# Genome-wide transcriptome profiling reveals the mechanisms underlying muscle group–specific phenotypic changes under different raising systems in ducks

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**ABSTRACT** Although a number of nongenetic factors have been reported to be able to modulate skeletal muscle phenotypes in meat-type birds, neither the underlying mechanisms nor the muscle group–specific phenotypic and molecular responses have been fully understood. In the present study, a total of 240 broiler ducks were used to compare the effects of floor raising system (**FRS**) and net raising system (**NRS**) on the physicochemical properties and global gene expression profiles of both breast and thigh muscles at the posthatching week 4 (**W4**), W8, and W13. Our results showed that compared with FRS, NRS generally induced higher pH, lower lightness (L*) and yellowness (b*), lower drip loss and cooking loss, and lower shear force in either breast or thigh muscles during early posthatching stages but subsequently showed less pronounced or even reverse effects. Meanwhile, it was observed that the raising system differently changed the myofiber characteristics depending on the muscle group and the developmental stage. Genome-wide transcriptome analysis showed that compared with FRS, NRS induced the most extensive gene expression changes in breast muscle (**BM**) at W4 but in thigh muscle (**TM**) at W13, suggesting the asynchronous molecular responses of BM and TM to the raising system and period. Most of differentially expressed genes in either BM or TM between NRS and FRS were enriched in the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes terms associated with regulation of muscle cellular functions, metabolic and contractile activities, and tissue remodeling, indicating similar molecular mechanisms principally responsible for the raising system-caused phenotypic changes in both muscle groups. Nevertheless, several crucial pathways (e.g., adipocytokine signaling, AGE-RAGE signaling, and apoptosis) and genes (e.g., ANO6, ACER2, UCP3, DTL, and TMEM120A) were tightly related to the muscle group–specific adaptive remodeling on different raising systems. These data could not only contribute to a better understanding of the molecular mechanisms behind meat quality but also provide novel insights into the molecular causes of the muscle group–specific adaptive remodeling in response to environmental stimuli.

**Key words:** duck, skeletal muscle, meat quality, transcriptome analysis, raising system

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## INTRODUCTION

In accordance with the data released by the FAO in 2019, poultry meat continues to be the most consumed animal source, accounting for approximately 40% of meat production worldwide; moreover, owing to the widespread outbreak of African swine fever, consumer demand for poultry meat is increasing rapidly, especially in Asia. Skeletal muscle, as a major contributor to body weight and the most economically valuable tissue in meat-type birds, enhancing its production efficiency, has been regarded as the first priority of poultry producers, researchers, and other related community over the past half-century (Petracci and Cavani, 2012). However, with the improved standard of living, meat quality has received tremendous attention and become one of the most important factors affecting consumer preferences in recent years (Petracci et al., 2017). It is well known that the quality of meat relies on the physicochemical properties of skeletal muscle, including pH, color, water holding capacity, cooking loss, and shear force, which are widely influenced by a number of genetic (e.g., species, breed, and sex) and nongenetic factors (e.g., age,



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nutrition, and raising system) and are finely regulated at the molecular level (Fletcher, 2002; Mir et al., 2017). Thus, a better understanding of the molecular mechanisms by which these factors affect the physicochemical characteristics of skeletal muscle will be of a great value to improve meat quality through genetic and environmental manipulations.

In poultry, breast muscle (**BM**) and thigh muscle (**TM**) are 2 principal groups of skeletal muscles and vary significantly in the physicochemical properties, metabolic activities, and nutrient values (Zhang et al., 2012; Chen et al., 2016). These differences have been previously demonstrated to be associated with muscle fiber number, density, cross-sectional area, and composition (Von Lengerken et al., 2002; Zierath and Hawley, 2004). In accordance with characteristic contractile and metabolic activities as well as distinct expression patterns of myosin heavy chain isoforms, muscle fibers are divided into 3 major subtypes: type I (slow-twitch, oxidative metabolism, and higher levels of capillaries, lipids, mitochondria, and myoglobin), type IIA (fast-twitch, oxidative/glycolytic metabolism, and intermediate levels of capillaries, lipids, mitochondria, and myoglobin), and type IIB (fast-twitch, glycolytic metabolism, and lower levels of capillaries, lipids, mitochondria, and myoglobin) (Zierath and Hawley, 2004; Talbot and Maves, 2016). These distinct characteristics impart each muscle fiber type's unique functional properties and differential responses to environmental stimuli. Intriguing, the proportions of fiber types constituting a particular muscle group are plastic, which enables muscles to adapt to different circumstances by remodeling muscle fiber composition (Talbot and Maves, 2016). For instance, in skeletal muscles of humans, mice, zebrafish, and chickens, it has been shown that extrinsic factors including exercise training, nutritional status, and disease states were able to alter the proportions of fiber types and hence the physicochemical properties (Zierath and Hawley, 2004; Handschin et al., 2007; Velleman, 2015). Because there is overwhelming evidence in poultry that BM predominantly comprises glycolytic (white, type IIB) fiber while the major type of TM is oxidative (red, type I) fiber (Xiong, 1994), it is supposed that these 2 skeletal muscle groups have distinct physicochemical and molecular responses to environmental stimuli. In support of this, a growing body of literature has indicated that the raising system, as a major nongenetic factor, not only affects poultry skeletal muscle yield and meat quality but also induces gene expression changes (Liu et al., 2011; Almasi et al., 2015; Xiang et al., 2018; Zhang et al., 2018). In addition, numerous protein-coding and noncoding RNA genes have been identified by high-throughput sequencing to play important roles in regulating either the embryonic or posthatching development of skeletal muscle (Li et al., 2012; Li et al., 2017), and particularly, key factors and pathways that act via the myogenic regulatory gene *MYOD1* have been well defined (Talbot and Maves, 2016). However, how genome-wide transcriptome changes are associated with distinct adaptive remodeling of BM and TM has not yet been deciphered.

To achieve this goal, we took Nonghua duck, one of the most economically valuable broiler duck breeds in Southwest China and showing superior meat quality and strong disease resistance, as the experimental model to compare the effects of 2 raising systems (i.e., floor raising system [**FRS**] and net raising system [**NRS**]) on both the physicochemical properties and genome-wide gene expression profiles of BM and TM during early posthatching development (i.e., at week 4, 8, and 13 after hatching). These results are expected to reveal crucial genes and pathways affecting meat quality and to provide novel insights into the molecular causes of the muscle group–specific adaptive remodeling in response to extrinsic stimuli.

## MATERIALS AND METHODS

### Ethics Statement

All experimental procedures involving the manipulation of birds were conducted in concordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). This study was reviewed and approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Chengdu campus, Sichuan, China).

### Experimental Birds, Design, and Tissue Collection

A total of 240 male Nonghua ducks (*Anas platyrhynchos*, one broiler duck breed in Southwest China), hatched from the same batch of fertilized eggs obtained at the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University (Ya'an campus, Sichuan, China) and during the first 13 wk after hatching, were used in the present study. All hatched birds were raised on the same indoor-net brooding systems during the first 2 wk after hatching, and thereafter, were randomly and equally assigned to 2 raising systems: indoor-floor raising system (FRS) and indoor-net raising system (NRS). In detail, ducks under FRS were raised in the indoor pens, whereas those under NRS were raised in the net beds made with stainless steel wires, with a height of 50 cm above the ground floor. These 2 raising systems were placed into the same house to ensure similar surrounding environmental conditions, and meanwhile, all birds were fed with the same diet formulated based on the nutrient requirements of broiler ducks and were provided with free access to food and water. Besides, the stocking density was set at 5 ducks per square meter for both systems throughout this experiment, and it was maintained stably after every sampling by shrinking the raising area. At week 4 (**W4**), 8 (**W8**), and 13 (**W13**) after hatching, 30 ducks from each system were randomly selected and slaughtered after a 12-hour period of fasting, and the left-side breast and thigh muscles were then removed from each duck and stored at −4°C or −80°C until further analyses.

