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Investigating the impact of pigmentation variation of breast muscle on growth traits, melanin deposition, and gene expression in Xuefeng black-bone chickens

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ABSTRACT The blackness traits, considered an important economic factor in the black-bone chicken industry, still exhibits a common phenomenon of significant difference in blackness of breast muscle. To improve this phenomenon, this study compared growth traits, blackness traits, and transcriptome of breast muscles between the High Blackness Group (**H group**) and Low Blackness Group (**L group**) in the Xuefeng black-bone chickens. The results are as follows: 1) There was no significant difference in growth traits between the H group and the L group ($P > 0.05$). 2) The skin/breast muscle L values in the H group were significantly lower than those in the L group, while the breast muscle melanin content exhibited the opposite trend ($P < 0.05$). 3) A significant negative correlation was observed between breast muscle melanin content and skin/breast muscle L value ($P < 0.05$), and skin L value exhibiting a significant positive correlation with breast muscle L value ($P < 0.05$). 4) The breast muscle transcriptome comparison between the H group and L group revealed 831 and 405 DEGs in female and male chickens, respectively. This included 37 shared DEGs significantly enriched in melanosome, pigment granule, and the melanogenesis pathway. Seven candidate genes (*DCT, PMEL, MLANA, TYRP1, OCA2, EDNRB2*, and *CALML4*) may play a crucial role in the melanin production of breast muscle in Xuefeng black-bone chicken. The findings could accelerate the breeding process for achieving desired levels of breast muscle blackness and contribute to the exploration of the mechanisms underlying melanin production in black-bone chickens.

Key words: breast muscle, blackness variation, histomorphological, pigmentation, Xuefeng black-bone chicken

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INTRODUCTION

The black-bone chicken is a highly prized breed in China due to its medicinal attributes, and it is often utilized in traditional Chinese medicinal dishes to promote health of body (Geng et al., 2010; Zhu et al., 2014; Zhang et al., 2015). Numerous studies have substantiated the medicinal qualities of black-bone chicken, attributing them to the abundant melanin content within the meat. Because melanin possesses antioxidant properties and contributes to enhanced immune function, radioprotective effects, and other beneficial functions (Chen et al., 2009; Tu et al., 2009; Premi et al., 2015). In comparison to other broiler chickens, black-bone chicken meat exhibits lower fat and cholesterol levels, along with higher protein and microelement content (Tu et al., 2009; Tian et al., 2011; Yang et al., 2019). Consequently, black-bone chicken holds substantial economic and market value in China.

The blackness traits is a pivotal economic characteristic in the black-bone chickens and occupies an important position in the breeding process. Consumers generally associate the blacker appearance with high nutritional value in black-bone chickens (Yu et al., 2018; Xu et al., 2023). However, compared to observable traits that can be directly measured or assessed instrumentally, such as feather color, skin pigmentation, body size, and more (Li et al., 2023; Lyu et al., 2023; Zheng et al., 2023), the blackness of breast muscle is not visually discernible and also cannot be directly detect in live body. And blackness variations significantly impact the commercial value of black-bone chickens, therefore, addressing this challenge remains a difficult problem in breeding processes today (Sun et al., 2022; Xu et al., 2023). RNA-seq, owing to its high-throughput and precision, demonstrates unique advantages in molecular breeding. It unravels the complexity of gene regulatory networks

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and facilitating the identification of key genes and pathways associated with phenotypic character (Deng et al., 2019; Saidi and Hajibarat, 2020). Several studies used transcriptome sequencing to discover gene ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathways and key genes related to meat color. This not only provides molecular markers for breeding but also establishes a theoretical foundation for understanding the functional molecular mechanisms associated with the meat color (Yu et al., 2018; Sun et al., 2022; Xu et al., 2023).

The Xuefeng Black-bone chicken is the only native black-bone chicken breed in Hunan Province of China. This breed exhibits substantial market potential within the province and its neighboring regions. Over an extended period of domestication, it has developed stable genetic traits and unique flavor. These traits encompass notable black features in the skin, bones, meat, beak, legs, cockscomb, and tongue (Deng et al., 2021; Deng et al., 2022). However, a noteworthy phenomenon was observed during the slaughter process, revealing significant variations in the blackness of breast muscles (**BBM**) among Xuefeng black-bone chickens. Scarce research has been conducted regarding the breeding of BBM in black-bone chickens. Does the augmentation of blackness traits impact the regular growth and development of chickens? Can observable correlated traits be leveraged to improve BBM selection? Are there molecular markers suitable for BBM breeding in black-bone chickens? Based on above problem, our objective is to expedite the breeding process of BBM by identifying molecular markers or correlated traits in black-bone chicken and enhance comprehension of the mechanism about melanin deposition. To achieve this, we conducted a comparative analysis of the growth traits, melanin deposition and transcriptomic variances between high blackness and low blackness group.

MATERIALS AND METHODS

Animals and Sample Collection

All the birds and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, Hunan, China (approval number: 2019022). Xuefeng black-bone chicken were obtained from Hunan Yunfeifeng Agricultural Commercial Company, Hunan, China. The study population was the Xuefeng black-bone chicken F2 resource population that was produced from reciprocal crosses of the high blackness line and low blackness line. All chickens were uniformly raised under consistent management conditions, with both management and nutritional requirements being overseen by company employees in accordance with established company procedures.

A total of 405 chickens, consisting of 194 female chickens at 180 d and 211 male chickens at 130 d, were slaughtered at the market age. Approximately 0.5 g breast muscle tissue were promptly collected and snap-frozen in liquid nitrogen for subsequent RNA-seq analysis. Additionally, roughly 2 cm × 1 cm × 1 cm breast muscle were preserved in 4% paraformaldehyde for morphological assessments, while the entirety of the breast muscle was collected for melanin content determination. All samples were extracted from the right breast muscle.

Growth and Blackness Traits

All chickens recorded the initial body weight (**IBW**) at one-day-old and the final body weight (**FBW**) at the market age. Skin lightness values (**SL**) were measured at different stages, including 6, 12, and 18 w, using a Minolta CR-400 colorimeter (Minolta Co. Ltd., Tokyo, Japan), the breast muscle under the wing has an area with less hairs, and this is where the SL was determined, each SL value measured 3 times. Subsequently, determinations were made for eviscerated weight (**EW**), breast muscle weight (**BMW**), breast lightness value (**BML**), and tibia weight (**TW**) post-slaughter, the BML were obtained from 3 areas of the breast major muscle.

