Journal of Heredity, 2021, 469–484
doi:10.1093/jhered/esab018
Special Issue Article
Advance Access publication May 24, 2021

Special Issue Article

Nile Tilapia: A Model for Studying Teleost Color Patterns

Chenxu Wang*, Baoyue Lu*, Tao Li, Guangyuan Liang, Mengmeng Xu, Xingyong Liu, Wenjing Tao, Linyan Zhou, Thomas D. Kocher, and Deshou Wang

From the Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Sciences, Southwest University, Chongqing 400715, China (Wang, Lu, Li, Liang, Xu, Liu, Tao, Zhou, D. Wang) and the Department of Biology, University of Maryland, College Park, MD 20742 (Kocher).

*These authors contributed equally to this work.

Address correspondence to D-S. Wang and T. D. Kocher at the address above, or e-mail: wdeshou@swu.edu.cn and tdk@umd.edu.

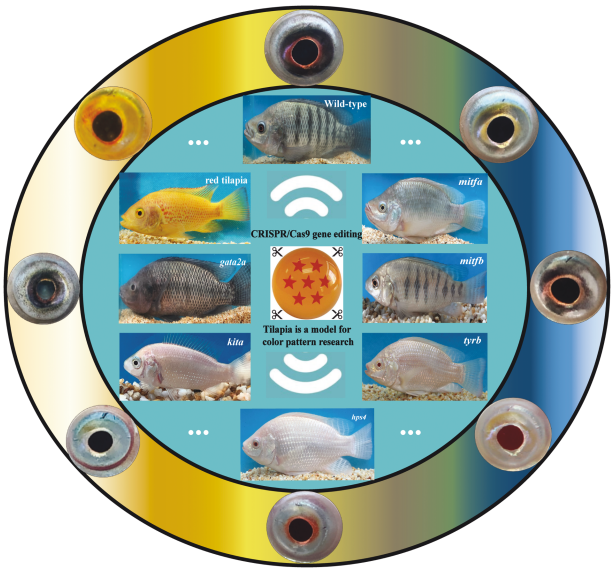
Received November 27, 2020; First decision March 11, 2021; Accepted April 8, 2021.

Corresponding Editor: Bridgett vonHoldt

Abstract

The diverse color patterns of cichlid fishes play an important role in mate choice and speciation. Here we develop the Nile tilapia (*Oreochromis niloticus*) as a model system for studying the developmental genetics of cichlid color patterns. We identified 4 types of pigment cells: melanophores, xanthophores, iridophores and erythrophores, and characterized their first appearance in wild-type fish. We mutated 25 genes involved in melanogenesis, pteridine metabolism, and the carotenoid absorption and cleavage pathways. Among the 25 mutated genes, 13 genes had a phenotype in both the F0 and F2 generations. None of F1 heterozygotes had phenotype. By comparing the color pattern of our mutants with that of red tilapia (*Oreochromis spp*), a natural mutant produced during hybridization of tilapia species, we found that the pigmentation of the body and eye is controlled by different genes. Previously studied genes like *mitf*, *kita/kitlga*, *pmel*, *tyrb*, *hps4*, *gch2*, *csf1ra*, *pax7b,* and *bco2b* were proved to be of great significance for color patterning in tilapia. Our results suggested that tilapia, a fish with 4 types of pigment cells and a vertically barred wild-type color pattern, together with various natural and artificially induced color gene mutants, can serve as an excellent model system for study color patterning in vertebrates.

Graphical Abstract



CRISPR/Cas9 gene editing is an efficient and convenient tool to create body color mutants of tilapia in a large scale. The RPE and trunk are possibly regulated by different genes. Tilapia is an excellent model for studying teleost color patterns.

Subject Area: Genotype to Phenotype
Key words: pigment cells, color genes, CRISPR/Cas9 gene editing, body color, tilapia

Body color is of great significance for protection against ultraviolet radiation, avoiding predation, species recognition, and mate choice (Protas and Patel 2008; Sköld et al. 2016). As the largest group of vertebrates, fish have a diversity of body colors and pigmentation patterns, and so are excellent models for the study of color patterning (Howe et al. 2013; O'quin et al. 2013; Patterson and Parichy 2019). The color patterning differences among teleost fish are mainly due to the types and movement of chromatophore/pigment cells originating from the neural crest (Fukamachi et al. 2004; Lopes et al. 2008; Kimura et al. 2014).

There are at least 6 kinds of pigment cells identified in teleost fish: melanophores, xanthophores, erythrophores, iridophores, leucophores, and cyanophores (Fujii 2000). Melanophores, which contain eumelanin, play a major role in the black/gray appearance. Xanthophores and erythrophores contain carotenoids and pteridines, and show orange, golden or red color appearance. Chromatophores that contain large amounts of yellow pteridine pigments are named xanthophores; those with excess red/orange carotenoids are termed erythrophores (Bagnara 1966). However, vesicles containing pteridine and carotenoids are sometimes found in the same cell, in which case the overall color depends on the ratio of red and yellow pigments (Matsumoto 1965). Therefore, the distinction between these chromatophore types is not always clear. Most chromatophores can generate pteridines from guanosine triphosphate, but xanthophores appear to have supplemental biochemical pathways enabling them to accumulate yellow pigment. In contrast, carotenoids are metabolized and transported to erythrophores. Iridophores contain guanine crystals, which create structural colors and iridescence. Leucophores contain uric acids, and appear white because they scatter light. Cyanophores appear blue, but the pigment they contain has not been identified (Sköld et al. 2016). Different fish species may have different sets of pigment cells types.

