Post-photostimulation energy intake accelerated pubertal development in broiler breeder pullets

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ABSTRACT The effect of ME intake (**MEI**) on the reproductive system was evaluated. Ross 308 broiler breeder pullets (n = 140) were assigned to 2 treatments from 22 to 26 wk of age: (1) Low-energy diet fed restricted (2,807 kcal/kg, low MEI) and (2) high-energy diet fed unrestricted (3,109 kcal/kg, high MEI). Daylength was increased from 8 to 14 h at 22 wk of age with a light intensity of 30 lux. Daily palpation was used to detect sexual maturity via the presence of a hard-shelled egg in the shell gland. Expression of gonadotropin releasing hormone-I (***GnRH***) and gonadotropin inhibitory hormone (***GnIH***) genes in the hypothalamus and GnRH receptor (***GnRH-RI***) and GnIH receptor (***GnIH-R***) genes in the anterior pituitary gland of each pullet was evaluated from 22 to 26 wk of age using quantitative real time-PCR. Blood samples were taken weekly and luteinizing hormone (**LH**), follicle stimulating-hormone (**FSH**), and 17-beta-estradiol (**E2**) determined using commercial ELISA kits. Carcass samples were used for determination of CP and fat content. Data were analyzed using the MIXED procedure in SAS, and differences were reported where $P \leq 0.05$. High MEI treatment pullets had 2.3-fold higher *GnRH* and 1.8-fold higher *GnRH-RI* mRNA levels than low MEI pullets. MEI affected neither expression of *GnIH* and *GnIH-R* nor carcass protein content. For high MEI (489 kcal/D) and low MEI treatments (258 kcal/D), respectively, from 22 to 26 wk of age ($P \leq 0.05$), LH concentration was 3.05 and 1.60 ng/mL; FSH concentration was 145 and 89.3 pg/mL; E2 concentration was 429 and 266 pg/mL, and carcass lipid was 13.9 and 10.3%. The onset of lay for pullets in the high MEI treatment advanced such that 100% had laid by 26 wk of age compared with 30% in the low MEI treatment. We concluded that higher MEI advanced the activation of the hypothalamic–pituitary–gonadal axis and also increased body lipid deposition, and moreover, stimulated reproductive hormone levels which overall accelerated puberty in broiler breeder pullets.

Key words: caloric restriction, metabolism, gene expression, reproductive hormone, carcass composition

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INTRODUCTION

The central nervous system (**CNS**) regulates energy utilization (Richards et al., 2010) and reproduction in avian species (Tsutsui, 2009; Tsutsui et al., 2010b). The hypothalamus is part of the CNS and plays an important regulatory role for feed intake and energy expenditure by interpreting information from internal physiological signals (hormones and nutrients) and external environmental cues (photoperiod, temperature, and stressors; Richards and Proszkowiec-Weglarz, 2007). Some aspects of the energy or feed intake regulated by the CNS and knowledge of the cellular and molecular mechanisms that can be affected by ME intake (**MEI**) in poultry is quite limited compared with mammals. The hypothalamic melanocortin system, which controls energy homeostasis, is composed of 2 populations of neurons. One set population expresses neuropeptide Y (***NPY***), whereas the second set that expresses proopiomelanocortin (***POMC***), with both having important roles in feed intake regulation (Richards et al., 2010). Increased expression of *NPY* stimulates feed intake and energy storage (anabolic; Kuenzel et al., 1987; Chen et al., 2016), whereas increased expression of *POMC* reduces energy intake (catabolic; Richards et al., 2010).

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Moreover, there are other genes that might have an important role in feed regulation. For example, the leptin receptor (***LEPR***) was shown to be expressed within *NPY* and *POMC* neurons in the arcuate nucleus of the hypothalamus (Elias et al., 1999).

The hypothalamus also plays a vital role in the reproductive system of birds. The hypothalamus controls the female reproductive life cycle by activating and inhibiting the hypothalamic–pituitary–gonadal (**HPG**) axis (Bédécarrats et al., 2009; Tsutsui et al., 2010a). In the hypothalamus, stimulatory and inhibitory neuropeptides are known as gonadotropin releasing hormone (**GnRH**) and gonadotropin inhibitory hormone (**GnIH**), respectively (Bédécarrats et al., 2009). In chickens, 2 GnRH have been characterized, GnRH-I and GnRH-II (Miyamoto et al., 1982, 1983). Radioimmunoassay measurement of GnRH in the brain of chickens confirmed that GnRH-I is significantly more abundant than GnRH-II in the hypothalamus (Katz et al., 1990). Two specific G-coupled protein receptors have been identified for GnRH-I in anterior pituitary gland (**GnRHR-I** and **GnRHR-II**, Sun et al., 2001; Shimizu and Bédécarrats, 2006). The GnRHR-II is a type III receptor; thus, GnRHR-II was renamed to **GnRHR-III** (Joseph et al., 2009). Upon photostimulation, increased release of GnRH binds to its receptors in the anterior pituitary to stimulate the synthesis and release of luteinizing hormone (**LH**) and follicle stimulating hormone (**FSH**) into the systemic circulation (Robinson and Etches, 1986; Bédécarrats et al., 2009). Both LH and FSH stimulate gametogenesis and the synthesis of sex steroid hormones such as estradiol (**E2**), thus initiating sexual maturation (Robinson and Etches, 1986; Bédécarrats, 2015, 2016). On the other hand, when GnIH is released into the hypothalamo–pituitary vascular portal system, it binds to its specific G-protein receptor (**GnIH-R**) in the anterior pituitary, where it inhibits the production of LH and FSH (Tsutsui et al., 2000; Maddineni et al., 2008; Shimizu and Bédécarrats, 2010).

Hypothalamic maturation (age) and achieving BW and carcass compositions thresholds are required for onset of lay in broiler breeders (Renema et al., 1999a). Similarly, laying hens with greater BW and greater lipid content entered into lay earlier than birds with lower BW and lower lipid content (Summers and Leeson, 1983). Thus, it seems that energy balance during sexual maturity can affect the rate of maturation of the neuroendocrine system which eventually influences the timing of puberty (Renema et al., 1999a).

The first objective of the current study aimed at assessing the effect of MEI on sexual maturity, specifically on (1) mRNA levels of *GnRH-I* and *GnIH* in the hypothalamus and their receptors in the anterior pituitary gland and (2) the plasma concentration of reproductive hormones (LH, FSH, and E2) in broiler breeders. The second objective was to evaluate the effect of MEI on the expression of *POMC*, *NPY,* and *LEPR* to understand how energy balance is regulated by the hypothalamus in broiler breeder pullets postphotostimulation and during sexual maturity. Finally, the third objective was to investigate the effect of MEI on carcass composition in broiler breeder pullets. It was hypothesized that higher MEI of broiler breeder pullets would increase *GnRH-I* mRNA levels in the hypothalamus and also increase the mRNA levels of *GnRH* receptors in the pituitary to advance the onset of lay. Alternatively, it was hypothesized that higher MEI of broiler breeder pullets would reduce *GnIH* mRNA levels in the hypothalamus and suppress the mRNA levels of *GnIH-R* in the pituitary at the onset of lay. Moreover, it was hypothesized that the advance in sexual maturation driven by higher mRNA levels of *GnRH-I* and lower expression of *GnIH* would translate into an increase in the concentrations of reproductive hormones after photostimulation. Furthermore, it was hypothesized that higher MEI in broiler breeder pullets would decrease the expression of *NPY* and increase the expression of *POMC* and *LEPR* to maintain energy homeostasis. Finally, it was hypothesized that higher MEI would increase carcass lipid and decrease carcass protein in broiler breeder pullets at the onset of lay.

MATERIALS AND METHODS

Experimental Design

All procedures in the present study were approved by the Animal Care and Use Committee for Livestock at the University of Alberta. The focus of the current experiment was sexual maturity, and all pullets were fed using a precision feeding (**PF**) system (Zuidhof et al., 2016, 2017). At 21 wk of age, 140 pullets were randomly and equally selected and assigned to 2 treatments (2 replicate pens with 35 birds each per treatment): (1) Low-energy diet fed restricted according to the breeder-recommended Ross 308 BW target (Aviagen, 2011) using a typical commercial diet (2,807 kcal/kg, low MEI, Table 1) and (2) high-energy diet fed unrestricted (3,109 kcal/kg, high MEI, Table 1). The current experiment was carried out from 22 to 26 wk of age. The target BW for the pullets was interpolated hourly, and the high MEI birds were provided access to feed on every visit to the feeding station. At 21 wk, before starting the current experiment, the BW of pullets in the high MEI and in the low MEI treatments were 2,489 ± 7 and 2,484 ± 7 g, respectively ($P > 0.05$). Individual birds were considered as the experimental unit because treatments were independently applied to them. The experiment was a completely randomized design, and pen was considered as a random effect. Diets were provided in pellet form; the grower diet for the low MEI treatment was formulated according to breeder recommendations (Table 1, Aviagen, 2013), and the grower diet for the high MEI treatment was the same as the low MEI diet and contained the same ingredients as the low MEI treatment except that the amount of canola oil was higher to increase the energy level.

