Transcriptome changes provide genetic insights into the effects of rearing systems on chicken welfare and product quality

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ABSTRACT: Farm animals raised under free-range (FR) systems are assumed to have improved welfare and higher-quality products that are better to eat than intensively reared animals. However, the modulations are limited in scientific investigations. In this study, we compared 2 rearing systems (FR and cage) and their effects on chickens, including production performance, product quality, body condition, physiological indicators, and gene expression. By using a match-mismatch design in which each treatment was transferred to the other treatment during the last period of the experiment, we aimed to understand the influence of current and former rearing conditions and the ability of individuals to adapt to the current environment. The results indicated that the FR system led to better chicken welfare (e.g., gait score,

feather condition, and physiological indicators, $P < 0.05$) and contributed to higher product quality ($P < 0.05$), although it resulted in poorer production performance ($P < 0.05$) and foot pad condition ($P < 0.05$) than that of the cage rearing system. Additionally, the FR system triggered a series of inner changes and genetic responses in chickens, such as the upregulation of calcium and GnRH signaling, actin and cytoskeleton regulations, immune functions, and developmental processes, and the downregulation of pathological regulations (q -value < 0.05 for all gene ontology terms and $P < 0.05$ for all Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways). In conclusion, rearing systems alter chicken gene expression patterns, which provide a genetic basis for the adaptability to rearing environments and ultimately affects chicken welfare and products.

Key words: chicken, product, rearing system, transcriptome, welfare

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INTRODUCTION

The worldwide demand for high productivity and quality of meat, eggs, milk, and other animal products is increasing, leading to an increasing requirement for natural rearing regimens and better welfare of animals. Product quality information now includes factors such as the welfare of animals during rearing (Broom, 2010, 2014).

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Therefore, livestock products must be produced in a responsible manner that prevents any harmful effects, is sustainable, and leads to high product quality.

For chicken production, consumers appreciate free-range (FR) systems and regard the products (eggs and meat) as being of high quality and good to eat, as well as involving better welfare for the hens (da Silva et al., 2017). Previous surveys have indicated that consumers expressed an interest in FR products for a brand that was marketed as having high welfare for the hens (Pettersson et al., 2016) and they are happy to pay a small premium for safety labeling to appear on chicken products (Ifft et al., 2012). However, the conclusions of studies relating to rearing systems on product quality are exclusive. Comparisons of meat quality between conventional and FR chickens have demonstrated that FR systems are able to modify the meat characteristics and oxidative stability of chickens (Castellini et al., 2002; Funaro et al., 2014). Higher cooking loss and lower slaughter weight of FR chickens have also been shown (Castellini et al., 2002; Tong et al., 2014; Chen et al., 2018). However, some studies have found no obvious changes in carcass traits or meat quality in the FR chickens compared to the conventionally produced chickens (Wang et al., 2009) and suggested that chicken genotypes played an important role in the fatty acid composition of meat in FR farming (Dal Bosco et al., 2012). Free-range rearing has an obvious effect on egg quality, with the eggs from caged chickens able to be differentiated from those from FR chickens by indicators such as shell breaking resistance, overrun, protein content, and shell thickness (Hidalgo et al., 2008). Regarding chicken welfare, studies have been extensively conducted. For example, extensive studies have revealed that FR systems promoted chicken welfare by contributing to more natural behavior including foraging, increasing their motivation to explore, and reducing the incidence of disturbances (Newberry, 1995; Cornetto et al., 2002; Dal Bosco et al., 2014; Rodriguez-Aurrekoetxea et al., 2015). Still, there is a lack of rigorous scientific study in some of these areas, especially regarding what inner changes and genetic responses occur and how they adapt to the rearing environment.

Hence, the present study aimed to provide a better understanding of the consequences of rearing systems by comparing hens produced in FR and cage rearing (CR) systems. During the study, an interconversion of rearing systems was conducted in the last period of the experiment for experimental verification. In brief, the experiment started by

raising 70-d-old Beijing You chickens under parallel FR and CR systems for 210 d. Then, FR chickens were transformed into CR chickens (FR-CR), and CR chickens were transformed into FR chickens (CR-FR) for 35 d. Finally, we analyzed the differences between FR and CR chickens, including production performance, product (egg and meat) quality, body condition, physiological indicators, and gene expression in thigh muscles to determine the inner changes and genetic basis behind their adaption to the FR or CR systems.

MATERIALS AND METHODS

Chickens and Experimental Design

The experimental protocols and animal care were approved by the China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Inspection (approval number: CAU20151205-5).

