7 mg. [2]The mineral premix contained zinc as zinc sulfate, 11.02%; manganese as manganese sulfate, 8.82%; iron as ferrous sulfate monohydrate, 5.29%; copper as copper sulfate, 1.03%; iodine as ethylenediamine dihydroiodide, 2,600 mg/kg; and selenium as sodium selenite, 400 mg/kg. [3]AME: Apparent metabolizable energy

Supplemental Table 1: List of genes and associated information

Gene symbol	Alias	RefSeq No.	Official gene name	RT^2 catalog number
LPL	–	NM_205282	Lipoprotein lipase	PPG01435
FAS	–	NM_001199487	Fas (TNF receptor superfamily member 6)	PPG00609
FABP2	FABP	NM_001007923	Fatty acid binding protein 2 (intestinal)	PPG07331
FABP5	–	NM_001006346	Fatty acid binding protein 5	PPG05517
CD36	–	NM_001030731	CD36 molecule (thrombospondin receptor)	PPG03630
APOA1	–	NM_205525	Apolipoprotein A-I	PPG01698
ACACA	ACAC	NM_205505	Acetyl-CoA carboxylase alpha	PPG01674
ACAA1	–	NM_001197288	Acetyl-CoA acyltransferase 1 (peroxisomal)	PPG11641
PPARG	–	NM_001001460	Peroxisome proliferator-activated receptor gamma	PPG00062
PPARA	–	NM_001001464	Peroxisome proliferator-activated receptor alpha	PPG00230
CROT	–	XM_418635	Carnitine O-octanoyltransferase	PPG05761
CPT1A	CPT1	NM_001012898	Carnitine palmitoyltransferase 1A (liver)	PPG07641
LRP2	–	XM_004942763	Low-density lipoprotein receptor-related protein 2	PPG08423
G6PC2	–	XM_422017	Glucose-6-phosphatase catalytic subunit 2	PPG08426
PKM2	PKM	NM_205469	Pyruvate kinase (muscle)	PPG01632
PCK1	PEPCK	NM_205471	Phosphoenolpyruvate carboxykinase 1	PPG01634
IVD	–	NM_001199561	Isovaleryl-CoA dehydrogenase	PPG07565
GPT2	–	XM_004944193	Glutamic pyruvate transaminase 2	PPG02193
BCAT1	–	XM_004937974	Branched-chain aminotransferase 1	PPG03992
RPL4	–	NM_001007479	Ribosomal protein L4	PPG02048
ACTB	Bact/actin	NM_205518	Beta-actin	PPG17308
UBC	UBB	XM_001234599	Ubiquitin C	PPG01607
HMBS	–	XM_417846	Hydroxymethylbilane synthase	PPG05149
H6PD	–	XM_425746	Hexose-6-phosphate dehydrogenase	PPG10346
BCKDHA	–	AB035083	Branched-chain keto acid dehydrogenase E1 alpha	PPG18530
GGDC	SA_00517	PPG17309	Genomic DNA contamination control	GGDC
RTC	SA_00104	PPX63340	Reverse transcription control	RTC
PPC	SA_00103	PPX63339	Positive PCR control	PPC

For maternal tissue sampling, three hens at 26 weeks and three hens at 35 weeks of age were randomly selected, individually weighed, and euthanized by cervical dislocation in accordance with approved animal care guidelines. Liver and abdominal fat (AF) were excised, weighed, rapidly frozen in liquid nitrogen, and stored at -80°C.

Ribonucleic acid isolation: Ribonucleic acid (RNA) was isolated using the miRNeasy Mini Kit (QIAGEN, 217004) following the manufacturer's protocol for tissue analysis. RNA quality was determined using an Agilent Bioanalyzer (Agilent) with RNA 6000 Nano Kits (Agilent, 5067-1511). Total RNA yield and 260/280 and 260/230 ratios were measured using a NanoDrop spectrophotometer (Thermo).

RT2 profiler PCR array gene expression: A total of 48 genes were profiled per sample. A custom RT2 Profiler polymerase chain reaction (PCR) Array was designed with QIAGEN to study the expression of target genes related to fatty acid and carbohydrate metabolism using real-time PCR. Primers used for these genes are listed in Supplementary Table S1. The procedure began with the conversion of 150 ng of experimental RNA into first-strand complementary deoxyribonucleic acid (cDNA) using the RT2 First Strand Kit (QIAGEN, 330401) according to the manufacturer's instructions. The cDNA was then mixed with RT2 SYBR Green Mastermix (QIAGEN, 330529) and aliquoted into the wells of the custom RT2 Profiler PCR Array. Real-time PCR was performed and relative gene expression was determined using data from the real-time cycler and the $\Delta\Delta C_t$ method. Samples were assigned to control and test groups. C_t values were normalized using the arithmetic mean C_t value of four housekeeping genes: RPL4, actin beta (ACTB), hydroxymethylbilane synthase (HMBS) and hexose-6-phosphate dehydrogenase (H6PD). Fold changes in expression for specific target genes were determined. Raw and normalized C_t values were used to generate heat maps in R[36].