Table1. Composition of broiler breeder pullet grower diets for low ME intake (low MEI) and high ME intake (high MEI) treatments.

Ingredient	Low MEI	High MEI
	g/kg	
Corn	327.6	312.0
Wheat	343.0	326.4
Soybean meal	65.3	62.1
Oats	60.7	57.1
Canola meal	78.4	74.6
Wheat Bran	49.0	46.6
Canola oil	9.8	57.0
Ground limestone	14.9	14.1
Dicalcium phosphate	14.7	14.0
Choline chloride premix	4.9	4.7
Vitamin premix[1]	2.5	2.5
Mineral premix[2]	2.5	2.5
NaCl	3.8	3.7
D, L-methionine	0.9	0.9
L-lysine	1.2	1.1
Threonine	0.3	0.2
Enzyme[3]	0.5	0.5
Celite[4]	20	20
Total:	1,000	1,000
Analyzed composition, as fed basis		
AME (kcal/kg)	2,807	3,109
CP (g/kg)[5]	147	143
Calculated composition, as fed basis		
AME (kcal/kg)	2,696	2,931
CP (g/kg)	154	152
Calcium (g/kg)	10	10
Nonphytate phosphorous (g/kg)	4.5	4.3
Available lysine (g/kg)	7.4	7.1
Available methionine (g/kg)	3.4	3.2
Available methionine + cysteine (g/kg)	6.3	6.3

[1]Premix provided per kilogram of diet: vitamin A (retinyl/acetate), 10,000 IU; cholecalciferol, 4,000 IU; Vitamin E (DL- α-tocopheryl acetate), 50.0 IU; vitamin K, 4.00 mg; pantothenic acid, 15.0 mg; riboflavin, 10.0 mg; folacin, 2.00 mg; niacin, 65 mg; thiamine, 4.00 mg; pyridoxine, 5.00 mg; vitamin B12, 0.02 mg; biotin, 0.20 mg.

[2]Premix provided per kilogram of diet: iodine, 1.65 mg; Mn, 120 mg; Cu, 20.0 mg; Zn, 100 mg; Se, 0.30 mg; Fe, 80.0 mg.

[3]Avizyme 1,302 feed enzyme for use in poultry diets containing at least 20% wheat (Danisco Animal Nutrition, Marlborough, Wiltshire, UK).

[4]Acid-insoluble ash marker (Celite 281, Lompoc, CA) was added to determine the AME content of the diets.

[5]Analyzed N using Leco TruMac (Leco Corporation, St. Joseph, MI).

Diet Analyses

The AME content of the diets was determined by adding 2% acid-insoluble ash marker (Celite, Celite 281, Lompoc, CA). Four birds in each pen (8 per treatment) were randomly selected at 22, 23, 24, 25, and 26 wk of age and euthanized by cervical dislocation. Ileal digesta samples were collected by gently squeezing the intestinal tract from Meckel's diverticulum to the ileal–cecal–colon junction. Digesta samples were pooled for each experimental pen and stored at −20°C until analysis. Diet and ileal digesta samples were oven dried at 60°C for 48 h and then ground. For acid insoluble ash, samples were digested with 4N HCL, and then the residues were ashed at 500°C (Vogtmann et al., 1975). Using bomb calorimetry, gross energy (**GE**) of feed and digesta samples were measured. The AME values were calculated as previously described by Scott and Boldaji (1997):

$$AME = GE_{diet} - GE_{digesta} \times \frac{Marker_{diet}}{Marker_{digesta}}$$

where GE = gross energy (kcal/kg of sample) and Marker = concentration of acid insoluble ash in sample. Apparent ME values were expressed on an as-fed basis. Nitrogen content of feed was determined by the combustion method using a Leco TruMac N machine (Leco Corporation, St. Joseph, MI) and dietary CP was estimated using a factor of 6.25 (Hossain et al., 2012; Mutucumarana et al., 2015).

Management

Each pen contained pine shavings as litter at a depth of approximately 5 cm. The stocking density was 2.1 birds/m^2. Two suspended nipple drinkers (2.5 pullets per nipple) provided water *ad libitum* throughout the experiment. The feeder in each PF station was 4.8 cm wide, and only one bird was provided access at a time. Temperature, measured hourly, was 21 ± 0.40°C during the entire experimental period. Daylength was increased from 8 to 14 h at 22 wk of age with a light intensity of 30 lux. The design and function of the PF stations are fully disclosed elsewhere (Zuidhof et al., 2016, 2017). Prior to start the current experiment birds were trained to become familiarized with the PF stations as has been explained by Zuidhof (2018) and all birds were identified with a unique radio frequency identification wing tag to be tracked by the PF system and were fed individually based on their BW. Briefly, each pullet was weighed by a built-in platform scale when it entered the PF station. If its BW was equal to or greater than the target BW, the pullet was gently ejected by the station. However, if its BW was lower than the target BW, the PF station provided access to approximately 25 g of feed for 1 min, after which the pullet was ejected from the station. Feed intake was calculated as the initial minus the final feed weight. For each visit, radio frequency identification, BW, and initial and final feed weight data were written to a database with a date and time stamp. Target BW was interpolated hourly. Thus pullets could access feed 24 h per day. Number of meals per day for the High MEI pullets and the Low MEI pullets were 24.81 ± 0.77 and 9.89 ± 0.53 respectively ($P < 0.001$). The meal size for the High MEI pullets and the Low MEI pullets were 6.97 ± 0.18 and 11.76 ± 0.16 g/meal respectively ($P < 0.001$).

Sample Collection

Dissection From 22 to 26 wk of age, 8 birds per treatment were dissected. In addition to the 8 birds per treatment, any pullet that entered into lay (had an egg in the shell gland) were also dissected for sample collection. Palpation was performed every morning to detect sexual maturity via the presence of a hard-shelled egg in the shell gland.

Hypothalamus and Pituitary Tissue Collection At 22 and 23 wk of age, 4 birds per treatment were randomly selected to collect hypothalamus and pituitary tissue samples. At 24, 25, and 26 wk of age, 4 birds per treatment that had and 4 birds per treatment that had

not laid an egg were randomly selected for tissue collection. The hypothalamus and the pituitary samples were immediately collected after cervical dislocation and dissection and snap frozen in liquid nitrogen then stored at −80°C until RNA extraction. The samples were used for gene expression analyses.

Blood Collection Blood samples were collected weekly from all birds, and approximately 2 mL of blood was taken by venipuncture from the brachial vein and collected in a sodium heparin blood vacutainer tubes (Evacuated glass tubes, Fisher Scientific, Hampton, NH). Blood plasma was recovered by centrifugation at 1,244 × g for 15 min at 4°C and stored at −20°C until hormone assay. The same subset of birds used for gene expression analyses was chosen for hormone assay.

Carcass Collection Each individual bird carcass was pressure-cooked for 2 h and homogenized using an industrial blender. The same subsample of birds used for gene expression analyses and hormone assays was chosen for carcass composition analyses.

