CELLULAR BIOLOGICAL AND EUMELANIN-RELATED GENE EXPRESSIONAL BASES OF PIGMENT DEVIATION OF *LEPTOBOTIA TAENIOPS*

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Abstract. Pigmentation is closely associated with various behaviors, and it is also an important feature that influences the popularity and price of ornamental fish. However, the molecular mechanism that regulates the pigmentation of fish is barely understood. There are two clearly different pigmentation phenotypes, i.e. light and dark phenotypes, in the same natural population of *Leptobotia taeniops*. Type and distribution of chromatophores is the cellular basis of the pigmentation. To analyze the composition of chromatophores in the light and dark phenotypes of *L. taeniops* and the correlation of the pigmentation to the expressions of pigment synthesis genes, we studied the pigment cell types and distribution in the skin of *L. taeniops* by microscope, and analyzed the expressions of genes that regulate melanin production using real-time qRT-PCR. Our results showed that the amount of melanophores in the skin was the major factor that caused the pigment deviation of *L. taeniops* living in the same habitat. The expressions of *TYR*, *Tyrp1*, and *Dct* genes were significantly higher in the skin of the dark phenotype than those in the light phenotype. This result implied that the amount of melanophores was influenced by the expression of these genes in the skin of *L. taeniops*.
Keywords: *chromatophores, ecological adaptation, fish skin, melanin-related gene, pigment cell, real-time fluorescent quantitative PCR*

Introduction

Pigmentation of fish is associated with various behaviors, such as warning, courtship, and hiding (Blakeslee et al., 2009; Culumber, 2013; Jiang et al., 2019). Additionally, in some species of fish, such as *Poecilia reticulate*, bright flashy colors may show a parasite infection (Houde and Torio, 1992) and the pigmentation has also been associated with immunocompetence in *Betta splendens* (Clotfelter et al., 2007). In ornamental fish market, pigmentation is an important feature that influences the popularity and price of ornamental fish. However, although cellular basis and development pattern of pigmentation of some fish species, such as *Betta splendens*, has been studied (Khoo et al., 2014; Carey et al., 2015), the molecular mechanism that regulates the pigmentation of fish is still rarely studied.

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Leptobotia taeniops is a small freshwater fish living in Yangtze River basin (Hunan Fisheries Science Institute, 1980; Ding, 1994; Ni and Wu, 2006; Fang et al., 2011). There are two obviously different pigment phenotypes, i.e. light and dark phenotypes, in the same natural population of *L. taeniops* (Jiang et al., 2019), and the difference is not influenced by heredity (Meng, 2011).

Type and distribution of chromatophores is cellular basis of the pigmentation (Khoo et al., 2014). It has been well documented that teleost fish have various chromatophore types, such as melanophores, xanthophores, erythrophores, iridophores, cyanophores, and leucophores (Jeon et al., 1993). Since the light phenotype of *L. taeniops* is of a near golden color and the dark one is yellowish-brown, and there are black stripes and splashes on their body (Jiang et al., 2019), melanophores and xanthophores were maybe the major chromatophores that influence the pigmentation of *L. taeniops*. In addition, melanin is the chromogenic substance in melanophores and tyrosinase plays an important role in synthesis of melanin (Lamoreux et al., 2001), and in our previous study, we have proved that the expression of tyrosinase gene in skin significantly higher in the light phenotype of *L. taeniops* than those in the dark phenotypes (Jiang et al., 2019). However, although it is important to elucidate the molecular mechanism that regulates the pigmentation of *L. taeniops* to adapt their external environment and protect wild *L. taeniops*, how the expression of tyrosinase gene in skin influences the pigmentation of *L. taeniops* and whether it associates with other genes is still unknown.

To analyze the composition of chromatophores in the light and dark phenotypes of *L. taeniops* and the correlation of the pigmentation to the expressions of pigment synthesis genes, we studied the pigment cell types and distribution by microscope, and analyzed the expressions of genes that regulate melanin production using real-time quantitative reverse transcription PCR (qRT-PCR). The results would provide important reference to elucidate the molecular mechanism that regulates the pigmentation of *L. taeniops* to adapt their external environment.

