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Unique feather color characteristics and transcriptome analysis of hair follicles in Liancheng White ducks

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ABSTRACT Avian feather color is a fascinating trait, and the genetic mechanism of duck plumage formation is still in the preliminary stage. In this study, feather color of Liancheng White ducks was analyzed by determination of melanin content and RNA-seq analysis. In this research, 9 ducks from Mallards (n = 3), Liancheng White (n = 3) and Pekin ducks (n = 3) were used by high performance liquid chromatography (**HPLC**) and Masson-Fontana staining to reveal the difference of feather melanin content. RNA-seq from 11 hair follicle tissues (1- and 8-wk-old) of Liancheng White ducks (n = 5) and Pekin ducks (n = 7) was used to analyze the candidate genes for the feather melanin synthesis, and Immunofluorescence experiment was used to show the protein expression in 6 black- and white-feathered ducks. Pectorale, skin, liver, fat, brain, heart, kidney, lung, spleen of an 8-wk-old black-feathered Mallard were collected for candidate gene expression. The results showed that the contents of feathers, beak, web melanin in Liancheng White ducks were higher than in Pekin ducks ($p < 0.05$). Melanin within hair follicles was located in the barb ridge and hair matrix of black feather duck, also we found that *TYRP1, TYR, SOX10* genes were differentially expressed between Liancheng White and Pekin ducks ($p < 0.05$), and these genes were mainly expressed showed in duck skin tissues. This study revealed the unique feather color phenotype of Liancheng White duck shedding light on the transcriptome that underlies it.

Key words: Liancheng White duck, RNA-seq, feather color, melanin, poultry

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INTRODUCTION

White feather is an important economic trait in ducks, and it can be processed into down jackets, down comforters and other products. After the duck down is washed, extracted and graded, the raw material formed is the main material of a down jacket, and the market demand is large (Gong et al. 2010). For these products, white feather ducks are favored by consumers. The variations of feather color in birds are related to the distribution, content and proportion of pigments (Liu et al., 2023a). Melanin is the main pigment, which mainly affects the color of feathers and the depth of skin color. Melanin production is associated with mutations in related genes, and the amount and distribution of melanin in ducks varies breeds (Zhou et al., 2018).

Mallards are the ancestors of many different types of ducks. After human domestication, domestic ducks have gradually developed a variety of different breeds and types. These duck breeds, although domesticated, still retain many of the characteristics of mallard ducks, but there are also significant differences, such as feather color, and body size (Zhou et al., 2018; Feng et al., 2021). As a typical local breed, the Liancheng White duck has white feathers but black beak and web, while the Pekin duck has white feathers and yellow beak and web. Both Pekin duck and Liancheng White Duck represent different aspects of Chinese food culture and are the treasures of Chinese duck breeds. Their feather color has attracted many researchers to conduct in-depth research, but no one has carried out a study on the comparison of melanin content and its link with feather color.

Duck is a good model to study the plumage color of birds, but there are only a few studies describing feather color. Researchers found *MC1R* are related to black

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feather in ducks, and single nucleotide polymorphism mutations in the *MC1R* regulatory region are found to be associated with duck melanism (Liu et al., 2023a). Some studies have shown that the dark spots in duck feathers are caused by the *EDNRB2* gene (Xi et al., 2021). The *MITF-M* gene is also considered responsible for white feathers (Zhang et al. 2018; Zhou et al. 2018). Although there is already more research performed on duck feather color compared to that of many birds, the research on duck feather color is still in the primary stage, and there are still many genetic mechanisms of duck feather color formation that deserve to be explored.