Ten thousand female Beijing You chicks, which is a Chinese native breed used for both meat and egg production, were randomly collected at day-old (day 0) and reared in a brooder house. At day 70, 200 chickens were equally assigned to 2 production systems: FR and CR. Free-range hens were raised with 10 even-aged cocks in a 4×5 m² indoor house, which was appended with a 5×6 m² outdoor playground fenced with hard wires. The house was equipped with a 50 cm depth fermentation bed and two 2-floor egg boxes containing 24 laying nests (length, width, and height: 0.3 m, 0.35 m, and 0.3 m, respectively) in total. The outdoor playground contained soil without plant cover, with a 2 m² sand bath area and 2 perches (length, width, and height: 2 m, 0.05 m, and 0.3 m, respectively). Free-range chickens had free access to the outdoor playground through a floor window (length and width: 0.8 m and 0.5 m). They had ad libitum access to a water tank (length and width: 1.5 m and 0.2 m) that was cleaned every day. The lighting schedule in the house was 16 h light and 8 h darkness with lights on at 6:00 a.m., and natural lighting in the outdoor area. For the CR system, 2 chickens were reared in each cage (length, width, and height: 0.66 m, 0.37 m, and 0.5 m, respectively) on the top tier of a 3-floor battery cage in a big house. Cage rearing chickens had ad libitum access to water by nipple drinkers. The lighting schedule for CR chickens was the same as the indoor condition for the FR chickens.

All chickens were fed with the same dietary program throughout the experiment. They were fed

twice at 9:00 and 16:00 under the diet composition of 64% corn, 19.6% soybean, 8% barn, 4% premix (i.e., amino acids, vitamins, and trace elements; New Hope Group, China), 4% limestone powder (i.e., heavy calcium carbonate power; New Hope Group, China), and 0.4% mealworm. In addition, finely cut chicory (*Cichorium intybus*) was added at a rate of 5 g of fresh matter per head per day.

On day 280, an interconversion of rearing condition was made. In brief, 30 FR chickens were randomly transferred into the CR system (FR-CR) for 35 d and 30 CR chickens were transferred into the FR system with 3 even-aged cocks (CR-FR) for 35 d. Meanwhile, 30 FR and 30 CR chickens remained until day 315 in their rearing unit during this time.

Measurements of Production Performance

We measured productive indicators during the laying period (day 140 to day 314). From day 140, production performance including feed consumption, egg number, soft-shell egg number, egg weight, and mortality of each group was recorded daily. Then, average daily feed consumption per chicken (g), average egg weight (g), egg feed conversion rate (%), egg yield (%), and mortality (%) were calculated.

Product Quality and Sensory Evaluation

Before the final test, fresh eggs from the same day were collected from each group to measure egg quality using an egg multi-tester EMT-5200 (Robotmation, Japan). On day 315, all hens in each group ($n = 30$) were slaughtered, and leg and pectoral muscles from 10 random hens in each group were collected. For drip loss, the fat and connective tissue attached to the surface were removed and the samples were trimmed to 5 cm $\times$ 3 cm $\times$ 2.5 cm in size and weighed (W1). The meat was stored vertically in an inflatable bag at 4 °C for 48 h and then reweighed (W2). Drip loss (%) = $[(W1 - W2)/W1] \times 100\%$. Additionally, part of the leg and pectoral muscles were stored at 4 °C. After 24 h, they were placed at room temperature (25 to 30 °C) and weighed for approximately 20 g samples (W3). The samples were placed into a water bath (80 °C) in a plastic bag. After the temperature reached 70 °C, the meat was removed and weighed (W4). The cooking loss (%) = $[(W3 - W4)/W3] \times 100\%$. The shear force (kg/cm²) was evaluated on cores (1.27 cm diameter and 3 cm length) obtained from the thickest part of the cooked samples by cutting them perpendicularly to

the direction of the fiber, using an Instron model 5542 (Instron, Boston, MA). Meat color was categorized into light (L*), red (a*), and yellow (b*) degrees. The measurements were taken on the medial surface of each right breast and then averaged using the CIELAB method with a colorimeter (Hunter, Scan XE, Reston, WA).

A total of 60 panelists performed the sensory evaluations. We used a 5-point descriptive scale for standard sensory evaluation on the eggs and meat, where 5 = excellent and 1 = extremely poor. The final products were evaluated for sensory attributes on the same day and cooked under the same conditions. The panelists evaluated samples for appearance, flavor, tenderness, and overall acceptability using scores ranging from 1 to 5.