### Assessment of Meat Quality Properties

All collected muscle samples were subjected to pH, meat color, drip loss, cooking loss, and shear force analyses. The initial (pH1) and final (pH2) values of each sample were measured in triplicate at 45 min and 24 h postmortem, respectively, using a portable pH meter (PH-STAR, Denmark) by placing the electrode midway through the thickest part. Meat color (L*, lightness; a*, redness; b*, yellowness) at 45 min postmortem was determined in triplicate for each sample using a colorimeter (CR-300; Minolta Camera, Osaka, Japan). For drip loss analysis, after removing the surface-attached fat and connective tissue, each muscle sample was manually trimmed into a rectangle shape and weighed (**W1**). Subsequently, each sample was placed into an inflatable bag and hanged vertically at 4°C for 24 h and ultimately weighed (**W2**). Drip loss (%) = [(W1 − W2)/W1] × 100%. For cooking loss analysis, the muscle samples were weighed and placed individually in the plastic bags and were subsequently cooked in water bath at 80°C for about 30 min to reach an internal minimal temperature of 75°C. Then, the samples were cooled in cold water and dried with tissue paper. The cooking loss was estimated as the percentage of the lost weight of the samples during cooking with respect to the initial weight. For shear force analysis, after removing the surface-attached fat and connective tissue, the muscle samples were manually trimmed into rectangle shapes along the direction of the fiber, with the size of at least 2 cm × 1 cm × 1 cm. Thereafter, the shear force values were measured using a digital texture analyzer produced by American FTC Company.

### Histological Analysis

The BM and TM tissues from 3 individuals per sampling time per raising system were subjected to histological analysis. Specifically, all muscle samples were 4% formaldehyde-fixed for 72 h at room temperature, dehydrated through a graded ethanol series, transferred to xylene, and embedded in paraffin wax. Paraffin sections of 5 μm thickness from each sample were stained with hematoxylin–eosin and photographed under a Nikon 90i microscope (Nikon, Japan). Myofiber characteristics were further analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD).

### RNA-seq Library Preparation, Sequencing, and Analysis

For genome-wide transcriptome analysis, the BM or TM tissues from 3 individuals at each sampling time per raising system were pooled for one biological replicate, and 3 replicates for each muscle group at each sampling time per raising system were processed for construction of RNA-seq libraries (36 libraries in total). Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Invitrogen, Carlsbad, CA) following the manufacturers' protocol. The libraries were prepared using the Illumina TruSeq mRNA Sample Preparation Kit (Illumina, San Diego, CA) following the manufacture's recommendation and were sequenced on an Illumina Hiseq X-Ten platform. The sequencing quality was assessed with the FastQC v0.11.8 software, and the clean reads were obtained by removing the adapter sequences, reads with >5% ambiguous bases, and low-quality reads with a Q-value < 20%. The clean reads were then aligned to the duck reference genome (https://www.ncbi.nlm.nih.gov/assembly/GCA_003850225.1) using the HISAT2 v2.0.0 software. The mRNA abundance was expressed as the fragments per kilobase of exon per million fragments mapped, and differentially expressed genes (**DEG**) between pairwise comparisons were identified using the DESeq2 (v1.16.0) package in R (v3.4.0) software, under the criteria of |fold change| ≥ 1.5 and false discovery rate < 0.05. Hierarchical clustering analysis was performed using R (v3.4.0). The Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) enrichment analyses of DEG were performed based on the GO (http://geneontology.org/) and KEGG (https://www.kegg.jp/) databases, respectively, and significant GO and KEGG terms were identified using the hypergeometric test. The transcriptomic data obtained in the present study are available in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) at NCBI, with the BioProject ID: PRJNA625897 and SRA Accession Number: SRR11560410-11560445.

### Statistical Analysis

All data were expressed as mean ± SEM. Statistical comparisons between NRS and FRS were analyzed by Student t-test using SAS 9.4 (SAS Institute, Cary, NC). *P*-values below 0.05 and 0.01 were considered statistically significant and extremely significant, respectively.

## RESULTS

### Comparison of the Physical Properties of Duck Breast and Thigh Muscles Under Two Raising Systems

The physical properties of duck muscles were assessed on the basis of pH values, meat color, drip loss, cooking loss, and shear force. As shown in Table 1, the raising system had significant but different effects on these physical indicators depending on the muscle group and the raising period. In detail, compared with FRS, NRS significantly increased ($P < 0.01$) pH1 and pH2 values of both muscle groups at W4 and those of TM at W8, did not change ($P > 0.05$) those of BM at W8 and those of TM at W13, and significantly decreased ($P < 0.01$) pH1 value of BM at W13. The BM of NRS ducks exhibited a lower L* value than that of FRS ducks at W8 ($P < 0.05$), whereas compared with that of FRS ducks, the TM of NRS ducks exhibited a lower L* value at W4 ($P < 0.01$) but a higher L* value at W13

**Table 1.** Effects of 2 raising systems on the physical properties of duck breast and thigh muscles.

| Indicator | Breast muscle | | | | | | | | Thigh muscle | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | W4 | | W8 | | W13 | | W4 | | W8 | | W13 | | | | | |
| | NRS | FRS | NRS | FRS | NRS | FRS | NRS | FRS | NRS | FRS | NRS | FRS | NRS | FRS | NRS | FRS |
| pH1 | 6.85 ± 0.05** | 6.56 ± 0.05 | 5.72 ± 0.03 | 5.62 ± 0.06 | 5.82 ± 0.03 | 5.98 ± 0.05** | 6.99 ± 0.06** | 6.42 ± 0.03 | 6.49 ± 0.04** | 5.95 ± 0.06 | 6.4 ± 0.04 | 6.45 ± 0.04 |
| pH2 | 6.75 ± 0.07** | 6.47 ± 0.04 | 5.92 ± 0.03 | 5.86 ± 0.03 | 6.16 ± 0.05 | 6.01 ± 0.04 | 6.9 ± 0.05** | 6.4 ± 0.04 | 6.55 ± 0.05** | 6.19 ± 0.05 | 6.72 ± 0.05 | 6.67 ± 0.05 |
| L* | 57.26 ± 0.91 | 57.26 ± 1.02 | 37.46 ± 0.68 | 39.28 ± 0.53* | 34.81 ± 0.56 | 34.8 ± 0.44 | 45.89 ± 0.48 | 51.38 ± 1.08** | 41.85 ± 0.67 | 41.97 ± 0.56 | 39.81 ± 0.59* | 37.84 ± 0.59 |
| a* | 10.42 ± 0.71 | 11.06 ± 0.95 | 17.45 ± 0.78 | 17.66 ± 0.21 | 16.08 ± 0.49 | 17.21 ± 0.28 | 13.95 ± 0.39 | 14.59 ± 0.85 | 16.54 ± 0.7 | 17.48 ± 0.5 | 17.04 ± 0.56 | 18.2 ± 0.42 |
| b* | 17.76 ± 0.54 | 17.56 ± 0.63 | 5.91 ± 0.25 | 7.79 ± 0.25** | 5.34 ± 0.31 | 5.88 ± 0.25 | 7.04 ± 0.44 | 8.33 ± 0.59 | 8.17 ± 0.48 | 8.82 ± 0.3 | 7.49 ± 0.41 | 7.59 ± 0.27 |
| Drip loss | 5.92 ± 0.72 | 8.1 ± 1.1** | 4.82 ± 0.4 | 5.17 ± 0.51* | 7.02 ± 1.15 | 7.81 ± 1.05 | 3.81 ± 0.36 | 5.34 ± 0.52** | 4 ± 0.44 | 4.65 ± 0.49 | 9.13 ± 1.06 | 10.65 ± 0.82 |
| Cooking loss | 31.98 ± 1.15 | 37.61 ± 1.46** | 24.98 ± 0.45 | 28.49 ± 0.81** | 26.03 ± 1.12 | 26.63 ± 1.36 | 29.15 ± 0.76 | 33.7 ± 0.8** | 25.72 ± 1.12 | 28.3 ± 0.94 | 26.84 ± 1.41 | 24.86 ± 1.62 |
| Shear force | 2,149.42 ± 97.6 | 2,514.65 ± 182.98 | 3,441.76 ± 149.87 | 3,300.16 ± 148.32 | 3,940.14 ± 140.35 | 3,797.26 ± 139.96 | 2,257.36 ± 163.05 | 3,639.23 ± 190.33** | 4,331.16 ± 175.99 | 5,775.64 ± 242.57** | 5,684.05 ± 277.27 | 5,101.25 ± 237.83 |

Note: * and ** indicate significant differences in either breast or thigh muscles between NRS and FRS at the same week at $P < 0.05$ and $P < 0.01$, respectively.

($P < 0.05$). Besides, the BM of NRS ducks exhibited a lower b* value than that of FRS ducks at W8 ($P < 0.01$). Compared with FRS, NRS significantly reduced drip loss of BM at W4 ($P < 0.01$) and W8 ($P < 0.05$) but showed no effect at W13 ($P > 0.05$); and meanwhile, the TM of NRS ducks exhibited less drip loss than that of FRS ducks at W4 ($P < 0.01$) but reached similar levels at either W8 or W13 ($P > 0.05$). Similarly, cooking loss from BM of NRS ducks was significantly lower than that of FRS ducks at W4 and W8 ($P < 0.01$) but was similar at W13($P > 0.05$), whereas cooking loss from TM of NRS ducks was significantly lower than that of FRS ducks at W4 ($P < 0.01$) but was similar at either W8 or W13 ($P > 0.05$). In addition, although there were no significant differences in shear force of BM between NRS and FRS throughout the raising period, NRS significantly decreased the shear force of TM than FRS at W4 and W8 ($P < 0.01$).