Western blot analysis: Yolk sac and LV samples from E17 were thawed and vortexed. Approximately 200 µL of sample was transferred to a new tube containing 200 µL of osmotic lysis buffer supplemented with protease inhibitor stock, phosphatase inhibitor stocks (I and II), and nuclease stock. To each sample, 400 µL of SDS boiling buffer without reducing agents was added and the samples were vortexed before treatment with 3 µL of Omnicleave (Lucigen, 7850K). The

samples were pulled through a 16-gauge needle and heated in a dry bath for 10 min at 95°C. Protein concentration was determined using the bicinchoninic acid assay[37]. All samples were diluted or re-dissolved to 2.5 µg/µL in SDS boiling buffer containing reducing agents and heated at 95°C for 10 min before loading.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing conditions[38], as modified by O'Farrell[39]. Samples were loaded into wells of 10% acrylamide slab gels (0.75 mm thick). SDS-PAGE was carried out for 4 hrs at 15 mA/gel. The following proteins (Millipore Sigma) were used as molecular weight standards: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000). These standards are visible in lanes on the Coomassie blue-stained gel or in images of the Coomassie blue-stained polyvinylidene difluoride (PVDF) membranes. After slab gel electrophoresis, the gel for blotting was placed in transfer buffer (10 mM CAPS, pH 11.0, 10% methanol) and trans-blotted onto PVDF membranes overnight at 200 mA and approximately 100 V per two gels.

Tris-acetate method for high molecular weight proteins: Samples were loaded into wells of a Bio-Rad 3-8% Criterion Tris-Acetate gel (Bio-Rad, 3450130). Precision Plus Protein Dual Color Standards (Bio-Rad, 161-0374) were loaded in lanes 7 and 12. Gels were run using XT Tricine running buffer (Bio-Rad, 161-0790) at a constant 80 V for 60 min, followed by 120 V for 45 min until the blue dye front was near the bottom of the gel. After slab gel electrophoresis, the gel was placed in pre-chilled transfer buffer (12.5 mM Tris, 192 mM glycine, pH 8.3, 10% methanol) and trans-blotted onto a PVDF membrane (Millipore, PVH00010) for 2 h at a constant 80 V for two gels in a Criterion Blotter with plate electrodes (Bio-Rad, 1704070) at 4°C on ice. All blots were stained with Coomassie Brilliant Blue R-250, cut into pieces at the dark lines, and scanned using a desktop scanner. The blots were then blocked for 2 hrs in 5% non-fat dry milk (NFDM) in Tween-20 Tris-buffered saline (TTBS).

Rabbit antibodies: Blots were incubated overnight in primary antibody diluted in 2% NFDM in TTBS and then rinsed three times for 10 min in TTBS. Blots were subsequently incubated with secondary antibody (anti-rabbit IgG-HRP; SeraCare, 5220-0337) diluted 1:20,000 in 2% NFDM in TTBS for 2 h, rinsed as described above, treated with enhanced chemiluminescence (ECL), and exposed to X-ray film.

Goat antibodies: Blots were incubated overnight in primary antibody diluted in 2% NFDM in TTBS and then rinsed three times for 10 min in TTBS. Blots were subsequently incubated with secondary antibody (anti-goat IgG-HRP; Invitrogen, A16142) diluted 1:30,000 in 2% NFDM in TTBS for 2 hrs, rinsed three times for 10 min in TTBS, treated with ECL, and exposed to X-ray film.

Heat map generation: C_t values were obtained from the primary results and prepared as two datasets: Raw C_t values and mean-normalized C_t values. Mean normalization was performed using the average of the positive PCR control (PPC) and reverse transcription control (RTC) values. Heat maps were generated in R using the *gdata* and *gplots* packages. The two datasets were imported and saved as matrices with values in string format. The heatmap.2 function was used to generate high-resolution grayscale heat maps (Figs. 1 and 2).

Pixel intensity quantification analysis: The films were digitized across the appropriate optical density range using a calibrated GE Healthcare Image Scanner III. Band volumes within the selected lanes were determined using Phoretix 1D software (version 11.2).

Statistical analysis: Data were analyzed as fold change and fold regulation using the $\Delta\Delta C_t$ method. The ΔC_t was calculated as the difference between the C_t value of the gene of interest (GOI) and the mean C_t value of the housekeeping genes (HKG), followed by calculation of the $\Delta\Delta C_t$. Fold change was then calculated as $2^{(-\Delta\Delta C_t)}$. The p-value was calculated using a Student's t-test of the replicate values for each gene between the control and treatment groups. Differences were considered significant at $p<0.05$, with a fold-regulation cut-off of 2.0.

Pre-amplification using the species- and pathway-specific RT2 PreAMP Primer Mix was not performed; corrections were made during data analysis to ensure accuracy. A consistent lower limit of detection was applied across all datasets, with a C_t cut-off of 35. Quality control procedures, including a check for genomic DNA contamination, were followed. To assess PCR array reproducibility, samples and groups passed quality control if the average PPC C_t values fell within 19 ± 3, with no two arrays exhibiting average PPC C_t values differing by more than two C_t units.

Animal use: All procedures were carried out in accordance with an Animal Use Protocol approved by the University of Arkansas Institutional Animal Care and Use Committee.