Body Condition Score

On day 314, all chickens were scored for body conditions, including gait, foot pad dermatitis, and feather condition, using different assessment systems. In particular, a 6-grade evaluation system was used for the assessment, with score 0 being the lowest score and representing the best welfare. For the gait score, the scores 0, 1, 2, 3, 4, and 5 represented normally walk, abnormally walk, obviously lame, be able walk under strong stimulation, unable to walk, and unable to stand, respectively. For foot pad dermatitis, the scores 0, 1, 2, 3, and 4 represent no injury, slight injury on <5% of the pads, a few injuries on 5% to 25% of the pads, moderate injury on 25% to 50% of the pads, and severe injury on >50% of the pads, respectively. The feathers on the head, neck, back, wing, vent, breast, paw, and abdomen were evaluated independently. The scores 1, 2, 3, 4, and 5 represent no harm, little harm but no bare, size of bare < 3 $\times$ 3 cm, size of bare > 3 $\times$ 3 cm, and totally bare, respectively.

Physiological Indicators

On day 315, blood was collected from 30 hens in each group. Approximately 1 mL of fresh blood was removed for blood routine measurement using an automated hematology analyzer HF-3800 (Healife, China). This included lymphocyte (LYM), monocyte (MON), granulocytes (GRA), hemoglobin (HGB), platelets (PLT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell count (RBC), and mean red cell volume (MCV). Additionally, approximately 7 mL of blood was immediately centrifuged at 6,000 $\times$ g for 10 min at room temperature

to obtain serum. Serum indicators including creatine kinase (CK), inorganic phosphorus (P), and immunoglobulins (IgA, IgG, and IgM) were measured with a semiautomatic biochemical analyzer HF-800B (Healife, China).

Transcriptome Sequencing

On day 315, 5 g of right thigh muscle was collected from all hens of each group for transcriptome analyses. Total RNA isolation was performed using TRIzol reagent (Invitrogen, San Diego, CA). After quality control, RNA samples from the same group were mixed and pooled into 3 equidensity solutions. Libraries were prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Beverly, MA) and were then subjected to an Illumina Hiseq platform to generate paired-end 150 base pairs reads.

The raw sequences were quality controlled by removing reads containing adapter and ploy-N and low-quality reads. The *Gallus_gallus*-5.0 (Ensembl release 86) was used as the reference genome and gene model annotation file. An index of the reference genome was built using Bowtie v2.2.3 (Langmead and Salzberg, 2012). Paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 (Trapnell et al., 2009) with 5% mismatches. Gene-level read counts were enumerated using the HTSeq v0.6.1 Python tool. Differential expression analyses were performed using the DESeq R package v1.18.0 (Anders and Huber, 2010). The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach (*q*-value) for controlling the false discovery rate. Genes with *q*-value < 0.05 were

assigned as differentially expressed. Gene ontology (GO) enrichment was implemented by the Goseq R package (Young et al., 2010), and GO terms with *q*-value < 0.05 were considered enriched. DAVID 6.8 software (<https://david.ncifcrf.gov/>) was used for KEGG pathway analyses using *P* < 0.05 as the statistical criteria.

Statistical Analyses

All data are shown as mean $\pm$ standard error (SE) and analyzed by SAS 9.2 (SAS Inst. Inc., Cary, NC). The data of production performance and products quality, and physiological characteristics were checked for normality and homogeneity of variance, of which data in line with the normal distribution were analyzed by 1-way analysis of variance, otherwise by the nonparametric test. Duncan's post hoc test was used to analyze the difference among groups when significance (*P* < 0.05) was detected. The Wilcoxon test was used to analyze body condition score. All values with *P* < 0.05 were regarded as being statistically significant.

RESULTS

Production Performance and Product Quality

Free-range hens had similar body weights to CR hens on day 314; however, they consumed more feed (*P* < 0.001) and produced fewer eggs (*P* < 0.001) with a lower feed conversion rate (*P* < 0.001). Free-range hens had lower mortality and less soft-shelled eggs than that of CR hens (Table 1).

Table 1. Production performance and aspects of product quality in FR and CR chickens

Items	FR	CR
Body weight (kg)	1.88 $\pm$ 0.03	1.83 $\pm$ 0.06
Average feed consumption (g per chicken per day)	101.12 ^b $\pm$ 0.08	84.93 ^a $\pm$ 0.09
Average laying rate (%)	43.09 ^a $\pm$ 0.89	52.34 ^b $\pm$ 1.01
Average egg weight (g)	44.51 $\pm$ 0.09	42.19 $\pm$ 0.07
Feed/egg	5.75 ^b $\pm$ 0.30	3.67 ^a $\pm$ 0.20
Mortality (%)	10	12.77
Soft-shell egg ratio (%)	0.06	3.72
Eggshell strength (kg/cm ²)	3.87 ^b $\pm$ 0.14	3.35 ^a $\pm$ 0.13
Yolk color (degree)	9.58 ^a $\pm$ 0.28	10.66 ^b $\pm$ 0.28
Egg sensory scores	291	194
Drip loss, thigh (%)	1.64 ^b $\pm$ 0.05	0.96 ^a $\pm$ 0.02
Drip loss, breast (%)	1.89 $\pm$ 0.04	1.26 $\pm$ 0.05
Cooking loss, thigh (%)	17.65 ^b $\pm$ 1.06	12.27 ^a $\pm$ 1.04
Cooking loss, breast (%)	16.06 ^b $\pm$ 1.00	12.03 ^a $\pm$ 1.09
Meat sensory scores	270	214

Values with different small letter superscripts mean statistically different (*P* < 0.05, 1-way ANOVA). FR = free-range chickens; CR = caged chickens.