## Comparison of the Myofiber Characteristics of Duck Breast and Thigh Muscles Under Two Raising Systems

Histological observations revealed that the raising system also exerted developmental stage-dependent effects on the myofiber characteristics of either BM or TM in ducks. For BM, compared with FRS, NRS failed to change both the myofiber diameter and density at either W4 or W13 ($P > 0.05$; Figures 1A, 1B, 1E and 1F) but significantly increased the myofiber diameter while decreased the myofiber density at W4 ($P < 0.01$; Figures 1C and 1D). For TM, compared with FRS, NRS significantly decreased the myofiber diameter while increased the myofiber density at W4 ($P < 0.01$; Figures 2A and 2B), significantly increased the myofiber diameter while decreased the myofiber density at W8 ($P < 0.05$; Figures 2C and 2D), and showed no effects on both the myofiber diameter and density at W13 ($P > 0.05$; Figures 2E and 2F).

## Comparison of the Global Transcriptome Profiles of Duck Breast and Thigh Muscles Under Two Raising Systems

To further compare the effects of 2 raising systems on global gene expression profiles of both muscle groups over a time course in ducks, a total of 36 libraries (i.e., 3 libraries per week per muscle group per raising system) were subjected to transcriptome sequencing. As shown in Supplementary Table 1, more than 6.2 billion clean bases and 41.7 million clean reads were yielded by each library. The guanine and cytosine content, Q20 ratio, and Q30 ratio varied from 52.93 to 55.74, 97.27 to 97.99, and 93.17 to 94.71%, respectively; and moreover, 53.24 to 62.35% clean reads from each library were uniquely mapped to the reference duck genome GCA_003850225.1 (https://www.ncbi.nlm.nih.gov/assembly/GCA_003850225.1). Fragments per kilobase

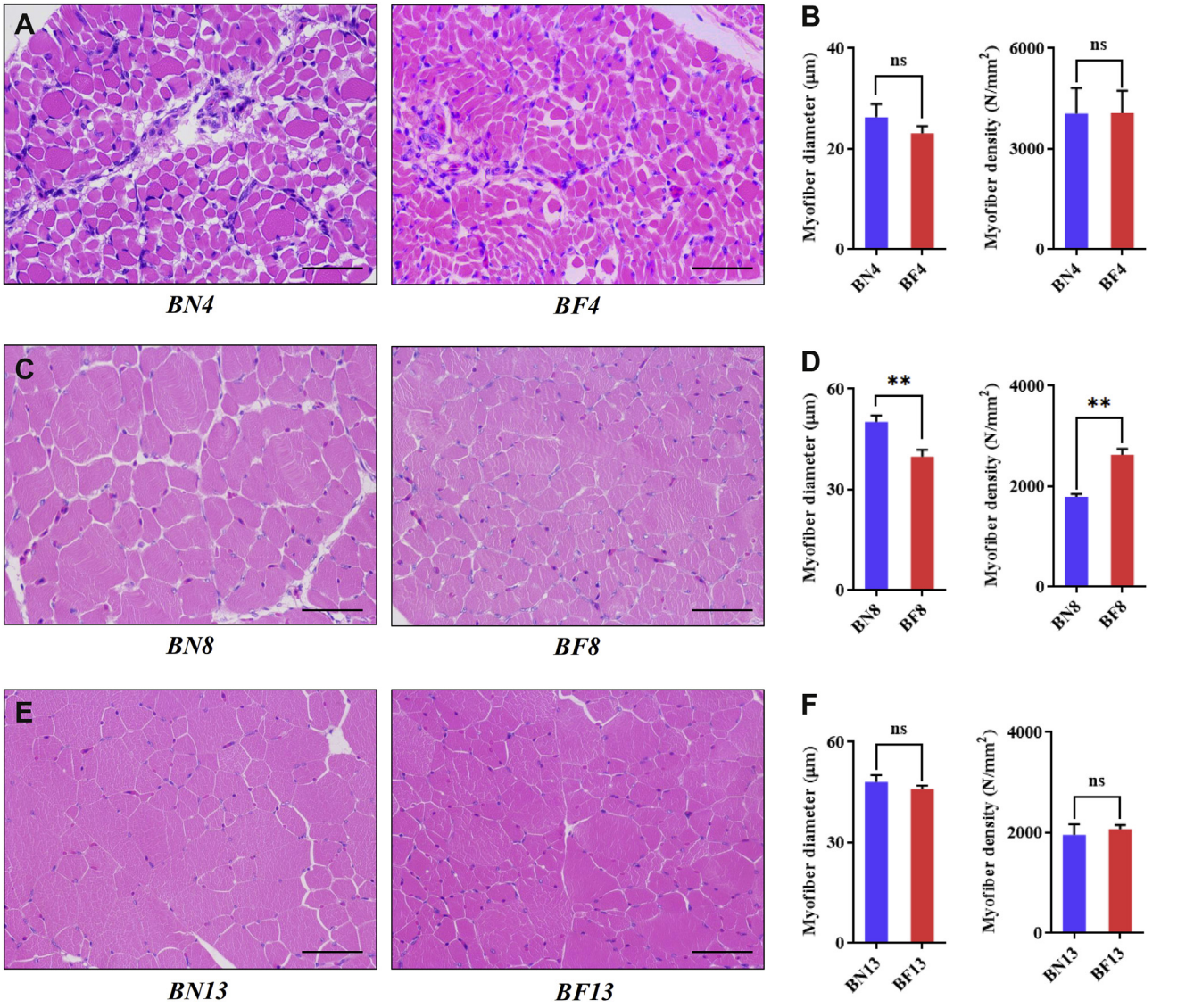


**Figure 1.** Differences in the myofiber characteristics of duck breast muscle (BM) between 2 raising systems at the the posthatching wk 4 (W4), 8 (W8), and 13 (W13). (A, C, and E) Representative images of HE-stained BM under 2 raising systems at W4, W8, and W13, respectively. (B, D, and F) Differences in the myofiber characteritics of BM under 2 raising systems at W4, W8, and W13, respectively. BN4, BN8, and BN13 represent BM under net raising system at W4, W8, and W13, respectively; whereas BF4, BF8, and BF13 represent BM under floor raising system at W4, W8, and W13, respectively. **Indicates significant differences between 2 groups at $P < 0.01$. Scale bar: 50 μm. Abbreviation: HE, hematoxylin–eosin.

of exon per million fragments mapped–based quantitative analysis was used to reveal developmental stage-dependent gene expression changes in duck BM and TM groups under 2 raising systems. As shown in Figure 3A, compared with BM of FRS ducks, 275 and 255, 69 and 95, and 198 and 226 genes were upregulated and downregulated in that of NRS ducks at W4, W8, and W13, respectively. Among them, there were 32 DEG commonly present between BN4 *vs.* BF4 and BN8 *vs.* BF8, and 14 DEG were commonly identified between the comparisons of BN8 *vs.* BF8 and BN13 *vs.* BF13 (Figure 3B). Moreover, there were 6 DEG present between all pairwise comparisons, and hierarchical clustering analysis suggested that the raising system had significant effects on the expression levels of these 6 DEG depending on the raising period and confirmed that more similar expression patterns existed among 3 biological replicates (Figures 3B and 3C). By comparison, for TM, there were 99 and 127, 56 and 50, and 321 and 218 genes were upregulated and downregulated by NRS when compared with FRS (Figure 3D). Among them, there were 8 DEG commonly present between TN4 *vs.* TF4 and TN8 *vs.* TF8, and 28 DEG were commonly identified between the comparisons of TN8 *vs.* TF8 and TN13 *vs.* TF13. Moreover, there were 2 DEG (i.e., *DTL* and *TMEM120A*) present between all pairwise comparisons (Figure 3E). As shown in Figure 3F, NRS significantly upregulated the expression levels of *DTL* and *TMEM120A* at week 4, 8, and 13 when compared with NRS.

