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# Gene expression profiling of hormonal regulation related to the residual feed intake of Holstein cattle

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ABSTRACT

An accumulation of over a decade of research in cattle has shown that genetic selection for decreased residual feed intake (RFI), defined as the difference between an animal's actual feed intake and its expected feed intake, is a viable option for improving feed efficiency and reducing the feed requirements of herds, thereby improving the profitability of cattle producers. Hormonal regulation is one of the most important factors in feed intake. To determine the relationship between hormones and feed efficiency, we performed gene expression profiling of jugular vein serum on hormonal regulation of Chinese Holstein cattle with low and high RFI coefficients. 857 differential expression genes (from 24683 genes) were found. Among these, 415 genes were up-regulated and 442 genes were down-regulated in the low RFI group. The gene ontology (GO) search revealed 6 significant terms and 64 genes associated with hormonal regulation, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) selected the adipocytokine signaling pathway, insulin signaling pathway. In conclusion, the study indicated that the molecular expression of genes associated with hormonal regulation differs in dairy cows, depending on their RFI coefficients, and that these differences may be related to the molecular regulation of the leptin-NPY and insulin signaling pathways.



## 1. Introduction

Feed efficiency affects the overall profitability of all livestock production systems [1], which can be measured as residual feed intake (RFI). Dairy cattle with low RFI have strong competitive advantages in production efficiency [2,3]. RFI is calculated as the difference between actual and expected feed intake required to support maintenance and production [2]. It should be a heritable indication of metabolism-related differences among animals rather than of differences in production [4]. Reported heritability estimates for RFI in lactating dairy cattle have ranged from 0.01 to 0.38 [3], indicating that RFI could be used as a index of selective breeding [5].

Although influenced by diet and prandial activity, systemic concentrations of key metabolic hormones associated with feed intake, growth, fat accumulation, nutrient repartitioning, and utilization have received attention as potential physiological markers of feed efficiency [6,7]. Perkins et al. (2014) found that neuropeptide-Y (NPY), relaxin-3 (RLN3), the melanocortin 4 receptor (MC4R), and gonadotropin-releasing hormone (GnRH) mRNA expression were 64%, 59%, 58%, and 86% lower, respectively, in the arcuate nucleus of low RFI steers, whereas gonadotropin inhibiting hormone (GnIH) and pro-opiomelanocortin (POMC) mRNA expression was 198% and 350% higher than the high ones. Serum tests among different RFI coefficients have been greatly researched because of the easily obtained samples in dairy cattle [8]. Kelly et al. (2010) measured concentrations of insulin-like growth factor-1 (IGF-1), insulin, and various metabolites in finishing heifers, and found that insulin concentrations were correlated with RFI [9]. Although the potential associations between hormone regulation and RFI might exist, additional comprehensive studies on dairy cows were needed.

Gene expression profiling has been recommended for finding candidate genes and identifying differentially expressed genes for genetically divergent characteristics, such as RFI [10]. The present study used a microarray with 24683 probes to profile the jugular vein serum transcriptome to discover genes that were differentially expressed in Chinese Holstein cattle selected for a high and low RFI. Functional analysis of differentially expressed genes was performed to reveal gene networks likely to contribute to the variation in RFI.

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The results provide deeper insight into the specific regulatory mechanisms underlying feed efficiency in lactating cows.

## 2. Materials and methods

### 2.1. The RFI feedlot tests

The RFI feedlot test was conducted from September 20 to mid-November in 2013 at the Taizhou Dairy Farm of the Milk Industry Group Company of Nanjing, in Jiangsu Province, China. The RFI coefficients of 84 healthy cows in the mid lactation were calculated based on the data of the 57 d feedlot tests. The ages of cows (2.99 ± 0.27, years) and the days in milk (DIM) (132.28 ± 6.08, day) were recorded before the experiment. Cows were managed in a tie-stall configuration and fed individually. They were fed total mixed rations (TMR) and allowed free access to food and water at all times. The feed intake of each animal was measured with feed bins (1.0 m length, 0.65 m wide and 0.5 m high). Daily feed offered and refused were measured (residual amount of feed ≥5%). Live weights (W, kg) of each cow were measured at 7:00 weekly during the experiment. The average daily gain (ADG, kg/d) was calculated by subtracting live weights on the first day of available feed intake data from live weights on the last day of available feed intake data and dividing by the number of days in the test period. Milk yields (kg/d) from all cows were recorded daily using milk flowmeters and milk fat percentage was measured with an infrared milk analyzer (Foss Electric Ltd., U.K.). The 4% fat-corrected milk yield (4% FCM, kg/d) was calculated using "4% FCM (kg) = (0.4 + 15 × milk fat percentage) × milk yield." All procedures involving the animals were approved by the Animal Research Ethics Committee of Nanjing Agriculture University.