RESULTS

Gene expression:

E17 LV tissue samples: Genes over-expressed in embryos from 26-wk-old breeders included fatty acid-binding protein 2 (FABP2), low-density lipoprotein receptor-related protein 2 (LRP2), apolipoprotein A-1 (APOA1) and phosphoenolpyruvate carboxykinase 1 (PCK1). The fold regulations for these genes were 7.08, 9.78, 4.25, and 2.07, respectively (Table 2). Genes under-expressed in embryos from 26-week-old breeders included pyruvate carboxylase (PC), glutamic pyruvate transaminase 2 (GPT2), lipoprotein lipase (LPL), branched-chain keto acid dehydrogenase E1 beta polypeptide (BCKDHB), fructose-1,6-bisphosphatase 1 (FBP1), phosphoglucomutase 1 (PGM1), branched-chain aminotransferase 1 (BCAT1), fatty acid elongase 6 (ELOVL6), adenylate cyclase 5 (ADCY5), carboxylesterase 1 (CES1), protein kinase AMP-activated non-catalytic subunit gamma 1 (PRKAG1), CD36 molecule (CD36) and protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1). The fold regulations for these genes were 0.01, 0.02, 0.03, 0.04, 0.13, 0.13, 0.06, 0.07, 0.08, 0.24, 0.21, 0.28, and 0.21, respectively (Table 3).

E17 YS tissue samples: Genes over-expressed in embryos from 26-wk-old breeders included PC, GPT2, LPL, peroxisome proliferator-activated receptor alpha (PPARA), carboxylesterase 1 (CES1), BCKDHB, isovaleryl-CoA dehydrogenase (IVD), FBP1, ELOVL6, PCK1 and branched-chain keto acid dehydrogenase E1 subunit alpha (BCKDHA). Their fold regulations, with $P < 0.05$, were 55.58, 27.08, 20.64, 19.29, 15.78, 10.44, 9.54, 5.94, 3.45, 3.41 and 2.65, respectively (Table 4). Genes under-expressed in embryos from 26-wk-old breeders were LRP2 and FABP2, with fold regulations of 0.01 (Table 5).

AF samples from hens: Genes over-expressed in AF from 26-week-old breeders included LPL, CD36, peroxisome proliferator-activated receptor gamma (PPARG), glucose-6-phosphatase catalytic subunit 2 (G6PC2) and BCKDHB. Their fold regulations were 4.14, 2.15, 2.11, 2.39 and 1.70, respectively (Table 6). Genes under-expressed in AF from 26-week-old hens compared with 35-week-old hens included acyl-CoA dehydrogenase family member 11 (ACAD11), FBP1, PC, GPT2, stearoyl-CoA desaturase (SCD), and phosphoenolpyruvate carboxylase (PPC). The fold regulations of these under-expressed AF genes were 0.01, 0.03, 0.07, 0.11, 0.26, and 0.56, respectively (Table 7).

Table 2: Over-expressed genes of E17 LV tissue samples in 26 versus 35-week-old hens

Gene symbol	Fold regulation	p-value
FABP2	7.08	0.013
LRP2	9.78	0.012
APOA1	4.25	0.049
PCK1	2.07	0.049

FABP2: Fatty acid-binding protein 2, LRP2: Low-density lipoprotein receptor-related protein 2, APOA1: Apolipoprotein A-1 and PCK1: Phosphoenolpyruvate carboxykinase 1

Table 3: Under-expressed genes of E17 LV tissue samples in 26 versus 35-week-old hens

Gene symbol	Fold regulation	p-value
PC	0.01	0.003
GPT2	0.02	0.002
LPL	0.03	0.005
BCKDHB	0.04	0.001
FBP1	0.13	0.001
PGM1	0.13	0.006
BCAT1	0.06	0.016
ELOVL6	0.07	0.014
ADCY5	0.08	0.023
CES1	0.24	0.028
PRKAG1	0.21	0.024
CD36	0.28	0.001
PRKAA1	0.21	0.010

PC: Pyruvate carboxylase, GPT2: Glutamic pyruvate transaminase 2, LPL: Lipoprotein lipase, BCKDHB: Branched-chain keto acid dehydrogenase E1 beta polypeptide, FBP1: Fructose-1,6-bisphosphatase 1, PGM1: Phosphoglucomutase 1, BCAT1: Branched-chain aminotransferase 1, ELOVL6: Fatty acid elongase 6, ADCY5: Adenylate cyclase 5, CES1: Carboxylesterase 1, PRKAG1: Protein kinase AMP-activated non-catalytic subunit gamma 1, CD36: CD36 molecule and PRKAA1: Protein kinase AMP-activated catalytic subunit alpha 1

Table 4: Over-expressed genes of E17 YS tissue samples in 26 versus 35-week-old hens

Gene symbol	Fold regulation	p-value
PC	55.58	0.0322
GPT2	27.08	0.0103
LPL	20.64	0.0049
PPARA	19.29	0.0261
CES1	15.78	0.0023
BCKDHB	10.44	0.0031
IVD	9.54	0.0001
FBP1	5.94	0.0019
ELOVL6	3.45	0.0132
PCK1	3.41	0.0131
BCKDHA	2.65	0.0092

PC: Pyruvate carboxylase, GPT2: Glutamic pyruvate transaminase 2, LPL: Lipoprotein lipase, PPARA: Peroxisome proliferator-activated receptor alpha, CES1: carboxylesterase 1, BCKDHB: Branched-chain keto acid dehydrogenase E1 beta polypeptide, IVD: Isovaleryl-CoA dehydrogenase, FBP1: Fructose-1,6-bisphosphatase 1, ELOVL6: Fatty acid elongase 6, PCK1: Phosphoenolpyruvate carboxykinase 1, BCKDHA: Branched-chain keto acid dehydrogenase E1 subunit alpha