Zebrafish (*Danio rerio*) have 3 basic types of pigment cell: melanophores, xanthophores and iridophores. Color mutants of this model fish have been collected from the pet fish trade, from ENU mutagenesis screens, and morpholino (MO) knockdown of specific genes (Kelsh et al. 1996; Rawls et al. 2001; Kelsh 2004; Parichy 2006, 2007). Recently, with the development of TALEN and the following CRISPR/Cas9 gene knockout methods, more color mutants have been obtained (Pickart et al. 2004; Spiewak et al. 2018; Cal et al. 2019a, 2019b, Camargo-Sosa et al. 2019). Other researchers have focused on pigment cell development in medaka (Kimura et al. 2014), carp (Wang et al. 2014; Liu et al. 2015; Zhang et al. 2017), guppy (Kottler et al. 2014) and clownfish (Salis et al. 2018). TALEN and CRISPR/Cas9 gene knockouts of pigmentation genes have been obtained in medaka (Kimura et al. 2014; Nagao et al. 2014, 2018), carp (Chen et al. 2018; Fang et al. 2018; Liu et al. 2019; Mandal et al. 2019), and the large-scale loach *Paramisgurnus dabryanus* (Xu et al. 2019). Many of the genes that affect fish pigmentation also affect color pattern development and maintenance in mammals (Lin and Fisher 2007; Kelsh et al. 2009; Yamaguchi and Hearing 2009; Ito and Wakamatsu 2011; Irion et al. 2016). Studies of these genes have enriched our understanding of the mysteries responsible for the dazzling body colors of fishes. However, even though over 150 genes have been mutated in teleosts, we still have a lot to learn about the gene networks regulating fish color patterns.

In teleost fish, pigmentation genes can be categorized into 3 groups based on their roles in pigment synthesis, pigment cell migration and differentiation, or pigment cell interaction. There are mainly 4 types of pigments, i.e., melanin, pteridine, carotenoids and guanine platelets. Melanin was the most widely researched, as this type of pigment is common in vertebrate (almost all vertebrate have melanophores). Thus genes in the melanogenesis pathway were also the most well studied (Hou et al. 2006; Braasch et al. 2007; Dooley

et al. 2013; D'Mello et al. 2016). Melanin is synthesized from tyrosine, and controlled by a series of genes, including the *tyrosinase* gene family (*tyr*, *tyrp1*, *tyrp2/dct*). Its biosynthesis includes 4 stages (Ito and Wakamatsu 2011). Pteridine is first identified in insects, and it is responsible for yellow pigmentation. It is synthesized from guanosine triphosphate (GTP) (Ziegler 2003; Le Guyader et al. 2005; Braasch et al. 2007), and controlled by several enzyme genes, including *gch2*, *pts*, *spr*. Carotenoids play a significant role for teleost fish body color formation, but teleost fishes are unable to synthesis carotenoid. They can only make use of carotenoids accumulate and modified from their diet. Reflective platelets are necessary for the iridescent colors in teleost fish. These platelets are synthesized from guanine, involving genes such as *pnp4a* and *mpv17* (Krauss et al. 2013; D'Agati et al. 2017; Kimura et al. 2017; Bian et al. 2019).

Studies in mammals and zebrafish have characterized the role of many genes involved in specifying pigment cells from neural crest lineages (Kelsh et al. 2009). It has been demonstrated that a diverse array of cell types during vertebrate development are generated from the neural crest cells (NCCs), a multipotent, migratory cell population originates in the central nervous system (CNS) primordium (Simões-Costa and Bronner 2015; Kalcheim and Kumar 2017; Vega-Lopez et al. 2017). The chromatoblasts, precursors of the highly specified pigment cells, are also from the NCCs. The pigment cell precursors migrate and distribute in the skin along different routes, to cover the surface of the whole trunk. The migration and differentiation of pigment cells are determined by both cell-intrinsic and extracellular signals (Parichy and Spiewak 2015; Simões-Costa and Bronner 2015; Patterson and Parichy 2019; Vandamme and Berx 2019).

More recently, studies have focused on the interactions among different types of pigment cells that establish color patterning. Various degrees of rejection or pursuit among pigment cells are acknowledged at both long and short ranges (Watanabe and Kondo 2012; Frohnhöfer et al. 2013; Irion et al. 2014). Cellular and molecular studies on color patterning have helped us to better understand the mechanisms of color patterning (Kelsh et al. 1996; Lister et al. 1999; Parichy et al. 2000; Iwashita et al. 2006; Singh and Nüsslein-Volhard 2015). Close interactions determined by gap junctions between each of the major 3 pigment cells control "run and chase" movements that determine stripe formation (Watanabe and Kondo 2012; Watanabe et al. 2012; Frohnhöfer et al. 2013; Patterson and Parichy 2013; Irion et al. 2014; Yamanaka and Kondo 2014; Eom et al. 2015; Patterson et al. 2014).

Cichlid fishes have attracted the interest of ecologists and evolutionists, due to their phenotypic diversity and explosive speciation. However, studies on the mechanisms of color pattern in cichlids have lagged far behind, despite a few studies demonstrated that they are good model for color patterning research (Kocher 2004; O'Quin et al. 2013; Santos et al. 2014; Kratochwil et al. 2018; Hendrick et al. 2019; Liang et al. 2020). Pigment cell types have also been characterized in several cichlids in relation to studies of color patterns (O'Quin et al. 2013; Santos et al. 2014; Hendrick et al. 2019; Liang et al. 2020). Even though melanophores, xanthophores iridophores and even red melanophores (erythrophores) have been reported to exist in tilapia (Avtalion and Reich 1989; Nandi 1997), a more detailed analysis of pigment cell types that responsible for their color patterning has not been reported. Although there are reports focused on the pigment cell patterning in cichlids, the genetic basis of color patterning remains to be fully characterized (O'Quin et al. 2013; Ding et al. 2014; Santos et al. 2014; Santos 2017).

Tilapia are commercially important cichlids that have the potential to act as an excellent model system to study the mechanisms of color patterning in development and evolution (Avtalion and Reich 1989; Nandi 1997; McAndrew et al. 1988; Lee et al. 2005; Zhu et al. 2016). Forward genetic analysis of body color in red tilapia, a natural color mutant with higher market price than those wild-type black fish, has mainly focused on crossbreeding, and the results showed that the red color is probably determined by a single dominant locus (Huang et al. 1988; Majumdar et al. 1997; McAndrew et al. 1988; Reich et al. 1990). Genetic linkage mapping suggests that the red locus lies on LG3 (Lee et al. 2005). Although tilapia are widely used in research, due to their short breeding cycle and the availability of several genome assemblies, there have been no reports using genome editing to produce specific color gene mutations.