### 2.2. RFI calculation and group

In the present study, RFI coefficients (kg/d) of all cows (n = 84) were computed using the actual feed intake (AFI, kg/d) minus the expected feed intake (EFI, kg/d). AFI was determined according to dry matter intake (DMI). The regression model was used to predict the EFI as an unknown dependent variable, given the values of the independent variables, included 4% FCM (4% fat-corrected milk yield), $W^{0.75}$ (metabolic body weight), ADG, DIM and age, and used the stepwise multiple linear regression analysis of SAS (Statistics Analysis System 9.2 Inst. Inc., Cary, NC) (adapted from Ref. [3]). The original statistical model is:

$$Y = \beta0 + \beta1 \times X1 + \beta2 \times X2 + \beta3 \times X3 + \beta4 \times X4 + \beta5 \times X5$$

where Y is EFI, β0 is the equation intercept, β1, β2, β3, β4, β5 are the coefficients of the equation, X1 is the 4% FCM, X2 is the $W^{0.75}$, X3 is the ADG, X4 is the DIM, and X5 is the age. Based on the standard of $P < 0.05$, independent variables, including 4% FCM ($P < 0.000$), $W^{0.75}$ ($P < 0.000$), ADG ($P = 0.02 < 0.05$) and age ($P = 0.04 < 0.05$), were selected by screening in the final regression model, and could explain 85% of the observed variation in EFI. DIM ($P = 0.96 > 0.05$) was rejected by the regression analysis, because it had no significantly effect to the model. Therefore, the best multitrait equation ($R^2 = 0.85$), taking collinearity among variables into account, was EFI = 10.201 + 0.227 × 4% FCM + 0.038 × $W^{0.75}$−7.941 × ADG + 0.134 × age.

The RFI averaged 0.0001 kg/day (n = 84, SD = 0.58) and ranged from −1.04 to +1.07 kg/day, representing a difference of 2.11 kg of feed per day between the least and most efficient animals. From a total of 84 animals, 29 multiparous lactating Holstein cows were selected for the follow-on experiments based on their RFI values. Of the 84 cows, 15 animals that showed lower RFI values (RFI < Mean−SD = −0.58, mean RFI = −0.84 kg/day, efficient) were grouped into the low RFI group. The 14 animals with higher RFI values (RFI > Mean + SD = 0.58, mean RFI = +0.86 kg/day, inefficient) were assigned to the high RFI group (Fig. 1). Table 1 shows the difference in the RFI coefficients between the two groups.

### 2.3. Samples and RNA isolation

Approximately 10 mL of blood samples were collected from the jugular vein of selected cows ($n = 29$) after the 57-d feedlot test period and placed on an ice slab within 5 min to decrease RNA degradation. The sample of serum were frozen in liquid nitrogen immediately after 4000 rpm centrifugation, and then stored at −80 °C until total RNA isolation. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, U.S.), according to the manufacturer's protocol. The 260/280 ratio was obtained to determine RNA concentration and the 260/230 ratio was used to assess purity in the samples. RNA integrity was assessed by electrophoresis on 2% agarose gels (m/v). After purification (QIAGEN RNeasy Kit), the total RNAs of the two RFI groups were separately mixed with equal amounts. The two mixtures of total RNA were used for the microarray assay.

### 2.4. Microarray assay

Gene chip analysis of the Bovine Genome Array was performed by an outside service provider (LC-Bio.CO., LTD, Houston, TX, USA). Total RNA from the serum specimens was individually hybridized with two gene chips. Briefly, in the first-strand cDNA synthesis reaction, 10 mg of total RNA were used for reverse transcription using a T7-oligo (dT) promoter primer. Then, the double−stranded cDNA was synthesized from the first-strand cDNA using One-cycle cDNA Synthesis Kit (Afymetrix). After that, cRNA was synthesized from ds cDNA by adding T7 RNA polymerase. Subsequently, the cRNA was labeled by Cyanine-3-CTP (Cy3). After the Cy3-labeled cRNA was purified (QIAGEN RNeasy Kit) and fragmented, the hybridization probe was obtained. After test, the probe was hybridized to the probe array at 65 °C for 17 h. Thereafter, the probe array was washed and stained on a Fluidics Station, and the microarrays were scanned using a Gene Chip Agilent Scanner G2505C (Agilent Technologies). The Agilent Micro Array Suite Genechip operating software (GCOS) GenePix pro 6.1 was used for quantity analysis of the hybridization.

The gene expression levels that fold change ≥2 and $P$ value ≤ 0.05 between the high and low RFI cattle serum were checked and further analyzed. The Molecule Annotation System (http://david.abcc.ncifcrf.gov/) was used to analyze the differentially expressed genes using the Kyoto encyclopedia of genes and genomes (KEGG) public pathway resource and the Gene Ontology (GO) consortium. The microarray data has been deposited in the Gene Expression Omnibus database (accession number: GSE67176).

### 2.5. RT-PCR

RT-PCR was performed to confirm the microarray results. The total RNA was extracted from the selected high and low RFI cattle ($n = 29$) serum as described above, and the total RNA was reverse transcribed using a Reverse Transcription Levels kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. The expression levels of 10 genes were measured. The 18S rRNA gene was used as an invariant control. Primers were designed using Primer Premier 5.0 (Premier, Canada) and are shown in Table 2. RT-PCR was performed with SYBR® Premix Ex TaqTM (TaKaRa Biotechnology Co., Ltd., Japan). The reaction solution was prepared on ice and consisted of: 10 μL of 2 × SYBR® Premix Ex