Grouping

This study primarily focusses on blackness of breast muscle (**BBM**) variations, so the BML results as the grouping criterion which have been obtained. The community evenness is defined as the percentage of number of individuals falling within the range of 0.9 to 1.1 times the mean value divided by the total number of individuals, indicating smaller differences among individuals in this range (Wang et al., 2017; Alfaro-Wisaquillo et al., 2021). Consequently, individuals falling outside the 0.9 to 1.1 times the mean range were categorized as extreme cases. Given the negative correlation between the lightness and the blackness, the groups were classified as follows: Female High Blackness group (**FH**) comprised 62 chickens with BML $<$ 28.62, Female Low Blackness group (**FL**) comprised 55 chickens with BML $>$ 34.98, Male High Blackness group (**MH**) included 43 chickens with BML $<$ 30.15, and Male Low Blackness group (**ML**) included 37 chickens with BML $>$ 36.73.

Determination of Melanin Content

The determination of melanin content followed the method described by Xu et al. (2023). In brief, the entire breast muscle, with the fascia removed, underwent vacuum freeze-drying (LGL-10 C, Sihuan Tech. Instrument Co. Ltd., Beijing, China). The melanin standard (CAS#8049-97-6, Sigma-Aldrich, Saint Louis, MO) was dissolved in sodium hydroxide and diluted to various concentrations to create a standard curve. The absorbance of a 10 mg sample was measured at 500 nm after dissolving in non-denaturing lysis buffer (Solarbio, Beijing, China) and sodium hydroxide for 20 min and 2 h, respectively. Melanin content was calculated using the absorbance of the sample and the melanin standard curve ($y = 0.1518x + 0.0012$, $R^2 = 0.9998$).

Table 1. Primer pairs used for real-time quantitative PCR.

Gene	Accession number	Sequence (5′-3′)	Product length (bp)
DCT	NM_204935.2	F: GCTGTTGGTGCACAGGAAAC R: ATCGAGGAACTGCTCCCTCT	149
PMEL	NM_205112.3	F: AGTTCAGCATCACCGACCAG R: CCCGAAGTCCCACGAATAGG	173
MLANA	XM_046935722.1	F: CAGGTCTGAAGGAGGGATAGGA R: AGCCGCTACGCCTTTTGTAA	186
EDNRB2	NM_204120.2	F: GCACTGGCATCTTCTACACCC R: TGACGAGGAGGAAACTGAGCA	237
CALML4	NM_001277629.2	F: CCATGGCCAAGTTTCTGTCC R: TCTGCATTGCGCTCGATCT	190
KITLG	NM_001105315.1	F: TGAAGAAGGCACAAACTTGGA R: ATCTGTCACTGGATTCCCGC	104
EDNRB	NM_001001127.2	F: TGGCCCTTTGGTGTCGAAAT R: CAACTGCTCGGTACCTGTCT	112
GAPDH	NM_204305.2	F: TCGGAGTCAACGGATTTGGC R: TTCCCGTTCTCAGCCTTGAC	181

Histology of the Breast Muscle

The breast muscle tissue fixed samples were embedded in paraffin, sectioned (4 mm), followed by hematoxylin-eosin staining. Morphological observation were performed by CaseViewer Image analysis software.

Total RNA Extraction, cDNA Library Preparation, and Sequencing

A total of 14 sample were sent to Novogene (Beijing, China) for RNA extraction, library preparation, and sequencing. Briefly, Total RNA was extracted from the breast muscles using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA). Library construction using the Next Ultra RNA Library Prep Kit for Illumina (NEB, Beijing, China) (Parkhomchuk et al., 2009). And then the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

Analysis of RNA-seq Data

Raw data of fastq format were firstly processed through fastp software to calculated Q20, Q30 and GC content. All the downstream analyses were based on the clean data with high quality. All clean reads were mapped to the chicken genome (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000002315.6/), using Hisat2 v2.0.5, and then compiled and evaluated. featureCounts v1.5.0-p3 were used to calculate the reads and FPKM mapped to each gene. Differential expression analysis of 2 groups was performed using the DESeq2 R package (1.20.0). The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value $<= 0.05$ found by DESeq2 were assigned as differentially expressed. Gene ontology (**GO**) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. Gene ontology terms with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes. We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

Real-Time Quantitative PCR Verification of RNA-seq Data

To verify the accuracy of the transcriptomic data, we randomly selected seven DEGs for RT-qPCR. The primers (Table 1) were synthesized by Tsingke Biotechnology Co., Ltd (Beijing, China). Three times were detected for each sample. The RNA samples from RNA-seq remained were reverse transcribed into cDNA using the cDNA Synthesis kit (Vazyme Biotech Co., Lted. Nanjing, China) according the operation manual. RT-qPCR reaction system and thermal cycling parameters are operated according to the SYBR qPCR Master Mix (Vazyme Biotech Co., Lted. Nanjing, China) instructions. Genes expression were calculated by the $2^{-\triangle\triangle Ct}$ method, using GAPDH as an internal control.

Statistical Analysis

All data except RNA-seq data were analyzed by t-test using SPSS 22.0 statistical software (SPSS Institute Inc., Chicago, IL). And correlation analysis of blackness characteristics was assessed by Spearman correlation. The results are expressed as arithmetic mean ± standard deviation, $P < 0.05$ was considered statistically significant.

RESULTS

Growth Traits

As presented in Table 2. There are no significant differences in IBW, FBW, EW, BMW, and TW between the H group and L group of Xuefeng black-bone chickens ($P > 0.05$).

Table 2. Comparative analysis of growth traits between H group and L group in Xuefeng black-bone chicken.[1]

	Group[2]				*P*-value	
Items/g[3]	FH	FL	MH	ML	FH vs. FL	MH vs. ML
IBW	26.91 ± 2.36	26.51 ± 1.80	26.63 ± 2.19	27.34 ± 2.55	0.301	0.193
FBW	1331.94 ± 150.51	1337.73 ± 141.18	1383.33 ± 140.85	1406.03 ± 167.84	0.830	0.518
EW	738.36 ± 84.84	738.14 ± 87.45	861.98 ± 96.14	875.45 ± 111.68	0.989	0.568
BMW	69.76 ± 10.20	72.10 ± 10.52	70.87 ± 10.10	72.49 ± 12.84	0.225	0.537
TW	18.92 ± 5.55	18.65 ± 2.54	25.08 ± 5.20	25.95 ± 3.75	0.731	0.393

[1]H group: high blackness group; L group: low blackness group.
[2]FH: female high blackness group; FL: female low blackness group; MH: male high blackness group; ML: male low blackness group.
[3]IBW: initial body weight; FBW: final body weight; EW: eviscerated weight; BMW: breast muscle weight; TW: tibia weight.