Materials and methods

Sample collection

The fish samples were collected from Yueyang Section (113.2051 N, 29.5140 E) of the Yangtze River during July to September, 2017, and transported to the Aquaculture Laboratory of Hunan Agricultural University to temporary culture in a circulating water system at 25 ± 1 °C. Healthy light and dark phenotype samples (6 specimens of each phenotype) were anaesthetized using MS-222, photographed, and measured body lengths and body weights. Subsequently, they were dissected and their sex was identified through sex gland. The skins at both sides of the dorsal fin base were collected using anatomical tools for microscopic examination of chromatophores and total RNA extraction.

Microscopic examination of chromatophores in skin of L. taeniops

The skins for microscopic examination of chromatophores were washed using phosphate buffer and made temporary slides. The temporary slides were examined and photographed using an Olympus BX53 microscope (Olympus, Japan). Amount of chromatophores in randomly selected 100 × 100 μm square regions of the photographs was counted and each phenotype was examined 10 times.

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Another 1 × 1 cm of skins were fixed 24 h using 4% paraformaldehyde tissue stationary fluid and paraffin sections of the skins were prepared and hematoxylin and eosin (HE) staining was conducted according to previous reports (Huang et al., 2018; Li et al., 2019). Then the skin tissue slices were examined and photographed using an Olympus BX53 microscope (Olympus, Japan).

Total RNA extraction and qRT-PCR

Total RNA was extracted from 50–80 mg of each skin sample using E.Z.N.A total RNA kit I (OMEGA, China) according to the manufacturer's introduction. The first stand of cDNA was synthetized using a RevertAid first strand cDNA synthesis kit (Fermentas, USA) with Oligo(dT)$_{18}$ primer according to the manufacturer's introduction (Jiang et al., 2019). Fluorescent quantitative PCR primers were designed using primer 6.0 software according to tyrosinase (*TYR*), tyrosinase-related protein 1 (*Tyrp1*), dopachrome tautomerase (*Dct*), Microphthalmia-associated transcription factor (*Mitfa*), Sox10 and melanocortin 1 receptor (*MC1R*) gene sequences of Cyprinidae fish in GenBank database (*Table 1*). β-Actin gene was used as endogenous reference (Jiang et al., 2019).

Table 1. *Real-time fluorescent quantitative PCR primers of melanin-related genes*

Gene name	Primer name	Primer sequence (5'-3')	Description	Reference
TYR	qRT-TYR-F	ATGCCTATTTGCTGGACCCC	qRT-PCR primer	Present study
TRY	qRT-TYR-R	TATGCCGACATCTTCCTGCG	qRT-PCR primer	Present study
Tyrp1	qRT-Tyrp1-F	ACCACCATATTACTCAACGTCCTCT	qRT-PCR primer	Present study
Tyrp1	qRT-Tyrp1-R	AGTCTGTCCACCTGTGCCATTAAG	qRT-PCR primer	Present study
Dct	qRT-Dct-F	GCAGAGGCATCACCGACAGAAC	qRT-PCR primer	Present study
Dct	qRT-Dct-R	CCTGAAGCTGAAGGTTGAGTTGGT	qRT-PCR primer	Present study
MITFa	qRT-Mitfa-F	TGCTCACGGACTTGCTGTAG	qRT-PCR primer	Present study
MITFa	qRT-Mitfa-R	AGAGATCGGAGGAGCAGTCT	qRT-PCR primer	Present study
Sox10	qRT-Sox10-F	CGATCAGTACCCGCACCTG	qRT-PCR primer	Present study
Sox10	qRT-Sox10-R	CTGTTTCCGCAAACGCTCG	qRT-PCR primer	Present study
MC1R	qRT-MC1R-F	CCATCTTTTACGCGCTTCGG	qRT-PCR primer	Present study
MC1R	qRT-MC1R-R	AACGTGACAAGACAGGCGAT	qRT-PCR primer	Present study
β-Actin	β-Actin-F	CTGGACTTGGCTGGTCGTG	Internal control primer	Jiang et al., 2019
β-Actin	β-Actin-R	CTCGAAGTCAAGGGCAACAT	Internal control primer	Jiang et al., 2019

To calculate the amplification efficiencies (*E%*) and the correlation coefficients (R^2) of the primers, the cDNA was gradiently diluted to 5, 25, 125, and 625 times and the E% and R^2 of the primers were calculated by qRT-PCR. The qRT-PCR was conducted as our previous description (Ni et al., 2018; Jiang et al., 2019). Three technical repetitions of each sample were set to exclude technical error. The results were collected and analyzed using CFX manager software 3.1. Relative expressions of the genes were calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Spivak et al., 2012).