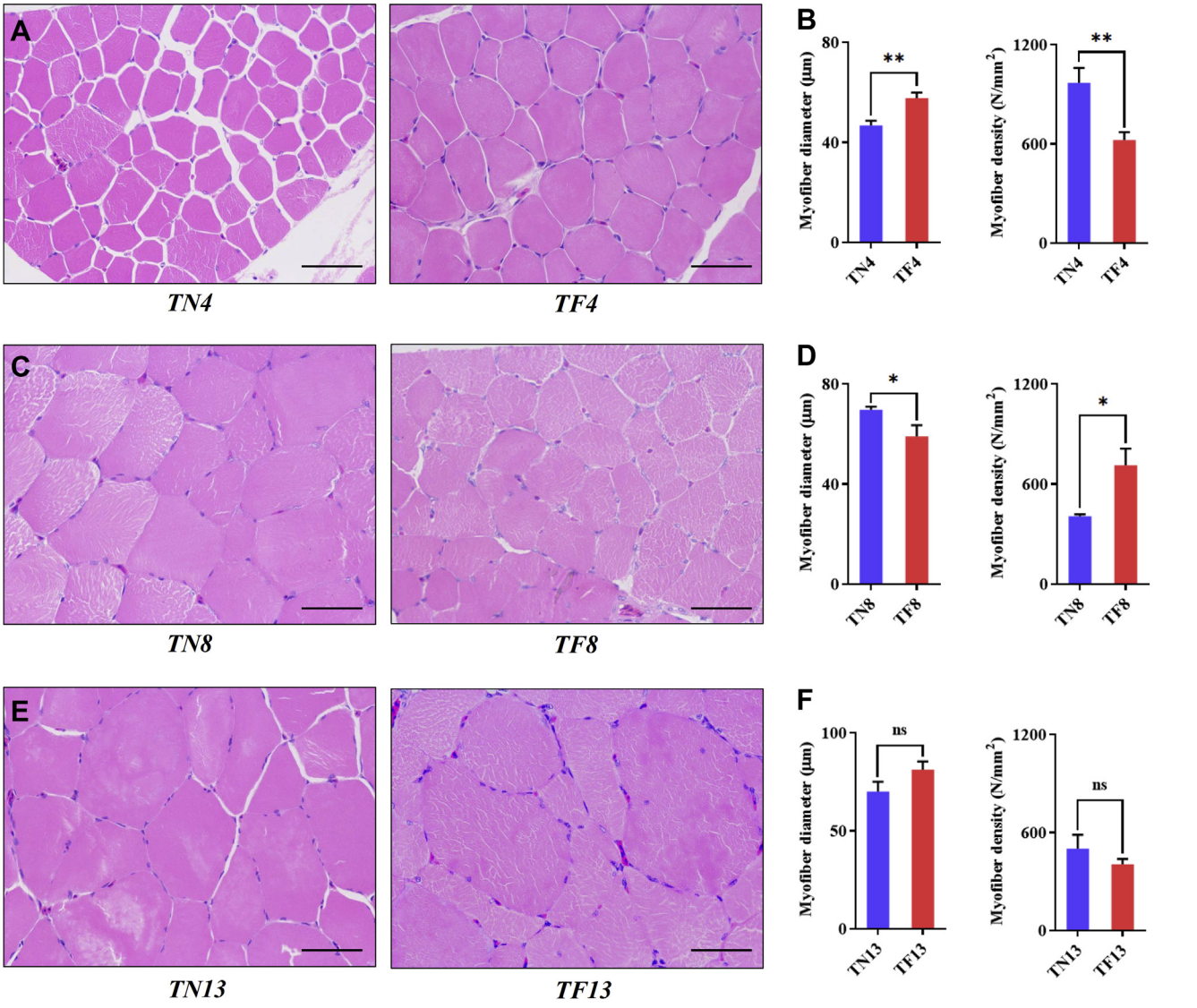


**Figure 2.** Differences in the myofiber characteristics of duck thigh muscle (TM) between 2 raising systems at W4, W8, and W13. (A, C, and E) Representative images of HE-stained TM under 2 raising systems at W4, W8, and W13, respectively. (B, D, and F) Differences in the myofiber characteritics of TM under 2 raising systems at W4, W8, and W13, respectively. TN4, TN8, and TN13 represent TM under net raising system at W4, W8, and W13, respectively; whereas TF4, TF8, and TF13 represent TM under floor raising system at W4, W8, and W13, respectively. * and ** indicate significant differences between 2 groups at $P < 0.05$ and $P < 0.01$, respectively. Scale bar: 50 μm. Abbreviation: HE, hematoxylin–eosin.

## GO Enrichment Analysis of Differentially Expressed Genes in Duck Breast and Thigh Muscles Between Two Raising Systems

All GO categories of biological process significantly enriched by DEG in duck BM groups between 2 raising systems over a time course are listed in Figure 4. At W4, compared with FRS, upregulated genes by NRS were enriched in the terms associated with autophagy and apoptosis (e.g., autophagosome assembly, autophagy, and negative regulation of extrinsic apoptotic signaling pathway), metabolic process (e.g., cellular response to starvation, reactive oxygen species metabolic process, and lipid storage), and tissue remodeling (e.g., vesicle transport along microtubule, Golgi organization, and cortical actin cytoskeleton organization; Figure 4A); by contrast, downregulated genes by NRS were enriched in the terms associated with muscle development and contractile activities (e.g., sarcomere organization, ventricular cardiac muscle tissue morphogenesis, and cardiac muscle contraction) and regulation of muscle cell functions (e.g., muscle cell cellular homeostasis and regulation of sodium ion transmembrane transporter activity; Figure 4B). Likewise, at W8, upregulated genes between NRS and FRS were enriched in lymphangiogenesis and protein targeting to Golgi (Figure 4C), whereas downregulated genes were enriched in extracellular fibril organization (Figure 4D). At W13, upregulated genes between NRS and FRS were enriched in the terms associated with regulation of muscle cell functions (e.g., cellular response to TNF and negative regulation of NF-kB activity), metabolic process (e.g., reactive oxygen species metabolic process and glucose homeostasis), and tissue remodeling (e.g., cartilage development and positive regulation of skeletal muscle tissue regeneration; Figure 4E); by contrast, downregulated genes were