The main mechanism of high-performance liquid chromatography (**HPLC**) is to oxidize eumelanin to pyrrole Pyrrole-2,3-dicarboxylic acid (**PDCA**) and Pyrrole-2,3,5-tricarboxylic acid (**PTCA**), respectively, and then perform liquid phase detection and quantitative conversion. Ultraviolet spectrophotometry can only achieve rough quantification of melanin content. There are problems of low separation degree and interference of similar substances in capillary electrophoresis. Thin layer chromatography (**TLC**) is a complicated and time-consuming process with low recovery rate. The HPLC method is simple, rapid and accurate to calculate the content of melanin. Masson staining is the most classic method of connective tissue melanin staining (Kwon-Chung et al., 1981), which are used for qualitative detection of skin tissue melanin in this study. At the same time, with the help of advanced sequencing techniques such as RNA-seq, more and more candidate genes related to feather color in birds can be revealed. RNA-seq results can be used to study the molecular mechanism of feather color formation (Domyan et al., 2019; Du et al., 2023), help us understand the biological process of feather formation of different colors, and provide more useful reference information for poultry breeding. More duck breeds should be investigated to reveal the gene expression profiles associated with duck plumage color. Liancheng White duck has a small amount of melanin in its feathers at birth, while Pekin duck has no melanin in its feathers. Therefore, it is worth exploring whether there are differences in feather melanin between adult Liancheng White ducks and Pekin ducks, and what the function would be of potential differentially expressed genes (**DEG**). In this study, we hope to analyze the formation principle of the unique feather color phenotype of Liancheng White duck, a rare local breed in China, so as to provide a theoretical basis for the study of bird feather color and contribute to research related to breeding of white feather in poultry and subtle phenotypic differences in biodiversity.

MATERIALS AND METHODS

Ducks and Sampling

All procedures used for this animal study fully complied with the guidelines for the care and use of experimental animals established by the Chinese Academy of Agricultural Sciences (IAS2022-103). The ducks were randomly selected from a duck farm and were half male and half female, all healthy, and fed in similar conditions. All the duck samples in the study met the requirements of animal ethics. Hair follicle and skin tissue for RNA-seq were collected from 11 ducks (1-wk-old, 8-wk-old), including 5 Liancheng White ducks, and 7 Pekin ducks. 3 Mallards, 3 Liancheng white ducks and 3 Pekin ducks were used for the determination of melanin for HPLC. Pectorale, skin, liver, fat, brain, heart, kidney, lung, spleen of an 8-wk-old black-feathered Mallard were collected for RNA -seq analysis, while skin tissues of 4 samples of black-feathered Mallards at 1 d, 2 wk, 4 wk, and 8 wk after birth were collected for time-course RNA-seq analysis. The sample size for each period is one. All samples of 3 breeds of ducks were immediately frozen in liquid nitrogen and stored at -80°C. Fresh skin tissue from each of the 3 breeds of ducks were soaked in 4% paraformaldehyde liquid and fixed for subsequent protein immunofluorescence analysis.

Determination of Melanin Content by High Performance Liquid Chromatography

Melanin can be oxidized and hydrolyzed to PDCA and PTCA in the alkaline environment of hydrogen peroxide (Bi et al., 2023). Their different concentrations of 5, 10, 25, 50, 100, 250, and 500 ng/mL were used to make quantitative standard curves. 0.01g plumage tissues of 6-wk-old of Liancheng White ducks, and Pekin ducks were accurately weighed into centrifugal tubes, and 0.5 mL of 30% hydrogen peroxide and 0.5 mL of 2M ammonia were added. The hydrolysate was gently mixed and hydrolyzed at 30°C for 12 h. First, 0.4 mL of 11.3% ammonium sulfite aqueous solution was added, placed for 5min, then 0.4 mL of 4M acetic acid was added, and 3,000 *g* of supernatant was collected by centrifugation. Then, the supernatant was added to the solid phase extraction column for adsorption. Third, 3 mL aqueous methanol was added for leaching, then 1 mL methanol solution containing 10% triethylamine was added twice for elution. The eluted liquid was dried by vacuum drying or nitrogen blowing. The dried liquid was redissolved in 1 mL methanol solution containing 0.5% formic acid. Finally, the filtrate was transferred to a 2 mL sample vial for HPLC detection (Wang et al., 2014). In this study, ultra-high pressure liquid phase (ACQUITY UPLC system, Waters, MA) was used.The mobile phase is A: water containing 0.1% acetic acid and B: acetonitrile containing 0.1% acetic acid. The chromatography was performed on Agilent ZORBAX C18 column (3.0 mm x 150 mm) with a flow rate of 0.35 mL/min and column temperature of 45°C. The acquisition mode of this study is multiheavy ion detection, and it is negative ion detection, and the ion source voltage and temperature are 5,500V and 500°C respectively (Wang et al., 2014).

Table 1. . Information on the primers used for qPCR.