Data analysis

Each parameter result is presented as means ± standard deviation (SD). Independent *t*-test was used to compare the significance of difference between the light and dark

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phenotypes. p-values < 0.05 were considered statistically significant. The statistical analyses and plotting were conducted using R 3.5.1 (R Core Team, 2017).

Results and discussion

Amount of melanophores caused pigment deviation of L. taeniops

Type and distribution of chromatophores is cellular basis of the pigmentation. Specific combinations of chromatophores in skin of *Betta splendens* formed the pigmentation patterns of the dark blue, ultra-maine, turquoise-bronze, and golden strains (Khoo et al., 2014). There were two major chromatophores, i.e. melanophores and xanthophores in the skin of *L. taeniops* (*Fig. 1*). There was no significant difference of the xanthophore amounts in the skin between light and dark phenotypes (Independent *t*-test, $t = 0.893$, $p = 0.384$). However, the melanophore amounts were significantly increased in dark phenotype skin than those in light phenotype skin (Independent *t*-test, $t = 10.622$, $p < 0.001$; *Fig. 1*). The result showed that the pigment deviation between light and dark phenotypes of *L. taeniops* mainly caused by the difference of melanophore amount.

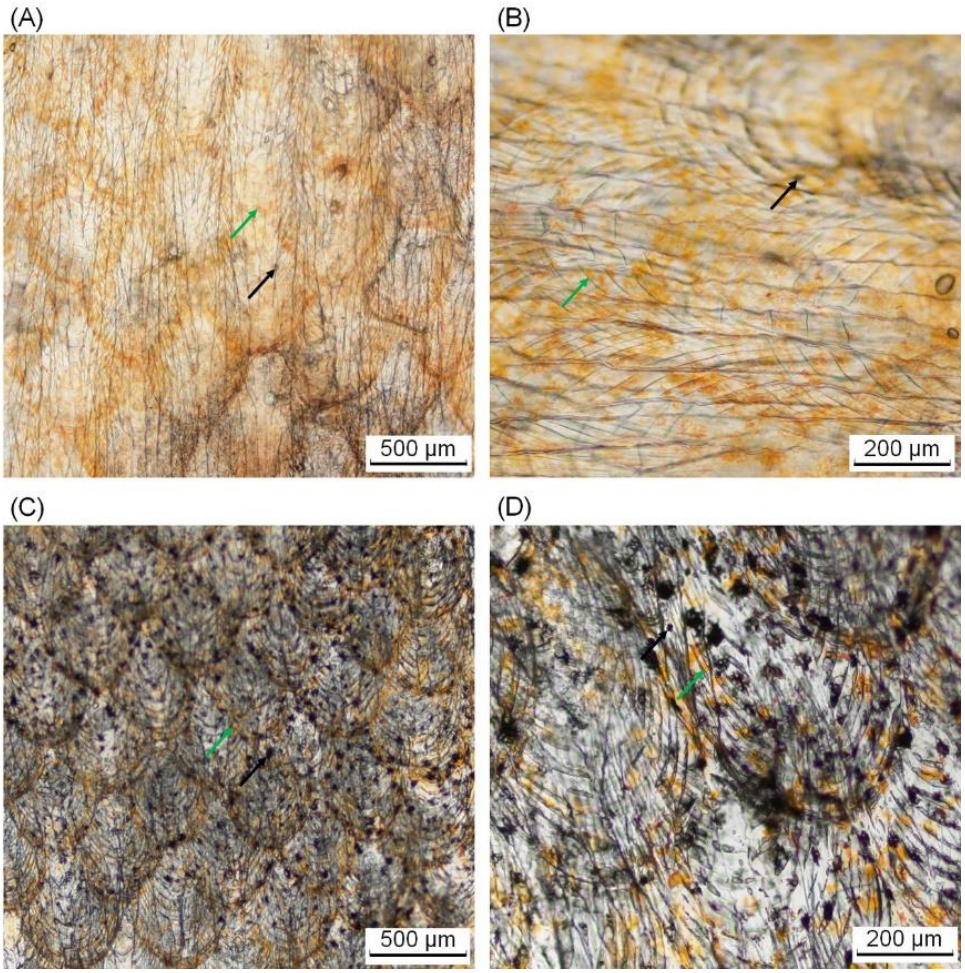

Figure 1. *Microscopic profiles of skin temporary slides showed pigment cells of Leptobotia taeniops. A and B: light phenotype of L. taeniops; C and D: dark phenotype of L. taeniops. The black arrows indicate the melanophores, and the green arrows indicate the xanthophores*