In relation to egg quality, FR hens produced brighter egg yolk colors ($P < 0.01$) and harder eggshells ($P < 0.05$) than those of CR hens (Table 1 and Supplementary Table S1). For meat quality, FR chickens had higher cooking losses for both thigh and breast muscles (both $P < 0.05$) than those of CR hens, and the same trend was found for drip loss in thigh muscles (Table 1 and Supplementary Table S1). Both eggs and meat of FR chickens received higher scores by the taster board than that of CR hens (Table 1).

The interconversion groups (CR-FR and FR-CR) displayed similar effects for chicken products (Supplementary Table S1). CR-FR chickens produced brighter egg yolk colors compared to CR chickens ($P = 0.051$), whereas egg yolk color was darker in FR-CR hens than that in FR hens ($P = 0.062$). The cooking losses for both thigh and breast muscles were higher in FR hens (CR-FR) than that in CR hens (FR-CR) (all $P < 0.05$).

Evaluations of Body Condition

Free-range hens usually showed normal gaits, whereas CR hens could hardly walk normally and some individuals were unable to stand up (Fig. 1a). For the interconversions, the gait scores improved

in CR-FR chickens but were worse in FR-CR hens (Fig. 1a). However, the FR chickens received higher scores for foot pad dermatitis than those in the CR group, and short-term living in the CR system did not restore injuries (Fig. 1b). The feather condition on the neck was better in FR chickens, whereas the feathers on heads and claws in the CR group were better (Fig. 1c). Feathers on both heads and claws got worse in CR-FR than in CR chickens, and were restored in FR-CR hens compared to the FR group (Fig. 1c).

Physiological Indicators

Free-range hens had similar levels for all physiological indicators to CR hens. For the interconversions, FR hens had higher CK levels than that of FR-CR hens ($P < 0.05$), whereas CR hens exhibited differences from CR-FR values for HGB, RBC, MCHC, MCV, and CK levels (all $P < 0.05$) (Supplementary Table S2).

Different Gene Expression Analysis of Thigh Muscle

In total, we obtained 55,220,048 to 69,055,504 paired-end reads for each sample (Supplementary

Figure 1. Body conditions of chickens in different rearing systems. (a) The gait score, (b) foot pad dermatitis score, and (c) feather condition score. The higher score indicates the poorer welfare level. FR = free-range chickens; CR = caged chickens; CR-FR = chickens transformed from CR to FR; FR-CR = chickens transformed from FR to CR.

Table S3). The raw data were deposited in the BIG Data Center (<http://bigd.big.ac.cn/>) with GSA accession no. CRA000838, CRA000839, CRA000841, and CRA000843. The Pearson correlation (R^2) of the biologically repeats of each group was higher than 0.99. A total of 23,831 genes were differentially analyzed between FR and CR, and 1,636 differentially expressed genes (DEGs) were identified (q -value < 0.05). Between CR-FR and CR, 23,623 genes were differentially analyzed and 944 DEGs were founded. In addition, 24,003 genes were differentially analyzed between FR and FR-CR, and 559 DEGs were founded. The up/down gene expression patterns of each comparison are displayed in Supplementary Table S4.

GO and Pathway Analyses

The DEGs between FR and CR were analyzed by GO enrichment, with 276 GO terms obtained (q -value < 0.05). The most significant GO terms that were upregulated in FR chickens were classed into immune function and developmental processes (Fig. 2), whereas the downregulated were mainly involved in the proteasome, several catabolic processes, and threonine-type metabolic enzymes (Fig. 3). Afterwards, the pathways relative to the immune system and developmental processes were upregulated in FR chickens to CR, including phagosome, focal adhesion, Wnt signaling, calcium signaling, and FoxO signaling (Table 2). Besides, a

hormone-relative pathway, the GnRH signaling pathway, was also upregulated in FR chickens (Table 2). The pathway of proteasome and insulin signaling, a metabolism-related pathway, was downregulated in FR compared to CR chickens (Table 2). The DEGs between CR-FR and CR were enriched onto 64 GO terms. CR-FR hens were upregulated in several cellular components, vasculature development, and factor binding (Supplementary Fig. S1), whereas they were downregulated in proteasome, steroid biosynthetic processes, several catabolic processes, and threonine-type metabolic enzymes (Supplementary Fig. S2). Similar to the comparison of FR and CR hens, CR-FR hens were found to upregulate in the KEGG pathways for focal adhesion, regulation of actin cytoskeleton, and the extracellular matrix (ECM)-receptor interaction, whereas they downregulated in the pathway of proteasome compared to CR hens (Table 2). Additionally, in CR-FR chickens there was downregulation of the metabolism-related pathway of steroid biosynthesis (Table 2).