Lab Analyses

RNA Isolation, cDNA Synthesis, and Real-time Quantitative PCR Two hundred fifty to 350 mg of hypothalamus sample was homogenized using trizol, and total RNA was extracted using Direct-zol RNA miniPrep plus kit (Zymo Research, Inc., Irvine, CA). Total RNA of hypothalamus sample was eluted in 40 μL nuclease-free water (Ambion, Austin, TX). Total RNA was extracted from 10 to 15 mg of pituitary sample, using Absolutely RNA miniprep kit (Agilent Technologies, Inc., Palo Alto, CA). Total RNA of pituitary sample was eluted in 24 μL elution buffer provided by the kit. The RNA extraction procedure for both kits was performed according to the manufacturer's instructions and included a DNase treatment on the purification column. Quantity and purity of total RNA isolated from all samples were determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Ratio of absorbance at 260 nm and 280 nm of RNA for all samples were between 1.8 and 2.0. Total RNA integrity (**RIN**) was evaluated using an Agilent 2,200 TapeStation (Agilent, Santa Carla, CA). As a subject to evaluate the RIN values for hypothalamus and pituitary samples, RNA of pullets within each treatment was selected randomly from 22 to 26 wk of age. The RIN value of RNA was above 8.5 and 6.5 for hypothalamus and pituitary samples, respectively. Total RNA (100 ng) from each individual hypothalamus and pituitary sample was reverse transcribed to cDNA with SuperScript VILO Master Mix (Invitrogen, Carlsbad, CA) in a final volume of 20 μL. Mixture was incubated for 10 min at 25°C, 60 min at 42°C, 5 min at 85°C, and cooled at 4°C. After reverse transcription, the cDNA was diluted 3 times with nuclease-free-water (1:3, Ambion). Real-Time PCR analysis was performed in duplicate. Two μL of the cDNA in 20 μL of PCR reaction was used as the template with the StepOnePlus Real-Time PCR System (Applied Biosystems StepOnePlus Real-Time PCR system), and Power SYBR Green Master Mix (Applied Biosystems., Inc., Foster City, CA). Thermal PCR cycler program was ran at 95°C for 20 s, 95°C for 3 s, and 60°C for 30 s then repeated 40 cycles. The PCR primers were designed for each gene transcript investigated using Integrated DNA Technologies PrimerQuest tool (http://www.idtdna.com/Primerquest/Home/Index). Specificity of the primer sequences (Gallus gallus) was then confirmed using Basic Local Alignment Search Tool (BLAST) at National Centre for Biotechnology Information (NCBI; Table 2). The amplification efficiencies of all genes were determined using serial dilution of hypothalamus and pituitary samples cDNA (1, 1:5, 1:10, 1:50, 1:100, 1:500, and 1:1000). The amplification efficiencies for all genes in both samples were above 95%, and slopes of the plots from the serial dilutions were close to -3.32.

Table 2. Sequences of the oligonucleotide primers used in quantitative real-time PCR and the publicly available sequences they were designed from.

Gene[1]	Primer sequence (5′– 3′)		Size for PCR product (bp)	GenBank accession no.
	Forward	Reverse		
ACTB	TGTTACCAACACCCACACCC	TCCTGAGTCAAGCGCCAAAA	110	NM_205518
EEF1A1	CTCTCACCTGGAACCAACTATTC	CCACTGTTTGGCATTGGTATTG	100	NM_204157.2
SDHA	GACAGAGGCATTGTGTGGAA	CGAGCCTCAGCACCATAAAT	98	NM_001277398.1
GnRH-I	CACCCAGCTGCTCCAATTA	CAGGTAATGCCACCTCATTCT	100	X69491.1
GnIH	GGAAGTCAGTGCCCATCAATC	ACGCTGCATCTTTTCCGAGT	130	NM_204363.1
GnRH-RI	GGGAGATCAGTAAGCAGCTAAAG	GCTGGCAACAATCACAATGG	113	NM_204653.1, XM_015292070
GnRH-RIII	ATGTACGCCTCCGCCTTCGT	GCAGGGTGACGGTGTGGAAG	178	AY895154
GnIH-R	GCATGTCTGTCTCCGCCTCT	GTGGACGATGCAGCGAAACC	71	AB120326
LH	TCGCCCCATAAACGTAACGG	CGTGGTGGTCACAGCCATAC	70	HQ872606.1
FSH	CCACGTGGTGCTCAGGATACT	AGGTACATATTTGCTGAACAGATGAGA	84	NM_204257.1
NPY	GAGGCACTACATCAACCTCATC	TCTGTGCTTTCCCTCAACAA	96	M87294.1
POMC	AGGAGACCCATCAAGGTGTA	TTCCTCCTCTTCTTCCTCCTC	135	NM_001031098.1
LEPR	ACCGAAGAATGAAGAAACTGCT	TGACAAAAAGGTGCTCAAAAGT	111	NM_204323.1

[1]Actin beta (*ACTB*), Eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*), Succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), Gonadotropin releasing hormone-I (*GnRH-I*), Gonadotropin inhibitory hormone (*GnIH*), Gonadotropin releasing hormone receptor-I (*GnRH-RI*), Gonadotropin releasing hormone receptor- III (*GnRH-RIII*), Gonadotropin inhibitory hormone receptor (*GnIH-R*), Luteinizing hormone beta subunit (*LH*), Follicle stimulating hormone beta subunit (*FSH*), Neuropeptide Y (*NPY*), Proopiomelanocortin (*POMC*), Leptin receptor (*LEPR*).

Real-time PCR efficiencies were calculated from the given slopes in step-one plus software. The corresponding real-time PCR efficiency (**E**) of 1 cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Pfaffl, 2001). Relative expression ratio of a target gene was calculated based on the E and threshold cycle deviation of control sample and expressed relative to the geometric mean of 2 stable housekeeping genes (Pfaffl, 2001).The minimum threshold cycle value for each gene was used as the control sample in the current study. Five housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase; actin beta (***ACTB***); succinate dehydrogenase complex flavoprotein subunit A (***SDHA***); ribosomal protein L19; and eukaryotic translation elongation factor 1 alpha 1 (***EEF1A1***), were tested for the hypothalamus and pituitary samples to select the most stable housekeeping genes in each tissue, and normalize target gene expression from hypothalamus and pituitary samples respectively. Two stable housekeeping genes, *ACTB* and *SDHA,* were selected to normalize expression measured in hypothalamus samples, and 2 stable housekeeping genes *ACTB* and *EEF1A1* were selected to normalize expression measured in pituitary samples, using the NormFinder Excel Add-In (Andersen et al., 2004).

Hormone Assay Plasma LH concentration was determined using a commercially available ELISA kit (E-EL-Ch1569, chicken LH; Elabscience Biotechnology, Hubei, China) according to the manufacturer's instructions. Assay was performed in duplicates for every individual sample. The optical density was measured with a microplate spectrophotometer at 450 nm (Molecular Devices, San Jose, CA). The standard curve and samples were plotted and analyzed using SoftMax Pro software. The intra-assay and interassay CV for LH were respectively 8.15 and 6.96%. Plasma FSH concentration was quantified using FSH ELISA kit (E-EL-Ch1365, chicken FSH; Elabscience Biotechnology) according to the manufacturer's instructions. Assay was performed in duplicates for every individual sample. The intra and inter-assay coefficients of variation for FSH were respectively 8.66 and 10.42%.

To quantify E2 plasma concentration, before ELISA assay, E2 was extracted from plasma using ethanol and according to the method suggested by Baxter et al. (2014). Briefly, thawed samples were diluted with ethanol at 5:1 (ethanol: plasma) ratio. Samples were then vortexed, centrifuged for 5 min at 20°C at 1,800 × *g*, and frozen at −80°C. The organic (ethanol) phase was recovered and transferred into new tubes and dried using a SpeedVac (Thermo Savant SpeedVac SC210 A Centrifugal Evaporator; Thermo Scientific, Waltham, MA). Samples were reconstituted in half the original volume with assay buffer and stored at −20°C until assay. Plasma E2 was quantified using E_2 ELISA kit (E-EL-0065, Elabscience Biotechnology) according to the manufacturer's instructions. Assay was performed in duplicates for every individual sample. The optical density was measured with a microplate spectrophotometer at 450 nm (Molecular Devices). The standard curve and samples were plotted and analyzed using SoftMax Pro software. The intra-assay and interassay coefficients of variation for E_2 were respectively 8.51 and 12.79%.

Carcass Composition 200-mL samples of the homogenate were collected and oven-dried at 55°C for 96 h and reground for homogeneity. A digital scale was used to calculate the difference (in milligram (0.001 g)) between the weight of the sample after oven drying and the weight of the sample before oven drying and was multiplied to 100 to calculate the dry matter percentage of the sample. Carcass fat was extracted using AOAC (2007), method number 906.39, and determined by Goldfisch method; diethyl ether ethyl was used as a solvent. Approximately 4 g samples were added to preweighed extraction thimbles. Samples were dried in a 100°C drying oven for 24 h, and after they cooled, dry weight was recorded. Samples were then placed into a Labconco goldfish ether extraction apparatus (Serial # 3500100, Kansas City, MO). Approximately 50 mL of diethyl ether per sample was used for fat extraction. Ether was allowed to drip through samples for 6 h. Samples were dried at 100°C in drying oven for 90 min and cooled to room temperature at which point weights were recorded. Fat percentage was determined indirectly by weight loss. Carcass nitrogen was determined by the combustion method using a Leco TruMac N determinator (Leco Corporation), and carcass CP was estimated using a factor of 6.25 (Hossain et al., 2012; Mutucumarana et al., 2015).