Table 3. Comparative analysis of blackness traits between H group and L group in Xuefeng black-bone chicken.[1]

	Group[2]				*P*-value	
Items[3]	FH	FL	MH	ML	FH vs FL	MH vs ML
BMMC/(mg/g)	1.82 ± 0.47[A]	1.22 ± 0.11[B]	1.58 ± 0.29[A]	1.13 ± 0.09[B]	<0.001	<0.001
BML	26.15 ± 2.02[B]	37.68 ± 2.29[A]	27.08 ± 2.48[B]	38.80 ± 1.74[A]	<0.001	<0.001
SL6	27.45 ± 2.48[B]	30.12 ± 1.84[A]	27.75 ± 1.68[B]	31.19 ± 2.15[A]	<0.001	<0.001
SL12	29.13 ± 2.19[B]	33.06 ± 2.29[A]	30.00 ± 2.47[B]	34.77 ± 2.52[A]	<0.001	<0.001
SL18	31.10 ± 2.27[B]	35.96 ± 2.53[A]	32.46 ± 2.61[B]	38.29 ± 2.82[A]	<0.001	<0.001

[A,B]Means within same sex with different superscripts differ highly significantly ($P < 0.001$).
[1]H group: high blackness group; L group: low blackness group.
[2]FH: Female high blackness group; FL: Female low blackness group; MH: Male high blackness group; ML: Male low blackness group.
[3]BML: breast muscle L value; BMMC: melanin content of breast muscle; SL6: 6w skin L value; SL12: 12w skin L value; SL18: 18w skin L value.

Blackness Traits

As showed in Table 3. The SL/BML in the H group were significantly lower than that in the L group while BMMC was higher than the L group ($P < 0.001$). And the SL increases as the chicken grows older, which indicated a decrease in skin blackness. We performed Spearman correlation to analyse the blackness traits of Xuefeng black-bone chickens (Figure 1). BML value was significantly positive correlated with different stage SL while significantly negative with BMMC ($P < 0.001$).

Histomorphological Characteristics of Breast Muscle

As presented at Figure 2. Melanin is primarily distributed unevenly between muscle fibers. Additionally, the H group exhibits higher melanin content and aggregation compared to the L group.

Sequencing Result and Differential Expression Genes

A total of 621,756,856 raw reads were obtained from 14 libraries, and the clean reads ratio was over 95% in each sample and the total map, Q20, Q30, and QC contents respectively exceeded 84, 97, 92, and 50%, which mean the the sequencing data can be used for subsequent bioinformatics analysis because of good quality and high coverage (Table 4). Meanwhile, a total of 831 differential expression genes (**DEG**) were discovered in FH vs. FL, including 476 upregulated genes and 355 downregulated genes, and in total of 405 DEGs consist of 244 upregulated genes and 161 downregulated genes were detected in MH vs. ML (Figure 3). In addition, 37 differential co-expressed genes were obtained from 2 comparisons (Figure 3).

GO Enrichment Analysis

We conducted GO enrichment analyses with DEGs of 2 comparisons and listed the top 30 most significant pathway (Figure 4). In the FH vs. FL comparisons, the results indicated that these genes were enriched to terms such as pigmentation, cytokine production, melanin biosynthetic process, and melanosome etc (Figure 4A). In the MH vs. ML comparisons, the results indicated these genes were enriched to terms such as contractile fiber part, myofibril, melanosome, and pigment granule etc (Figure 4B). As presented in Table 5. We primary focus on melanin-related pathways, including pigmentation, melanin biosynthetic process, melanin metabolic process, melanosome organization, pigment granule organization, melanosome, and pigment granule. And we screened 5 upregulated genes (*DCT, PEML, MLALA, TYRP1, and OCA2*) in the melanin-related pathway that may influence the blackness trait of Xuefeng black bone chicken.

KEGG Enrichment Analysis

We also performed KEGG enrichment analyses with DEGs of 2 comparisons and listed the top 30 pathway (Figure 5). In the FH vs. FL comparisons, the results indicated that these genes were significantly enriched to