LV tissue samples from hens: Genes over-expressed in LV from 26-week-old breeders included SCD, ELOVL6, acetyl-CoA carboxylase alpha (ACACA), LPL, FBP1, PPC, APOA1, G6PC2, GPT2, BCKDHA, PGM1, liver glycogen phosphorylase (PYGL),

Table 5: Under-expressed genes of E17 YS tissue samples in 26 versus 35-week-old hens

Gene symbol	Fold regulation	p-value
LRP2	0.01	0.0035
FABP2	0.01	0.0007

LRP2: low-density lipoprotein receptor-related protein 2 and FABP2: fatty acid-binding protein 2

Table 6: Over-expressed Genes of adipose tissue samples in 26 versus 35-wk-old hens

Gene symbol	Fold regulation	p-value
LPL	4.14	0.006
CD36	2.15	0.013
PPARG	2.11	0.020
G6PC2	2.39	0.034
BCKDHB	1.70	0.045

LPL: Lipoprotein lipase, CD36: CD36 molecule, PPARG: Peroxisome proliferator-activated receptor gamma, G6PC2: Glucose-6-phosphatase catalytic subunit 2, BCKDHB: Branched-chain keto acid dehydrogenase E1 beta polypeptide

Table 7: Under-expressed genes of adipose tissue samples in 26 versus 35-week-old hens

Gene Symbol	Fold regulation	p-value
ACAD11	0.01	0.003
FBP1	0.03	0.015
PC	0.07	0.031
GPT2	0.11	0.011
SCD	0.26	0.028
PPC	0.56	0.041

ACAD11: Acyl-CoA dehydrogenase family member 11, FBP1: Fructose-1,6-bisphosphatase 1, PC: Pyruvate carboxylase, GPT2: Glutamic pyruvate transaminase 2, SCD: Stearoyl-CoA desaturase and PPC: Positive PCR control

LRP2, CES1, and BCKDHB (Table 8). Genes under-expressed in LV from 26-wk-old breeders were PCK1 and hexose-6-phosphate dehydrogenase (H6PD), with fold regulations of 0.01 (Table 9).

Heat map distribution: The most abundant genes from the breeders and progeny were clustered and plotted using R (Figs. 1 and 2). Grayscale shading in the heat maps represents C_t values, with higher C_t values indicating lower gene expression. Differentially expressed genes are labeled to the right of each heat map.

Protein quantification and western blot: The YS from E17 embryos produced from eggs of 35-week-old hens had higher protein abundance of FABP2 but lower abundance of LRP2, GPT2, PC and LPL. Conversely, the YS from E17 embryos produced from eggs of 26-week-old hens showed higher protein abundance of LRP2, GPT2, PC, and LPL (Table 10 and Fig. 3). The LV of E17 embryos from eggs of 26-week-old hens had higher protein abundance of FABP2, PKM2, and PCK1 but lower abundance of LRP2 and GPT2. The LV of E17 embryos from 35-wk-old hens had higher protein abundance of LRP2 and GPT2 but lower abundance of FABP2, PKM2, and PCK1 (Table 11 and Fig 4).

Table 8: Over-expressed genes of LV tissue samples in 26 versus 35-wk-old hens

Gene symbol	Fold regulation	p-value
SCD	1.64	0.015
ELOVL6	1.07	0.028
ACACA	1.48	0.017
LPL	2.10	0.031
FBP1	1.28	0.030
PPC	5.35	0.036
APOA1	1.11	0.036
G6PC2	1.79	0.004
GPT2	1.04	0.029
BCKDHA	1.07	0.017
PGM1	1.38	0.017
PYGL	1.28	0.036
LRP2	1.25	0.019
CES1	1.02	0.004
BCKDHB	1.07	0.023

SCD: Stearoyl-CoA desaturase, ELOVL6: Fatty acid elongase 6, ACACA: Acetyl-CoA carboxylase alpha, LPL: Lipoprotein lipase, FBP1: Fructose-1,6-bisphosphatase 1, PPC: Positive PCR control, APOA1: Apolipoprotein A-1, G6PC2: Glucose-6-phosphatase catalytic subunit 2, GPT2: Glutamic pyruvate transaminase 2, BCKDHA: Branched-chain keto acid dehydrogenase E1 subunit alpha, PGM1: Phosphoglucomutase 1, PYGL: Liver glycogen phosphorylase, LRP2: Low-density lipoprotein receptor-related protein 2, CES1: Carboxylesterase 1 and BCKDHB: Branched-chain keto acid dehydrogenase E1 beta polypeptide

Table 9: Under-expressed genes of LV tissue samples in 26 versus 35-week-old hens

Gene symbol	Fold regulation	p-value
PCK1	0.01	0.003
H6PD	0.01	0.021

PCK1: Phosphoenolpyruvate carboxykinase 1, H6PD: Hexose-6-phosphate dehydrogenase

Table 10: Protein quantification of overexpressed genes in the YS of the embryo at day 17 in 26 versus 35-week-old hens[1]

	Breeder age group	
Gene symbol	26-week-old hens	35-week-old hens
FABP2	802.02	1076.85
LRP2	849.32	748.65
GPT2	742.17	130.95
PC	1264.82	191.6
LPL	407.58	269.24