Statistical Analysis

Pearson Correlation The Pearson correlation coefficient was estimated to measure the strength of the linear relationship between the expression of genes. The Pearson correlation was estimated in overall considering all birds, and also it was estimated within individuals that had laid or had not laid an egg. The Pearson correlation was estimated using the CORR procedure in SAS software (version 9.4; SAS Inst. Inc., Cary, NC). The Pearson correlation coefficient "r" ranges from −1 to 1, where r = −1 indicates a perfect negative linear relationship, and r = 1 indicates a perfect positive linear relationship. The Pearson correlation was reported as significant where $P \leq 0.05$.

Analysis of Variance Treatment effects for the mRNA levels of all genes (*GnRH-I*, *GnIH*, *GnRH-RI*, *GnRH-RIII*, *GnIH-R*, *LH*, *FSH*, *POMC*, *NPY*, and *LEPR*), the ratio of *GnRH-RI* to *GnRH-RIII*; the ratio of *GnRH-RI* to *GnIH-R*; the ratio of *GnRH-RIII* to *GnIH-R*; BW; MEI; reproductive hormones (LH, FSH, E2); carcass compositions; and percentage of flock in lay were evaluated using 2-way ANOVA using the MIXED procedure in SAS, with age and treatment as fixed effects. Moreover, since birds started to lay eggs at different ages from 24 wk onward, the presence of an egg was included as a covariate for the mRNA levels of all genes; the ratios of *GnRH-RI* to *GnRH-RIII*, *GnRH-RI* to *GnIH-R*, and *GnRH-RIII* to *GnIH-R*; reproductive hormones; and carcass composition. The covariate took

the value of either 0 or 1, respectively, for birds that did not or did enter into lay and estimated the magnitude of the change in the expected value for each variable with respect to reproductive status. The PDIFF option of the LSMEANS statement used to estimate pairwise differences between means and least significant difference test was applied to multiple mean comparisons. Differences between means were reported as significant where $P \leq 0.05$. Trends were reported where $P < 0.1$.

RESULTS AND DISCUSSION

Metabolizable Energy Intake and BW

Because the high MEI treatment birds were fed whenever they entered the stations, it was not surprising that the high MEI pullets had higher MEI compared with the low MEI pullets during the entire experimental period (Table 3). Higher MEI of pullets in the high MEI treatment resulted in greater BW relative to the pullets in the low MEI treatment from 22 to 26 wk of age (Table 3). Pearson and Herron (1981) explained surplus of energy intake is mainly stored as fat. In the current experiment, the high MEI pullets with higher MEI than the low MEI pullets partitioned extra nutrients for carcass lipid deposition (it will be discussed further in the next sections) and also for the ovary development. The high MEI pullets compared with the low MEI pullets had greater ovary weight as a percentage of BW from 22 to 26 wk of age (0.62% ± 0.07 and 0.32% ± 0.07 respectively; $P = 0.003$; data not shown). The greater ovary weight of the high MEI pullets indicates that they partitioned more energy toward developing reproductive tissues for the onset of egg production than the low MEI pullets, and this is consistent with observed egg production data (Table 3).

Gene Expression

Effect of MEI on Genes of the Reproductive Axis

The high MEI treatment had higher hypothalamic mRNA level of *GnRH-I* compared with the low MEI treatment at 23 wk of age (1.77 vs. 0.63 respectively, Table 4) and also overall from 22 to 26 wk of age (0.74 vs. 0.32 respectively, Table 4). Moreover, the high MEI treatment had also higher pituitary mRNA level of *GnRH-RI* compared with the Low MEI treatment at 25 and 26 wk of age and overall from 22 to 26 wk of age (2.84 vs. 1.54 respectively, Table 4). The hypothalamic mRNA level of *GnRH-I* increased at 23 wk of age compared with other ages; however, the mRNA level of *GnIH* reduced with age from 23 to 26 wk of age (Table 4). The pituitary mRNA level of *GnRH-RI*

Table 3. Body weight (BW)[1], ME intake (MEI), and percentage of flock in lay for broiler breeder pullets on low ME intake (low MEI) and high ME intake (high MEI) feeding treatments from 22 to 28 wk of age.

			BW	SEM		MEI	SEM	Hens in lay	SEM
Treatment	Age (wk)	Age (D)	g		Age (wk)	kcal/D		%	
Low MEI			2,943^{s}	5		258^{s}	3.8	32.1^{s}	3.6
High MEI			3,569^{r}	8		489^{r}	6.4	80.1^{r}	3.0
	22	160	2,738^{e}	6	21 to 22	362	4.5	-	-
	23	167	3,032^{d}	6	22 to 23	373	4.8	-	-
	24	174	3,340^{c}	7	23 to 24	367	5.2	13.7^{d}	3.8
	25	181	3,547^{b}	9	24 to 25	378	6.8	48.3^{c}	3.8
	26	188	3,623^{a}	19	25 to 26	386	14.8	65.7^{b}	3.8
	27	195	-	-	26 to 27	-	-	73.5^{a}	3.8
	28	202	-	-	27 to 28	-	-	79.7^{a}	3.8
Low MEI	22	160	2,641^{o}	8	21 to 22	266^{j}	6.3	-	-
	23	167	2,789^{n}	9	22 to 23	243^{k}	6.7	-	-
	24	174	2,928^{l}	9	23 to 24	228^{k}	7.3	6.6^{m}	4.6
	25	181	3,095^{k}	11	24 to 25	271^{j}	8.8	20.2^{l}	4.6
	26	188	3,263^{j}	16	25 to 26	279^{j}	12.1	30.0^{l}	4.6
	27	195	-	-	26 to 27	-	-	45.6^{k}	4.6
	28	202	-	-	27 to 28	-	-	58.1^{j}	4.6
High MEI	22	160	2,834^{m}	8	21 to 22	458^{i}	6.4	-	-
	23	167	3,276^{j}	9	22 to 23	502^{h}	6.8	-	-
	24	174	3,751^{i}	10	23 to 24	506^{h}	7.4	20.8^{l}	4.2
	25	181	4,000^{h}	13	24 to 25	486^{h}	10.4	76.6^{i}	4.2
	26	188	3,983^{h}	34	25 to 26	493^{h,i}	27.1	101^{h}	4.2
	27	195	-	-	26 to 27	-	-	101^{h}	4.2
	28	202	-	-	27 to 28	-	-	101^{h}	4.2
Source of variation						*P*-value		*P*-value	
Treatment			<0.001			<0.001		<0.001	
Age			<0.001			0.16		<0.001	
Treatment x Age			<0.001			<0.001		<0.001	

[a–e]Means within column within age with no common superscript differ ($P \leq 0.05$).
[h–o]Means within column within treatment x age with no common superscript differ ($P \leq 0.05$).
[r–s]Means within column within treatment with no common superscript differ ($P \leq 0.05$).
[1]At 21 wk of age (153 D), before starting the current experiment, the BW of pullets in the high MEI and in the low MEI treatments did not differ (2,489 ± 7 and 2,484 ± 7 g respectively; $P > 0.05$).

Table 4. Relative expression of gonadotropin releasing hormone (*GnRH-I*) and gonadotropin inhibitory hormone (*GnIH*) in hypothalamus and GnRH-I receptor-I (*GnRH-RI*), GnRH-I receptor-III (*GnRH-RIII*), GnIH receptor (*GnIH-R*) in anterior pituitary of broiler breeder pullets on low ME intake (low MEI) and high ME intake (high MEI) feeding treatments from 22 to 26 wk of age.