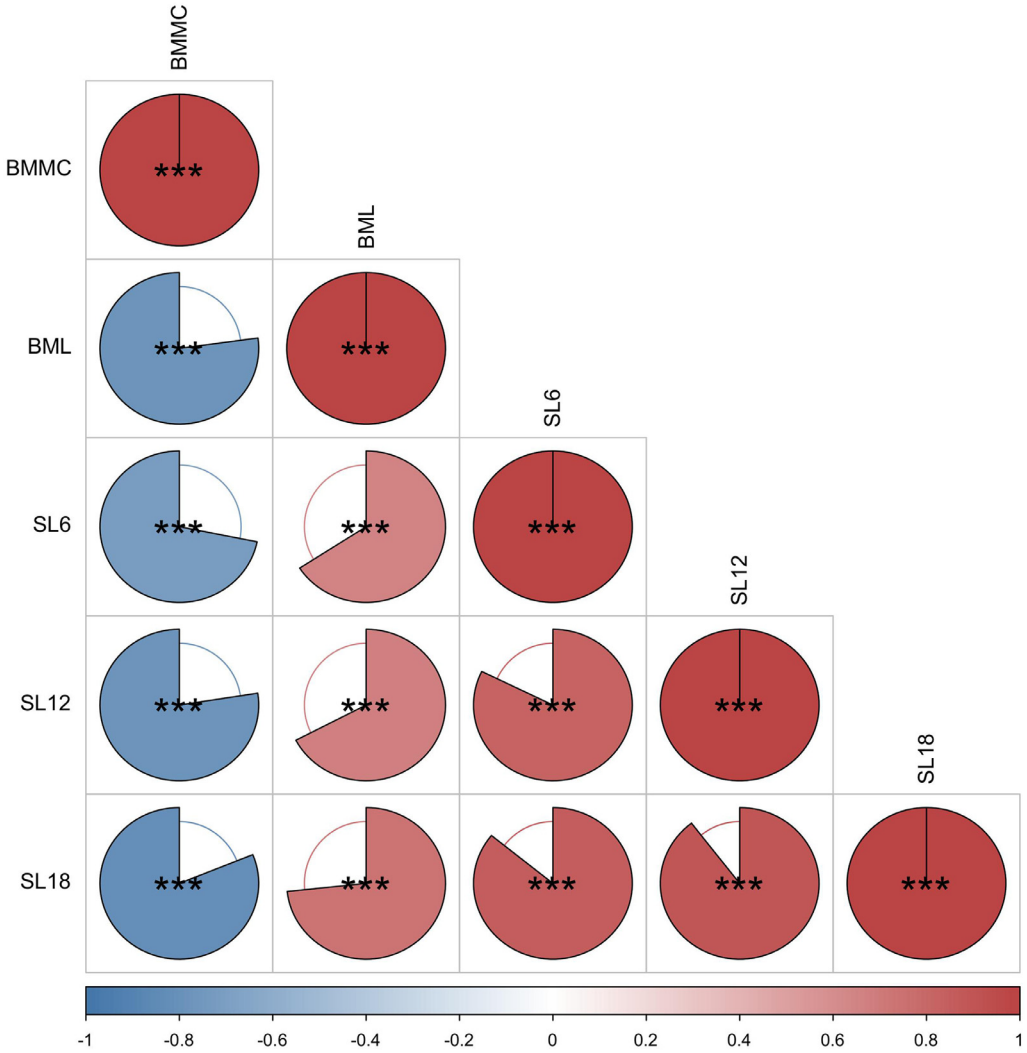

Figure 1. Correlation analysis of blackness traits in Xuefeng black-bone chicken. BML: breast muscle L value; BMMC: melanin content of breast muscle; SL6: 6w skin L value; SL12: 12w skin L value; SL18: 18w skin L value. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

terms such as lysosome, other glycan degradation, melanogenesis, sphingolipid metabolism, and nucleotide metabolism (Figure 5A). In the MH vs ML comparisons, the results indicated that these genes were significantly enriched to terms such as ribosome, TGF-beta signaling pathway, and motor proteins (Figure 5B). As showed in Table 6, we listed melanin-related pathways, including melanogenesis and tyrosine. And we screened 4 upregulated genes (*DCT, EDNRB2, TYRP1, and CALML4*) in the melanin-related pathway that may influence the blackness trait of Xuefeng black bone chicken. In addition, according to GO and KEGG pathway results, there are 2 downregulated genes (*KITLG* and *EDNRB*) which may be potential candidate genes for blackness trait of female chicken.

Real-Time Quantitative PCR Verification

To validate RNA-seq data, the mRNA levels of 7 DEGs were analyzed by RT-qPCR (Figure 6), including 2 downregulated genes (*KITLG* and *EDNRB*) and 5 upregulated genes (*MLANA, EDNRB2, CALML4, DCT*, and *PMEL*). The expression patterns observed in the RT-qPCR data consistently aligned with the findings from RNA-Seq analysis, thus confirming the credibility and reliability of the transcriptome results.

DISCUSSION

As the economic trait of the black-bone chicken, the blackness traits are influenced by many factors, including environment, nutrition and genetics (Yu et al., 2018; Li et al., 2019; Cheng et al., 2023). Although the melanin formation process and some candidate genes for pigmentation have been defined, the regulatory mechanism and the key candidate genes in the breast muscle are still unclear (Singh et al., 2021; Zhou et al., 2021). Therefore, there is still a common phenomenon of significant variation of breast muscle in black-bone chicken (Yu et al., 2018; Xu et al., 2023). Our study found that the blackness variations not affect the growth performance of black-bone chicken, indicating that strengthening the breeding of blackness traits does not influence the original economic value. Meanwhile, there was a significant

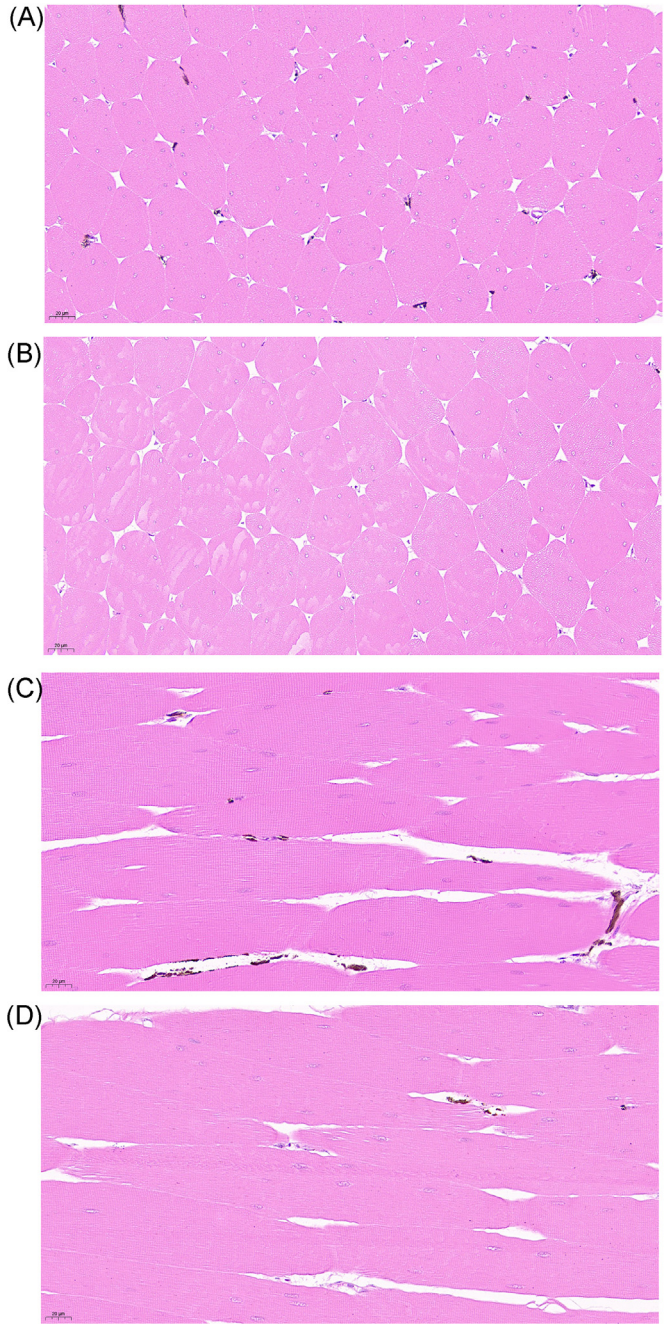

Figure 2. Histomorphological of breast muscle in Xuefeng black-bone chickens (40 ×). (A) Cross-section of high blackness group; (B) Cross-section of low blackness group; (C) Longitudinal-section of high blackness group; (D) Longitudinal-section of low blackness group.

