Peroxisome proliferator-activated receptor gamma upregulation and dietary fat levels in laying hens

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ABSTRACT The present study was conducted to investigate the effect of feeding the different levels of the dietary fat on the expression of genes encoding proteins involving energy metabolism, oxidative phosphorylation, and lipid synthesis including peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) of laying hens in the intestine. Birds fed diets with 3 levels of fat, that is, low (LF), medium (MF), and high fat (HF) were reared from 22 to 42 wk of age. Jejunum tissue was collected at week 42 for gene expression analysis. Dietary fat content as ether extract, net energy to AME ratio, and CP content of 3 treatment groups were as follows: LF: 25, 0.735, 187 (g/kg, DM); MF: 61, 0.739, 185 (g/kg, DM); HF: 73, 0.752, 181 (g/kg, DM). The BW, fat pad weight (g), fat pad–to–BW ratio (%) was the same for all the treatments (\(P > 0.05\)). Birds fed a diet containing HF increased the AME daily intake per metabolic BW (BW\(^{0.75}\)) (\(P < 0.05\)). The expression of jejunal PPAR\(\gamma\) was increased in the birds fed MF than that fed LF (\(P < 0.05\)). Dietary fat level did not affect the expression of other genes: protein kinase AMP-activated noncatalytic subunit gamma 2, NADH dehydrogenase subunit 2, succinate dehydrogenase complex flavoprotein subunit A, ubiquinol-cytochrome c reductase Rieske iron-sulfur polypeptide 1, cytochrome c oxidase subunit III, ATP synthase subunit alpha, avian adenine nucleotide translocator, and acetyl-CoA carboxylase alpha (\(P > 0.05\)). The mitochondrial count per cell showed no difference among the 3 groups with different dietary treatments (\(P > 0.05\)). The results suggest that PPAR\(\gamma\) may be important to the energy expenditure during nutrient absorption, digestion, and metabolism, and respiratory chain complexes, and other genes involving mitochondrial energy metabolism and lipogenesis may be less responsive to dietary treatment.

Key words: dietary fat, PPAR\(\gamma\), gene expression, laying hen

INTRODUCTION

The energy balance is defined by 2 main components as energy intake and energy expenditure. In endotherm animals, the main constituents of energy expenditures are basal metabolism, physical activity, and body thermoregulation. Peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) plays an important role in energy metabolism by regulating fatty acid storage and glucose metabolism by mediating the expression of fat-specific genes in adipocyte differentiation and function in mammals (Tontonoz et al., 1995). Any changes to adenosine diphosphate/adenosine triphosphate (ATP) ratio in mitochondria will be sensed to activate the PPAR\(\gamma\) coactivators (Puigserver and Spiegelman, 2003). Adenosine monophosphate-activated protein kinase (AMPK) is an enzyme that plays a leading role in cellular energy homeostasis and appetite regulation.

Dietary nutrients after breakdown will be oxidized through metabolic pathways such as oxidative phosphorylation or electron transport chain (ETC) resulting in releasing ATP as an active form of energy via ETC inside mitochondria. Both acetyl-CoA carboxylase alpha and AMPK genes are involved in energy metabolism and fatty acid synthesis. The extra amount of energy stores as triacylglycerol and it breaks down again into glycerol and fatty acids (\(\beta\)-oxidation) and transports into mitochondria to generate acetyl-CoA when the cell energy reserves deplete. Acetyl-CoA is a fuel for Krebs cycle for ATP production.

Sato et al. (2004) reported PPAR\(\gamma\) as a pivotal gene for energy partitioning as fat deposition and egg production in laying hens. High-fat (HF) diets increased energy consumption through increased ATP production, which resulted in increased body weight gain and egg production.
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MATERIALS AND METHODS

Birds and Diets

The study was approved by the Animal Ethics Committee of the University of New England and designed to follow the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013).