[1]Stain density in individual protein bands as a % of total stain per lane was quantified, FABP2: Fatty acid-binding protein 2, LRP2: Low-density lipoprotein receptor-related protein 2, GPT2: Glutamic pyruvate transaminase 2, PC: Pyruvate carboxylase and LPL: Lipoprotein lipase

DISCUSSIONS

Numerous studies have demonstrated that breeder hen age has a measurable and economically significant impact on hatchability, chick quality, and subsequent broiler performance. Eggs produced by young breeder flocks (≤30 weeks of age) consistently result in lighter chicks at hatch (3-8 g less), lower hatchability (5-20% reduction), increased first-week mortality (2-4% greater) and poorer flock uniformity compared with eggs produced by prime-aged breeders[14,28,40-44]. From an economic standpoint, increased

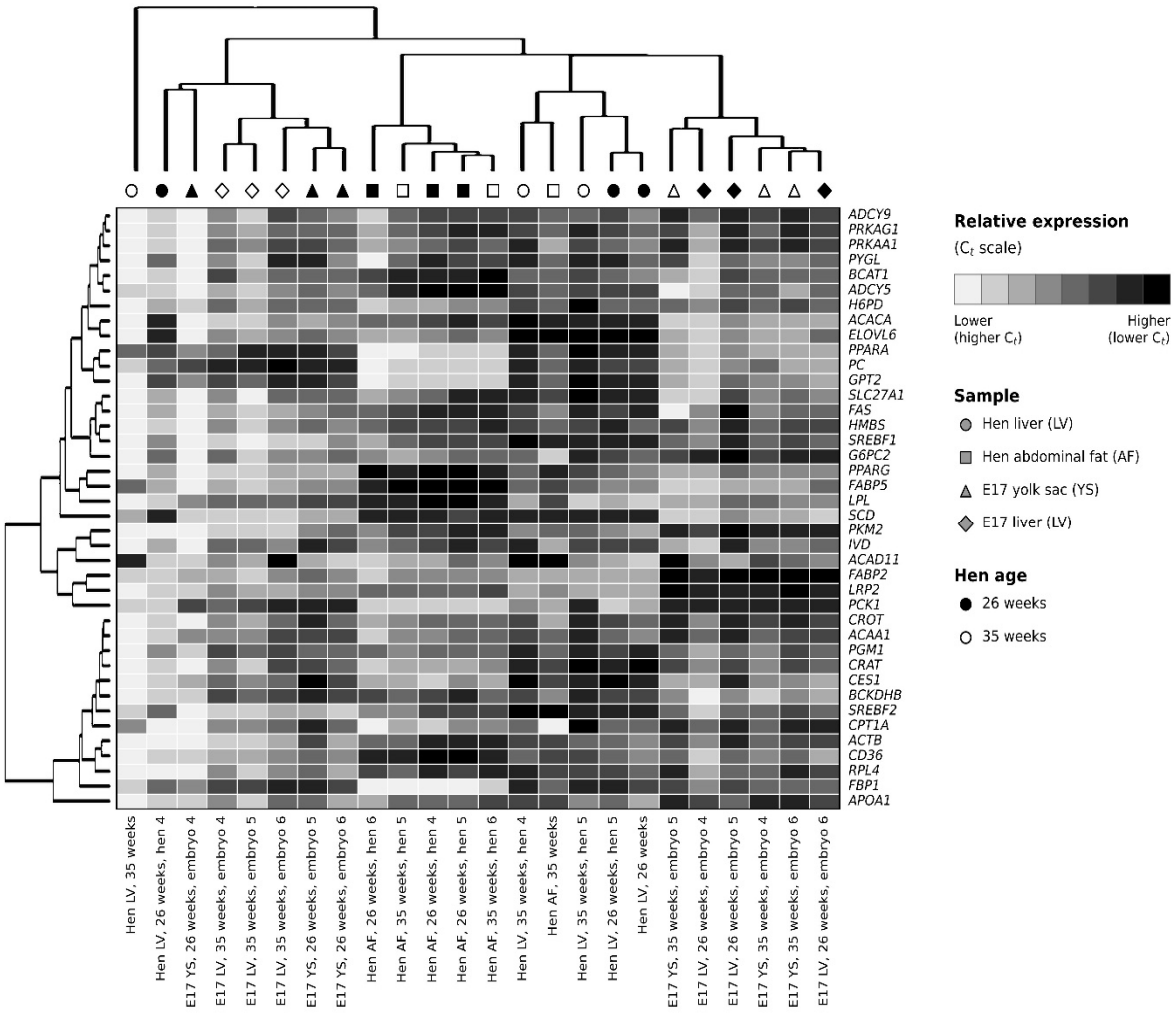

Fig. 1: Heat map of mean-normalized cycle threshold (C_t) values in the liver (LV) and yolk sac (YS) of embryonic day 17 (E17) embryos and in the LV and abdominal fat (AF) of breeder hens at 26 and 35 weeks of age. Columns are samples (3 embryos or 3 hens per tissue and age) and rows are genes. Darker cells indicate lower C_t (higher expression) and lighter cells higher C_t (lower expression). Symbols identify tissue and hen age (filled, 26 weeks; open, 35 weeks). Dendrograms show hierarchical clustering of genes (left) and samples (top)

early mortality, lighter placement weights, delayed growth, and reduced uniformity directly increase production costs through poorer feed conversion, longer grow-out periods, and increased processing downgrades[45].