In the present study, we have characterized the types of pigment cells and their detailed temporal and spatial appearance in wild-type tilapia. We then mutated dozens of the genes using CRISPR/Cas9 gene editing, and characterized the color patterns of these mutants. The results confirm the roles of the melanophore and xanthophore-specific genes in the body color formation of tilapia. Our results confirm that tilapia are an excellent model system to study the mechanisms of color patterning and body color formation in cichlids, as suggested in a recent review (Patterson and Parichy 2019).

Materials and Methods

The founder strain of wild-type black Nile tilapia was obtained from Prof. Nagahama (Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki, Japan), thus the homozygosity for the parental fish can be well guaranteed. All fish were reared in recirculating aerated freshwater tanks and maintained at ambient temperature (26 °C) under a natural photoperiod. Prior to the experiments, the fish were kept in laboratory aquariums under 15:9 h light: dark conditions at temperature of 26 ± 1 °C for 1 week to control the experimental conditions. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Committee for Laboratory Animal Experimentation at Southwest University, China.

Pigment Cell Type Analysis of Nile Tilapia

Skin, scale, and caudal fin samples were used for pigment cell type analysis. Skin samples collected were around 1 cm^2 on average. Scales were separated directly from the skin with forceps. Skin and scales were soaked in 0.65% Ringers' solution under cover slip, and were observed under microscope (Germany, Leica EM UC7). The tip of the caudal fins were removed with medical scissors, soaked in 0.65% Ringers' solution and directly observed with stereomicroscope (Olympus SZX16) without cover slip under transparent or bright field. Analyses of pigment cells were conducted as quickly as possible after putting them in the Ringers' solution as the preparations are not stable.

Nile tilapia at embryonic, larval and juvenile stages was used to study the first appearance of the major types of pigment cells under stereomicroscope (Olympus SZX16). The embryos were observed every 0.5 h (for the first 150 h). From the first day post fertilization (dpf), the larval and juvenile fish were observed at 15:00 every day.

gRNA Design and Transcription

Genome sequences/assemblies of the Nile tilapia have been published (Brawand et al. 2014; Conte et al. 2019). Selected gene

sequences were downloaded from the NCBI and Ensemble databases. The gDNA target sites were selected from sequences corresponding to GGN18NGG on the sense or antisense strand of DNA (Chang et al. 2013). Candidate target sequences were compared to the entire tilapia genome using the Basic Local Alignment Search Tool (BLAST) to avoid cleavage of off-target sites. Any candidate sequences with perfectly matched off-target alignments (i.e., the final 12 nt of the target and NGG protospacer adjacent motif [PAM] sequence) were discarded (Cong et al. 2013). For detailed information about the guide RNAs we used in this study, see Table 1.

For *in vitro* transcription of gRNAs, the DNA templates were obtained from the pMD19-T gRNA scaffold vector (kindly provided by J. W. Xiong, Peking University, Beijing, China) by polymerase chain reaction (PCR) amplification (Chang et al. 2013). The forward primer contained the T7 polymerase binding site, the 20-bp gRNA target sequence, and a partial sequence of gRNA scaffold. The reverse primer was located at the 39 end of the gRNA scaffold. In vitro transcription was performed with the Megascript T7 Kit (Ambion, United States) for 4 h at 37 °C using 500 ng purified DNA (PCR products) as template. The transcribed gRNA was purified and quantified using a NanoDrop-2000 (Thermo Scientific), diluted to 150 ng/µl in RNase-free water and stored at −80 °C until use (Li et al. 2014; Li et al. 2015).

Cas9 Messenger RNA In Vitro Transcription

The Cas9 nuclease expression vector pcDNA3.1 (+) (Invitrogen) was used for in vitro transcription of the Cas9 messenger RNA (mRNA) as previously described (Chang et al. 2013). Plasmids templates were prepared using a plasmid midi kit, linearized with *Xba*I, and purified by ethanol precipitation. Cas9 mRNA was produced by in vitro transcription of 1 µg DNA using a T7 mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's instructions. The resulting mRNA was purified using the MegaClear Kit (Ambion), suspended in RNase-free water and quantified using a NanoDrop-2000 (Li et al. 2014; Li et al. 2015).

Microinjection, Genomic DNA Extraction, and Mutation Detection Assay

The gRNA (150 ng/µL) and cas9 mRNA (500 ng/µL) was mixed at a molar ratio of 1:1. About 500 PG of the mixture was directly microinjected into XX or XY fertilized egg at one cell stage for target gene editing, while Cas9 mRNA or gRNA was injected as control. About 400 fertilized eggs were used for gene editing and 100 for control. The injected embryos were incubated at 26 °C. Twenty injected embryos were collected 72 h after injection. Mutated fish were identified by loss of the restriction enzyme site. Mutation efficiencies and sequences of the mutated targets were evaluated by restriction enzyme digestion and Sanger sequencing as follows: the DNA fragments spanning the target for each fish were amplified. The recovered PCR products were purified and digested by restriction enzyme within the target. The uncleaved bands were recovered, sequenced, and then aligned with the wild type. In addition, the percentage of uncleaved band (i.e., potential mutations in target site) was measured by quantifying the band intensity of the restriction enzyme digestion with Quantity One Software (Bio-Rad, United States). The indel frequency was calculated by dividing uncleaved band intensity to the total band intensity (Li et al. 2015).