		GnRH-I	SEM	*GnIH*	SEM	*GnRH-RI*	SEM	*GnRH-RIII*	SEM	*GnIH-R*	SEM
Treatment	Age (wk)	Relative expression									
Low MEI		0.32^{s}	0.12	1.50	0.16	1.54^{s}	0.29	1.25	0.13	3.51	1.48
High MEI		0.74^{r}	0.13	1.63	0.17	2.84^{r}	0.30	1.17	0.14	2.27	1.56
	22	0.51^{b}	0.26	$1.43^{a,b}$	0.34	$1.65^{b,c}$	0.57	0.87	0.26	1.71	2.93
	23	1.20^{a}	0.23	2.12^{a}	0.30	$1.35^{b,c}$	0.57	1.08	0.26	1.67	2.93
	24	0.40^{b}	0.15	2.09^{a}	0.20	1.62^{c}	0.38	0.99	0.17	3.29	1.96
	25	0.38^{b}	0.15	1.36^{b}	0.20	$2.72^{a,b}$	0.38	1.51	0.17	2.73	1.96
	26	0.16^{b}	0.22	0.81^{b}	0.26	3.59^{a}	0.49	1.58	0.22	5.04	2.54
Low MEI	22	0.34^{i}	0.36	0.98	0.48	1.06^{j}	0.78	0.91	0.35	2.20	4.02
	23	0.63^{i}	0.31	1.89	0.42	1.44^{j}	0.78	1.08	0.35	1.68	4.02
	24	0.25^{i}	0.22	1.95	0.29	1.59^{j}	0.54	1.21	0.24	4.91	2.77
	25	0.14^{i}	0.22	1.25	0.29	1.61^{j}	0.54	1.38	0.24	1.02	2.77
	26	0.24^{i}	0.22	1.16	0.29	1.98^{j}	0.54	1.65	0.24	7.73	2.77
High MEI	22	0.68^{i}	0.36	1.88	0.48	$2.24^{i,j}$	0.78	0.83	0.35	1.22	4.02
	23	1.77^{h}	0.31	2.36	0.42	1.26^{j}	0.78	1.09	0.35	1.66	4.02
	24	0.55^{i}	0.22	2.24	0.29	1.66^{j}	0.54	0.77	0.24	1.67	2.77
	25	0.62^{i}	0.22	1.48	0.29	$3.84^{h,i}$	0.54	1.65	0.24	4.44	2.77
	26	0.09^{i}	0.36	0.47	0.43	5.20^{h}	0.81	1.52	0.37	2.35	4.17
Egg[1](covariate)		0.34		0.48		-0.59		0.38		0.35	
Source of variation		*P*-value									
Egg		0.081		0.07		0.22		0.08		0.89	
Treatment		0.021		0.59		0.003		0.70		0.57	
Age		0.031		0.002		0.01		0.09		0.91	
Treatment x Age		0.32		0.28		0.05		0.70		0.66	

[a–c]Means within column within age with no common superscript differ ($P \leq 0.05$).
[h–j]Means within column within treatment x age with no common superscript differ ($P \leq 0.05$).
[r–s]Means within column within treatment with no common superscript differ ($P \leq 0.05$).
[1]The covariate egg was 0 for birds that had not laid an egg and was 1 for birds that had laid an egg.

reached the maximum at 25 and 26 wk of age (Table 4). Similarly, the mRNA level of *LH* (beta-subunit) increased with age from 22 to 26 wk of age. Interestingly, the mRNA level of *FSH* (beta-subunit) was greater at 22 and 23 wk of age compared with other ages (Table 5). On the other hand, the high MEI and the low MEI treatments did not impact the mRNA levels of *GnIH*, *GnRH-RIII*, *GnIH-R*, *LH*, and *FSH* from 22 to 26 wk of age (Table 4 and Table 5). Ratio of the *GnRH-RI* to *GnRH-RIII* was 1.63 times higher in the high MEI than the low MEI treatment from 22 to 26 wk of age (Table 6). Moreover, ratio of the *GnRH-RI* to *GnIH*-R was 1.38 times higher in the high MEI than the low MEI treatment from 22 to 26 wk of age (Table 6). However, ratio of the *GnRH-RIII* to *GnIH-R* was not significantly different between the high MEI and the low MEI treatments, but the trend showed that this ratio was 1.41 times higher in the low MEI than the high MEI treatment from 22 to 26 wk of age (Table 6). For the mRNA level of every gene, the covariate egg effect was estimated and presented in Table 4 and 5. Without considering the egg effect as the covariate, the relative expression of the genes would be biased systematically. If an egg had been laid, the relative expression of *GnRH-I*, *GnIH*, *GnRH-RIII*, and *LH* were different by 0.34, 0.48, 0.38, and −1.03 respectively.

In birds, reproduction is mainly regulated by stimulatory GnRH-I and inhibitory GnIH hypothalamic neuropeptides, on interaction with their receptors in anterior pituitary (Bédécarrats et al. (2009). Photostimulation increases the mRNA level of GnRH-I in the hypothalamus of chickens (Bédécarrats et al., 2006); however, under short day length, the mRNA level of GnIH is increased in quails (Ubuka et al., 2005). In the current experiment, in addition to higher mRNA level of *GnRH-I* for the high MEI treatment compared with the low MEI treatment, the mRNA level of *GnRH-RI* was also higher in the high MEI treatment from 22 to 26 wk of age. Moreover, the ratio of *GnRH-RI* to *GnRH-RIII* was 1.6 times higher in the high MEI treatment than the low MEI treatment. This indicated that *GnRH-I* interacted with *GnRH-RI* and caused the higher mRNA level of *GnRH-RI* in the high MEI treatment compared with the low MEI treatment. Interestingly, it was previously shown that *GnRH-RI* is barely expressed in the pituitary, and *GnRH-RIII* expression in pituitary was 1373-fold that of *GnRH-RI* in broiler chicken (Joseph et al., 2009). Similarly, it was reported that a significant increase in pituitary *GnRH-RIII* mRNA in white leghorn birds only occurs after photostimulation, whereas levels of *GnRH-RI* mRNA were found constant regardless of the reproductive stage (Shimizu and Bédécarrats, 2006). In the current study, it seems that high MEI along with photostimulation increased *GnRH-RI* expression, whereas Joseph et al. (2009) and Shimizu and Bédécarrats (2006) found that photostimulation increased *GnRH-RIII* expression. In the current study, the greater ratio of *GnRH-RI* to *GnIH-R* and the lower ratio of *GnRH-RIII* to *GnIH-R* in the high MEI treatment than the low MEI treatment showed

Table 5. Relative expression of luteinizing hormone (*LH*) and follicular stimulating hormone (*FSH*) in anterior pituitary and neuropeptide Y (*NPY*), proopiomelanocortin (*POMC*), and leptin receptor (*LEPR*) in hypothalamus of broiler breeder pullets on low ME intake (low MEI) and high ME intake (high MEI) feeding treatments from 22 to 26 wk of age.

Treatment	Age (wk)	*LH*	SEM	*FSH*	SEM	*NPY*	SEM	*POMC*	SEM	*LEPR*	SEM
		Relative expression									
Low MEI		0.63	0.12	0.58	0.08	1.93	0.24	0.94^{s}	0.13	1.26	0.08
High MEI		0.80		0.63	0.09	2.46	0.25	1.47^{r}	0.13	1.43	0.08
	22	0.06^{c}	0.13	0.76^{a,b}	0.16	1.50	0.51	1.49^{a}	0.26	1.06	0.17
	23	0.04^{c}	0.24	1.05^{a}	0.16	2.50	0.45	1.98^{a}	0.25	1.64	0.15
	24	0.16^{c}	0.24	0.49^{b,c}	0.11	1.92	0.30	1.54^{a}	0.15	1.34	0.10
	25	1.12^{b}	0.16	0.33^{c}	0.11	2.38	0.30	0.74^{b}	0.15	1.30	0.10
	26	2.21^{a}	0.16	0.40^{b,c}	0.14	2.67	0.39	0.28^{b}	0.20	1.37	0.13
Low MEI	22	0.06	0.21	0.74	0.22	0.89	0.71	0.98^{j,k,l,m}	0.36	0.98	0.23
	23	0.05	0.33	1.16	0.22	1.81	0.62	1.22^{i,j,k,l}	0.36	1.49	0.20
	24	0.16	0.33	0.33	0.15	1.88	0.42	1.48^{i,j,k}	0.22	1.24	0.14
	25	0.85	0.22	0.30	0.15	2.18	0.42	0.60^{l,m}	0.22	1.25	0.14
	26	2.04	0.22	0.36	0.15	2.88	0.42	0.45^{l,m}	0.22	1.34	0.14
High MEI	22	0.07	0.22	0.78	0.22	2.10	0.71	2.01^{h,i}	0.36	1.14	0.23
	23	0.02	0.33	0.95	0.22	3.19	0.62	2.75^{h}	0.32	1.78	0.20
	24	0.16	0.33	0.64	0.15	1.97	0.42	1.61^{i,j}	0.22	1.45	0.14
	25	1.38	0.22	0.35	0.15	2.58	0.42	0.87^{k,l,m}	0.22	1.36	0.14
	26	2.38	0.22	0.44	0.23	2.47	0.64	0.11^{m}	0.32	1.41	0.21
Egg[1](covariate)		−1.03	0.34	0.08		0.09		0.23		−0.08	
Source of variation		*P*-value									
Egg		<0.001		0.56		0.80		0.24		0.51	
Treatment		0.33		0.67		0.13		0.005		0.14	
Age		<0.001		0.01		0.29		<0.001		0.11	
Treatment x Age		0.73		0.72		0.49		0.03		0.97	

[a–c]Means within column within age with no common superscript differ ($P \leq 0.05$).
[h–m]Means within column within treatment x age with no common superscript differ ($P \leq 0.05$).
[r–s]Means within column within treatment with no common superscript differ ($P \leq 0.05$).
[1]The covariate egg was 0 for birds that had not laid an egg and was 1 for birds that had laid an egg.

that the MEI changed the ratio of *GnRH-RI* to *GnIH-R* in favor of *GnRH-RI* from 22 to 26 wk of age by sexual maturity and the onset of lay.

