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Gene expression patterns in Korean native ducks (*Anas platyrhynchos)* with different apparent metabolisable energy (AME) levels

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ABSTRACT

Quantitative energy changes in the feeding regimens affect metabolic efficiency and product quality of an animal. Apparent metabolisable energy (AME) is used to evaluate energy utilization such as the difference between the energy consumed and the energy lost. Given the importance of the diet in the profitability we focused on determination of AME levels using biomarkers based on molecular techniques. qRT-PCR was performed to determine gene expression differences in ducklings with different metabolisable energy (ME) levels and potential biomarker genes from circulating mRNA in the body fluid based on estimated AME values. Muscle tissues and cell-free plasma samples were obtained from ducklings fed at three different ME levels (basal ME: 2300; AME: 2900; high ME: 3300 kcal/kg diet). We first identified that 18 S ribosomal RNA was the most suitable endogenous reference gene for determining gene expression analysis. Next, we investigated expression levels of genes related to cell growth, proliferation, oxidative stress, myogenic differentiation, adipocyte differentiation, and mitochondrial function. qRT-PCR assay demonstrated that *MAPK8*, *MAP2K4*, *HADHA*, *CSRP3*, and *MYL1* were gradually up-regulated at the AME and high ME levels. However, *PRKAG2*, *CRH*, and *PPARG* transcript levels were significantly increased in the muscle and the cell-free plasma at the excessive high ME level but not at the AME and the basal ME level. Finally, *HSP90AA1* and *HSPB7* were not different between AME and high ME level. Our findings suggest that altered patterns of gene expression in response to different levels of energy are useful biomarkers to evaluate the AME value with growing ducklings. Upregulation of genes including *MAPK8*, *PRKAA2*, and *PRKAG2* at the excessive high ME and their enrichment for lipid and fatty acid biosynthetic process in gene network analysis also supported our molecular approach using circulating nucleic acid in the body fluid.

1. Introduction

Global meat production and consumption have increased rapidly in developing countries over the past decades (Cao and Li, 2013). Particularly, in Korea the proportion of production and consumption of duck meat and the consumption per capita have gradually increased in the past ten years (Kim et al., 2013). The first outbreaks of avian influenza virus (AI) adversely affected meat consumption in 2003 and 2004, but others had no impact on production and consumption (www.kosis.kr; Korean statistical information service). The domestic expanding production and consumption of Korean native ducks has created a need not only to provide suitable feed ingredients, but also to assess the nutrient requirement to maximise farm profitability. Apparent metabolisable energy (AME) requirement is the most commonly used method to evaluate energy utilization for feed efficiency, but AME requirements for Korean native duck were not determined, and AME for the white Pekin ducks has been used because of limited studies (Fan et al., 2008; Xie et al., 2010) and genetic similarity between Pekin and Korean native ducks (*Anas platyrhynchos domestica*) that have a diverse genetic pool, but common genetic background such as Mallard(*Anas platyrhynchos*), and spot-billed duck, (*Anas zonorhyncha*) (He et al., 2008; Jin et al., 2014). However, growth performance, carcass yield and meat quality of Korean native duck are different from those of Pekin Duck (Hong et al., 2014). Therefore, the AME requirements for the white Pekin ducks may not be suitable to maximise feed efficiency and utilization of metabolisable energy (ME) in production of Koran native ducks (Fan et al., 2008; Xie et al., 2010).

A recent study reported that the estimated AME requirements for Korean native ducklings from hatch to 21 days of age are 2,953, 3007, and 2,50 kcal/kg diet for maximum daily gain, feed intake, and minimum feed conversion ratio, respectively (Wickramasuriya et al., 2016). The optimum AME requirements were calculated by measuring several parameters including body weight, average daily gain, and average daily feed intake. However, there are significant differences in

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energy utilization between individual birds although they have similar genetic background and are raised together under identical condition (Konsak et al., 2013). Thus, analysis of expression of genes involved in energy utilization efficiencies is a very useful for the poultry producers to select birds with consistent performance because the analysis using molecular tools such as qRT-PCR is relatively rapid and easy, compared to traditional assessment such as measuring body weight, average daily gain, and average daily feed intake. Although extensive studies focusing on global gene expression related to feed efficiency such as feed intake, and energy expenditure have been done in chickens (Bottje et al., 2012; Bottje and Kong, 2013; Fang et al., 2014), little is known about the genes governing regulation of energy utilization efficiency in ducks. Moreover, most studies in duck were limited to genetic diversity or gene expression in terms of breast muscle development (Xu et al., 2012, 2014; Jin et al., 2014).

To evaluate growth and efficiency of dietary energy of utilization by ducklings using quantification of gene expression levels, we first selected genes associated with muscle development (Xu et al., 2012, 2014), and expressed differentially in the phenotypic variation of feed efficiency traits (Bottje et al., 2012) because gaining weight(growth including muscle) and balances of energy storage and expenditure (energy utilization) are coordinated and tightly regulated by genes encoding key regulatory factors such as hormones, transcription factors, and metabolic pathways in poultry (Richards, 2003; Richards and Proszkowiec-Weglarz, 2007; Fang et al., 2014).