Table 1. Detailed information of the targets used in this study

gRNA	Target	Exon	Forward primer	Reverse primer	Product length
mitfa	CCGGTCCTGGCAGCAGTGCACCC	2	GTCCAGACCCACCTGGAAAGTCC	AGAACATCTTCGTTGTAGCTTGACTC	356 bp
mitfb	*CC*TGCCATCAATGTCAGTGTCCC	2	CTTGACCTCTCCTTCCACATTTGAC	GACCATTCACGCTGCACCAAT	530 bp
gata2a	CCCGGACTCTCACCACCACAGCC	1	GCAGATCAGTCTCGGTGGAT	TCGTCCTGCGGCAGTAAAGG	125 bp
kita	GGATACAGTGGAAGCGGGAG*AGG*	2	GACACAAAGCCCACTATCACA	GCAAAGTCACAAACACCTTCTT	406 bp
kitlga	CCGATGACATCGCTAAACTCTCC	3	GCCGAGTTGTTAAGCATGGA	TGTAGATTGTTCAGTGGTAGCC	446 bp
pmela	CCCACCAAACCAGACGGTGCTCC	3	CTTGACCTCTCCTTCCACATTTGAC	TGCTGCTAAGCTCTTATGGGG	468 bp
pmelb	GGTGCTGCCTGATGGAG*ACGTGG*	3	CCAAAAACCGCTTCACTCGTT	TCTTCCTGTGGGGACTGACC	477 bp
tyrb	*CC*ACTTCAGACGGACTGCGGACC	1	TCTCTCTAACCTTCTGTGCG	CTGAAACCACCACGTCTGT	278 bp
hps4	CCGGCGTGGGCCGCTGCGTCTCC	3	AGGTCTGGCTGACACAAAGTG	TAGGGACATCCACCGAGCAG	482 bp
gch2	GGGGAGGACACCGACCGGGAG*GG*	1	TCTCGGTCCTCAGCAGAGTC	CCAGGTGACTCACCATCGAT	320 bp
csf1ra	CCGCGGCATTCTCATCCACAGCC	3	GGTGGTAGTGAAGGAGGGTGAG	CAGAGACAGAAGCAGCCAGTGA	365 bp
pax7b	GGACAGAACTATCCCGCAC*TGG*	1	ATGTCGTCTTTGCCAGGAACA	AATTTCCAGGCGCGCAGAAC	515 bp
bco2b	GGGCCCGGAAAGTTTGAGATC*GG*	1	GCACTCAAGAAATCTAGGTCTGC	GCGATTGTGCTCCTTGTTGG	409 bp

Image Recording and Body Color Observation of the Mutants

The wild-type and mutant fish were shifted to a glass aquarium (20 × 20 × 5 cm) with clean water (28 °C) before image recording, and then photographed by digital camera (Nikon D7000, Japan) against a blue background. ACDSee Official Edition software and Adobe Illustrator CS6 were used to format the pictures.

Results and Discussion

Identification of the Pigment Cell Types of Nile Tilapia

So far, at least 6 types of pigment cells have been characterized in teleosts (Fujii 2000; Sköld 2016). The first appearance of melanophores, iridophores and xanthophores in zebrafish has been carefully described (Taylor et al. 2011; Patterson et al. 2014).

Nile tilapia has 4 types of pigment cells: melanophores, xanthophores, iridophores and erythrophores (Avtalion and Reich 1989; Nandi 1997). Under bright field, we detected melanophores with abundant mature melanosomes in the anterior dorsal scales in bright fields (Figure 1a). The melanophores can change appearance from stellate to punctate by the movement of melanosomes, which contributes to rapid color change (Figure 1a,b). It should be noted that we detected both big melanophores and smaller, punctate melanophores on the head of the young embryos (Figure 2d), but in the trunk there were only smaller-sized melanocytes, suggesting that different groups of melanophores are responsible for body color formation in different patterning areas of wild-type Nile tilapia.

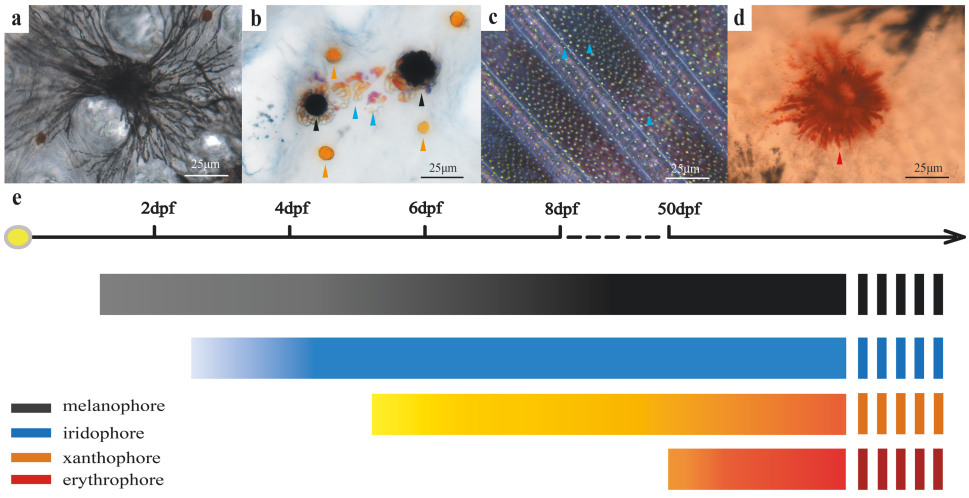
We also detected some round but smaller xanthophores separated from the melanophores in the dorsal scales (Figure 1b). They displayed orange color and round shape, which were possibly a result of the melanophore restriction. We also detected young stellate xanthophores, which were not fully pigmented with orange pigment in the head and lateral trunk of the young embryos (Figure 2d,d').

We observed the appearance of auto fluorescent pigment cell (iridophore-like pigment cells) in the dorsal fin ray (Figure 1c) and even 2–3 dpf embryos (Figure 2b'). In the area close to the melanophores we found some iridophores surrounded by or attached with the central area of melanophores, which turned out to be important for rapid color change of tilapia. Erythrophores were the second-largest chromatophore observed, also with obvious clustered branches like melanophore, but filled with red pigments (Figure 1d). The erythrophores appeared late in development and contributed to reddish pigmentation of the anal and caudal fins in male tilapia (Figure 1e). Tilapia males display more reddish body color than females. We speculate that sexual selection has favored the development of erythrophores in males to produce fins more attractive to females. In addition, we observed that erythrophores always stay together with xanthophores (Supplementary Figure 1), the close relationship between them may matter in mate choice.

With various pigment cells gathering and interacting with each other, we can finally see the specific color patterns of tilapia. The first to appear in development, melanophores, xanthophores, and iridophores, are responsible for most aspects of body color and color pattern formation in tilapia.