Older breeder hens produce larger eggs that yield heavier and more uniform chicks at hatch, which contributes to improved early growth and livability[45,46]. Chicks from 49-week-old breeders have been reported to be approximately 4-6 g heavier at hatch than chicks from 32-week-old breeders[47]. However, breeder age effects are not strictly linear with chick quality. Very old breeder flocks (>45 weeks) produced a greater proportion of low-quality chicks compared with 35-week-old breeders[8] and progeny from 49-week-old breeders showed an increased incidence of red hocks[47]. These findings indicate that although early-lay eggs are disadvantaged, optimal chick quality may be achieved within a defined window of breeder age, typically between 32 and 40 weeks.

Hepatic gene expression and protein abundance in E17 embryos: The present study demonstrates that breeder age significantly alters the expression of genes related to lipid and carbohydrate metabolism in the E17 embryo, but gene expression differences do not always correspond directly to protein abundance. In the embryonic LV at E17, FABP2 mRNA

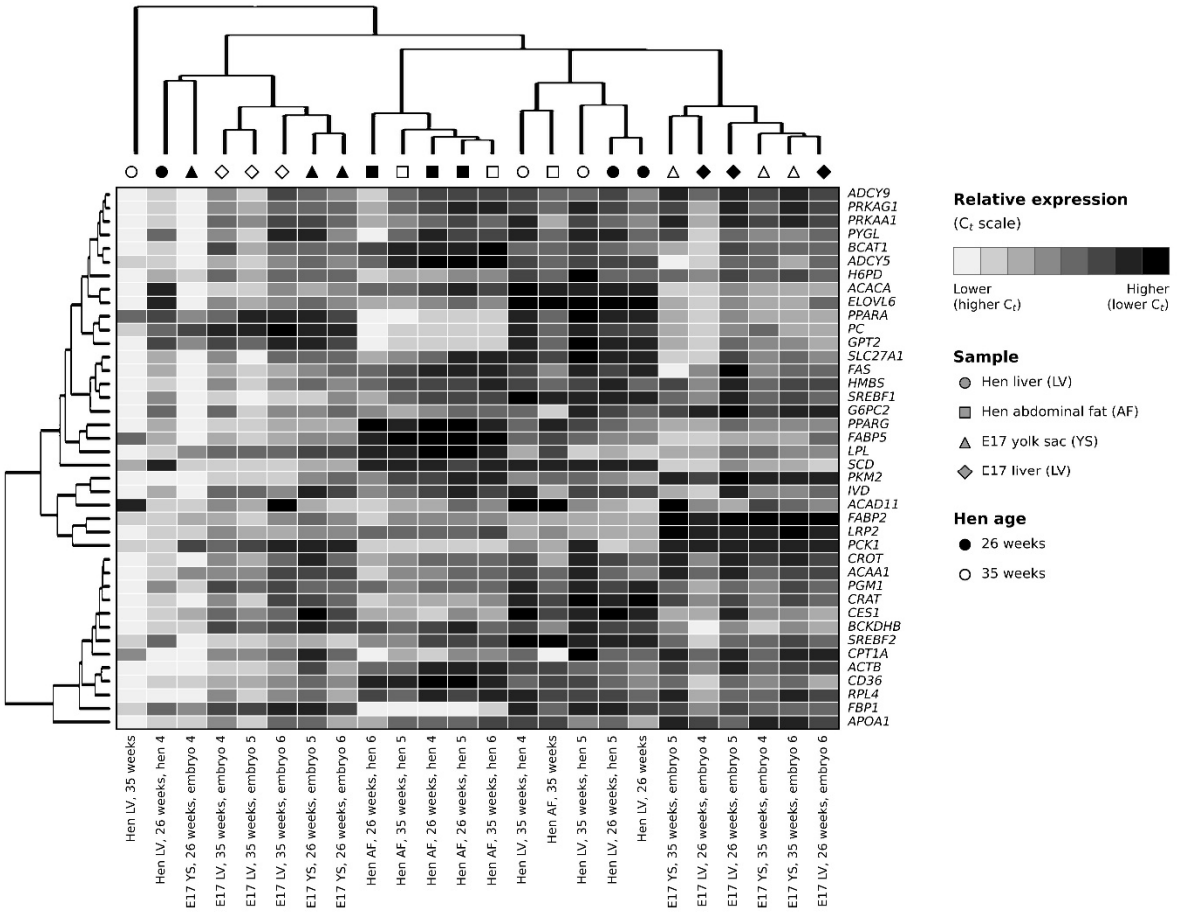

Fig. 2: Heat map of raw cycle threshold (C_t) values in the liver (LV) and yolk sac (YS) of embryonic day 17 (E17) embryos and in the LV and abdominal fat (AF) of breeder hens at 26 and 35 weeks of age. Columns are samples (3 embryos or 3 hens per tissue and age) and rows are genes. Darker cells indicate lower C_t (higher expression) and lighter cells higher C_t (lower expression). Symbols identify tissue and hen age (filled, 26 weeks; open, 35 weeks). Dendrograms show hierarchical clustering of genes (left) and samples (top)

Table 11: Western blot films of over expressed genes in the LV of the embryo at day 17 in 26 versus 35-week-old hens[1]

	Breeder age group	
Gene symbol	26-week-old hens	35-week-old hens
FABP2	1099.21	1049.28
LRP2	175.54	206.86
GPT2	409.84	576.19
PKM2	377.06	71.50
PCK1	519.08	452.62

[1]Stain density in individual protein bands as a % of total stain per lane was quantified, FABP2: Fatty acid-binding protein 2, LRP2: Low-density lipoprotein receptor-related protein 2, GPT2: glutamic pyruvate transaminase 2, PKM2: Pyruvate kinase (muscle), PCK1: Phosphoenolpyruvate carboxykinase 1

expression was significantly higher ($p<0.05$) in embryos from 26-week-old hens than in embryos from 35-week-old hens. However, FABP2 protein abundance did not differ significantly between ages, indicating that transcriptional upregulation in embryos from young breeders does not necessarily result in increased protein abundance.