There was a sharp increase in the mRNA level of *GnRH-I* at wk 23 compared with other ages which is most likely because of the photostimulation. Photostimulation was started at the beginning of wk 22 and results are consistent with the hypothesis that broiler breeders are most sensitive to circulating estrogen levels (estrogenic) and follicle development between 2 to 4 wk after photostimulation (Robinson et al., 2003). Increased *LH* expression with age and also the greater *FSH* expression at 22 and 23 wk of age were also consistent with photostimulation and maximum responsiveness of *GnRH-I* gene between 2 to 4 wk after photostimulation. We hypothesized that *GnRH-RI* may be more prevalent on the gonadotropes synthesizing *LH* as the increased expression of *LH* and *GnRH-RI* occurred concomitantly with age. Therefore, these observations indicated that the expression of *GnIH* and *GnRH-I* in the hypothalamus may shift toward increasing *GnRH-I* by sexual maturation.

Effect of MEI on Genes Involved in the Regulation of Energy Balance The mRNA levels of *NYP* and *LEPR*

Table 6. The ratios of relative expression of gonadotropin releasing hormone (*GnRH-I*) receptor-I (*GnRH-RI*), GnRH-I receptor-III (*GnRH-RIII*), and gonadotropin inhibitory hormone receptor (*GnIH-R*) in anterior pituitary of broiler breeder pullets on low ME intake (low MEI) and high ME intake (high MEI) feeding treatments from 22 to 26 wk of age.

Treatment	Age (wk)	*GnRH-RI:GnRH-RIII* ratio	SEM	*GnRH-RI:GnIH-R* ratio	SEM	*GnRH-RIII:GnIH-R* ratio	SEM
Low MEI		1.69^{s}	0.26	1.06^{s}	0.14	0.89	0.10
High MEI		2.75^{r}	0.28	1.46^{r}	0.14	0.63	0.10
	22	2.85	0.52	1.54	0.27	0.6791	0.19
	23	1.78	0.52	1.17	0.27	0.7030	0.19
	24	1.83	0.35	0.85	0.18	0.7044	0.13
	25	1.72	0.35	1.18	0.19	1.0279	0.13
	26	2.89	0.45	1.56	0.24	0.6939	0.17
Low MEI	22	2.15	0.72	0.84^{j}	0.37	0.67	0.26
	23	1.81	0.72	1.18^{i,j}	0.37	0.70	0.26
	24	2.17	0.49	0.72^{j}	0.26	0.52	0.18
	25	2.27	0.49	1.45^{h,i,j}	0.27	0.65	0.18
	26	3.94	0.74	1.12^{i,j}	0.26	0.60	0.27
High MEI	22	3.55	0.72	2.25^{h}	0.37	0.69	0.26
	23	1.81	0.72	1.16^{i,j}	0.37	0.70	0.26
	24	2.17	0.49	0.99^{j}	0.26	0.52	0.18
	25	2.27	0.49	0.92^{j}	0.26	0.65	0.18
	26	3.94	0.74	2.00^{h,i}	0.39	0.60	0.27
Egg[1] (covariate)		-0.46	0.44	0.02	0.23	0.12	0.16
Source of variation		*P*-value					
Egg		0.30		0.93		0.45	
Treatment		0.008		0.049		0.071	
Age		0.11		0.10		0.32	
Treatment x Age		0.58		0.030		0.32	

[a–b]Means within column within age with no common superscript differ ($P \leq 0.05$).
[h–j]Means within column within treatment x age with no common superscript differ ($P \leq 0.05$).
[r–s]Means within column within treatment with no common superscript differ ($P \leq 0.05$).
[1]The covariate egg was 0 for birds that had not laid an egg and was 1 for birds that had laid an egg.

did not differ between the high MEI and the low MEI treatments from 22 to 26 wk of age (Table 5). On the other hand, the mRNA levels for *POMC* were greater in the high MEI treatment compared with the low MEI at 22 and 23 wk of age (Table 5).

AMP-activated protein kinase (**AMPK**) is a serine-threonine kinase that regulates cellular metabolism (Carling, 2004; Proszkowiec-Weglarz et al., 2006a; Proszkowiec-Weglarz and Richards, 2007). AMP-activated protein kinase maintains energy homeostasis and regulates food intake by changing the expression of orexigenic (stimulates feeding behavior) neuropeptides such as *NPY* and anorexigenic (inhibits feeding behavior) and neuropeptides such as *POMC* in the hypothalamus (Long and Zierath, 2006). Activation of AMPK in the hypothalamus stimulates energy producing pathway (catabolic) results in oxidation of glucose and fatty acids, and it inhibits energy-consuming pathways (anabolic; Proszkowiec-Weglarz et al., 2006a,b; Proszkowiec-Weglarz and Richards, 2007; Winder and Thompson, 2007). Moreover, activation of AMPK in the hypothalamus in response to lowered energy status stimulated the activity of the *NPY* expressing neuron (anabolic) and resulted in increasing feed intake and reduced energy expenditure which consequently increased energy status (Minokoshi et al., 2004; Long and Zierath, 2006). However, in the current experiment, *NPY* expression did not differ between treatments. Moreover, in the current experiment, the mechanism for downregulation of *POMC* by the low MEI was not assessed. It appears that downregulation of *POMC* expression in the low MEI treatment compared with the high MEI treatment occurred via AMPK activation. The AMPK activation in the low MEI treatment was probably because of the response to energy balance to enhance catabolic pathways and inhibit anabolic pathways.

The *LEPR* in birds has been found in the hypothalamus (Horev et al., 2000; Paczoska-Eliasiewicz et al., 2003), in the pituitary (Paczoska-Eliasiewicz et al., 2003), and in the ovary (Ohkubo et al., 2000; Paczoska-Eliasiewicz et al., 2003). This may suggest a role for leptin in reproductive system activity. In humans, ICV injection of leptin decreased food intake, decreased adipose tissue and decreased BW, and regulated energy stores (Friedman and Halaas, 1998). Similarly, ICV injection of leptin suppressed feed intake in broiler chickens and in leghorns (Denbow et al., 2000). The results of the current experiment showed that although *LEPR* was expressed in the hypothalamus of broiler breeder pullets from 22 to 26 wk of age; however, MEI did not change its expression.

The Correlation Between Gene Expression

Overall, GnRH-I and GnIH mRNA levels were positively correlated (r = 0.36; $P = 0.01$). This positive correlation between GnRH-I and GnIH gene expression was observed within individuals that entered lay (r = 0.42; $P = 0.05$) as well as within individuals that had not (r = 0.37; $P = 0.03$). Similarly, the overall expression of the POMC and GnRH-I were positively correlated

(r = 0.31; $P = 0.04$) both within individuals that entered lay (r = 0.38; $P = 0.08$) and within individuals that had not laid (r = 0.31; $P = 0.08$). Moreover, expression of the POMC and GnIH were also positively correlated overall (r = 0.50; $P = 0.005$) within individuals that had laid (r = 0.86; $P < 0.001$) or not (r = 0.49; $P = 0.004$). Interestingly, overall the mRNA levels of POMC were inversely related to the mRNA levels of LH (r = −0.40; $P = 0.006$), within individuals that entered lay (r = −0.61; $P = 0.002$) and within individuals that did not (r = −0.48; $P = 0.005$).