Timing of Pigment Cell Appearance in Nile Tilapia

In Nile tilapia, melanophores, xanthophores, and iridophores first appear during the early embryonic stages just like zebrafish, but not in high numbers and some are not heavily pigmented (Figure 2). Among them, melanophores first appeared on the yolk sac near animal pole (Figure 2a) at around 27.5 hours post fertilization (hpf), which was earlier than reported previously (Fujimura and Okada 2007). Later, melanophores appeared in the eyes and sharply arose symmetrically, at the same time, melanophores on the yolk sac also sharply arose from 3 dpf. Simultaneously, iridophore appeared in the eyes (Figure 2b,b'). At 5 dpf, almost all of the melanophores on the yolk sac migrated to the area near the peritoneum and heart. New melanophore appeared continuously on the dorsal head, and the eyes were filled with melanophore and iridophore (Figure 2c). At 6 dpf, we detected the appearance of light colored xanthophores, both on the head and trunk (Figure 2d). From 3 to 12 dpf, melanophores appeared in several waves and spread to the entire trunk surface, and bars began to form



AQ32 **Figure 1.** Identification of the pigment cell types of Nile tilapia and the time when they first arise during development. (**a**) Melanophore with branching clusters on scales (black or gray). (**b**) Xanthophore (blond/orange round shape, yellow triangle) and iridophore (orange/purple/blue, blue triangle) with smaller sizes than melanophore or erythrophore, melanophore (round shape as a result of aggregation of pigment granules, black triangle) on scales. (**c**) Fluorescent iridophore (green/iridescent, blue triangle) "shining" like twinkle stars in dorsal fins. (**d**) Erythrophore (red, red triangle) with branching clusters, but smaller than the neighboring melanophore in the skin. (**e**) Time table of the first appearance of every types of pigment cell.

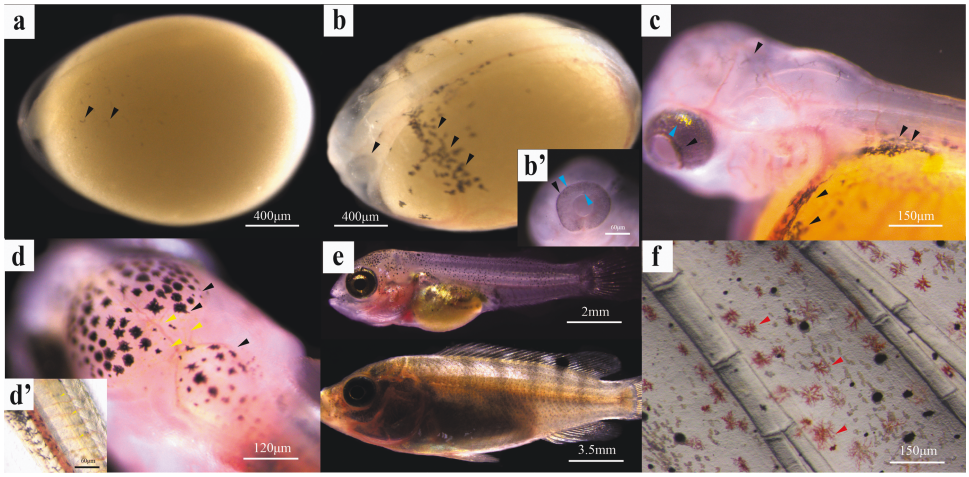


Figure 2. The temporal-spatial appearance of 4 types of pigment cells in Nile tilapia. (**a**) A few slightly pigmented melanophores appeared on the yolk sac near the animal pole (black triangle) at 27.5 hpf. (**b**) Auto fluorescent iridophores appeared in eyes (blue triangle) and melanophores (black triangle) sharply arose on the yolk sac at 3 dpf. (**c**) Melanophores on the yolk sac concentrated near the area of peritoneum, heart, and eyes, and appeared on the heads (black triangle), iridophores sharply increased to cover the eyes (blue triangle) at 5 dpf. (**d**) Melanophores sharply increased on head (black triangle) and xanthophores appeared in head and trunk skin (yellow triangle). (**e**) Larvae without bars even until 14 dpf and juvenile fish with complete vertical bars at 27 dpf. (**f**) Erythrophores with clusters appeared first in the caudal fin around 80 dpf, with the development of caudal fin rays.

slowly since 15 dpf. However, no erythrophores were observed during the embryonic or larval stage. In fact, we did not observe any erythrophores even when the 7–9 black vertical bars and the light inter-bars had formed (around 27 dpf) on the trunk surface (Figure 2e,f). Two to four weeks after fertilization is the key time point for vertical bar pattern formation during metamorphosis. But the actual time point for pigment cell patterning in the trunks is probably much earlier, as migration of pigment cells precursors happens before the pigment cells were significantly pigmented. We could easily detect the juvenile tilapia mark, a heavily pigmented black round spot in the dorsal fin.

The adult phenotype of Nile tilapia is a pattern of 7–9 black vertical bars separated by lighter inter-bar regions. The retinal pigmented epithelium (RPE) color was black (Figure 4a). Around 50 (45–55) dpf, erythrophores were detected in the caudal fin of the fish, and they kept growing in number with the development of fin rays (Figure 2f). Later on, the erythrophores became more abundant, like the other types of pigment cells (Figure 1e). They were concentrated in the caudal fin and anal fins together with xanthophores, and functioned as nuptial coloration beginning 150–180 dpf. (Supplementary Figure 1). We observed that erythrophores were found together with xanthophores, especially in fin regions associated with signaling sexual attractiveness of males. The 2 groups of pigment cells possibly attract each other by communicating with their synaptic junctions.

The Pigmentation Genes Mutated in This Study

Pigment metabolism pathways are thought to be similar across teleosts. In this study, we used CRISPR/Cas9 genome editing to mutate 25 genes. Most of them were involved in melanogenesis, pteridine metabolism, the carotenoid absorption and cleavage pathway, as well as some genes specific to the xanthophore and iridophore lineages. In Supplementary Figure 2–4, the targeted genes are highlighted in pink, and genes that displayed mutant phenotypes are highlighted in red. For 3 genes, we have so far obtained only the F0 generation. For 9 genes, we have generated an F1 generation, and for 13 genes we have developed an F2 generation. Establishment of homozygous mutant lines of the other color genes is ongoing.