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The tissue structure of *L. taeniops* skin was analogous to other fish, including epidermis (EP; Approximately 50-100 μm), dermis (D; Approximately 130-200 μm), and subcutaneous layer (SL). The epidermis did not keratinization and covered by multilayer epithelial cells. The epithelial cells were flat and arranged closely. Under the epithelial cells, glandular layer occupied the majority of the epidermis. It was composed by goblet-cell (GC), club cell (CC), and epithelial cell (EC). The malpighian layer was close to the basement membrane. The dermis was constituted by dense connective tissue and was rich in fiber. The dermis was divided into the loose layer (SS) and the compact layer (SC) according to the fiber arrangement. The loose layer occupied a large part of the dermis, and the fibers in the loose layer arranged loosely. The fibers in the compact layer arranged closely. The subcutaneous layer located below the dermis, and contained a large number of fat cells, vessels and nerves (*Fig. 2*). The pigment cells of *L. taeniops* mainly distributed in the dermis, and a small amount of the pigment cells distributed in the epidermis. The pigment cells were stained into deep color by HE staining as the cells contain pigment granule. Shapes of the pigment cells were diversiform, and were mainly long strip and asteroid. A large number of pigment cells distributed in the loose layer and their distribution was not continuously and evenly (*Fig. 2*). This result implied that the pigment cells were not even-distributed in the skin of *L. taeniops*, in accordance with the results of the skin temporary slides.

The dermis of light *L. taeniops* was thicker than those of dark phylotype, while the epidermis and subcutaneous layer of light *L. taeniops* was thinner. The epithelial cells in the skin of light *L. taeniops* were more than those of dark phylotype (*Fig. 2*). These results implied that although the amount of melanophores caused the pigment deviation of *L. taeniops*, there was a deeply histological basis that influenced the amount of melanophores. This may be influenced by expression of the pigment genes.