Positive correlation of *GnIH* and *GnRH-I* expressions suggests some relationship is maintained between *GnRH-I* and *GnIH* neurons. Within the hypothalamus, *GnIH* neurons directly contact *GnRH-I* neurons suggesting that *GnIH* may directly regulate the synthesis and release of GnRH-I at the level of the cell body (Bentley et al., 2003). Positive correlation of *POMC* with *GnRH-I* and *GnIH* and the negative correlation of *POMC* with *LH* provides a new insight for *POMC* function. It is hypothesized that POMC may play a role to link the control of reproduction with the control of energy status in broiler breeders. Proopiomelanocortin gene possibly signals to modulate *GnRH*, *GnIH*, and *LH* expression and integrate this information with energy storage information to allow or prevent reproduction to proceed.

Reproductive Hormones

The concentration of plasma LH was higher in the high MEI treatment compared with the low MEI treatment at 22 wk of age (7.10 vs. 0.93 ng/mL, Table 7). Similarly, the concentration of plasma FSH was higher in the high MEI treatment compared with the low MEI treatment at 22 wk of age (336 vs. 90.6 pg/mL, Table 7). The overall concentration of plasma E2 was higher in the high MEI treatment relative to the low MEI treatment from 22 to 26 wk of age (429 vs. 266 pg/mL, Table 7). The LH and FSH plasma concentrations decreased with age, and their levels decreased sharply after wk 22 (Table 7). The E2 plasma concentration was at the highest at 25 wk of age compared with the other ages for both MEI treatments (Table 7), indicating that timing of the activation of the ovarian follicular pool was not affected, but rather the amplitude was increased under high MEI. For the concentration of each reproductive hormone, the covariate egg was estimated, and it was not significant (Table 7). This indicated that reproductive status did not affect the concentrations of the reproductive hormones from 22 to 26 wk of age.

The major source of E2 is small white follicles in the ovary of a hen; however, large white follicles and small yellow follicles also produce E2 in small amounts (Robinson and Etches, 1986). Moreover, E2 is produced from theca cells of large yellow follicles in small amounts

Table 7. Plasma concentrations of luteinizing hormone (LH), follicular stimulating hormone (FSH), and 17 beta-estradiol (E2) of broiler breeder pullets on low ME intake (low MEI) and high ME intake (high MEI) feeding treatments from 22 to 26 wk of age.

		LH	SEM	FSH	SEM	E2	SEM
		ng/mL		pg/mL		pg/mL	
Treatment	Age (wk)						
Low MEI		1.60^{s}	0.30	89.3^{s}	13.6	266^{s}	47.5
High MEI		3.05^{r}	0.31	145^{r}	14.4	429^{r}	50.1
	22	4.02^{a}	0.59	213^{a}	27.0	262^{b}	94.0
	23	1.80^{b}	0.59	101^{b}	27.0	191^{b}	94.0
	24	2.25^{b}	0.40	81.7^{b}	18.1	339^{b}	63.1
	25	1.80^{b}	0.40	99.1^{b}	18.1	671^{a}	63.1
	26	1.75^{b}	0.51	90.6^{b}	23.4	274^{b}	81.5
Low MEI	22	0.93^{i}	0.81	90.6^{i}	37.0	126	129.1
	23	1.04^{i}	0.81	59.7^{i}	37.0	142	129.1
	24	2.18^{i}	0.56	84.9^{i}	25.5	294	88.8
	25	2.01^{i}	0.56	116^{i}	25.5	610	88.8
	26	1.82^{i}	0.56	95.1^{i}	25.5	157	88.8
High MEI	22	7.10^{h}	0.81	336^{h}	37.0	398	129.1
	23	2.55^{i}	0.81	143^{i}	37.0	240	129.1
	24	2.33^{i}	0.56	78.4^{i}	25.5	385	88.8
	25	1.60^{i}	0.56	82.1^{i}	25.5	731	88.8
	26	1.69^{i}	0.84	86.1^{i}	38.4	390	133.8
Egg[1] (covariate)		0.27		30.7		37.2	
Source of variation		*P*-value					
Egg		0.59		0.18		0.64	
Treatment		0.001		0.007		0.022	
Age		0.028		0.004		0.001	
Treatment x Age		0.002		0.005		0.89	

[a–b]Means within column within age with no common superscript differ ($P \leq 0.05$).
[h–i]Means within column within treatment x age with no common superscript differ ($P \leq 0.05$).
[r–s]Means within column within treatment with no common superscript differ ($P \leq 0.05$).
[1]The covariate egg was 0 for birds that had not laid an egg and was 1 for birds that had laid an egg.

(Etches and Duke, 1984; Robinson and Etches, 1986). The greater concentration of plasma E2 for the high MEI birds relative to the low MEI birds suggests a more pronounced activation of the ovary in the high MEI birds. This was consistent with the results of the ovary weight as a percentage of live BW which was higher for the high MEI treatment than the low MEI treatment from 22 to 26 wk of age (0.62% ± 0.07 and 0.32% ± 0.07 respectively; $P = 0.003$; data not shown). Estradiol increased with age in *ad libitum* fed broiler breeder hens and peaked at 25 wk (Onagbesan et al., 2006). In restricted broiler breeder hens, the concentration of E2 at peak was significantly lower than the *ad libitum* fed birds (Onagbesan et al., 2006). Similarly, in the current experiment, the E2 peaked at 25 wk of age (age effect), and pullets in the low MEI treatment had lower plasma E2 levels compared with the high MEI pullets. In the current experiment, the peak of E2 concentration occurred after the peaks of gonadotropins, and this indicated that the gonadotropins drove the E2 secretion. Similarly, it was observed in previous studies in broiler breeder hens that peak of E2 concentration happened after peaks of the gonadotropins (Renema et al., 1999b; Onagbesan et al., 2006) and also similar result was reported in Leghorn hens (Imai and Nalbandov, 1978).

In the current experiment, the overall higher plasma LH and FSH concentrations in the high MEI treatment relative to the low MEI treatment showed that the MEI enhanced reproductive development as the ovary weight was higher in the high MEI treatment than the low MEI treatment. Interestingly, considering an age effect, the peaks of FSH coincided with the peaks of LH at 22 wk of age. This suggested that there was a synergic effect between LH and FSH as it was reported by Imai and Nalbandov (1978) and that both could be stimulated by GnRH. Plasma LH and FSH concentrations increased to peak levels within 3 D of photostimulation and subsequently declined as sexual maturity proceeded in broiler breeder hens (Renema et al., 1999b). It was reported that FSH peak occurred at 20 wk in *ad libitum* fed birds when photostimulation was started at 19 wk of age (Liu et al., 2015). In the current experiment, photostimulation was started at the beginning of wk 22, and the plasma LH and FSH peaked at the end of wk 22 (6 D postphotostimulation), and subsequently, their values declined with age as the plasma E2 increased with age. Similarly, it was demonstrated that plasma LH concentration declined with age in White Leghorn chickens as the plasma steroid concentration increased with age (Vanmontfort et al., 1995). Renema et al. (1999b) suggested that the reduction in plasma LH and FSH after the peak could be because of the negative feedback effects of E2 on hypothalamic stimulation of LH and FSH. It was shown that in humans, E2 negative feedback on plasma LH and FSH occurs at the hypothalamus level (Welt et al., 2003) and also at the pituitary level (Shaw et al., 2010). However, in broiler breeders, there is lack of information whether E2 has a direct negative feedback effect on plasma LH and FSH either at the hypothalamus or at the pituitary level. Sun et al. (2001) suggested that estrogen played an inhibitory effect on hypothalamic neurons by reducing *GnRH-I* mRNA in juvenile cockerels. Maddineni et al. (2008) reported that estrogen decreased pituitary *GnIH-R* mRNA levels in female white Leghorn birds, and *GnIH-R* gene probably mediated inhibitory effect of *GnIH* on LH and FSH secretion. It was shown in mice that E2 has a direct inhibitory effect on plasma LH at the hypothalamus level (Couse and Korach, 1999). Moreover, it was reported that in ewe (Clarke and Cummins, 1984) and rat, E2 has a direct inhibitory effect on plasma LH and FSH at the pituitary level (McLean et al., 1975). In the current study, it seems that plasma E2 have probably a negative feedback effect on the stimulation of LH and FSH either at the hypothalamus or pituitary level.

Carcass Composition

The high MEI and the low MEI treatments did not differ for protein, ash, and water from 22 to 26 wk of age. However, pullets in the high MEI treatment had higher lipid than the pullets in the low MEI treatment from 23 to 26 wk of age (Table 8). Overall, protein increased with age, and fat also increased with age, whereas water decreased with age (Table 8). A nonsignificant covariate indicated that reproductive status did not affect the estimation for the carcass compositions from 22 to 26 wk of age.