Gene	Primer information (5′-3′)	Size (bp)	TM (°C)
TYRP1	F: TCTCAGCAGAGGAGAGGTACCAAAA R: GCCGTCTCTTTCTGAGGGCTG	113	62
TYR	F: AAAGAACACCCCCAGCCAGG R: GTCCCGCCACACATTGGACC	169	62
SOX10	F: CCGGCTTCCTTGCGATCAGT R: AGCGGGGAAGCAAGCGTAAG	104	62
GAPDH	F: GGTAGTGAAGGCTGCTGCTGATG R: GGAGGAATGGCTGTCACCGTTG	100	63

Masson-Fontana Staining to Determine Melanin Distribution

Fresh 1 × 1 cm^2 skin tissue from 6-wk-old of black-feathered Mallard, Liancheng White duck, and Pekin duck were soaked in 4% paraformaldehyde. The samples were placed in the fixing solution of 20 times the sample volume and fixed for more than 24h. Dewaxing and hydration of paraffin sections (Lecia CM1900, Lecia, China) involves the following steps: Firstly, dewaxing in xylene (II) for 10 min. Then 70, 80, 95% anhydrous ethanol was used to wash for 2 min each time, and finally anhydrous ethanol washed for 5min and distilled water washed for 2 min. Put it in Fontana ammonia-silver solution, stain it against light for 12 to 24 h or incubate it at 56°C for 30 to 40 min. Soak in distilled water for 5 to 6 times, 1 to 2 min each time. The sections were treated with sea wave solution for 1 to 5 min. Treat with tap water for 3 to 5 min. A total of 95% ethanol, anhydrous ethanol dehydration, and xylene transparent, neutral gum seal (Kwon-Chung et al., 1981). Finally, Seal with neutral gum and observe under a Panoramic scanner (3DHISTECH P250 FLASH, 3DHISTECH, Hungary).

RNA Extraction, Library Construction, and Sequencing

Using an RNA extraction kit (Vazyme, China), total RNA of the hair follicles of 1- and 8- wk old ducks was extracted and converted into cDNA, including 5 Liancheng White ducks and 7 Pekin ducks, as well as 4 different periods of Mallard skin tissues and 9 multi-tissues. In total, 25 libraries were finally produced for the RNA-seq experiment. The average output was 6 Gb per library. RNA-seq paired-end reads from each of 25 libraries were mapped against the above-mentioned Pekin duck reference genome using Hisat. The constructed library was sequenced on the Illumina Hiseq X Ten sequencing platform at 150bp both ends (Gai et al., 2023).

Comparative Analysis of the Sequence of RNA Data

According to the Perl script, we will analyze the raw fastq data to filter the raw data in fastq format. This script deletes reads with adapters, low-quality reads, and reads with poly-N. The latest duck reference genome ZJU1.0 (GCF_015476345.1)) and gene model annotation files (GCF_015476345.1) from the NCBI database. The fragments per kilobase per million values were calculated by Hisat (Zhou et al., 2018). The clean data were then aligned to the reference genome using HTSeq (Liu et al. 2022), and the counts per million mapped sequence read (**CPM**) for each gene were calculated. Differential expression for each gene in different samples were calculated using edgeR version 3.20.9 software package (Gai et al., 2023; Liu et al., 2023b).

Validation of RNA-Seq Data Via Quantitative Real-Time PCR

The differentially expressed genes (**DEG**) in 1- and 8-wk-old tissues of Liancheng White ducks and Pekin Ducks were identified. With the help of qPCR technology, gene expression analysis was performed on the selected candidate genes, and the primers are shown in Table 1. qPCR was performed using SYBR Green Real-time PCR Master Mix (TaKaRa, China). $2^{-\Delta\Delta Ct}$ method and Student's t tests were used for comparative analysis of gene differential expression (Wang et al., 2022a, b). $p < 0.05$ and $p < 0.01$ were considered significant and highly significant, respectively.

Immunofluorescence Experiment

Skin samples of each one of 6-wk-old Mallard, Liancheng White, Pekin duck were embedded in paraplast, fixed in 4% buffered paraformaldehyde, and cut into 5 cm sections, and 4% paraformaldehyde fixed overnight at 4°C. Each skin tissue contains multiple hair follicles. The fixed time should not be too long, to less than 24 h is appropriate. These slices were dewaxed, rehydrated, in order to make the section adhere more firmly and not easy to take off, and is also conducive to dewaxing. The sections incubated for 20 min at 100°C with EDTA (Servicebio). Section antigens were fixed in Tris-EDTA and then the sections were washed 3 more times with phosphate-buffered saline (**PBS**) before being washed once more. The sections were then saturated with the protein antibody (Abclonal) at 4°C 12h after being treated with 3% bovine serum albumin (Solarbio) for 30 min. SOX10 antibody used was anti-rabbit antibody, and the hair follicle tissues with different plumage colors were counterstained with DAPI, and the experimental results were photographed and recorded.