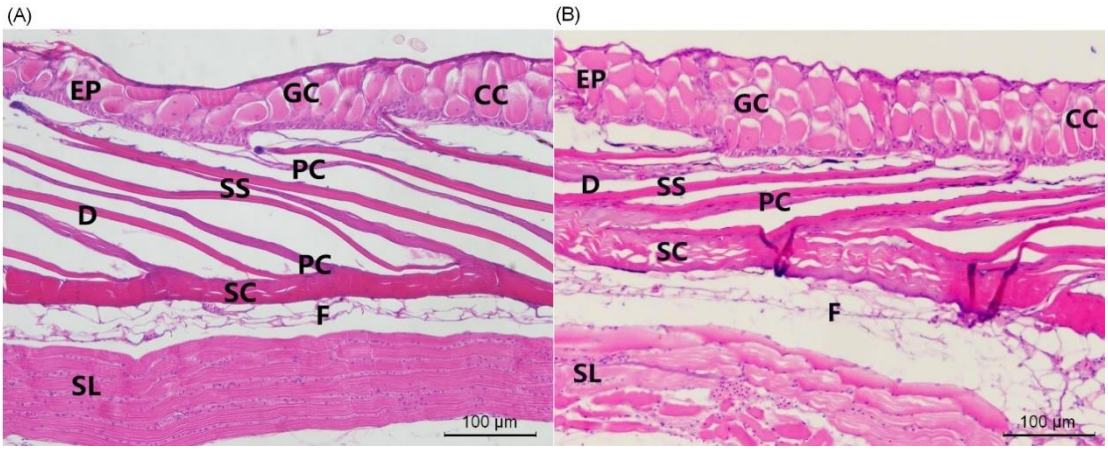

Figure 2. *Microscopic profiles of the skin paraffin sections with H-E dyeing of Leptobotia taeniops. A: Light phenotype; B: dark phenotype. EP, epidermis; D, dermis; SL, subcutaneous layer; SS, loose layer; SC, compact layer; GC, goblet cells; CC, club cell, EP, epithelial cells; PC, pigment cells; F, fat cells*

TYR, Tyrp1, and Dct genes were higher expressed in light than in dark L. taeniops

The first step of melanin production involves *TYR* which catalyzes the transformation of tyrosine to dopaquinone and then dopachrome in the eumelanin pathway. Then, *Dct*

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catalyzes the transformation of dopachrome to dihydroxyindole carboxylic acid (DHICA) and *Tyrp1* catalyzes the oxidation of DHICA and finally biosynthesis of eumelanin (*Fig. 3*; Murisier and Beermann, 2006). In addition, *MC1R* protein shows high affinity for α-melanocyte-stimulating hormone (α-MSH) and bingding of α-MSH to *MC1R* activated adenylate cyclase and increased cyclic adenosine monophosphate production, thus activating a variety of intracellular pathways which promote a switch in melanin synthesis from pheomelanin to eumelanin (Sturm et al., 1998). *Mitf* is critically involved in melanin synthesis, which enhances transcription of the genes encoding major enzymes in the melanin synthesis pathway, including *TYR*, *Tyrp1*, *Tyrp2*, and *Dct* (Yasumoto et al., 1994, 1997; Udono et al., 2000; Saito et al., 2003). Sox10 regulates the development of neural crest-derived melanocytes and the early development of the prospective neural crest (Aoki et al., 2003; Honoré et al., 2003). Therefore, we analyzed the expression of these genes in the skin of the light and dark *L. taeniaps*. The *E%* of TYR, Tyrp1, Dct, Mitfa, Sox10, and MC1R primers were 104.8%, 101.8%, 102.6%, 103.8%, 101.4%, and 95.5%, respectively; and the R^2 were 0.991, 0.998, 0.997, 0.997, 0.994, and 0.987, respectively. All of them were achieved the technical requirements of qRT-PCR that 90% < *E%* < 105% and $R^2 > 0.98$. The results showed that these primers could be used to quantify the expressions of these genes in the skins of *L. taeniops* with different pigmentation.