positive correlation between skin L value (**SL**) and breast muscle L value (**BML**). In addition, we also identified seven key candidate genes associated with melanin deposition in the breast muscle of Xuefeng black-bone chicken at market-age, and 2 potential candidate genes only for female chickens, which can be used as molecular markers for breeding. This result will help us accelerate the breeding efficiency of breast muscle blackness traits in black-bone chicken industry and understand the regulatory mechanisms of melanin deposition better.

Our studies demonstrated that there are no difference in the initial body weight (**IBW**), final body weight (**FBW**), eviscerated weight (**EW**), breast muscle weight (**BMW**), tibia wight (**TW**) between the high blackness group (H group) and the low blackness group (L group) in same sex. The body weight as a important economic trait can directly reflect the growth performance of chickens, so we recorded the one-day-age, the market-age and the eviscerated weight (Li et al., 2018). The breast muscle is one of the major meat-producing sites and the breast muscle rate was roughly 18.42% in Xuefeng black-bone chicken (Maharjan et al., 2021; Xu et al., 2023). And we also focused on the BMW because the most obvious changes in our research were the blackness of breast muscle (**BBM**), but there are no affect in BMW. The TW are positively correlated with body weight of chickens, which can react the potential growth trends of body (Kolakshyapati et al., 2019; Chew et al., 2021). Wang et al. (2021a) reported that genetics and breeding of body weight and skin color are not affect each other. There are no paper reported about the relationship between the BBM and the growth performance at present but our results showed that BBM changes can not effect the growth performance of black-bone chickens.

Melanin content is the main influencing factor that leads to the different degree of blackness (Nganvongpanit et al., 2020; Jian et al., 2021), our results maybe proof this point. Since the melanin content of breast muscle (**BMMC**) in the H group was significant higher than the L group. And the breast muscle histomorphological presented that melanin distributed unevenly between muscle fibers, and the H group can be observed more melanin content and aggregation compared to the L group. There are many results similar with our finding (Nganvongpanit et al., 2020; Kriangwanich et al., 2021). The SL/BML of the H group was significantly lower than the L group and the SL/BML was significantly negative correlated with the melanin content in this study. It indicates that L value can be used as one of the criteria for judging blackness in Xuefeng black-bone chicken, and the L value is easier to obtain in routine breeding process. A large number of studies evaluate the blackness of black-bone chickens by using colorimeter to measure the L value (Li et al., 2019; Wang et al., 2021a; Zi et al., 2023). And there are significantly positive correlation between the SL and BML. This suggests that perhaps the SL can be used as an indirect breeding index of the breast muscle blackness. In addition, we found an interesting phenomenon that the SL increased with the growth of black-bone chickens. It is mean the blackness was become lower along with growth, the reason maybe that the number of melanocytes and the melanin deposition ability was affected by age (Wang et al., 2021a; Zi et al., 2023).

We performed transcriptome sequencing analysis of breast muscle tissues from roosters and hens. There are 831 and 405 DEGs were discovered in FH vs. FL and MH vs. ML comparison, respectively. But only 37 differential co-expressed genes were obtained from 2 comparisons, which may be due to different market-age and sex. And these DEGs included seven candidate gene (*DCT*,

Table 4. Sequencing data for breast muscle samples in Xuefeng black-bone chickens.

Sample[1]	Raw reads	Clean reads	Total map	Q20	Q30	GC_pct
MH1	44168884	42678944	36355893(85.18%)	97.26%	93.05%	52.35%
MH2	45267180	43956246	38929352(88.56%)	97.63%	93.87%	50.32%
MH3	46332722	44211802	38744528(87.63%)	97.88%	94.46%	51.76%
MH4	43878464	42568726	37089713(87.13%)	97.48%	93.50%	51.33%
ML1	46953370	45410886	40154823(88.43%)	97.66%	93.91%	50.60%
ML2	40939768	39197944	34157427(87.14%)	97.49%	93.40%	51.50%
ML3	43945952	42790528	36608247(85.55%)	97.49%	93.56%	52.16%
ML4	44817096	43525082	37198343(85.46%)	97.10%	92.74%	51.89%
FH1	43630796	42175002	35775386(84.83%)	97.44%	93.50%	53.38%
FH2	43940412	42779666	36912851(86.29%)	97.25%	92.96%	52.31%
FH3	45499310	44051084	39182153(88.95%)	97.44%	93.40%	51.38%
FL1	44083926	42830290	37937133(88.58%)	97.59%	93.72%	51.49%
FL2	43931098	42673912	38245440(89.62%)	97.61%	93.72%	50.23%
FL3	44367878	42918108	37657478(87.74%)	97.69%	93.96%	51.59%

[1]FH: female high blackness group; FL: female low blackness group; MH: male high blackness group; ML: male low blackness group.

PMEL, *MLANA*, *TYRP1*, *OCA2*, *EDNRB2*, and *CALML4*) were mainly enriched in the pigment granule, melanosome, and melanogenesis pathway, which involved in the regulation of melanin biosynthesis. Melanin deposition is mainly divided into the following 3 processes, melanocytes formation and development, melanin synthesis and melanin transport (Cieslak et al., 2011; Vandamme and Berx, 2019). First, the embryonal neural crest cells differentiate into melanoblast which migrate to specific sites along the dorsolateral and ventral migratory pathways and thus differentiate into melanocytes (Mort et al., 2015; Vandamme and Berx, 2019). Endothelin receptor B subtype 2 (**EDNRB2**) is play an important role in above process. Numerous