Six hundred Hy-Line Brown pullets obtained from Glenwarrie Farm in Tamworth were housed at 16 wk of age in the cages located in a shed at the University of New England in Australia. The experiment was conducted from 22 to 42 wk of age when the hen day production (HDP) was 78% from start and up to 96% at peak

Table 1. Ingredients composition of diets (g/kg; as-is basis).

| Treatment | LF | MF | HF |
|-----------|----|----|----|
| Ingredient |    |    |    |
| Wheat     | 616| 434| 419|
| Barley    | 100| 116| 114|
| Wheat bran| 20 | 120| 120|
| Soybean meal | 100 | 54 | 59 |
| Canola meal – cold pressed | 30 | 150| 150 |
| Canola oil | 3.3 | 19.2| 31.8 |
| Limestone | 95.1| 94.4|  |
| Dicalcium phosphate | 2.0 | 0.6 | 0.7 |
| Salt | 2.0 | 1.8 | 1.8 |
| Choline 60% | 0.4 | 0.6 | 0.6 |
| UNE vitamin and mineral premix1 | 1.0 | 1.0 | 1.0 |
| Na bicarbonate | 2.0 | 2.0 | 2.0 |
| Pigment-Jabiru Red (10%) | 0.04 | 0.04 | 0.04 |
| Pigment-Jabiru Yellow (10%) | 0.03 | 0.03 | 0.03 |
| Phytase (Axtra TPT 10000)2 | 0.10 | 0.10 | 0.10 |
| Xylanase (Axtra XB)3 | 0.08 | 0.08 | 0.08 |
| L-lysine HCl 78.4 | 2.7 | 2.2 | 2.2 |
| DL-methionine | 2.0 | 1.6 | 1.6 |
| L-threonine | 1.2 | 0.9 | 0.9 |
| L-isoleucine | 1.0 | 1.0 | 1.0 |
| L-valine | 0.8 | 0.5 | 0.5 |

1UNE laying hens premix supplied per tonne: 10.0 MIU vitamin A, 3.0 MIU vitamin D, 20.0 g vitamin E, 3.0 g vitamin K, 35.0 g nicotinic acid, 12 g pantothentic acid, 1 g folic acid, 6 g riboflavin, 0.02 g cyanocobalamin, 0.10 g biotin, 5.0 g pyridoxine, 2.0 g thiamine, 8.0 g copper, 0.20 g cobalt, 0.50 g molybdenum, 1.0 g iodine, 0.30 g selenium, 60.0 g iron, 60.0 g zinc, 90.0 g manganese, 20.0 g Oxicap E2 (antioxidant).

2Matrix values for phytase (Axtra TPT 10,000, 500 FTU) were: 2,866% P avail, 2,844% Ca, 720,000 kcal/kg AMEn, 240% lysine, 72% methionine, 210% methionine 1 cystine, 214% threonine, 174% isoleucine, 64% tryptophan, 212% valine, and 204% arginine with amino acids on a digestibility basis.

3No matrix values were used for xylanase in any formulation.

The objective of the study was to find any potential link between dietary fat levels and correspondent dissipated heat on the genes involved in cellular energy homeostasis in intestinal mitochondria of laying hens as the primary sites for nutrient digestion, energy metabolism, and ATP production.

Table 2. Nutrient composition of experimental diets (g/kg, DM).

| Treatment | LF | MF | HF |
|-----------|----|----|----|
| Nutrients assayed |    |    |    |
| DM % | 90.3 | 89.9 | 90.0 |
| CP | 187 | 185 | 181 |
| Ether extract | 25 | 61 | 73 |
| Starch | 449 | 370 | 365 |
| Crude fiber | 33 | 61 | 48 |
| Calcium | 45 | 47 | 43 |
| Phosphorus, total | 4.7 | 6.2 | 5.7 |
| Sodium | 1.7 | 2.4 | 1.9 |
| Lysine | 9.6 | 10.8 | 9.5 |
| Methionine | 5.4 | 4.9 | 3.7 |
| Cysteine | 3.5 | 3.8 | 3.7 |
| Threonine | 6.9 | 7.6 | 6.9 |
| Isoleucine | 7.8 | 8.4 | 7.7 |
| Arginine | 9.1 | 10.2 | 9.4 |
| Valine | 8.7 | 9.3 | 8.4 |
| Tryptophan | 2.1 | 2.2 | 2.2 |

Energy values (measured)

| Treatment | LF | MF | HF |
|-----------|----|----|----|
| AME (kcal/kg, DM) | 2,968 | 2,992 | 3,129 |
| NE (kcal/kg, DM) | 2,182 | 2,211 | 2,352 |
| NE/ AME | 0.735 | 0.739 | 0.752 |
## Table 3. Sequences of primers used for quantitative real-time PCR.