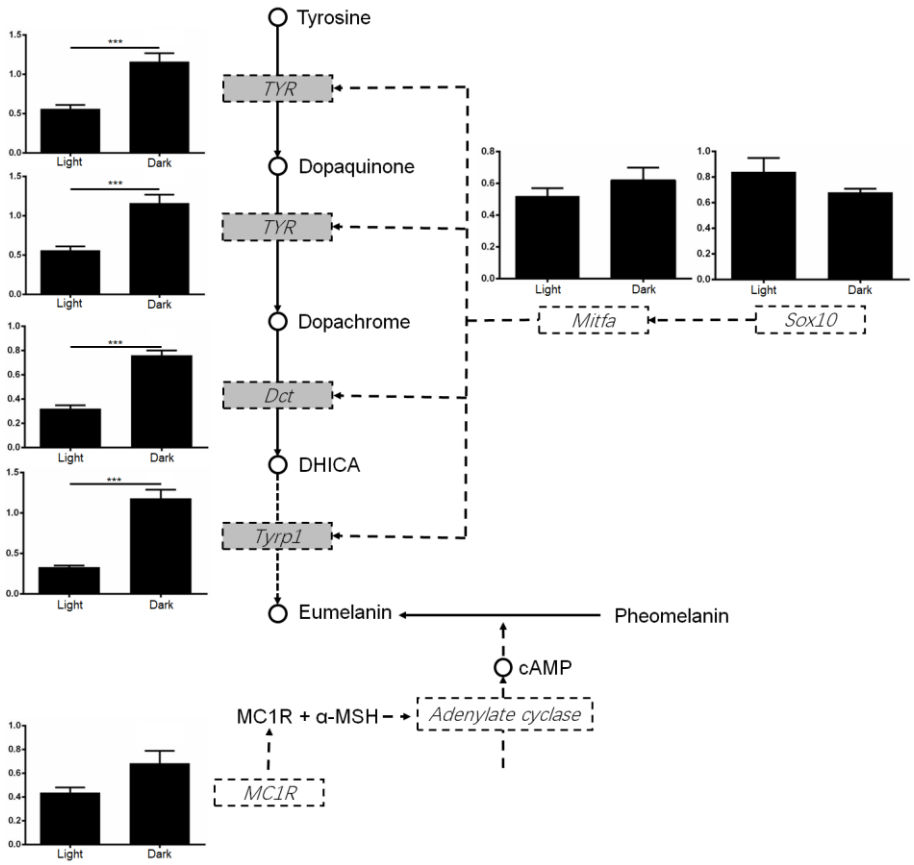

Figure 3. *Regulatory pathway of eumelanin biosynthesis in the skin tissue of Leptobotia taeniaps. TYR, tyrosinase; Tyrp1, tyrosinase-related protein 1; Dct, dopachrome tautomerase; MC1R, melanocortin 1 receptor; α-MSH, α-melanocyte-stimulating hormone; cAMP, cyclic adenosine monophosphate; Mitfa, microphthalmia-associated transcription factor. *p < 0.05; **p < 0.01; **p < 0.001*

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Although only six specimens of each phenotype were analyzed through qRT-PCR, qRT-PCR experiments with small sample size had been reported in previous reports, and their results were also considered reliable (Varkonyi-Gasic et al., 2007; Vieth et al., 2007). In the present study, the expressions of *TYR* (Independent *t*-test, $t = 4.966$, $p < 0.001$), *Tyrp1* (Independent *t*-test, $t = 7.603$, $p < 0.001$), and *Dct* (Independent *t*-test, $t = 8.800$, $p < 0.001$) genes in the skin of the dark phenotype were significantly higher than those in the light phenotype, while the expressions of Mitfa (Independent *t*-test, $t = 1.060$, $p = 0.314$), Sox10 (Independent *t*-test, $t = 1.403$, $p = 0.191$), and *MC1R* (Independent *t*-test, $t = 1.986$, $p = 0.075$) were not detected significant difference (*Fig. 3*). These results showed that the eumelanin biosynthesis was the major reason that caused the pigment deviation of *L. taeniops*. However, the expressions of the upstream regulation factors, i.e. *Mitfa*, *Sox10*, and *MC1R*, were not significantly different. This result implied that there was probably another undiscovered way to regulate the expression of *TYR*, *Dct*, and *Tyrp1* genes and the eumelanin biosynthesis.

Sox10 is a major regulator of neural crest formation (Honoré et al., 2003) and it is reported that regulates the development of neural crest-derived melanocytes in *Xenopus* (Aoki et al., 2003). In addition, MITF expression is maintained in about 90% of melanomas and a genomic amplification of MITF has been frequently observed in metastatic melanomas (Beuret et al., 2011). However, our results showed that both *Sox10* and *Mitfa* genes were not significantly upregulated in the skin of *L. taeniops*. These results implied that the developments of neural crest melanocytes and skin melanocytes were through different pathways, and pigmentation was probably not a direct factor that causes melanomas. In addition, our results also implied that through regulating *TYR* pathway, but not *Sox10* pathway, probably changed the production of melanophores and produced novel ornamental fish species.

Conclusion

In summary, amount of melanophores in the skin was the major factor that caused the pigment deviation of *L. taeniops* living in the same habitat. The amount of melanophores was influenced by the expression of *TYR*, *Tyrp1*, and *Dct* genes in the skin of *L. taeniops*. The expressions of these genes were significantly higher in the skin of the dark phenotype than those in the light phenotype. However, what and how external factors regulate the expression of these genes still need to be further studied in future.

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