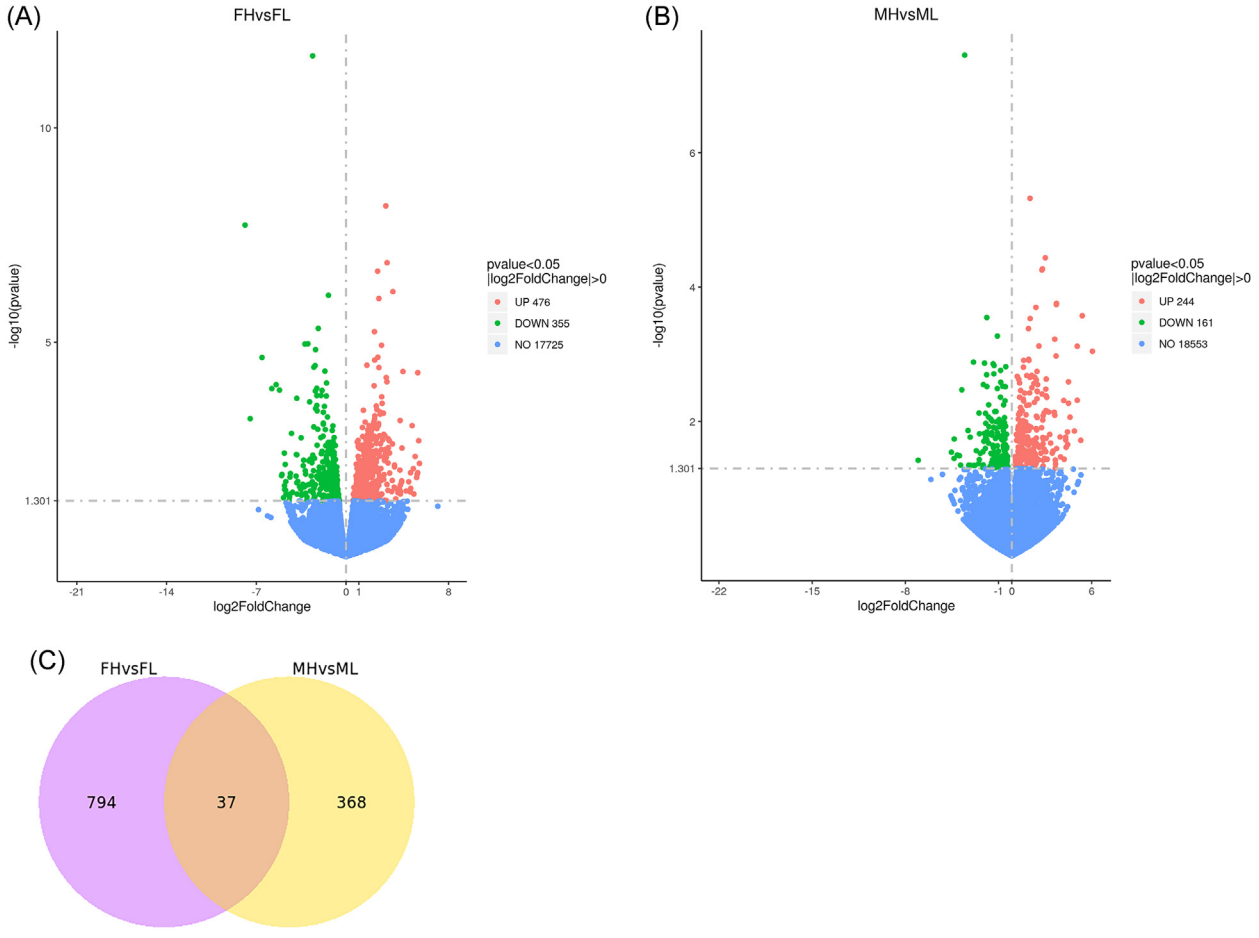

Figure 3. The volcano plot and venn diagram of the differential expression genes. (A) FH vs. FL: the high blackness group compared with the low blackness group in female chicken; (B) MH vs. ML: the high blackness group compared with the low blackness group in male chicken; (C) Venn diagram of the differential co-expression genes in 2 comparisons (FH vs FL and MH vs. ML). Red dots mean upregulated genes; green dots mean downregulated genes; blue dots mean non-differential genes.

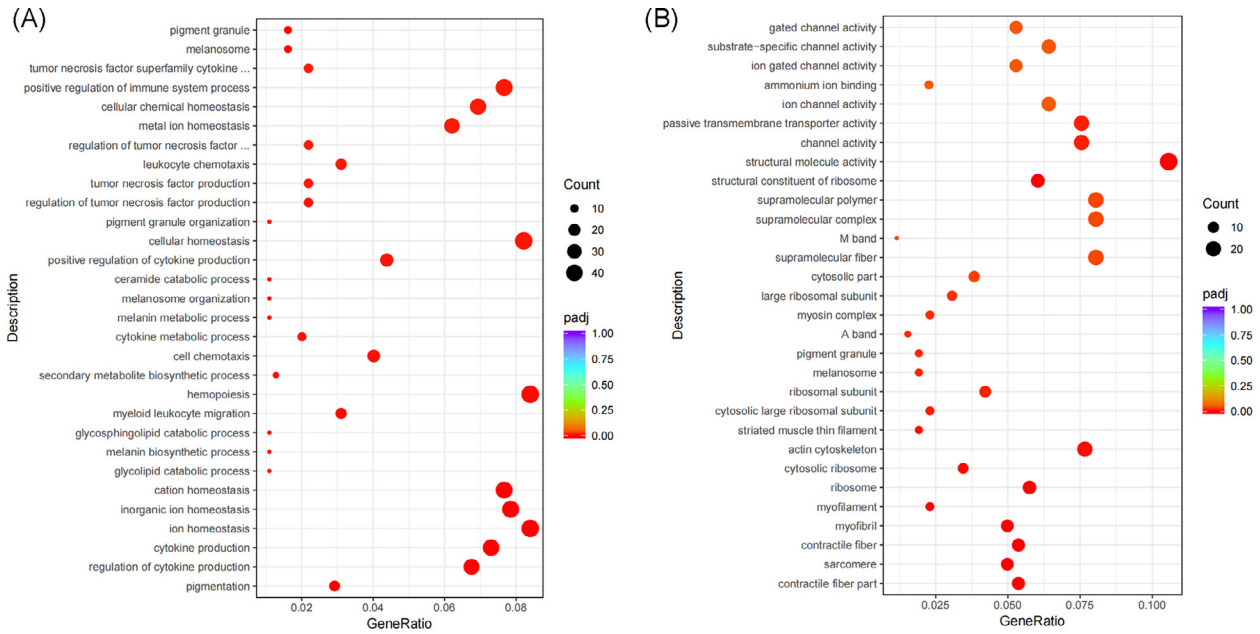

Figure 4. The 30 most significant GO pathway in the 2 comparisons. (A) FH vs. FL: the high blackness group compared with the low blackness group in female chicken; (B) MH vs. ML: the high blackness group compared with the low blackness group in male chicken.