As a result of high target cleavage efficiency, some of the color genes displayed obvious phenotypes even in F0 mutants. Among the 25 mutated genes, 13 genes had phenotype in both the F0 and F2 generations (Table 2). None of the F1 heterozygotes had a phenotype, thus we deduced that these mutations are all recessive in tilapia. Among the 13 genes for which we have made homozygous mutants, 9 genes had phenotype. Some homozygous mutants have no obvious phenotype, which may be due to the compensation effect of other genes or they are not in the pigment synthesis pathway, which is quite different from the results of model organism.

Mutation of the Key Genes Responsible for Melanophores Differentiation, Melanoblast Migration, and Melanin Biosynthesis Led to Body Color or Color Pattern Changes

We mutated a total of 13 genes in the melanophores differentiation, melanoblast migration and melanin biosynthesis. Seven of these showed obvious phenotypes in the F0 or F2.

Mitf

Mitf plays a central role in the melanogenesis pathway (Cheli et al. 2010; Yamaguchi and Hearing 2009; Vachtenheim and Borovansky 2010; Goding and Arnheiter 2019). In vertebrates, this gene is the most widely investigated and it is closely related to albinism, vitiligo, or even melanoma. Loss of function studies in zebrafish and mouse suggested that *mitf* is critical for yellow skin and white hair formation. In mice, *mitf* mutants displayed hypopigmentation as reflected by obvious albinism with white furs, and pink RPE. However, a few of the mutants displayed black patterns (Cheli et al. 2010). As a crucial transcription factor in pigment cells, the phenotypic effects depend on whether a given mutation affects the coding sequence or whether there is a regulatory mutation. White spotting in dogs are caused by an allelic series at the MITF locus (Irish spotting, Piebald, and Extreme white), all caused by non-coding changes (Baranowska Körberg et al. 2014). In vertebrates, *mitf* mediates the expression of many melanophore specific genes, such as *pmel*, *tyr*, etc., that

Table 2. A summary of the genes mutated in this study

Gene	Linkage group	Function	F0	F1	F2	Latest	Mutant body phenotype	Mutant eye phenotype
bco2a	10	Carotenoids cleavage	+	?	?	F0	No obvious phenotype	Not detected
bco2b	14	Carotenoids cleavage	m	m	m	F2	Pericardial edema, hyperpigmentation	Not detected
cbsb	16	Pheomelanin biosynthesis	+	+	+	F2	No obvious phenotype	Not detected
csf1ra	2	Xanthophore differentiation	m	m	m	F3	Xanthophroe absence	Not detected
csf1rb	10	Xanthophore differentiation	+	+	+	F2	No obvious phenotype	Not detected
dct	16	Dopachrometautomerase	+	+	?	F1	No obvious phenotype	Not detected
gata2a	5	Hematopoiesis and cardiac development	m	m	m	F2	Hyperpigmentation	Microphthalmia
gch2	8	Melanoblast and xanthoblast shared maker	m	m	?	F1	Disrupted color pattern	Not detected
hps4	12	Melanosome biosynthesis	m	?	m	F2	Albinism	Not detected
kita	23	Melanophore migration	m	+	m	F2	Golden body color	Hypopigmentation in iris
kitlga	7	Ligand of kita	m	+	?	F1	Bars loss	Not detected
mitfa	5	Melanophore differentiation	m	m	m	F3	Hypopigmentation	Hypopigmentation in iris
mitfb	20	Melanophore differentiation	m	m	m	F2	Hyperpigmentation	Hypopigmentation in iris
mitfa;mitfb	5,20	Melanophore differentiation	m	m	m	F2	Hypopigmentation	Hypopigmentation in iris
pax3a	18	Melanoblast and xanthblast shared precursor	+	?	?	F0	No obvious phenotype	Not detected
pax3b	14	Melanoblast and xanthblast shared precursor	+	+	?	F1	No obvious phenotype	Not detected
pax7a	5	Melanophore and xanthophore	+	+	?	F1	No obvious phenotype	Not detected
pax7b	5	Melanophore and xanthophore	m	m	m	F2	Absence of color pattern	Not detected
pmela	5	Melanosome biosynthesis	m	+	?	F1	Disrupted color pattern; hypopigmentation	Not detected
pmelb	20	Melanosome biosynthesis	m	+	?	F1	Disrupted color pattern; hypopigmentation	Not detected
pts	8	Pteridine biosynthesis enzyme	m	+	?	F1	Not confirmed	Not confirmed
shdb	23	Iridophore survival	+	+	+	F2	Not confirmed	Not confirmed
sox10a	6	Neural crest differentiation	+	+	?	F1	No obvious phenotype	Not detected
sox5	17	Neural crest differentiation	+	?	?	F0	No obvious phenotype	Not detected
tyra	10	Melanin biosynthesis	+	+	+	F2	No obvious phenotype	Not detected
tyrb	14	Melanin biosynthesis	+	+	+	F2	Albinism	Hypopigmentation in iris and RPE
tyra;tyrb	10,14	Melanin biosynthesis	m	m	m	F2	Albinism	Hypopigmentation in iris and RPE

directly involved in melanin biosynthesis. A genome duplication in early teleosts produced 2 paralogs of *mitf*. In zebrafish only *mitfa* is important for melanophores differentiation and function, while *mitfb* appears to be necessary for eye and olfactory bulb development and is highly expressed in these tissues (Lister et al. 1999; Lister 2002).

In tilapia, *mitfa* homozygous mutants displayed light yellow body color together with gray vertical bars (Figure 4b). The RPE color was black, while iris and the surrounding tender surface was light pigmented, and the iris even displayed more carotenoids contributed orange pigmentation (Figure 4b'). In the *mitfb* homozygous mutant tilapia, bars were still detected, but were often disrupted into round black spots in the horizontal line (Figure 4c). The RPE color was also black, while the iris displayed a hypopigmentation phenotype (Figure 4c').

We also created *mitfa;mitfb* double mutant fish, which displayed pink body color with few melanophores and no bars, but occasionally a few black spots (Figure 4d). The RPE color was still black, but the iris and surrounding tender surface showed a greater loss of pigmentation in double mutants than *mitfa* homozygous mutant (Figure 4d'). These results confirmed the role of *mitf* in tilapia color patterning and the subfunctionalization of the 2 *mitf* genes derived from the third round of vertebrate genome duplication.