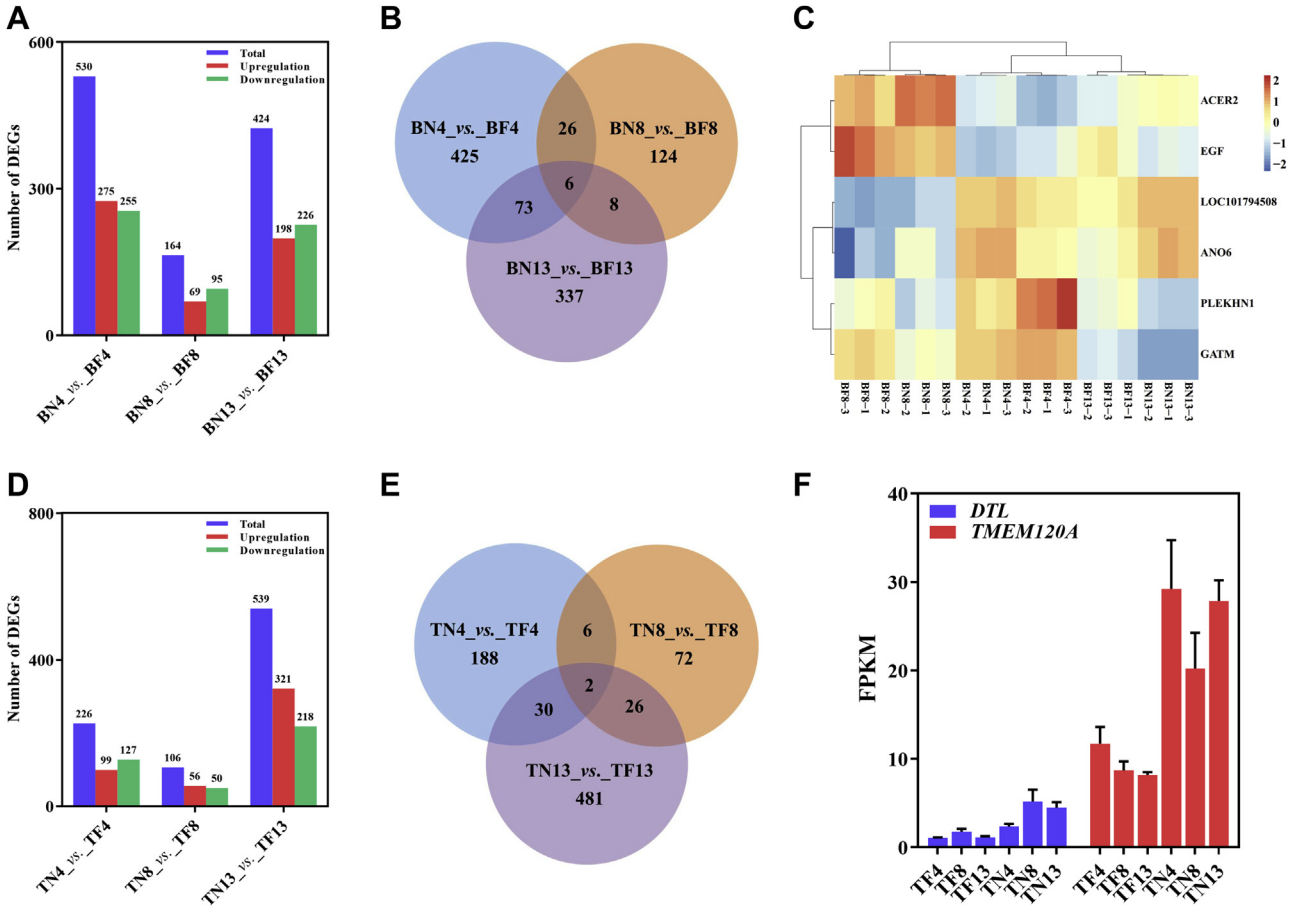


**Figure 3.** RNA-seq reveals genome-wide transcriptome changes in duck muscles between 2 raising systems at W4, W8, and W13. (A) The number of differentially expressed genes (DEG) in BM between 2 raising systems. (B) Venn diagram indicating the number of DEG in BM between 3 pairwise comparisons. (C) Hierarchical clustering of the 6 DEG overlapped in BM among 3 pairwise comparisons. (D) The number of DEG inTM between 2 raising systems. (E) Venn diagram indicating the number of DEG in TM between 3 pairwise comparisons. (F) Expression profiling of the 2 DEG overlapped in TM among 3 pairwise comparisons. Abbreviations: BM, breast muscle; TM, thigh muscle.

enriched in the terms associated with regulation of muscle cell functions (e.g., regulation of calcium ion import and positive regulation of ERK1 and ERK2 cascade) and tissue remodeling (e.g., collagen fibril organization and cartilage development; Figure 4F).

As for duck TM groups, as shown in Figure 5A, upregulated genes at W4 between NRS and FRS were enriched in positive regulation of peptidyl-tyrosine phosphorylation, whereas downregulated genes were enriched in the terms associated with metabolic process (e.g., protein polymerization, glycolytic process, and carbohydrate metabolic process), tissue remodeling (e.g., positive regulation of heterotypic cell–cell adhesion, positive regulation of vasoconstriction, microtubule-based process, and cell–matrix adhesion), and regulation of muscle cell functions (e.g., positive regulation of peptide hormone secretion and positive regulation of ERK1 and ERK2 cascade). Likewise, upregulated genes at W8 between NRS and FRS were mainly enriched in the terms associated with regulation of muscle development and cell functions (e.g., adipose tissue development, positive regulation of bone resorption, and cellular respiration; Figure 5B). At W13, upregulated genes between NRS and FRS were enriched in the terms associated with regulation of muscle cell functions (e.g., negative regulation of insulin receptor signaling, response to reactive oxygen species, and negative regulation of cysteine-type endopeptidase activity) and tissue remodeling (e.g., skeletal system morphogenesis, fibroblast migration, and positive regulation of angiogenesis; Figure 5C); by contrast, downregulated genes were enriched in the terms associated with tissue remodeling and regulation of muscle cell functions (e.g., embryonic skeletal system morphogenesis, anterior or posterior pattern specification, and positive regulation of PI3K signaling; Figure 5D).

## *KEGG Enrichment Analysis of Differentially Expressed Genes in Duck Breast and Thigh Muscles Between Two Raising Systems*

All KEGG pathways significantly enriched by DEG in duck BM groups between 2 raising systems over a time course were listed in Figure 6. At W4, upregulated genes between NRS and FRS were mainly enriched in the pathways related to autophagy and apoptosis (e.g., regulation of autophagy, apoptosis, lysosome, and phagosome) and metabolic activities (e.g., FoxO signaling, adipocytokine signaling, insulin signaling, and mTOR signaling; Figure 6A); by contrast, downregulated genes

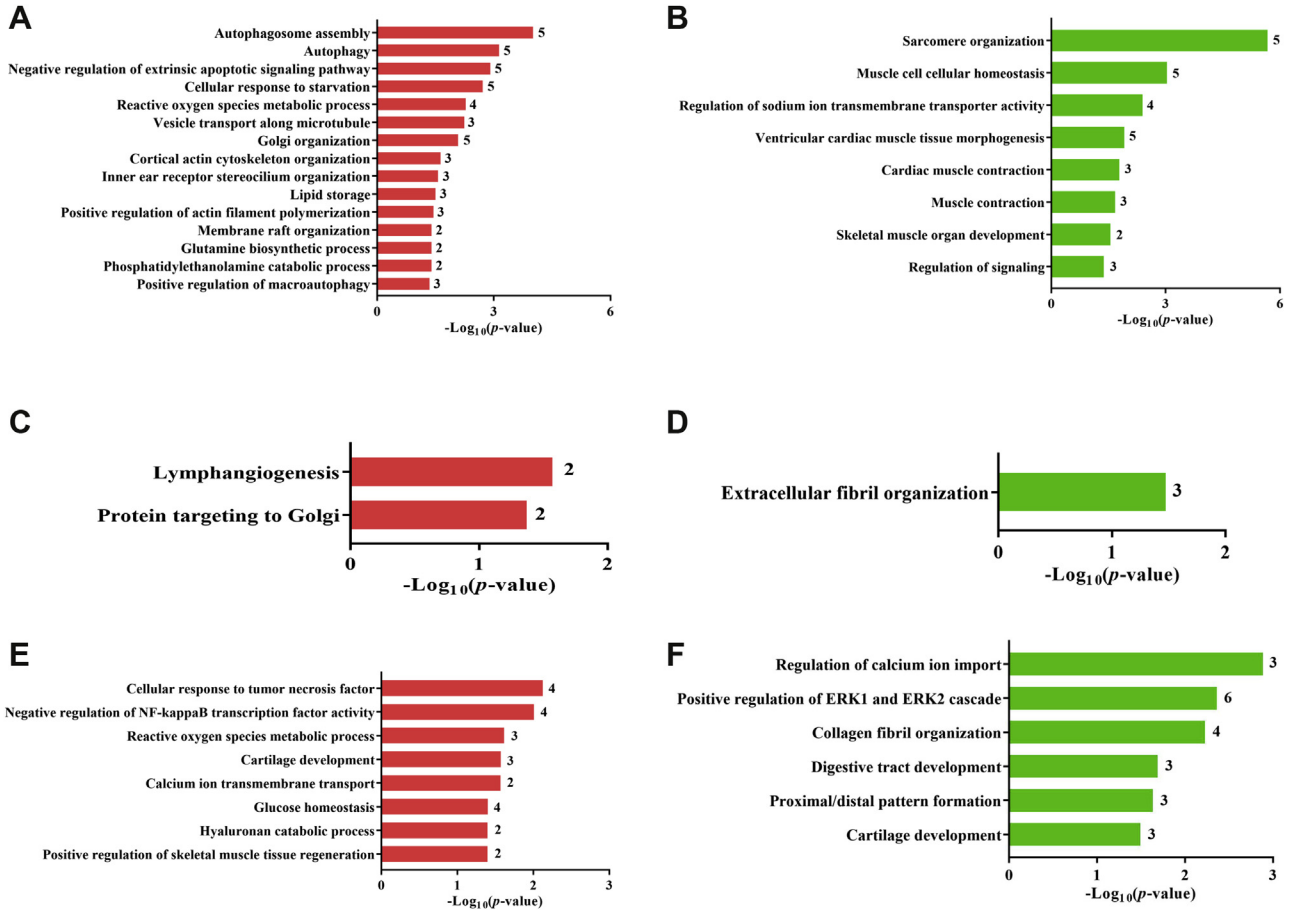


**Figure 4.** GO enrichment analysis of DEG in duck BM between 2 raising systems at W4, W8, and W13. (A, C, and E) GO terms significantly enriched by upregulated genes between BN4 *vs.* BF4, BN8 *vs.* BF8, and BN13 *vs.* BF13, respectively. (B, D, and F) GO terms significantly enriched by downregulated genes between BN4 *vs.* BF4, BN8 *vs.* BF8, and BN13 *vs.* BF13, respectively. Abbreviations: BM, breast muscle; DEG, differentially expressed genes; GO, Gene Ontology.

were mainly enriched in the pathways associated with muscle contractile activities (e.g., adrenergic signaling in cardiomyocytes and cardiac muscle contraction), metabolic activities (e.g., metabolic pathways, glycolysis, gluconeogenesis, and biosynthesis of amino acids), and tissue remodeling (e.g., focal adhesion, gap junction, and regulation of actin cytoskeleton; Figure 6B). Likewise, upregulated genes at W8 between NRS and FRS were mainly enriched in adipocytokine signaling, herpes simplex infection, and PPAR signaling pathways (Figure 6C). At W13, upregulated genes between NRS and FRS were mainly enriched in the pathways related to autophagy and apoptosis (e.g., apoptosis and regulation of autophagy) and metabolic activities (e.g., FoxO signaling and metabolic pathways; Figure 6D); by contrast, downregulated genes were mainly enriched in the pathways related to tissue remodeling (e.g., focal adhesion, cytokine–cytokine receptor interaction, and ECM–receptor interaction), metabolic activities (e.g., biosynthesis of amino acids andglycine, serine and threonine metabolism), and muscle contractile activities (e.g., vascular smooth muscle contraction and adrenergic signaling in cardiomyocytes; Figure 6E).