The gene FABP2 encodes intestinal-type fatty acid-binding protein, which facilitates the intracellular transport of long-chain fatty acids[48]. The lack of a corresponding increase in protein abundance suggests that the elevated FABP2 transcript levels in embryos from young hens are not accompanied by an increase in FABP2 protein abundance. These findings emphasize the importance of evaluating both gene expression and protein abundance when interpreting potential differences in metabolic capacity.

Other lipid-related genes were also differentially expressed between age groups. LRP2 mRNA was upregulated in E17 embryos from 26-week-old hens, whereas LRP2 protein

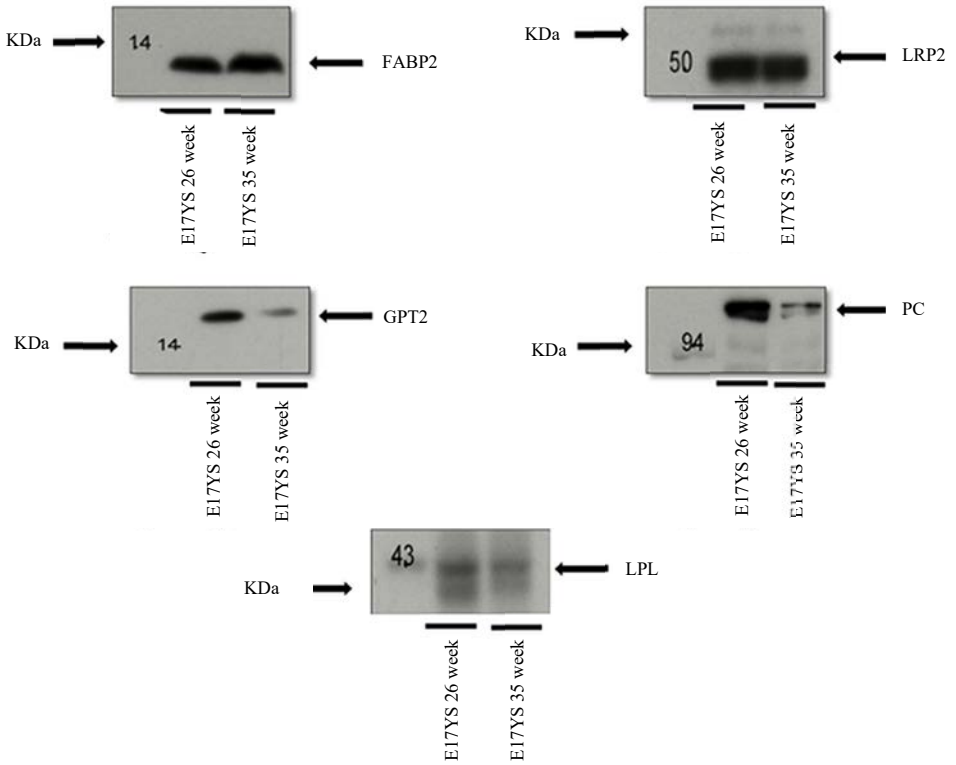

Fig. 3: Western blot films of overexpressed genes in the YS of the embryo at day 17 in 26 versus 35-wk-old hens