Pullets in the high MEI treatment had higher MEI and also greater BW compared with the pullets in the low MEI treatment. Extra energy intake is mainly stored as fat (Pearson and Herron, 1981), thus higher lipid content of pullets in the high MEI treatment may be related to the difference in MEI and BW. Lipid contains more energy (9.1 kcal/g; Johnston, 1970) compared with other energy sources. Pullets in the high MEI treatment could advance the onset of lay relative to the pullets in the low MEI treatment because they had more energy available in the body and sufficient metabolic status to stimulate the onset of lay (it will be discussed further in the next section). It was reported that BW and lipid percent of BW for *ad libitum*-fed broiler breeder birds were greater than feed-restricted pullets at sexual maturity, and sexual maturity was advanced in the *ad libitum*-fed birds than the feed-restricted birds for 13.6 D ($P \leq 0.05$, Renema et al., 1999a).

In the current experiment, considering age effect, pullets had higher lipid and protein at wk 26 compared with wk 22. This indicated that broiler breeder pullets partitioned more energy toward protein and lipid deposition with age as sexual maturation proceeded. Lipid and protein contain more energy compared with the water (9.1, 5.5, and 0 kcal/g respectively; Johnston, 1970; Pullar and Webster, 1977), and the pullets are required to partition energy toward egg production as sexual maturity proceeded. Therefore, the metabolism of pullets changed with age, toward increasing protein and fat deposition.

Table 8. Proportional carcass composition of broiler breeder pullets on low ME intake (low MEI) and high ME intake (high MEI) feeding treatments from 22 to 26 wk of age.

Treatment	Age (wk)	Protein	SEM	Lipid	SEM	Ash	SEM	Water	SEM
		% of live BW							
Low MEI		20.5	0.3	10.3^{s}	0.4	3.17	0.12	65.5	0.6
High MEI		19.8	0.3	13.9^{r}	0.4	3.24	0.13	64.0	0.6
	22	17.8^{c}	0.5	9.5^{b}	0.8	2.89	0.24	69.3^{a}	1.1
	23	20.1^{b}	0.5	12.4^{a}	0.8	3.06	0.24	65.1^{b}	1.1
	24	20.2^{b}	0.4	13.4^{a}	0.6	3.26	0.17	64.1^{b,c}	0.8
	25	21.9^{a}	0.4	11.9^{a}	0.5	3.59	0.16	62.4^{c}	0.7
	26	20.8^{a,b}	0.5	13.3^{a}	0.7	3.24	0.21	62.6^{b,c}	1.0
Low MEI	22	17.5	0.7	9.3^{k}	1.1	2.61	0.34	69.7	1.5
	23	20.3	0.7	9.7^{k}	1.1	3.15	0.34	65.8	1.5
	24	21.0	0.6	10.3^{k}	0.8	3.49	0.25	65.2	1.1
	25	22.5	0.5	10.6^{k}	0.7	3.44	0.22	62.7	1.0
	26	21.1	0.5	11.7^{j,k}	0.8	3.18	0.23	63.8	1.0
High MEI	22	18.2	0.7	9.8^{k}	1.1	3.17	0.34	68.9	1.5
	23	19.8	0.7	15.1^{h,i}	1.1	2.97	0.34	64.5	1.5
	24	19.4	0.6	16.5^{h}	0.8	3.03	0.25	63.0	1.1
	25	21.4	0.5	13.2^{i,j}	0.8	3.74	0.23	62.0	1.0
	26	20.5	0.8	14.8^{h,i}	1.2	3.30	0.35	61.5	1.6
Egg[1] (covariate)		0.32		1.05		-0.20		−1.16	
Source of variation		*P*-value							
Egg		0.49		0.14		0.35		0.22	
Treatment		0.11		<0.001		0.71		0.07	
Age		<0.001		0.005		0.16		0.001	
Treatment x Age		0.46		0.033		0.38		0.93	

[a–c]Means within column within age with no common superscript differ ($P \leq 0.05$).
[h–k]Means within column within treatment x age with no common superscript differ ($P \leq 0.05$).
[r–s]Means within column within treatment with no common superscript differ ($P \leq 0.05$).
[1]The covariate egg was 0 for birds that had not laid an egg and was 1 for birds that had laid an egg.

The Onset of Sexual Maturity

All pullets in the high MEI treatment entered lay by 26 wk of age; however, only 30% of the pullets in the low MEI treatment entered lay by 26 wk of age (Table 3). Heavier laying hens with greater lipid content have been shown to enter lay earlier (Summers and Leeson, 1983). It was also reported that broiler breeder hens from 1980 with 5.38% abdominal fat pad entered lay 19.2 D earlier than hens from 2000 and with 2.65% of abdominal fat pad (Eitan et al., 2014). Similarly, in the current experiment, in the high MEI treatment relative to the low MEI treatment, lipid carcass was 1.3 times higher from 22 to 26 wk of age; thus, the high MEI pullets had more energy available to start the onset of sexual maturity. It was recently shown that modern broiler breeder pullets are restrictedly fed according to the standard breeder-recommended target BW, mostly deposited lean tissues (Hadinia et al., 2018). Interestingly, it was reported that although growth potential of broiler breeders increased during the last 30 yr (Renema et al., 2007), feed restriction became more severe with continued selection program for broiler growth traits (van Emous, 2015). Severe feed restriction decreases the nutrients available in the body such as fat, which eventually affects the body composition and delays the onset of lay in modern broiler breeders. It was reported that broiler breeder hens that followed the standard breeder-recommended target BW delayed the onset of lay for 18.9 D compared with hens with target BW increased by 22% reaching the standard 21 wk BW at 18 wk (van der Klein et al., 2018). Moreover, total egg production was higher for the hens on the high target BW treatment compared with the standard target BW treatment at the end of wk 55 (129.4 vs. 92.8, respectively; van der Klein et al., 2018). The onset of sexual maturity can be affected by the concentration of reproductive hormones. The peak concentration of E2 for broiler breeder hens provided either restricted or *ad libitum* access to feed occurred between 5.69 and 6.63 D before sexual maturity, respectively (Renema et al., 1999b). Interestingly, the concentration of plasma LH and FSH increased in *ad libitum* fed broiler breeder hens relative to restricted birds and indicated that energy balance may interact with BW and age threshold (HPG axis maturation) to initiate sexual maturity (Renema et al., 1999b). In the current study, in the high MEI treatment compared with the low MEI treatment, the plasma LH was 1.9 times higher from 22 to 26 wk of age. Moreover, in the high MEI treatment compared with the low MEI treatment, the plasma FSH and E2 were both 1.6 times higher from 22 to 26 wk of age. The increased reproductive hormones indicated more robust ovary development in the high MEI pullets compared with the low MEI pullets. In addition, in the high MEI treatment compared with the low MEI treatment, the *GnRH-I* mRNA level was 2.3-fold higher in the hypothalamus, and the *GnRH-RI* mRNA level was 1.8-fold higher in the pituitary from 22 to 26 wk of age. These results indicated that pullets in the high MEI treatment were better prepared to respond to photostimulation with a more responsive HPG axis leading to advanced onset of lay compared with the low MEI pullets. In addition, these results are

consistent with the hypothesis that current broiler breeder BW recommendations may restrict pullets from entering lay because the proportional degree of feed restriction is increasing, whereas little change has occurred to broiler breeder BW recommendations (Zuidhof, 2018). Therefore, the results of the current experiment would suggest increasing the target BW for the broiler breeder pullets to help them to obtain all the cascades for the sexual maturity earlier and advance the onset of lay and also increase the productivity.

CONCLUSION

High MEI along with photostimulation can increase *GnRH-I* expression in the hypothalamus and *GnRH-RI* and *LH* expression in the pituitary. Therefore, in addition to increasing body lipid deposition, higher MEI enhanced the activation of the HPG axis which accelerated puberty in broiler breeder pullets. Whether the effect of MEI was mediated through the orexigenic/anorexigenic pathways or by directly acting on the hormones of the HPG remains to be determined. However, the decreased mRNA levels of *POMC* in the low MEI treatment compared with the high MEI treatment does indicate that *POMC* (anorexigenic) is involved in energy balance in broiler breeder pullets to prevent a decrease in energy intake. Furthermore, the downregulation of *POMC* in the low MEI treatment appears to occur via AMPK activation; however, further studies are required to confirm this pathway.

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