Free-range hens had 559 DEGs to the FR-CR group. Further, 73 GO terms were enriched, all of which were upregulated in FR chickens and mainly involved in developmental processes (Supplementary Fig. S3). Similar to the previous comparisons, the pathway of the proteasome was downregulated, whereas the pathways of focal adhesion and phagosome were upregulated in FR chickens (Table 2). Furthermore, the developmental process-related pathway, dorsoventral axis formation, was upregulated in FR chickens (Table 2).

Figure 2. The most upregulated GO terms in a comparison of free-range chickens (FR) with caged chickens (CR) in thigh muscle. Asterisk (*) indicates q -value < 0.05 .

Figure 3. The most downregulated GO terms in a comparison of free-range chickens (FR) with caged chickens (CR) in thigh muscle. Asterisk (*) indicates q -value < 0.05.

Table 2. Comparison of different KEGG pathways across free-range and caged chickens

Comparison	KEGG pathway	Enrichment fold	P-value	Regulation
FR vs. CR	Phagosome	2.05	0.003	Up
	Focal adhesion	1.88	0.002	Up
	Wnt signaling pathway	1.83	0.020	Up
	Calcium signaling pathway	1.67	0.023	Up
	FoxO signaling pathway	1.71	0.043	Up
	GnRH signaling pathway	2.00	0.034	Up
	Insulin signaling pathway	1.79	0.031	Down
	Proteasome	7.13	0.000	Down
CR-FR vs. CR	Focal adhesion	2.94	0.000	Up
	Regulation of actin cytoskeleton	2.09	0.020	Up
	ECM-receptor interaction	3.90	0.001	Up
	Proteasome	9.48	0.000	Down
	Steroid biosynthesis	8.37	0.002	Down
FR vs. FR-CR	Phagosome	3.63	0.001	Up
	Focal adhesion	2.46	0.018	Up
	Dorsoventral axis formation	8.24	0.011	Up
	Proteasome	5.04	0.043	Down

FR = free-range chickens; CR = caged chickens; CR-FR = chickens transformed from CR to FR; FR-CR = chickens transformed from FR to CR. The down/up regulation is shown as free-range chickens (FR and CR-FR) to caged (CR and FR-CR).

DISCUSSION

The present study revealed that the FR system contributed to better chicken welfare and product qualities and flavors, including better gait score, lower mortality, less soft-shelled eggs, brighter egg yolk color, higher drip loss and cooking loss of muscles, and higher sensory scores for eggs and meat. However, the FR system led to lower egg production and higher feed consumption by chickens (Table 1 and Supplementary Table S1).

The disabilities of CR hens in walking and even standing up have been shown to be related to bone damage and weakness (Knowles and Broom, 1990; Tuytens et al., 2008; Yilmaz Dikmen et al., 2016), which reflects poor welfare levels. The wire materials used in the CR system result in poor conditions of feathers on the head and claws. Cocks were present in the FR group that provided the opportunity for mating behavior in FR chickens. This was evidenced by the upregulation of the GnRH signaling pathway, which is related to gonadal hormonal

levels (Kraus et al., 2001), in FR compared to CR chickens (Table 2). In addition, because of this the neck and anus feather conditions of the FR chickens were underscored compared to the CR chickens. Inappropriate management such as heavy moisture of padding (Dawkins et al., 2004) and the opportunity to encounter extreme weather affects the foot pad dermatitis in FR chickens (Stadig et al., 2017). These results reflect the apparent ethological patterns related to more outdoor activities for FR hens (Yang et al., 2017) or confinement in cramped cages for CR chickens. Indeed, higher serum CK levels were found in FR chickens than in the interconverted CR chickens (FR-CR), which indicated more exercise under the FR rearing system (Hicks et al., 2017).