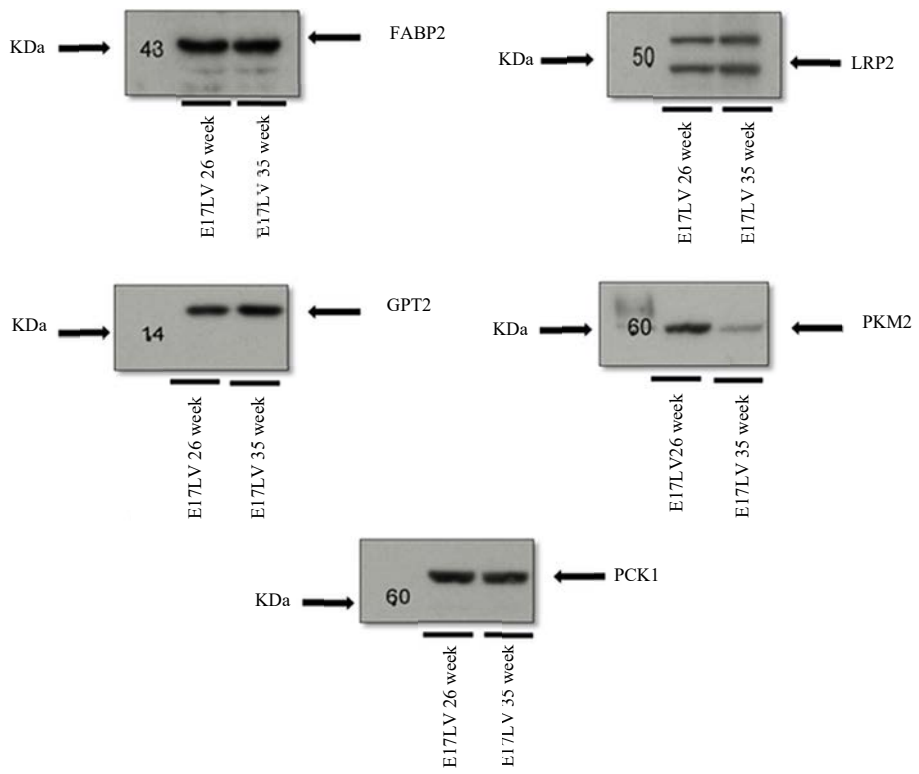

Fig. 4: Western blot films of overexpressed genes in the LV of the embryo at day 17 in 26 versus 35-week-old hens

abundance was significantly greater ($p<0.05$) in embryos from 35-week-old hens. LRP2 encodes megalin, a multiligand endocytic receptor involved in the uptake of lipoprotein-associated ligands. The greater protein abundance of LRP2 in embryos from 35-week-old breeders suggests a potentially greater capacity for receptor-mediated nutrient uptake at the cellular level, which may be consistent with differences in embryonic development and chick quality associated with breeder age[49].

Importantly, these findings do not demonstrate inefficient lipid utilization in embryos from young hens but rather indicate age-related differences in the expression and abundance of proteins involved in nutrient transport. Such differences may reflect developmental or metabolic adaptations associated with early-lay eggs.

Yolk sac metabolism and carbohydrate utilization: The YS represents the primary interface for nutrient transfer from the yolk to the developing embryo[27]. In the present study, gene expression analysis of the YS revealed that PC was the most highly upregulated gene ($p<0.05$) in E17 embryos from 26-wk-old hens. The gene PC encodes pyruvate carboxylase, an enzyme that converts pyruvate to oxaloacetate, a critical anaplerotic reaction supporting gluconeogenesis and the tricarboxylic acid cycle[50].

Similarly, GPT2 expression and protein abundance were significantly greater ($p<0.05$) in the YS of embryos from 26-week-old hens. GPT2 encodes mitochondrial alanine aminotransferase, which facilitates the conversion of alanine to pyruvate, thereby supplying substrate for gluconeogenesis. Together, the coordinated upregulation of PC and GPT2 suggests that embryos from 26-week-old hens may have greater reliance on pathways utilizing carbohydrate- and amino acid-derived substrates to support glucose production during late embryogenesis.

Maintaining glucose availability is critical during the perihatch period, as emerging embryos require glucose to sustain muscular activity for pipping and shell breakthrough and to stabilize circulating glucose concentrations prior to exogenous feed intake[51]. Previous work has shown that embryos can experience glucose depletion during late incubation, which negatively affects hatch success and chick vigor[11,52]. Thus, the present findings are consistent with a model in which embryos from young breeders exhibit increased expression of genes involved in gluconeogenic pathways, potentially reflecting an adaptive response to developmental or substrate limitations.

Previous studies have demonstrated that lipid transfer from the YS to the embryo during late incubation is less efficient in eggs from young breeders than in eggs from older breeders, largely because of reduced yolk size and altered yolk lipid composition[18,19]. Those studies quantified lipid transfer dynamics but did not evaluate gene expression or protein abundance. The present study builds on that foundational work by showing that embryos from young breeders exhibit distinct patterns of metabolic gene expression that differ from those of embryos produced by prime-aged hens.

Unified interpretation and application: Taken together, the results of this study do not demonstrate impaired fatty acid oxidation but rather reveal age-specific differences in metabolic pathways at the molecular level during late embryogenesis. Embryos from 26-week-old breeders exhibit increased expression and abundance of proteins associated with carbohydrate metabolism and gluconeogenesis, whereas embryos from 35-wk-old breeders exhibit greater abundance of proteins associated with lipid transport[8].

These findings suggest differences in relative metabolic pathway utilization, potentially reflecting developmental or substrate-related constraints associated with early-lay eggs. From an applied perspective, these results support future investigations into *in ovo* nutritional interventions, such as targeted carbohydrate supplementation or optimized fatty acid profiles, aimed at improving embryonic energy availability and chick quality from young breeder flocks[53].

CONCLUSION

In conclusion, breeder hen age was associated with distinct differences in the expression of genes and abundance of proteins involved in lipid, carbohydrate, and amino acid metabolism in E17 embryos and maternal tissues. Embryos from 26-week-old breeders exhibited marked changes in yolk sac expression of genes involved in gluconeogenesis, carbohydrate metabolism, lipid transport, and branched-chain amino acid metabolism, particularly PC, GPT2, LPL, and PPARA. In contrast, embryonic liver showed a different expression pattern, including greater expression of FABP2, LRP2, APOA1, and PCK1 in embryos from 26-wk-old breeders. Western blot analysis further demonstrated age-dependent differences in FABP2, LRP2, GPT2, PC, LPL, PKM2, and PCK1 protein abundance. Collectively, these findings indicate that breeder age is associated with tissue-specific differences in metabolic gene expression and protein abundance during late embryonic development. These molecular differences may contribute to age-related variation in embryonic nutrient utilization and developmental outcomes, although further functional studies are required to establish their physiological significance.

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