As for duck TM groups, as shown in Figure 7A, upregulated genes at W4 between NRS and FRS were mainly enriched in the adrenergic signaling in cardiomyocytes and glycerolipid metabolism pathways; by contrast, downregulated genes were mainly enriched in the pathways related to metabolic activities (e.g., glycolysis, gluconeogenesis, and metabolic pathways), autophagy and apoptosis (e.g., phagosome and apoptosis), and muscle contractile activities (e.g., adrenergic signaling and cardiac muscle contraction; Figure 7B). Likewise, at W8, upregulated genes between NRS and FRS were mainly enriched in the AGE-RAGE signaling in diabetic complications and adipocytokine signaling pathways (Figure 7C), while downregulated genes were mainly enriched in the apoptosis and phagosome pathways (Figure 7D). At W13, upregulated genes between NRS and FRS were mainly enriched in the pathways related to metabolic activities (e.g., mTOR signaling, FoxO signaling, and insulin signaling), autophagy and apoptosis (e.g., regulation of autophagy and apoptosis), and tissue remodeling (e.g., GnRH signaling and regulation of actin cytoskeleton; Figure 7E); in contrast, downregulated genes were mainly enriched in muscle contractile activities (e.g., vascular smooth muscle contraction and adrenergic signaling in cardiomyocytes), tissue remodeling (e.g., cytokine-cytokine receptor interaction, gap junction, and endocytosis), and

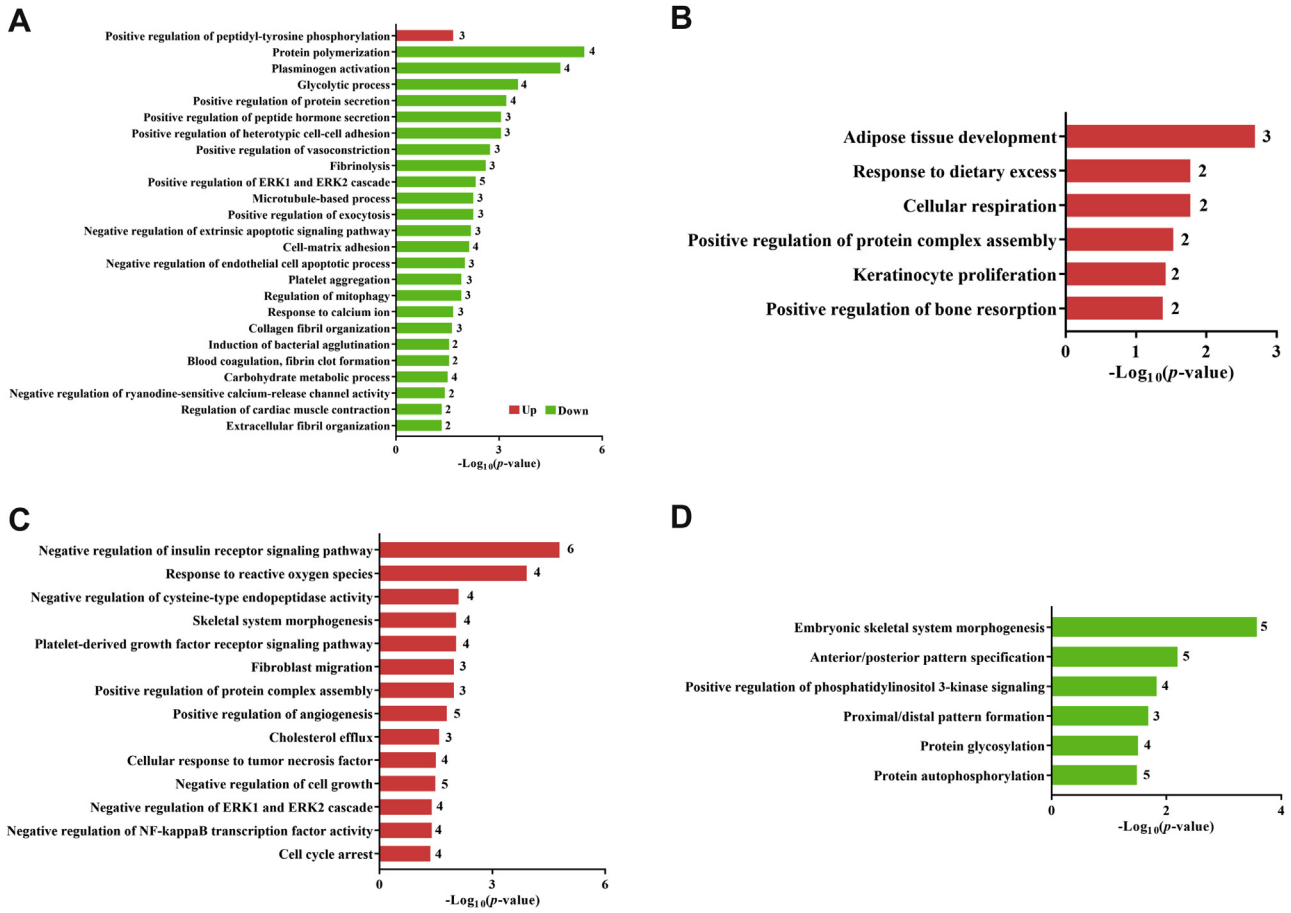


**Figure 5.** Gene Ontology (GO) enrichment analysis of DEG in duck TM between 2 raising systems at W4, W8, and W13. (A) GO terms significantly enriched by upregulated or downregulated genes between TN4 *vs.* TF4. (B-C) GO terms significantly enriched by upregulated genes between TN8 *vs.* TF8 and TN13 *vs.* TF13, respectively. (D) GO terms significantly enriched by downregulated genes between TN13 and TF13. Abbreviations: TM, thigh muscle; DEG, differentially expressed genes.

metabolic activities (e.g., metabolic pathways and glutathione metabolism; Figure 7F).

## DISCUSSION

Skeletal muscle yield and meat quality are the 2 most economically important production traits in meat-type birds, and both traits have been reported to be greatly affected by many environmental factors including the raising system (Fletcher, 2002; Mir et al., 2017). Nevertheless, the physicochemical and molecular responses of different skeletal muscle groups (e.g., BM and TM) to different raising systems have not been fully understood, especially in broiler ducks. In the present study, we systematically compared the effects of NRS and FRS on the meat quality properties, histological characteristics, and global gene expression profiles of BM and TM over a time course in Nonghua duck, one of the most economically valuable broiler duck breeds in Southwest China. In accordance with the observations in other domestic birds such as chickens and ducks (Almasi et al., 2015; Zhang et al., 2018), our results confirmed and extended the notion that the raising system can significantly affect the physicochemical properties of skeletal muscles depending on the muscle group and the raising period. Regardless of the raising system and period, the pH values of duck BM at 45 min and 24 h postmortem were generally lower than those of TM, which was consistent with the results from several previous studies (Chang and Chou, 2010; Huda et al., 2011), suggesting higher glycolytic metabolism and consequently higher lactic acid concentration in poultry BM. Meanwhile, duck BM generally exhibited a lower redness (a*) value than TM, implying that fewer capillaries exist in BM than in TM, which could at least by part result in a lower pH value in BM because of the retention of lactic acid (Velleman, 2015). Besides, compared with TM, both drip loss and cooking loss of BM were generally higher at W4 and W8 but lower at W13, whereas the shear force of BM was constantly lower throughout the raising period, suggesting differences in water holding capacity and tenderness between duck BM and TM.