We performed quantitative real time polymerase chain reaction (qRT-PCR) to determine differentially expressed genes in muscle tissues obtained from ducks fed at different ME levels (basal ME: 2300; AME: 2900; high ME: 3300 kcal/kg diet), and compare them with transcript profiles in circulating cell-free nucleic acid from collected blood plasma. Finally we analysed the altered expressed gene network and suggested biomarker genes for evaluation of different feeding regimes.

2. Materials and methods

All practices and procedures were performed under Korean Food & Drug Administration personal license (No.16-00996) and approved from the Animal Ethics committee of the Chungam National University (CNU-00558).

2.1. Collection of muscle tissue and plasma from ducklings at 21 days

Birds, housing and diets, and post mortem procedures were formed as previously described (Wickramasuriya et al., 2016). Briefly, 336 one-day-old male Korean native ducks obtained from a local hatchery were randomly allocated to eight dietary treatments (n = 6 replicate per treatment and 7 ducklings per pen) from 2600 ~3300 kcal/kg in 100 kcal/kg scale (Table 1), and the birds were fed the experimental diet on an *ad libitum* basis for 21days and subjected to a 12- h feed withdrawal period. The ducklings were euthanized via cervical dislocation followed by manual evisceration after bleeding. Six different muscle tissues were obtained from skinless drumsticks of each duckling and immediately stored at liquid nitrogen until use. Six cell-free-plasma samples were separated from whole blood by centrifugation at 594.2 × *g* for 10 min at 4 °C and freezed with liquid nitrogen and stored at −70 °C until use.

2.2. Isolation of total RNA and synthesis of cDNA

Three different dietary treatment levels (basal ME: 2300; AME: 2900; high ME: 3300 kcal/kg diet) were selected from the eight dietary treatments based on previous estimated AME for Korean native duck (Wickramasuriya et al., 2016) and total RNA was extracted from 100 mg muscle and 500 μl of cell-free plasma using Qiagen RNesy mini kit (Qiagene Inc., Valencia, CA, USA) or AccuPrep®Viral RNA Extraction kit (Bioneer, Daejeon, Republic of Korea). Concentration and quality of RNAs(the ratio of absorbance at 260 nm and 280 nm) isolated from muscle and plasma (two birds per pen, six biological replicates, tree group; The basal ME, AME, higher ME level) were measured by NanoDrop®ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). All the isolated RNA samples were then run on 2% formaldehyde agarose gel to confirm RNA integrity. Non-degraded muscle (n = 8) and plasma RNA samples (n = 8) per each treatment were selected and pooled, respectively. cDNAs were synthesized by the Superscript Reverse Transcriptase Enzyme (Invitrogen, Grand Island, NY, USA) using the pooled 500 ng/μl of total RNAs from muscle and plasma, respectively.

Table 1
Composition of experimental diets (% as fed).

Item	Dietary energy content (AME, kcal/kg)							
	2600	2700	2800	2900	3000	3100	3200	3300
Corn	17.65	22.66	27.68	32.69	37.7	42.71	47.73	52.74
Soybean meal	18	19	20	21	22	23	24	25
Wheat	15	15	15	15	15	15	15	15
Wheat bran	24	20.57	17.14	13.71	10.29	6.86	3.43	–
Oats	12.8	10.97	9.14	7.31	5.49	3.66	1.83	–
Barley	7	6	5	4	3	2	1	–
Vegetable oil	1.4	1.65	1.91	2.16	2.42	2.67	2.93	3.18
Limestone	1.7	1.61	1.53	1.44	1.36	1.27	1.19	1.1
Monocalcium Phosphate	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Salt	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin-mineral premix	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Lysine-HCL	0.2	0.19	0.17	0.16	0.14	0.13	0.11	0.1
L-Arginine	–	0.02	0.04	0.06	0.09	0.11	0.13	0.15
DL-Methionine	0.2	0.22	0.24	0.26	0.29	0.31	0.33	0.35
L-Threonine	0.05	0.07	0.08	0.1	0.12	0.14	0.15	0.17
L-Isoleucine	–	0.01	0.02	0.03	0.05	0.06	0.07	0.08
L-Valine	–	0.02	0.04	0.06	0.07	0.09	0.11	0.13
Calculated composition								
ME, kcal/kg	2600	2700	2800	2900	3000	3100	3200	3300
CP,%	18	18	18	18	18	18	18	18
Available phosphorus, %	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Lysine, %	1	1	1	1	1	1	1	1
Methionine, %	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.6
Analysed composition								
GE, kcal/kg	3089	3180	3270	3389	3490	3579	3690	3806
CP, %	18.2	18.1	18.1	18.2	18.3	18.1	18.2	18.1

2.3. Analysis of gene expression and interaction

qRT-PCR was carried out utilizing a StepOnPlus real-time PCR system (SYBR Green Master Mix; Applied Biosystems, Foster City, CA, USA) and gene-specific designed primers (Bioneer) with four biological and two technical replicates. The primer sequences are listed in Table 2. The relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method. 18S rRNA was used as an endogenous internal control for quantification of target genes. For prediction of gene set function and interaction of differentially expressed genes, gene network analysis was performed using Gene Multiple Association Network Integration Algorithm (GeneMANIA)(http://www.genemania.org/) with automatically selected weighting method (query-dependent weighing) based on human database. In addition, to determine functional enrichments in the differentially expressed genes, Search Tool for the Retrieval of Interacting Genes (STRING) version 10.0 (http://string-db.org/) was used in the *Gallus gallus* database with medium confidence score (0.400).