Pmel

Pmel lies downstream of *mitf*, is a key genetic factor for melanosome/melanin biosynthesis and maturation (Hellström et al. 2011; Ishishita et al. 2018). *Pmel* is one of the most widely known genetic factors in vitiligo, an autoimmune condition in humans characterized by loss of melanophores from patches of skin (Yuan et al. 2019; Lei et al. 2020). In mouse, mutations in *pmel* have been reported to affect melanosome/melanin shape, but have only subtle influence on visible coat color (Hellström et al. 2011; Kwon et al. 1994; Hellström et al. 2011). In *Japanese quail*, the gene has proved to be responsible for the yellowish plumage color (Ishishita et al. 2018). In chickens, *pmel* has been shown to play a critical role for melanosome function, loss-of-function mutations of this gene have a milder phenotype than dominant negative allele, the latter of which showing complete white plumage color, thus used for billions of commercial chickens worldwide for egg and meat production (Kerje et al. 2004). To our knowledge, this gene has never been mutated in any teleost fish despite the observation that it is expressed more highly in black vertical bars than light inter-bars in a cichlid (Liang et al. 2020).

In *pmel* F0 mutant tilapia, we detected hypopigmentation in iris and varying levels of bars loss in the trunk and fins, indicating that the color patterns of the trunk surface and the fins might be controlled by the same genetic factors. *Pmel* F0 mutant fish showed various levels of bar loss, which indicates that *pmel* is important for bar formation. Additionally, in those fish with disrupted patterns, melanophores were significantly reduced, while the remaining bars were still located in the original area of the fish (Figure 3d,e). We also found that the fish with greater loss of bars and black pigmentation were under more stress and had higher risk of being attacked by other fish (Supplementary Figure 5).

Tyr

Tyr encodes a key enzyme in the melanogenesis pathway, which catalyzes tyrosine hydroxylation to L-DOPA in the biosynthesis of melanin. Tyrosinase is a key rate-limiting enzyme in the process of melanogenesis, and its expression is regulated by *mitf* (Yasumoto et al. 1994). It has been widely studied as a cause of albinism in the animal kingdom. Mutation of this gene resulted in an albinism phenotype in medaka, zebrafish, and carp (Koga and Hori 1997; Park et al. 2016; Fang et al. 2018; Liu et al. 2019). CRISPR/Cas9 knockout of *tyr* resulted in an albino phenotype in large-scale loach, with hypopigmentation in F0 and complete albinism in F2 homozygous mutants (Xu et al. 2019). Previous analysis has revealed that tyr mutant in zebrafish still has melanophores, as melanoblast migration and melanophore differentiation markers can be still detected, but the melanophores contain no mature melanosome (Taylor et al. 2011).

No phenotypes were observed in tilapia *tyra* F0 and homozygous mutants. In contrast, *tyrb* F0 mutants were highly variable in body color and color pattern (Figure 3f–h). However, the F1 heterozygous mutant fish displayed no obvious body color differences when compared with the wild-type fish. The *tyrb* homozygous mutant fish displayed a phenotype of complete albinism, with pink RPE (Figure 4e,e'). The *tyra/tyrb* double mutants displayed albinism phenotype, with no differences compared to *tyrb* homozygous mutants.

Hps4

Defects in Hermansky–Pudlak syndrome 4 protein are responsible for disorders of organelle biosynthesis, including the biogenesis of melanosomes and lysosomes. In mammals, *hps4* is reported to be important in the transformation of melanosomes from stage I to stage II. Mutation of this gene in mammals led to red RPE and increasing amounts of pheomelanin, a pigment responsible for yellow and red hair colors (Li et al. 2017; Dorgaleleh et al. 2020). In channel catfish it was reported to be a candidate gene for a natural albinism mutant through GWAS mapping (Li et al. 2017).

Tilapia *hps4* mutants exhibited variable coloration (red with black blotches, black with white patches/spots), quite similar to the situation observed in *tyrb* disrupted F0 fish (Figure 3i–k). In some *hps4* F0 mutant fish, melanophores and iridophores were seriously reduced in some areas and some of them were even partially transparent in the lateral view (Supplementary Figure 6). Loss of black and iridescent pigment cells in the peritoneum are responsible for the transparent phenotype of fish (White et al. 2008; D'Agatia et al. 2017; Martorano et al. 2019; Bian et al. 2019) and melanophores and iridophores in both trunk surface and peritoneum originate from the same NCCs (Lopes et al. 2008; Bian et al. 2019). The gene *hps4* turned out to be important for both melanophore and iridophore survival and pigment biosynthesis. It should be highlighted that in most of the mutant fish with abnormal vertical bars, the remaining bars were irregularly placed in not the initial pigment precursors' final landing area. Mutants of both *tyrb* and *hps4* displayed varying levels of albinism from hypo- to hyper-pigmentation, indicating that disruption of the 2 genes may result in abnormal biosynthesis of melanosome (Figure 3f–k). The *hps4* homozygous mutant fish displayed similar phenotype with *tyrb* mutants, and the RPE (wine red) was heavily pigmented, when compared with the *tyrb* albino (Figure 4f,f'). In the iris of the 2 albinism mutants, obvious carotenoids contributing orange coloration were detected (Figure 4e, e', f, and f').