The upregulation of calcium signaling in the gene expression of thigh muscles (Table 2) provides a molecular explanation for the harder eggshell, better meat quality, and foraging activities of FR chickens. The freedom to move outdoors produces better calcium metabolism from the benefit of sunlight and intake from the environment. Additionally, vitamin D₃ content, which is good for calcium absorption, has been proven to be 3- to 4-fold higher in the egg yolks of FR chickens than that of CR chickens (Kuhn et al., 2014). In turn, higher calcium intake suggests that FR hens undertake more foraging activities and have higher feed consumption (Christensen et al., 2003), which makes their bones stronger (Leyendecker et al., 2005; Shipov et al., 2010; Aguado et al., 2015). On the contrary, due to the stress encountered, CR hens have increased physiological responses and biochemical changes, which leads to abnormal sarcoplasmic calcium ion homeostasis and bone-related calcium metabolism or PSE-like meat (Xing et al., 2017; Sun et al., 2018). In addition, the results provide genetic understanding for the higher cooking loss and drip loss of meat in the FR hens (Table 1). Meat with higher cooking loss and drip loss suggests better water-holding capacity, which means the meat is healthier with less fat and contains a higher content of n-3 polyunsaturated fatty acids (Castellini et al., 2002; Funaro et al., 2014). In addition, the upregulation of the actin cytoskeleton, which is fundamental for various motile and morphogenetic processes in cells (Hilpela et al., 2004), was found in the short-term transformation from CR-FR to CR chickens in thigh muscles (Table 2). This reflects the benefit to meat quality and health due to increased exercise in the FR system. Moreover, these findings give a reasonable understanding on the lower production performance of FR hens, especially by considering the upregulation of pathways related to metabolism, absorption, and biosynthesis in FR hens (Table 2).

In contrast, the CR hens had upregulations of genes that involved GO terms and pathways of steroidogenesis, proteasome, and insulin signaling, which may lead to acute or chronic regulations of pathological changes (Ghayee and Auchus, 2007). It has been reported that the ubiquitin-proteasome system is involved in disease pathogenesis, including Alzheimer's disease and amyotrophic lateral sclerosis in humans and also in chickens (Ciechanover and Brundin, 2003). Further, caged hens are found to have downregulations of the insulin signaling pathway, which is demonstrated for Alzheimer's disease as well (Liu et al., 2011). Meanwhile, the downregulation of immune genes and pathways makes caged hens susceptible to potential pathogenic factors, which relate to the previous studies of chickens (Erhard et al., 2000).

Traceability is well known to use for animal well-being (Smith et al., 2005) and precise agriculture (Kondo, 2010), and has recently been demonstrated to be driven by food safety regulations (Lavelli, 2013). In the present study, the different aspects of production performance, product quality, body condition, and physiological indicators between CR and FR chickens have been well documented by a match-mismatch designed experiment and reasonably explained by their gene expression profiles. Therefore, the gait score, feather condition, and serum CK levels could be used as biological markers to validate traceability systems of FR chicken products and chicken welfare.

CONCLUSIONS

A FR rearing system implicates better chicken welfare (e.g., gait score, feather condition, and physiological indicators) and contributes to better product quality, although it creates poorer production performance and foot pad condition. The FR system triggers a series of inner changes and genetic responses in chickens, such as the upregulations of calcium and GnRH signaling, actin and cytoskeleton regulations, immune functions and developmental processes, and downregulations of pathological regulations. In conclusion, rearing systems alter chicken gene expression patterns, which provides genetic basis to the adaptability to rearing environments and ultimately affects chicken welfare and products.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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LITERATURE CITED