| Gene          | Gene full name                                      | Primer sequence (5’-3’)                                                                 | Ta  | Size (bp) | Accession no. | Reference                  |
|---------------|-----------------------------------------------------|-----------------------------------------------------------------------------------------|-----|-----------|----------------|----------------------------|
| PPARG        | Peroxisome proliferator-activated receptor gamma    | F-TGGTTGACACAGAAATGCGTG                                                                 | 60  | 234       | NM_001001460.1 | This study                 |
|              |                                                     | R-CCATTTTGATTTGCACTTGTG                                                                 |     | 60        |                |                            |
| PRKAG2       | Protein kinase AMP-activated non-catalytic subunit gamma 2 | F-ACGCTCGAACATAATGCGTG                                                                 | 60  | 73        | NM_001278143.1 | This study                 |
|              |                                                     | R-CCATTTTGATTTGCACTTGTG                                                                 |     | 60        |                |                            |
| ND2          | NADH dehydrogenase subunit 2                       | R-AGGCTGCACTCCTACTG                                                                   | 60  | 147       | J00970529.1    | This study                 |
| SDHA         | Succinate dehydrogenase complex flavoprotein subunit A | F-ACGCTGCACTCCTACTG                                                                   | 60  | 74        | NM_001277398.1 | This study                 |
|              |                                                     | R-AGGCTGCACTCCTACTG                                                                   |     | 60        |                |                            |
| UQCRFS1      | Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 | F-TGGTTGACACAGAAATGCGTG                                                                 | 60  | 90        | NM_001005843.1 | This study                 |
|              |                                                     | R-CCATTTTGATTTGCACTTGTG                                                                 |     | 60        |                |                            |
| COX III      | Cytochrome c oxidase subunit III                    | F-ACGCTGCACTCCTACTG                                                                   | 60  | 72        | KC847880.1     | This study                 |
| ATP15 W      | ATP synthase subunit alpha                          | R-AGGCTGCACTCCTACTG                                                                   | 60  | 232       | XM_429118.5    | This study                 |
| avANT        | ATP/ADP antiporter                                  | F-ACGCTGCACTCCTACTG                                                                   | 60  | 147       | AB088686.1     | This study                 |
| ACACA        | Acetyl-CoA carboxylase alpha                        | R-AGGCTGCACTCCTACTG                                                                   | 60  | 181       | NM_005505.1    | This study                 |
| GAPDH        | Glyceraldehyde-3-phosphate dehydrogenase            | R-AGGCTGCACTCCTACTG                                                                   | 60  | 66        | NM_204305.1    | (Kuchipudi et al., 2012)   |
| ND4¹         | NADH dehydrogenase subunit 4                       | R-AGGCTGCACTCCTACTG                                                                   | 60  | 137       | NC_001323.1    | (Samiullah et al., 2017)   |
| GAPDH²       | Glyceraldehyde-3-phosphate dehydrogenase            | R-AGGCTGCACTCCTACTG                                                                   | 60  | 137       | NC_006088.3    | (Samiullah et al., 2017)   |
| HMBS         | Hydroxymethylbilane synthase                        | R-AGGCTGCACTCCTACTG                                                                   | 60  | 131       | XM_417846.2    | (Yin et al., 2011)         |

¹Gene was used to amplifying the fragment of the mitochondrial DNA.
²Gene was used to amplifying the fragment of genomic DNA.
The main ingredients used for making diets were wheat, barley, wheat bran, soybean meal, and cold-pressed canola meal (Table 1). Canola oil was used to provide energy. The ingredients were analyzed for nutrient content by NIRS (Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany) before formulation. Diets were formulated as per the minimum digestible amino acid specifications of Hy-Line Brown (Hy-Line, 2016) (Table 2). Diet 1 contained 187 and 25 g/kg (DM) CP and ether extract (EE), respectively, in all diets. Birds were fed ad libitum with nase (Axtra XB) with the dosages at 0.08 and 0.10 g/kg, respectively. Diets were formulated with phytase (Axtra TPT 10,000) and xylanase (Axtra XB) with the dosages at 0.08 and 0.10 g/kg, respectively, in all diets. Birds were fed ad libitum with free access to water.