Regarding the impact of the raising system, NRS induced higher pH values in both muscle groups at W4 and W8 but showed no significant effects on or even reduced pH values at W13 when compared with FRS, which implied that NRS could exert the most pronounced inhibitory effects on glycolytic metabolism of duck skeletal muscles during early posthatching development. In the meantime, NRS decreased the lightness

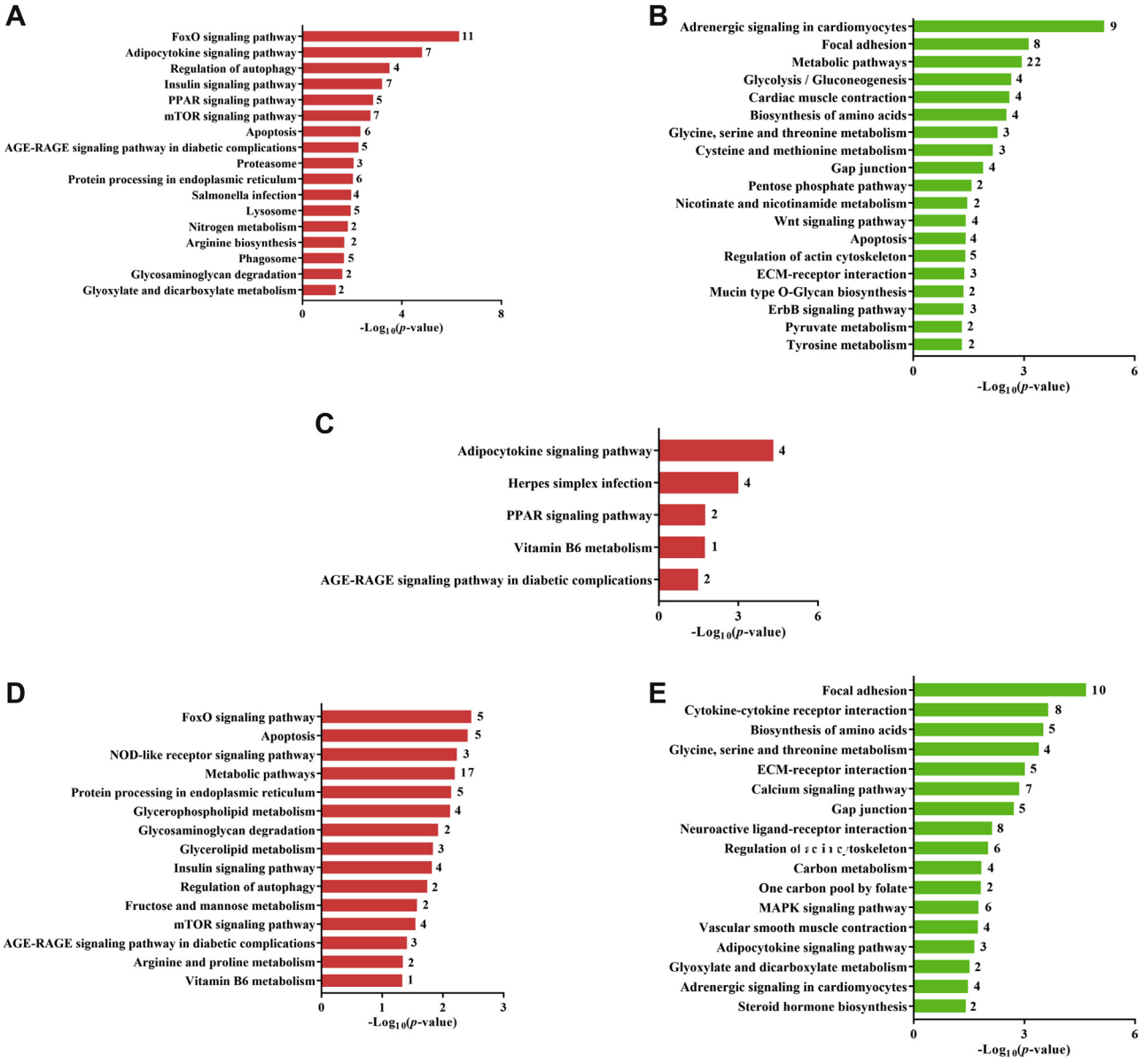


**Figure 6.** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEG in duck BM between 2 raising systems at W4, W8, and W13. (A, C, and D) KEGG pathways significantly enriched by upregulated genes between BN4 *vs.* BF4, BN8 *vs.* BF8, and BN13 *vs.* BF13, respectively. (B and E) KEGG pathways significantly enriched by downregulated genes between BN4 *vs.* BF4 and BN13 *vs.* BF13, respectively. Abbreviations: DEG, differentially expressed genes; BM, breast muscle.

(L*) and yellowness (b*) values not only in duck BM at W8 but also in TM at W4, but did not significantly alter or even increased both values in either muscle group at W13. However, there were no significant differences in the a* value of either muscle group between NRS and FRS throughout the raising period. In addition, drip loss and cooking loss of both muscle groups were generally lower in ducks under NRS than those under FRS throughout the raising period, especially at early stages of development, and NRS remarkably reduced shear force of duck TM at W4 and W8. These results were in agreement with the previous observations that meat with a higher L* value was positively correlated with increased drip loss (Chen et al., 2013; Zhang et al., 2018). Because it is generally accepted that meat with a higher L* value, a lower a* value, and higher drip loss is more susceptible to become the pale, soft, and exudative meat (Van Laack et al., 2000; Zhang et al., 2018), we postulated that compared with FRS, NRS could markedly improve duck meat quality by affecting glycolytic metabolism (pH and color), water holding capacity (juiciness), and shear force (tenderness), which are known as key determinants of meat quality (Mir et al., 2017), during early stages of posthatching development. In support of this, our histological results showed that compared with FRS, NRS induced a higher myofiber diameter but a lower myofiber density in duck BM at W8; and for TM, NRS induced a lower myofiber diameter but a higher myofiber density at W4 while induced a higher myofiber diameter but a lower myofiber density at W8. Because the histological parameters of muscle fibers have been shown to be strongly correlated with meat quality traits (Fletcher, 2002; Petracci and Cavani, 2012), it was inferred that the raising system