Table 5. GO enrichment pathways related to melanin.

Sex	Category[1]	GO ID	Description	Gene name	*P*-adj
Female	BP	GO:0043473	Pigmentation	Up: *PMEL, OCA2, TYR, DCT, TYRP1, EDN3, SOX10, ASIP, RAB32, GPR143, FIG4, RAB17, HPS1, MC1R* Down:*KITLG, EDNRB*	<0.001
	BP	GO:0042438	Melanin biosynthetic process	Up: *OCA2, TYR, DCT, TYRP1, ASIP, MC1R*	0.002
	BP	GO:0006582	Melanin metabolic process	Up: *OCA2, TYR, DCT, TYRP1, ASIP, MC1R*	0.003
	BP	GO:0032438	Melanosome organization	Up: *PMEL, TYRP, ASIP, RAB32, GPR143, HPS1*	0.003
	BP	GO:0048753	Pigment granule organization	Up: *PMEL, TYRP, ASIP, RAB32, GPR143, HPS1*	0.004
	CC	GO:0042470	Melanosome	Up: *PMEL, OCA2, MLANA, TYR, DCT, TYRP1, RAB32, GPR143, RAB17*	0.001
	CC	GO:0048770	Pigment granule	Up: *PMEL, OCA2, MLANA, TYR, DCT, TYRP1, RAB32, GPR143, RAB17*	0.001
Male	CC	GO:0042470	Melanosome	Up: *DCT, PMEL, MLANA, TYRP1, OCA2*	0.010
	CC	GO:0048770	Pigment granule	Up: *DCT, PMEL, MLANA, TYRP1, OCA2*	0.010

[1]BP: biological process; CC: cellular component.

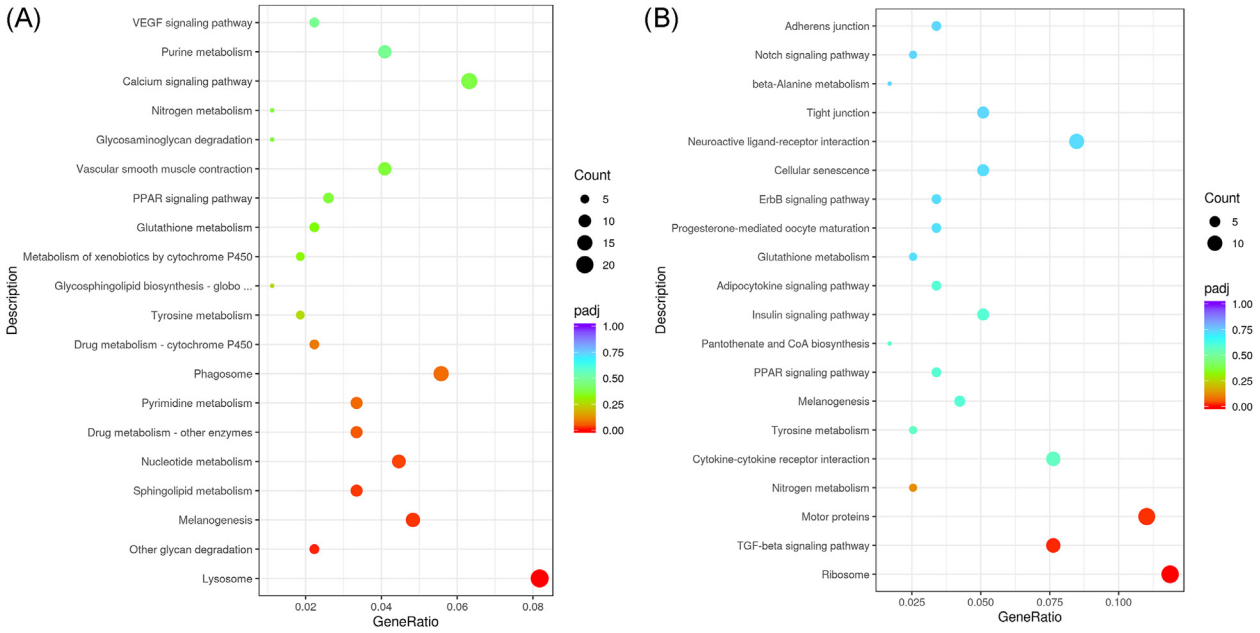

Figure 5. The KEGG enrichment pathway. (A) FH vs. FL: the high blackness group compared with the low blackness group in female chicken; (B) MH vs. ML: the high blackness group compared with the low blackness group in male chicken.

Table 6. KEGG enrichment pathways related to melanin.

Sex	KEGG ID	Description	Gene name	*P*-adj
Female	gga04916	Melanogenesis	Up: *EDNRB2, CALML4, TYR, DCT, TYRP1, ASIP, CAMK2A, PRKCB, GNAO1, MC1R* Down:*KITLG, EDNRB, WNT9B*	0.018
	gga00350	Tyrosine metabolism	Up: *TYR, DCT,TYRP1* Down: *ADH1C*	0.258
Male	gga04916	Melanogenesis	Up: *DCT, EDNRB2, TYRP1, CALML4* Down: *CREBBP*	0.594
	gga00350	Tyrosine metabolism	Up: *DCT, TYRP1*	0.566

studies have shown that aberrant *EDNRB2* mainly causes hypopigmentation through restraining the migration of melanoblasts to other places (Li et al., 2015; Xi et al., 2020; Xi et al., 2021). Xi et al. (2021) revealed that *EDNRB2* is the key genes influenced the duck body surface spot size, and found 2 allele locus as molecular markers. And Xi et al. (2020) demonstrated that a 14-bp insertion in *EDNRB2* exon 3 related with white plumage in Chinese geese.