RESULTS

Plumage Melanin Content and Melanin Distribution in Hair Follicle of Ducks

The head, back, abdomen, wing, tail plumages of Mallards, Liancheng White ducks and Pekin ducks were determined by HPLC method. Meanwhile, tissue samples from the beak and web tissues of Liancheng White ducks and Pekin ducks were collected for the determination of melanin content. The results showed that the content of feather melanin in Liancheng White ducks was significantly higher than Pekin ducks ($p < 0.01$) (Figure 2), but its melanin content were lower than that of Mallards. The melanin contents of Liancheng White ducks in the beak and web were very significantly higher than Pekin ducks ($p < 0.001$). Masson-Fontana staining showed the melanin was located in the barb ridge and hair matrix of black-feathered Mallards, and there was almost no obvious melanin distribution in the hair follicles of Liancheng White and Pekin ducks (Figure 3). The results showed that there was a small amount of melanin in Liancheng White duck feathers, and the head feathers had the highest amount of melanin in all feathers.

Differentially Expressed Genes in Hair Follicles of Liancheng White and Pekin Ducks

After filtering DEGs with an FDR ≤ 0.05 and a | Log2 (fold change) | ≥ 1, 659 DEGs were found between 1-wk-old Liancheng White and Pekin ducks, while 2093 DEGs were found between 8-wk-old Liancheng White and Pekin ducks. Among these differential genes, we found genes including *TYRP1, TYR* and *SOX10*, that were higher expressed in the hair follicles of Liancheng White ducks than in those of Pekin ducks. These genes participate in the melanin synthesis pathway, indicating that the feather color of Liancheng White duck and Pekin duck is indeed different in the synthesis of melanin. The RNA-seq results showed that the CPM expression of these candidate genes in hair follicles of Liancheng White ducks was higher than that in Pekin ducks (Figure 4). qPCR results confirmed the RNA-seq results (Figure S1). These results indicated that *TYRP1, TYR* and *SOX10* genes were involved in the synthesis of melanin in Liancheng White duck feathers.

Immunofluorescence Revealed Protein Expression in Black and White Hair Follicle of Ducks

Immunofluorescence was verified by using SOX10 proteins in skin tissues of each one of black-feathered Mallards, Liancheng White ducks, and Pekin ducks (Figure 6). The results showed that the expression of SOX10 was high in the hair follicles of black-feathered Mallards, but almost no fluorescence was detected in the hair follicles of Liancheng White ducks and Pekin ducks, indicating that SOX10 protein was differently expressed in the black hair follicles, and the expression was very low or almost not existent in the white feather follicles.

Melanin Synthesis Related Gene Expression of Different Tissues and Periods in Hair Follicle of Black-Feathered Mallards

The expression of differentially expressed *TYRP1, TYR*, and *SOX10* genes varied across various tissues. However, these genes demonstrated heightened expression within skin tissues compared to other tissues, such as pectorales, liver, fat, brain, heart, kidney, lung, and spleen (Figure 5A). The results showed that *TYRP1, TYR*, and *SOX10* were highly expressed in the skin tissue of black-feathered Mallards. Meanwhile, the expression levels of these candidate genes had variational tendency in different growth stages of black-feathered Mallards (Figure 5B). The expression levels were higher in hair follicle tissues at the early stage, and reached a peak at 2-wk-old, which was consistent with the early molting behavior of ducks. These results indicated that *TYRP1, TYR,* and *SOX10* genes were highly expressed in duck dark-feathered skin tissues, while their expression levels were generally low in other tissues.

DISCUSSION

Ducks are a good model for studying feather color since they have a variety of feather color patterns (Zhou et al., 2018). Different breeds of ducks have different feather colors and patterns, and these differences are mainly due to the interaction of genetic factors. As a typical local breed, the Liancheng White duck has white feathers but black beak and web, while the Pekin duck has white feathers and yellow beak and web, but the plumage color of both the Liancheng White and Pekin duck is white (Figure 1), and so far, no research has been carried out comparing the feather melanin of Liancheng White and Pekin ducks. In this study, we first discovered that the content of feather melanin in Liancheng White duck was higher than in Pekin duck ($p < 0.01$) (Figure 2). Masson-Fontana staining showed that the melanin was located in the barb ridge and hair matrix of black-feathered Mallards, which is consistent with previous findings (Koch et al., 2019), and there was almost no obvious melanin distribution in the hair follicles of Liancheng White ducks and Pekin ducks (Figure 3). This result indicated that the feather of Liancheng white ducks contained a small amount of melanin, and the white feather trait of it was caused by the low content of melanin. Through RNA-seq analysis, we found that there are differences in the expression of genes related to the synthesis of melanin in the feathers of Liancheng White Duck and Pekin duck, including *TYRP1, TYR*, and *SOX10* (Figure 4, S1). These genes have been widely reported to be involved in the melanin synthesis pathway (Sultana et al., 2018; Yun et al., 2019).