### Performance, Fat Pad, and Energy of Feed

The performance of laying hens fed different diets was measured. The HDP was measured as the average hen day production (%). Egg weight was the average egg weight (g) for the total experimental period. Egg mass was calculated as the average egg weight multiple by average HDP (g of egg/bird/day); Fat pad (g), abdominal fat pad weight; Fat pad/BW (%), the ratio of the fat pad to the correspondent BW; FCR (g/g), feed conversion ratio as total feed intake (g/hen/d as is) divided by total egg mass (g); HDP, average hen day production (%). Egg weight was the average egg weight (g) for the total experimental period. Egg mass, average egg weight × average HDP (g of egg/bird/day); Fat pad (g), abdominal fat pad weight; Fat pad/BW (%), the ratio of the fat pad to the correspondent BW; FCR (g/g), feed conversion ratio as total feed intake (g/hen/d, as is) divided by total egg mass (g); HDP, average hen day production (%).

### DNA and RNA Extraction

Total DNA was extracted from approximately 65 mg of proximal jejunum tissue using an ISOLATE II Genomic DNA Kit (Bioline, Sydney, Australia) as per the manufacturer’s protocol. The quantity and purity of total DNA were determined using NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham). The extracted DNA was stored at −20°C until required for downstream applications.

Total RNA was extracted from approximately 90 mg of proximal jejunum tissues at week 42 using TRIzol (Bioline, Sydney, Australia) following the manufacturer’s instructions. The total RNA was further purified using ISOLATE II RNA Mini Kit (Bioline, Sydney, Australia) as per the manufacturer’s instructions. For each RNA sample, a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to analyze the purity and quantity of the RNA. RNA integrity was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany) using RNA 6000 Nano kit as per the manufacturer’s protocol. The RNA integrity number values of the samples > 7.5 were considered high in quality (ranged from 7.7 to 9.7 in this study).

### The cDNA Synthesis

Approximately 1 μg RNA was reversely transcribed into cDNA using the SensiFAST cDNA Synthesis Kit following the manufacturer’s instructions. The cDNA was diluted 10 times with nuclease-free water and stored at −20°C for further analysis.

### Primers

The National Center for Biotechnology Information primer tool (https://www.ncbi.nlm.nih.gov) was used to design the primers for target genes in this study.
The primers for the reference genes and mitochondrial quantification were sourced from previously published studies in chickens (Yin et al., 2011; Kuchipudi et al., 2012; Samiullah et al., 2017). Table 3 shows the primers that were used in the present study. The specificity for each pair of primers was evaluated with an Agilent DNA 1000 Kit (Agilent Technologies, Inc., Germany) after PCR amplification with a subset of template samples. The amplification efficiency of each pair of primers was also evaluated and only the primer pairs with high specificity and amplification efficiency were used in the present study.

**Quantitative PCR**

Quantitative PCR was performed in a Rotor-Gene 6000 real-time PCR machine (Corbett Research, Sydney, Australia) using a SYBR Green kit SensiFAST SYBR No-ROX (Bioline, Sydney, Australia). The quantification cycle (Cq) value for each gene was imported into qBase+ version 3.0 (Biogazelle, Zwijinbeke, Belgium) software and analyzed against 2 optimized reference genes (glyceraldehyde 3-phosphate dehydrogenase and hydroxymethylbilane synthase) as the internal normalizers in this study. The relative quantification of the target genes obtained by arithmetic means method in qBase+ was exported to SAS statistics, version 9 (SAS, 2010), for further analysis.

**Mitochondria Quantification**

Mitochondria were enumerated as per the method described by Samiullah et al. (2017). Briefly, quantitative PCR was performed to enumerate mitochondrial DNA counts using the SensiFAST SYBR No-ROX Kit (Bioline, Sydney, Australia). The quantitative PCR reaction was performed in a total volume of 20 μL with a Rotor-Gene 6000 thermocycler (Corbett Research, Sydney, Australia). The reaction consisted of 10 μL 2× SensiFAST SYBR No-ROX mix, 400 nM each of the primers, 6.4 μL RNase-free water, and 2 μL of 10^-2 diluted DNA. Serial dilutions of linearized plasmid DNA (TOPO TA Cloning Kit for sequencing, Thermofisher Scientific, Australia) inserted with ND4 and glyceraldehyde 3-phosphate dehydrogenase amplicons were used to construct a standard curve. The cloned plasmid DNA amplification cycle (Cq) values were then used to quantify the mitochondrial DNA and genomic DNA copies in the sample. The mitochondrial DNA copy numbers per cell were calculated by the equation (count of mitochondrial DNA)/(count of genomic DNA/2).