Melanin is synthesized and secreted by melanosomes in melanocytes, and then transported to the surrounding keratinocytes to exercise the pigment function (Crawford et al., 2020; Zhou et al., 2021). The melanosome, as a lysosome-associated organelles, mature through 4 stages including the immature step (stage I and II) and mature step (stage III and IV) (Costin and Hearing, 2007; Bissig et al., 2016). Premelanosomal protein (***PMEL***) as a type I transmembrane glycoprotein, it mainly maintains the structural morphology during the immature step of melanosomes (Mcglinchey et al., 2009; Knaust et al., 2020). Premelanosomal protein begin to hydrolyze form the *PMEL* fibrils/melanosome matrix at the stage I melanosomes, and Orderly and structured *PMEL* fibrils turn melanosomes into a typical ellipsoid shape at the stage II melanosomes (Bissig et al., 2016; Knaust et al., 2020). Melan-A (***MLANA***) also known as melanoma antigen recognized by T-cells 1 (***MART-1***), which could form a complex with *PMEL* to regulating its expression, stability, and processing (Hoashi et al., 2005). We can observe coat color faded and morphological structure of melanosomes altered in mice when silence *MLANA* expression or knockout this gene (Hoashi et al., 2005; Aydin et al., 2012). Oculocutaneous albinism type 2 (***OCA2***), encoded a melanosome-specific transmembrane protein, may participate melanosome maturation at the stage I and II. This protein forms a transmembrane channel crucial for the modulation of melanosome pH and transport of tyrosine, which is essential for melanin synthesis (Bellono et al., 2014; Klaassen et al., 2018; Le L et al., 2020). And *OCA2* abnormal expressed along with the changes in the number, morphology and type of melanosomes (Park et al., 2015), but the specific mechanism is not clear. The melanogenesis process is start in stage III melanosomes, the L-tyrosine oxidation to dopaquinone and eventually generate eumelanin under the catalysis of tyrosinase, tyrosinase-related proteins1 (***TYR1***), dopachrome tautomerase (***DCT***) (Pillaiyar et al., 2017; Lu et al., 2021). Mutations in *TYRP1* usually can result in oculocutaneous albinism type 3 with rufous or brown phenotype in skin, hair and irises (Patel et al., 2021). And recently studies defined *DCT* as a disease-causing gene in oculocutaneous albinism type 8 (Tingaud-Sequeira et al., 2022). No candidate genes related to melanin transport were found in this study, but there are lots of studies proved all of the above genes play necessary roles in the process of melanin production in animals (Bovo et al., 2023; Cheng et al., 2023; Li et al., 2023).

In addition, we found calmodulin-like protein 4 (***CALML4***) also involved in melanogenesis pathway, but there are no paper reported that its functional properties about melanin-related at present. Calmodulin-like protein 4, which functions as a light chain for myosin-7a, is a novel component of the intermicrovillar adhesion complex and Usher complex. It may play a significant role in both intestine and inner ear biology (Choi et al., 2020; Kapustina and Cheney, 2020). We speculated that *CALML4* maybe need combined with other melanin-related proteins to form a complex that participates in melanin production, similar to how melanophilin requires binding with Rab27a and myosin Va to affect melanosomes transport (Park et al., 2019). And we found 2 potential candidate genes (*KITLG* and *EDNRB*) in female chicken, which could help us exhaustive understand the mechanisms of melanin production. Receptor tyrosine kinase (***KIT***) / *KIT* ligand (***KITLG***) signaling pathway is necessary for the survival of melanocytes. Ligand-induced dimerization is triggered after *KITLG* binds the *c-KIT* receptor, and it startover signal

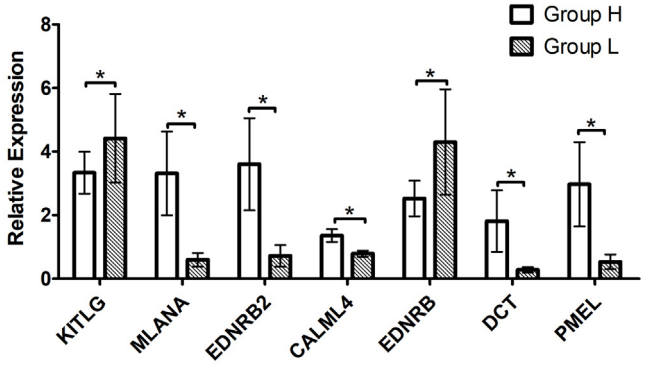

Figure 6. Validation of candidate DEGs using real-time quantitative PCR. Sample size in each group was seven, include female and male chickens. $^*P < 0.05$.

transduction through the *RAS/MAPK* pathway to increased melanoblast reproduce (Gorenjak et al., 2021). Mutation in *KITLG* are responsible for causing familial progressive hyper- and hypopigmentation in human (Wang et al., 2021b). And *KITLG* expression was lower in light color animals than in dark color animals (Song et al., 2017; Wu et al., 2021). Endothelin (***EDN***)/Endothelin Receptor Type B (***EDNRB***) signaling pathway was similar with *KIT*/*KITLG* signaling pathway, which mainly affecting the migration and proliferation of melanoblasts (Regazzetti et al., 2015). Therefore, abnormal expression in *EDNRB* cause a reduction in the number of melanoblasts and pigment dilution (Imokawa and Ishida, 2014; Bovo et al., 2023).

CONCLUSIONS

In conclusion, our research revealed that blackness variations in breast muscle do not affect growth traits in Xuefeng black-bone chicken. And the skin L value demonstrates a significant positive correlation with breast muscle L value, suggesting skin L value can serve as an indirect indicator for breast muscle breeding. Additionally, we identified seven candidate genes (*DCT, PMEL, MLANA, TYRP1, OCA2, EDNRB2,* and *CALML4*) associated with melanin production at market-age of Xuefeng black-bone chicken, and 2 potential candidate genes (*KITLG* and *EDNRB*) only for female chicken. These genes are beneficial to explore the genetic mechanism of black traits and could serve as molecular markers for breeding of breast muscle blackness. However, there maybe some shortage to deeper explore mechanism of melanin deposition due to the different market-age, we will adopt the same age black-bone chicken to research further. This study provides valuable insights for the breast muscle breeding of black-bone chicken, contributing to the advancement of the black-bone chicken industry.

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Integrative analysis of Genome Re-sequencing and 10 × Genomics scRNA-Seq Data Elucidate the Molecular Mechanisms Regulating Melanin Transfer of Pectoral Muscle in Black-boned Chickens (32072711) and Identification of SNPs Affected Blackness of Breast Muscle in Xuefeng Black-bone Chicken by Combining of Bulked Segregant Analysis and RNA Sequencing (2021JJ30322).

DISCLOSURES

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103691.

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