- Aguado, E., F. Pascaretti-Grizon, E. Goyenvale, M. Audran, and D. Chappard. 2015. Bone mass and bone quality are altered by hypoactivity in the chicken. *PLoS One* 10:e0116763. doi:10.1371/journal.pone.0116763
- Anders, S., and W. Huber. 2010. Differential expression analysis for sequence count data. *Genome Biol.* 11:R106. doi:10.1186/gb-2010-11-10-r106
- Broom, D. M. 2010. Animal welfare: an aspect of care, sustainability, and food quality required by the public. *J. Vet. Med. Educ.* 37:83–88. doi:10.3138/jvme.37.1.83
- Broom, D. M. 2014. Sentience and animal welfare. CABI, Wallingford, United Kingdom.
- Castellini, C., C. Mugnai, and A. Dal Bosco. 2002. Effect of organic production system on broiler carcass and meat quality. *Meat Sci.* 60:219–225. doi:10.1016/S0309-1740(01)00124-3
- Chen, S., H. Xiang, X. Zhu, H. Zhang, D. Wang, H. Liu, J. Wang, T. Yin, L. Liu, M. Kong, et al. 2018. Free dietary choice and free-range rearing improve the product quality, gait score, and microbial richness of chickens. *Animals* 8:84. doi:10.3390/ani8060084
- Christensen, J. W., B. L. Nielsen, J. F. Young, and F. Noddegaard. 2003. Effects of calcium deficiency in broilers on the use of outdoor areas, foraging activity and production parameters. *Appl. Anim. Behav. Sci.* 82:229–240. doi:10.1016/S0168-1591(03)00056-X
- Ciechanover, A., and P. Brundin. 2003. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron* 40:427–446. doi:10.1016/S0896-6273(03)00606-8
- Cornetto, T., I. Estevez, and L. W. Douglass. 2002. Using artificial cover to reduce aggression and disturbances in domestic fowl. *Appl. Anim. Behav. Sci.* 75:325–336. doi:10.1016/S0168-1591(01)00195-2
- da Silva, D. C. F., A. M. V. de Arruda, and A. A. Gonçalves. 2017. Quality characteristics of broiler chicken meat from free-range and industrial poultry system for the consumers. *J. Food Sci. Technol.* 54:1818–1826. doi:10.1007/s13197-017-2612-x
- Dal Bosco, A., C. Mugnai, A. Rosati, A. Paoletti, S. Caporali, and C. Castellini. 2014. Effect of range enrichment on performance, behavior and forage intake of free-range chickens. *J. Appl. Poult. Res.* 23:137–145. doi:10.3382/japr.2013-00814
- Dal Bosco, A., C. Mugnai, S. Ruggeri, S. Mattioli, and C. Castellini. 2012. Fatty acid composition of meat and estimated indices of lipid metabolism in different poultry genotypes reared under organic system. *Poult. Sci.* 91:2039–2045. doi:10.3382/ps.2012-02228
- Dawkins, M. S., C. A. Donnelly, and T. A. Jones. 2004. Chicken welfare is influenced more by housing conditions than by stocking density. *Nature* 427:342–344. doi:10.1038/nature02226
- Erhard, M. H., H. Ozpinar, T. Bilal, I. Abas, C. Kutay, H. Eseceli, M. Stangassinger, and Y. Abbas. 2000. The humoral immune response and the productivity of laying hens kept on the ground or in cages. *Altern. Lab. Anim.* 28:699–705.
- Funaro, A., V. Cardenia, M. Petracci, S. Rimini, M. T. Rodriguez-Estrada, and C. Cavani. 2014. Comparison of meat quality characteristics and oxidative stability between conventional and free-range chickens. *Poult. Sci.* 93:1511–1522. doi:10.3382/ps.2013-03486
- Ghayee, H. K., and R. J. Auchus. 2007. Basic concepts and recent developments in human steroid hormone biosynthesis. *Rev. Endocr. Metab. Disord.* 8:289–300. doi:10.1007/s11154-007-9052-2
- Hicks, K. M., G. L. Onambele-Pearson, K. Winwood, and C. I. Morse. 2017. Muscle-tendon unit properties during eccentric exercise correlate with the creatine kinase response. *Front. Physiol.* 8:657. doi:10.3389/fphys.2017.00657
- Hidalgo, A., M. Rossi, F. Clerici, and S. Ratti. 2008. A market study on the quality characteristics of eggs from different housing systems. *Food Chem.* 106:1031–1038. doi:10.1016/j.foodchem.2007.07.019
- Hilpela, P., M. K. Vartiainen, and P. Lappalainen. 2004. Regulation of the actin cytoskeleton by PI(4,5)P-2 and PI(3,4,5)P-3. *Curr. Top. Microbiol. Immunol.* 282:117–163. doi:10.1007/978-3-642-18805-3_5
- Ifft, J., D. Roland-Holst, and D. Zilberman. 2012. Consumer valuation of safety-labeled free-range chicken: results of a field experiment in Hanoi. *Agr. Econ.* 43:607–620. doi:10.1111/j.1574-0862.2012.00607.x
- Knowles, T. G., and D. M. Broom. 1990. Limb bone strength and movement in laying hens from different housing systems. *Vet. Rec.* 126:354–356. doi:10.1136/vr.126.15.354
- Kondo, N. 2010. Automation on fruit and vegetable grading system and food traceability. *Trends Food Sci. Tech.* 21:145–152. doi:10.1016/j.tifs.2009.09.002
- Kraus, S., Z. Naor, and R. Seger. 2001. Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor. *Arch. Med. Res.* 32:499–509.
- Kuhn, J., A. Schutkowski, H. Kluge, F. Hirche, and G. I. Stangl. 2014. Free-range farming: a natural alternative to produce vitamin D-enriched eggs. *Nutrition* 30:481–484. doi:10.1016/j.nut.2013.10.002
- Langmead, B., and S. L. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9:357–359. doi:10.1038/nmeth.1923
- Lavelli, V. 2013. High-warranty traceability system in the poultry meat supply chain: a medium-sized enterprise case study. *Food Control.* 33:148–156. doi:10.1016/j.foodcont.2013.02.022
- Leyendecker, M., H. Hamann, J. Hartung, J. Kamphues, U. Neumann, C. Sürie, and O. Distl. 2005. Keeping laying hens in furnished cages and an aviary housing system