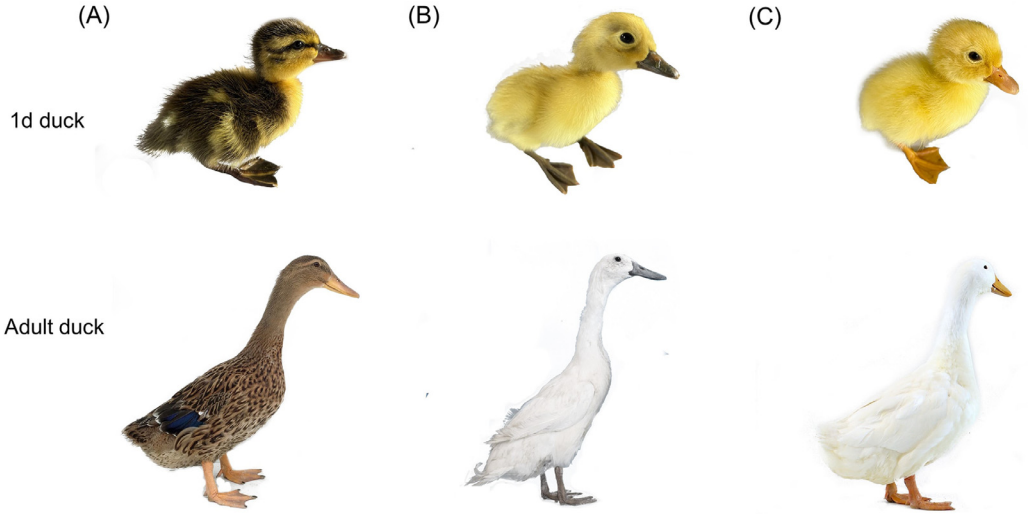

Figure 1. One-week-old and adult periods of Mallards, Liancheng White ducks and Pekin ducks. The plumage of Liancheng White ducks and Pekin ducks change from light yellow to white after birth.

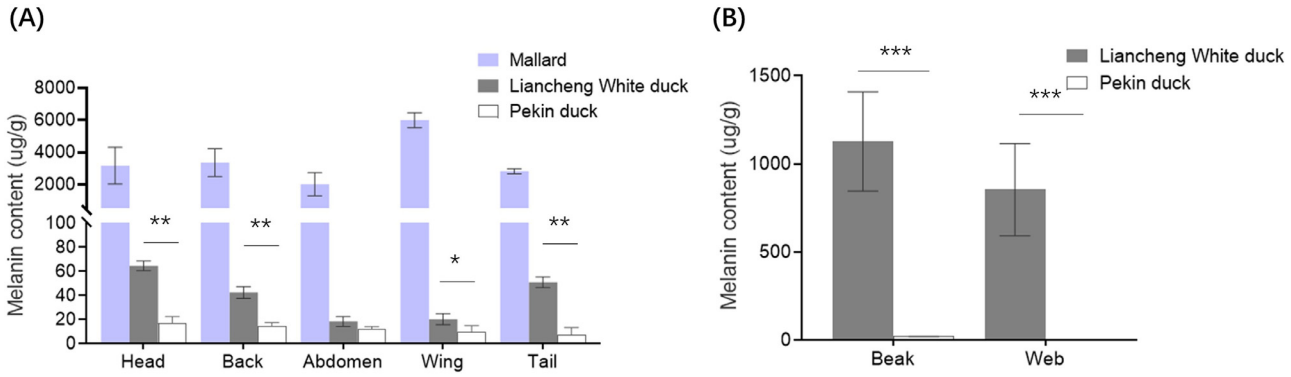

Figure 2. Determination of melanin content in duck tissues. (A) Melanin content in head, back, abdomen, wing, tail feathers of Mallards (n = 3), Liancheng White (n = 3) and Pekin ducks (n = 3). (B) Content of melanin in beak and web tissues of Liancheng White (n = 3) and Pekin ducks (n = 3). Data are presented as mean ± SD (n = 3).