**Statistical Analysis**

All the data of performance parameters, mRNA gene expression, and mitochondrial counts were examined for their distribution normality with the Shapiro-Wilk test. The data were then subjected to a 1-way ANOVA using PROC CORR, PROC GLM, and Tukey’s multiple-range test (SAS, 2010) for paired comparison. Difference was declared if P < 0.05.

**RESULTS AND DISCUSSION**

In the present study, feeding the HF diet had no effect on the HDP (P > 0.05) while it increased the egg weight (P < 0.01) and egg mass (P < 0.05). In addition, the birds fed the higher dietary fat levels improved the feed conversion ratio (P < 0.001). It was also observed that birds fed medium-fat (MF) and HF diets had higher AME intake (P < 0.05) than those fed a low-fat diet (Table 4). Furthermore, it was shown that the MF diet upregulated the PPARγ expression compared with the low-fat diet (P < 0.05), whereas the MF values were numerically higher than the HF diet (Table 5). On the other hand, the dietary treatments did not change BW (g), abdominal fat (g), and abdominal fat pad/BW (%) (P > 0.05) (Table 5), the expression of genes PRKAg2, ND2, SDHA, UQCRFS1, COXIII, ATP15W, avANT and ACACA (P > 0.05), and the mitochondrial count per cell (P > 0.05) (Table 5).

It has been reported that feed restriction and low energy intake reduced PPAR-γ2 mRNA levels in rats, mice, and humans (Vidal-Puig et al., 1996, 1997; Arai et al., 2004). These observations are in agreement with our findings in relation to AME intake and PPARγ expression level. As MF and HF diets were higher in EE, the dietary fat content likely contributes to the expression of PPARγ. Dietary fats are important modulators of PPARγ, and this has been related to the regulation of energy balance (Cecil et al., 2006). Kliewer et al.
(1997) suggested that PPAR α and γ are physiological sensors for lipid homeostasis which can be triggered by dietary fatty acids. Sato et al. (2004) reported that PPARγ expression was higher when chickens fed linoleic than that in those fed oleic acid; furthermore, the level of PPAR expression was higher in the liver compared with adipose tissue during the laying period which might be because of more demands for lipogenesis and fat deposition in the developing egg. The same researchers also observed that the body fat deposition as a depot tissue can be affected by PPARγ function in body.

The AMPK is the fundamental regulator of energy balance and food intake within the cell of the animal body (Minokoshi et al., 2004). In the present study, dietary treatments did not result in the change of AMPK expression in the jejunum.

Different levels of fat or possibly the fatty acid profile between diets might not be big enough to provoke the effect of fat on PRKAY2 gene regulation or the effect may not be present in the jejunum. Cho et al. (2017) observed that PRKAY2 in the muscle and cell-free plasma did not differ by feeding ducks the diets with low and medium levels of AME (2,300 and 2,900 kcal/kg diet); on the other hand, high AME level (3,300 kcal/kg diet) (with higher dietary fat) upregulated PRKAY2 in those tissues possibly to maintain energy homeostasis.

In the present study, oxidative phosphorylation was not affected by dietary compositions. It might be possible that oxidative phosphorylation in the jejunum of laying hens is not sensitive to relatively subtle differences present in diets. Lemieux et al. (2008) reported that long-term feeding diets with different fat and fatty acid profile resources were not able to change the mitochondrial respiration rate at ETC complex I, II, or IV in the rat heart. Furthermore, the mRNA expression of avANT and COX III did not differ in broilers with different genetic lines (Ojano-Dirain et al., 2007).

The dietary fat level which affects the ratio of net energy/AME corresponds to the amount of heat increment of feed. This heat dissipation can be used by chickens for energy balance and food intake: the role of PPARγ gene polymorphisms. Physiol. Behav. 88:227–233.

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