- enhances their bone stability. *Br. Poult. Sci.* 46:536–544. doi:10.1080/00071660500273094
- Liu, Y., F. Liu, I. Grundke-Iqbal, K. Iqbal, and C. X. Gong. 2011. Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. *J. Pathol.* 225:54–62. doi:10.1002/path.2912
- Newberry, R. C. 1995. Environmental enrichment - increasing the biological relevance of captive environments. *Appl. Anim. Behav. Sci.* 44:229–243. doi:10.1016/0168-1591(95)00616-Z
- Pettersson, I. C., C. A. Weeks, L. R. M. Wilson, and C. J. Nicol. 2016. Consumer perceptions of free-range laying hen welfare. *Brit. Food J.* 118:1979–1993.
- Rodriguez-Aurrekoetxea, A., E. H. Leone, and I. Estevez. 2015. Effects of panels and perches on the behaviour of commercial slow-growing free-range meat chickens. *Appl. Anim. Behav. Sci.* 165:103–111. doi:10.1016/j.applanim.2015.02.004
- Shipov, A., A. Sharir, E. Zelzer, J. Milgram, E. Monsonego-Ornan, and R. Shahar. 2010. The influence of severe prolonged exercise restriction on the mechanical and structural properties of bone in an avian model. *Vet. J.* 183:153–160. doi:10.1016/j.tvjl.2008.11.015
- Smith, G. C., J. D. Tatum, K. E. Belk, J. A. Scanga, T. Grandin, and J. N. Sofos. 2005. Traceability from a US perspective. *Meat Sci.* 71: 174–193. doi:10.1016/j.meatsci.2005.04.002
- Stadig, L. M., T. B. Rodenburg, B. Ampe, B. Reubens, and F. A. M. Tuytens. 2017. Effects of shelter type, early environmental enrichment and weather conditions on free-range behaviour of slow-growing broiler chickens. *Animal* 11:1046–1053. doi:10.1017/S1751731116002172
- Sun, Z. W., Q. H. Fan, X. X. Wang, Y. M. Guo, H. J. Wang, and X. Dong. 2018. High stocking density alters bone-related calcium and phosphorus metabolism by changing intestinal absorption in broiler chickens. *Poult. Sci.* 97:219–226. doi:10.3382/ps/pex294
- Tong, H. B., Q. Wang, J. Lu, J. M. Zou, L. L. Chang, and S. Y. Fu. 2014. Effect of free-range days on a local chicken breed: growth performance, carcass yield, meat quality, and lymphoid organ index. *Poult. Sci.* 93:1883–1889. doi:10.3382/ps.2013-03470
- Trapnell, C., L. Pachter, and S. L. Salzberg. 2009. Tophat: discovering splice junctions with RNA-seq. *Bioinformatics* 25:1105–1111. doi:10.1093/bioinformatics/btp120
- Tuytens, F., M. Heyndrickx, M. De Boeck, A. Van Nuffel, E. Van Poucke, E. Van Coillie, S. Van Dongen, and L. Lens. 2008. Broiler chicken health, welfare and fluctuating asymmetry in organic versus conventional production systems. *Livest. Sci.* 113:123–132. doi:10.1016/j.livsci.2007.02.019
- Wang, K. H., S. R. Shi, T. C. Dou, and H. J. Sun. 2009. Effect of a free-range raising system on growth performance, carcass yield, and meat quality of slow-growing chicken. *Poult. Sci.* 88:2219–2223. doi:10.3382/ps.2008-00423
- Xing, T., X. Zhao, P. Wang, H. Chen, X. Xu, and G. Zhou. 2017. Different oxidative status and expression of calcium channel components in stress-induced dysfunctional chicken muscle. *J. Anim. Sci.* 95:1565–1573. doi:10.2527/jas.2016.0868
- Yang, H., X. Wu, and M. Wang. 2017. The effect of three different meditation exercises on hypertension: a network meta-analysis. *Evid. Based Complement. Alternat. Med.* 2017:9784271. doi:10.1155/2017/9784271
- Yilmaz Dikmen, B., A. İpek, Ü. Şahan, M. Petek, and A. Sözcü. 2016. Egg production and welfare of laying hens kept in different housing systems (conventional, enriched cage, and free range). *Poult. Sci.* 95:1564–1572. doi:10.3382/ps/pew082
- Young, M. D., M. J. Wakefield, G. K. Smyth, and A. Oshlack. 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol.* 11:R14. doi:10.1186/gb-2010-11-2-r14