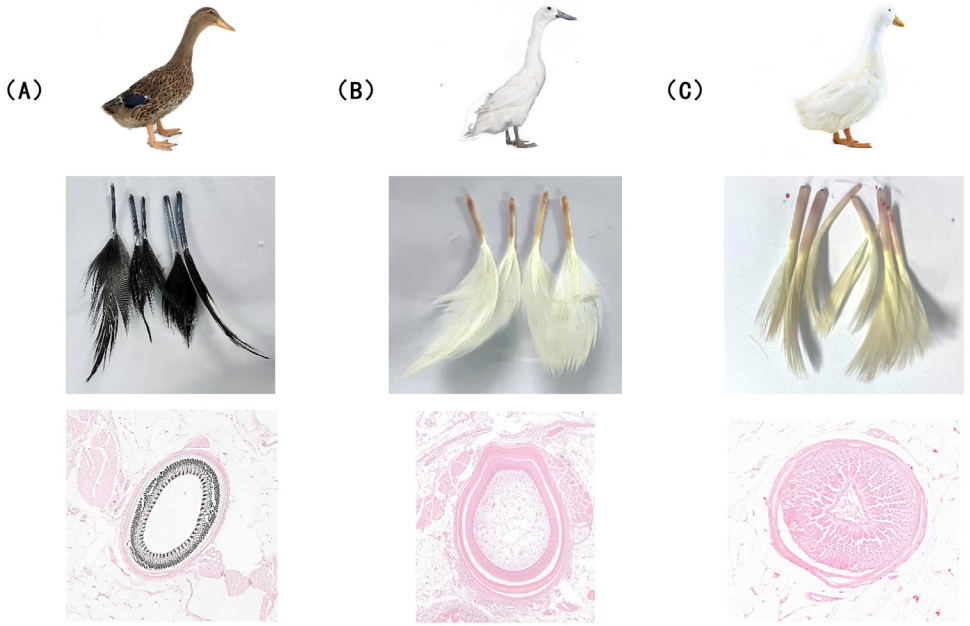

Figure 3. Masson-Fontana staining of melanin in the skin tissues of black-feathered Mallard (A), Liancheng White duck (B) and Pekin duck (C), and their hair bundles, and hair follicles in the back skin.

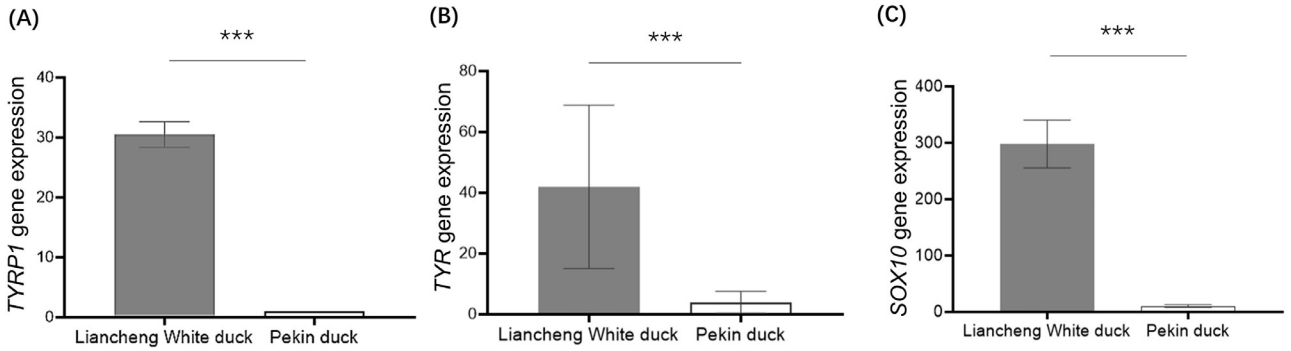

Figure 4. Expression levels of candidate genes that are differentially expressed in hair follicles of Liancheng White (n = 5) and Pekin ducks (n = 7). *** $p < 0.001$.