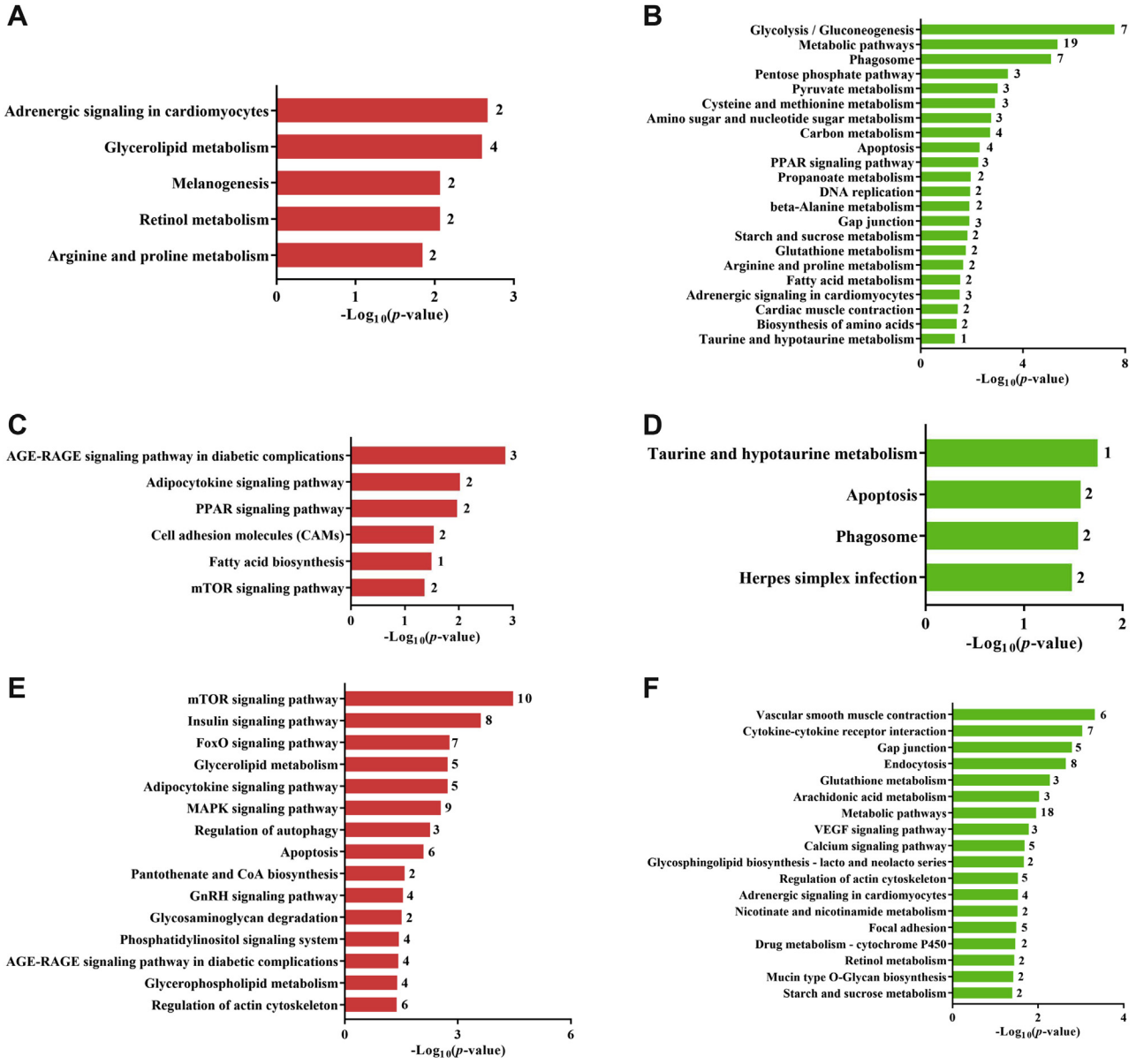


**Figure 7.** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEG in duck TM between 2 raising systems at W4, W8, and W13. (A, C, and E) KEGG pathways significantly enriched by upregulated genes between TN4 *vs.* TF4, TN8 *vs.* TF8, and TN13 *vs.* TF13, respectively. (B, D, and F) KEGG pathways significantly enriched by downregulated genes between TN4 *vs.* TF4, TN8 *vs.* TF8, and TN13 *vs.* TF13, respectively. Abbreviations: DEG, differentially expressed genes; TM, thigh muscle.

influences the physicochemical properties of duck BM and TM through manipulation of muscle fiber characteristics.

To further explore the molecular mechanisms underlying the raising system-caused phenotypic differences in duck muscles, genome-wide transcriptomic differences in both BM and LM between NRS and FRS were analyzed using RNA-seq. Compared with FRS, NRS induced the most extensive transcriptomic changes in duck BM at W4 while in TM at W13, implying different molecular mechanisms underlying the asynchronous adaptive remodeling of BM and TM in response to the raising system and period. As for BM, upregulated or downregulated genes by NRS at W4 were mainly enriched in the GO or KEGG terms associated with autophagy and apoptosis, metabolic activities, tissue remodeling, muscle development and contractile activities, and regulation of muscle cell functions. The DEG at W8 between NRS and FRS were mainly related to tissue remodeling and metabolic activities, whereas those at W13 were mainly related to autophagy and apoptosis, metabolic activities, tissue remodeling, and muscle contractile activities. These results indicated that the raising system changed the phenotype of duck BM principally by modulating expression of genes involved in control of muscle cellular functions, metabolic and contractile activities, and tissue remodeling. Of particular note, the adipocytokine and AGE-RAGE signaling pathways were commonly enriched by either upregulated or downregulated genes among the comparisons of BN4 *vs.* BF4, BN8 *vs.* BF8, and BN13 *vs.* BF13. Because these 2 pathways were known as key regulators

of cellular energy metabolism and inflammation (Lin et al., 2009; Cao, 2014), we conjectured that altered expression of metabolism and inflammation-related genes could be the core transcription machinery directing BM-specific adaptive remodeling in response to the raising system. In addition, throughout the raising period, six genes were identified to be constantly differentially expressed between NRS and FRS. Among them, pleckstrin homology domain-containing family N member 1 (PLEKHN1) contains 2 pleckstrin-homology domains at the N terminus that can specifically recognize phosphatidylinositol lipids and recruit them to different cellular compartments (Dowler et al., 2000), and recently it was shown to have a role in regulating the inflammatory response (Yu et al., 2018). *GATM* encodes L-arginine:glycine amidinotransferase catalyzing the rate-limiting step in the synthesis of creatine, which is primarily produced by skeletal muscles and plays a central role in control of muscle cellular energy metabolism (Choe et al., 2012), and moreover, it was highly expressed in chicken cells (Jang et al., 2013). *ANO6*, also called *TMEM16F,*is widely recognized as a key regulatory of phospholipids translocation between membrane leaflets by serving as a $Ca^{2+}$-dependent phospholipid scramblase and a $Ca^{2+}$-activated $Cl^-$ channel (Kunzelmann et al., 2014), and it was also reported to be involved in regulating cell apoptosis and innate immunity (Juul et al., 2014; Ousingsawat et al., 2015). Epidermal growth factor plays an important role in phenotypic modulation of both vascular smooth muscle and skeletal muscle cells via interaction with its receptor (Yamanaka et al., 2001; Hamdi and Mutungi, 2010), whereas alkaline ceramidase 2 (***ACER2***) is widely involved in regulating ceramides hydrolysis, growth, apoptosis, and adhesion in a number of cells (Sun et al., 2009; Sun et al., 2010; Wang et al., 2017). *LOC101794508*, also called uncoupling protein 3 (**UCP3**), is predominantly expressed in the mitochondria of skeletal muscles and plays important roles in regulating glucose and fatty acid metabolism by uncoupling adenosine triphosphate production from mitochondrial respiration (Schrauwen, 2002). However, the physiological significance of these genes in the duck skeletal muscle remains extremely scarce to date and warrants further investigations. Hence, it could be concluded that the adaptive remodeling of duck BM on environmental stimuli be the result of coordinate regulation of a multitude of genes related to energy metabolism, inflammation, growth, and apoptosis.

As for TM, most of DEG at W4 between NRS and FRS were enriched in the GO or KEGG terms associated with metabolic activities, tissue remodeling, autophagy and apoptosis, and muscle contractile activities. The DEG at W8 were mainly related to regulation of muscle development and cell functions, metabolic activities, autophagy and apoptosis, and inflammation, whereas those at W13 were mainly associated with regulation of muscle cell functions, tissue remodeling, muscle contractile and metabolic activities, and autophagy and apoptosis. These results indicated that the raising system–caused phenotypic changes in duck TM could be mainly mediated by the pathways involving regulation of muscle cellular functions, metabolic and contractile activities, autophagy and apoptosis, and tissue remodeling. Notably, there was only the apoptosis pathway commonly enriched by either upregulated or downregulated genes among these 3 pairwise comparisons, suggesting its key roles in directing TM-specific adaptive remodeling in response to the raising system. Indeed, there is evidence that apoptosis occurs in the injured muscles and overaccumulation of apoptotic cells results in maladaptive muscle remodeling (Sciorati et al., 2016). In addition, denticleless protein homolog (*DTL*) and *TMEM120A* were identified to be constantly differentially expressed in duck TM between NRS and FRS throughout the raising period. *DTL*, also called retinoic acid-regulated nuclear matrix-associated protein (*RAMP*), or DNA replication factor 2 (***CDT2***), encodes a nuclear and centrosome protein that acts as a regulator of cell proliferation, cell cycle arrest, and cell invasion in multiple tumor cells (Pan et al., 2006; Song et al., 2010), whereas TMEM120A, also called nuclear envelope transmembrane protein 29 (NET29), is a key member of the NET superfamily, which have been reported to be able to direct chromosome position and gene expression during adipogenesis and myogenesis (Robson et al., 2016) and act as an ion channel involved in sensing mechanic pain (Beaulieu-Laroche et al., 2020). However, nothing is known about the physiological functions of these 2 genes in avian skeletal muscles, which are required to be further determined. Therefore, it was speculated that although the principle pathways involved in mediating the raising system–caused phenotypic changes in duck BM and TM were similar, several unique pathways (e.g., adipocytokine signaling, AGE-RAGE signaling, and apoptosis), and genes (e.g., *ANO6*, *ACER2*, *UCP3*, *DTL*, and *TMEM120A*) could be part of the regulatory mechanisms underlying the muscle group–specific adaptive remodeling.

In conclusion, our results demonstrated that the raising system can differently affect glycolytic metabolism, water holding capacity, and shear force of duck breast and thigh muscles through manipulation of myofiber characteristics. Although these effects were exerted principally by regulating expression of a series of genes involved in control of muscle cellular functions, metabolic and contractile activities, and tissue remodeling, several crucial pathways (e.g., adipocytokine signaling, AGE-RAGE signaling, and apoptosis), and genes (e.g., *ANO6*, *ACER2*, *UCP3*, *DTL*, and *TMEM120A*) could, at least by part, contribute to the muscle group–specific adaptive remodeling in response to extrinsic stimuli.

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## DISCLOSURES

The authors declare no conflicts of interest.

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.09.027.

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