Melanin is synthesized by melanocytes and is mainly found in the skin, feathers and eyes of birds. In this study, the expression levels of the *TYRP1, TYR*, and *SOX10* genes in different tissues of the black-feathered Mallards were analyzed, and it was found that they were mainly highly expressed in the skin tissues (Figure 5A). Meanwhile, we found that the expression levels of these genes were peaked around 2 wk old of age (Figure 5B), indicating that these genes played an important role in the early growth and development of duck feathers. The differences in the dynamic expression of these genes may be related to the molting behavior of ducks (Zhang et al., 2023). The early feather development is more vigorous, and later the melanin remains in the feathers, and the feathers are no longer replaced.

The melanin formation pathway is relatively conserved, which means that the basic processes and molecular mechanisms involved in melanin formation are similar across species and individuals. The melanin formation pathway mainly includes the catalysis of tyrosinase and a series of subsequent oxidation and polymerization reactions. The structure and function of these enzymes are conserved between different species and individuals, thus ensuring the stability and reliability of melanin formation pathways (Cooper. 2017). The melanin synthesis pathway is conservative, and melanocytes in animals produce melanocytes, which lead to the formation of melanin, a process that requires the involvement of multiple functional genes. Eumelanin and phemelanin make up the melanin of mammals, with eumelanin appearing brown or black and phemelanin yellow or red, so eumelanin is the main source of duck feather color. Eumelanin can be decomposed into PDCA and PTCA by oxidation. The formation of eumelanin is closely related to the tyrosine pathway. Tyrosine is converted into 3,4-dihydroxyphenylalanine (**dopa**) in melanocytes, and then further oxidation, decarboxylation and other reactions are converted into indolequinone, and finally indolequinone is polymerized into eumelanin (D'Mello et al., 2016; Koch et al., 2019). The key enzyme in this process is tyrosinase (**TYR**), which catalyzes the conversion of tyrosine into melanin. Tyrosine is ubiquitous in the body, but the amount and activity of tyrosinase determines whether melanin is eventually formed (Barriales et al., 2023). In addition, this pathway contains some genes that affect the amount of melanin. The *TYRP1* gene encodes tyrosinase-related protein 1, a putative membrane-bound, coppercontaining enzyme that oxidizes the 5,6-dihydroxyindole-2-carboxylic acid (**DHICA**) into eumelanin pigment in the last step of melanin synthesis (Choudhury et al., 2024). *SOX10* can promote the synthesis of melanin and proliferation of melanocytes. It activates the *TYR* core enhancer, which helps to regulate the tyrosinase gene in melanocytes

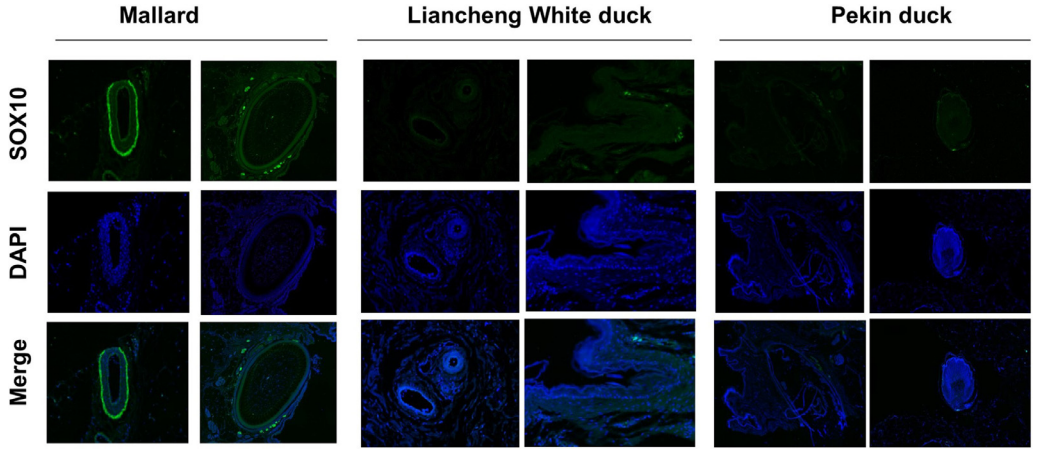

Figure 5. Immunofluorescence of the SOX10 protein in skin tissues of the black-feathered Mallard, Liancheng White, and Pekin duck.

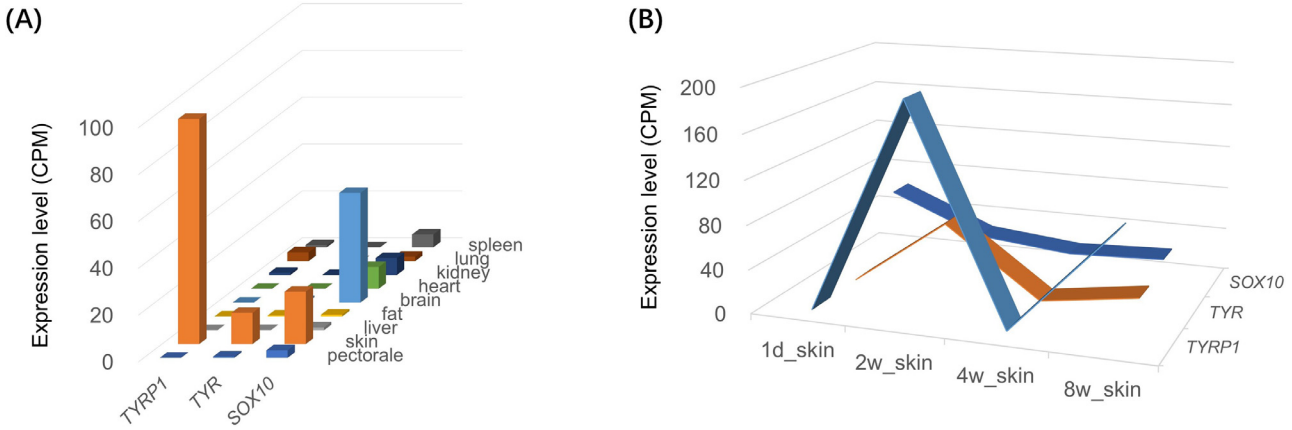

Figure 6. . Expression levels of melanin synthesis gene *TYRP1, TYR*, and *SOX10* in different tissues and different periods of skin tissues of black-feathered Mallards.

(Yun et al., 2019). At present, the predominant candidate genes related to duck feather color are *TYR* (Sultana et al., 2018), *TYRP1* (Xi et al., 2020), *DCT* (Sultana et al., 2018), *MC1R* (Liu et al., 2023a), *EDNRB2* (Guo et al., 2022), and *MITF* (Zhou et al., 2018; Lin et al., 2019; Wang et al., 2022a).

In this study, *TYRP2*, a key gene in the tyrosine pathway, was not detected in either feather color. This is consistent with another study in human (Dolinska et al. 2022). The loss of *TYRP2* gene expression in melanin-producing melanocytes of black hair follicles showed eumelanogenesis do not require *TYRP2* expression. Meanwhile, the lack of *TYRP2* in melanin production in duck hair follicles was consistent with previous study, so the function of *TYRP2* in ducks and the mechanism of melanin synthesis in duck hair follicles need further study. Taken together, these results suggest that lack of expression of *TYR* and *TYRP1* leads to inadequate biosynthesis of melanin in white hair follicles, which is the direct cause of duck white feathers.

Microphthalmia-associated transcription factor (**MITF**) transcription factor regulates the expression of *TYR* and *TYRP1*, and *TYRP1* is also involved in the process of melanin generation, and the MITF binding site "M-box" structure has the common characteristics of tyrosinase gene family (Wang et al., 2023). It regulates the differentiation and function of melanocytes through its interaction with transcription factors such as *MITF*. *TYRP1* can also interact with *TYR* to maintain its stability and regulate its catalytic activity. This study did not distinguish the different spliceosomes of *MITF*, so we did not find difference in the expression of *MITF-M* in the hair follicles of Liancheng White ducks and Pekin ducks. In the future, the genetic mechanism of duck feather color formation still needs to be further explored, and we believe that there are still many unanswered questions.

In conclusion, this study suggested that the content of feather melanin in Liancheng White duck was higher than that in Pekin duck, and it is much lower than black-feathered ducks. It indicated that the feathers of Liancheng White ducks had a small amount of melanin content, resulting in a unique white feather phenotype. Moreover, *TYRP1, TYR, SOX10* genes were involved in the synthesis of melanin in Liancheng White duck feathers, and mainly expressed in the skin of ducks. This research showed that we should pay attention to the subtle differences between avian feather coloration and help reveal the genetic mechanism behind different avian feather coloration. In actual production, it also helps breeders to quickly select pure white high-quality down and improve production efficiency. However, the formation of feather color phenotypes in birds is complex. Our research is still superficial, and further in-depth and more systematic research should be carried out.

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DISCLOSURES

The authors declare 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.2024.103794.

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