Kita / kitlga

kita and *kitlga* are an important membrane receptor tyrosine kinase and corresponding ligand, respectively, necessary for melanophore development and function in vertebrates (Parichy et al. 1999; Rawls and Johnson 2000; Mills et al. 2007; Dooley et al. 2013; Otsuki et al. 2020). This signaling system is important to pigment patterning of medaka and zebrafish by directing the pigment cell precursor's migration and differentiation. Disruption of either of these 2 genes leads to changes in the number of melanophores or even loss of

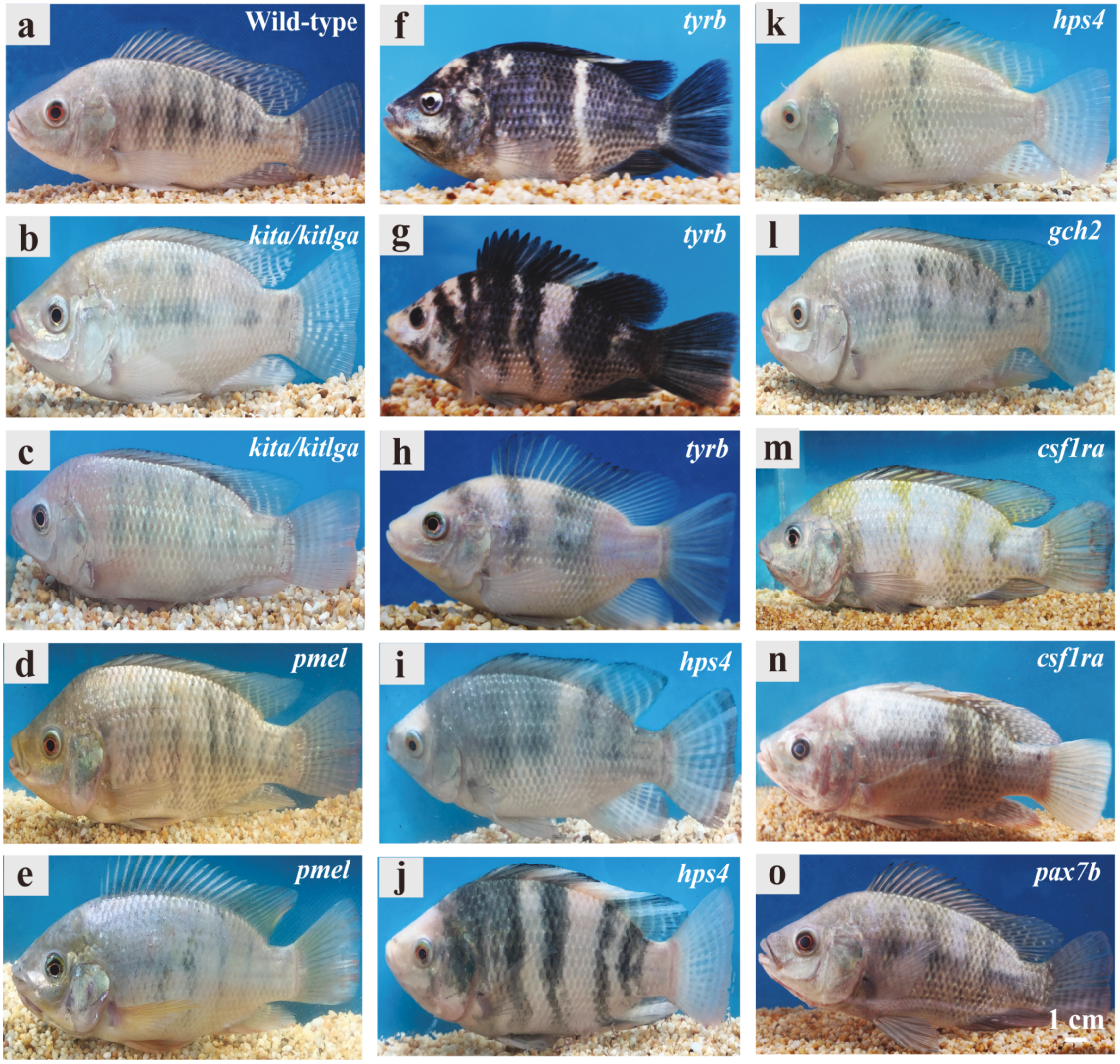


Figure 3. Representative phenotypes of the melanophore and xanthophore specific color gene mosaic mutants (F0). (**a**) Wild-type fish with 7–9 obvious black vertical bars. (**b and c**) Phenotype of the *kita/kitlga* mutants showed serious hypopigmentation in bars and inter bars. (**d and e**) Phenotype of the *pmel* mutants showing different levels of bar loss and obvious vitiligo phenotype. (**f–h**) Phenotype of the *tyrb* mutants showing disrupted body color and color patterns. (**i–k**) Phenotype of the *hps4* mutants showing pink or white background body color and disrupted color patterns. (**l**) Phenotype of the *gch2* mutants showing disrupted color patterns in trunk. (**m–o**) Phenotype of the *csf1ra* or *pax7* mutants showing different levels of bar loss and disruption of yellow and black pigmentation.

other pigment cell types (Rawls and Johnson 2000; Mills et al. 2007; Dooley et al. 2013; Otsuki et al. 2020).

In *kita* and *kitlga* F0 mutant tilapia, serious hypopigmentation in bars or inter bars (mainly black pigmentation loss) was detected in trunk and fins (Figure 3b,c). *kita* F2 homozygous mutant tilapia were golden in color with no bars and only a few scattered melanophores. The RPE was still black, which made the fish look similar to red tilapia. The *kita* homozygous mutants displayed red body color with no bars. In *kita* F2 homozygous mutant fish, strong golden body color with a few melanophores but no bars was detected. The RPE color did not change much when compared with the wild-type fish despite black pigmentation loss in whole trunk and fins of *kita* homozygous mutants (Figure 4g,g' and Supplementary Figure 7).

Gata2a

In humans, deficiency of *gata2* has been found to be related to melanoma, a deadly skin disease (Nguyen et al. 2018). However, the relationship between *gata2* and the melanogenesis pathway has not been revealed yet. In Lake Malawi cichlids, *gata2a* lies within the candidate interval for the orange-blotch phenotype characterized by a reduction in melanophores (Roberts et al. 2009).

Tilapia *gata2a* homozygous mutants displayed very serious hyper-pigmentation that even covered up the original vertical bars, inter bars, and nuptial coloration. *gata2a* homozygous mutants also displayed microphthalmia phenotype with abnormal pigmentation in RPE (Figure 4h,h').

Melanophore Mutants and Eye Color

It should be noted that most of the melanophore-related homozygous mutants were either white/golden or dark black, and some of the homozygous mutants even looked like the red tilapia (Figure 4d,e–h). In the red tilapia strains, the iris color ranged from yellow to silver white, matching the color of their body surface. Carotenoids composed color was also detected in iris of some red tilapia, mainly determined by their genetic backgrounds. Few melanophores were detected in iris and surrounding tender surface

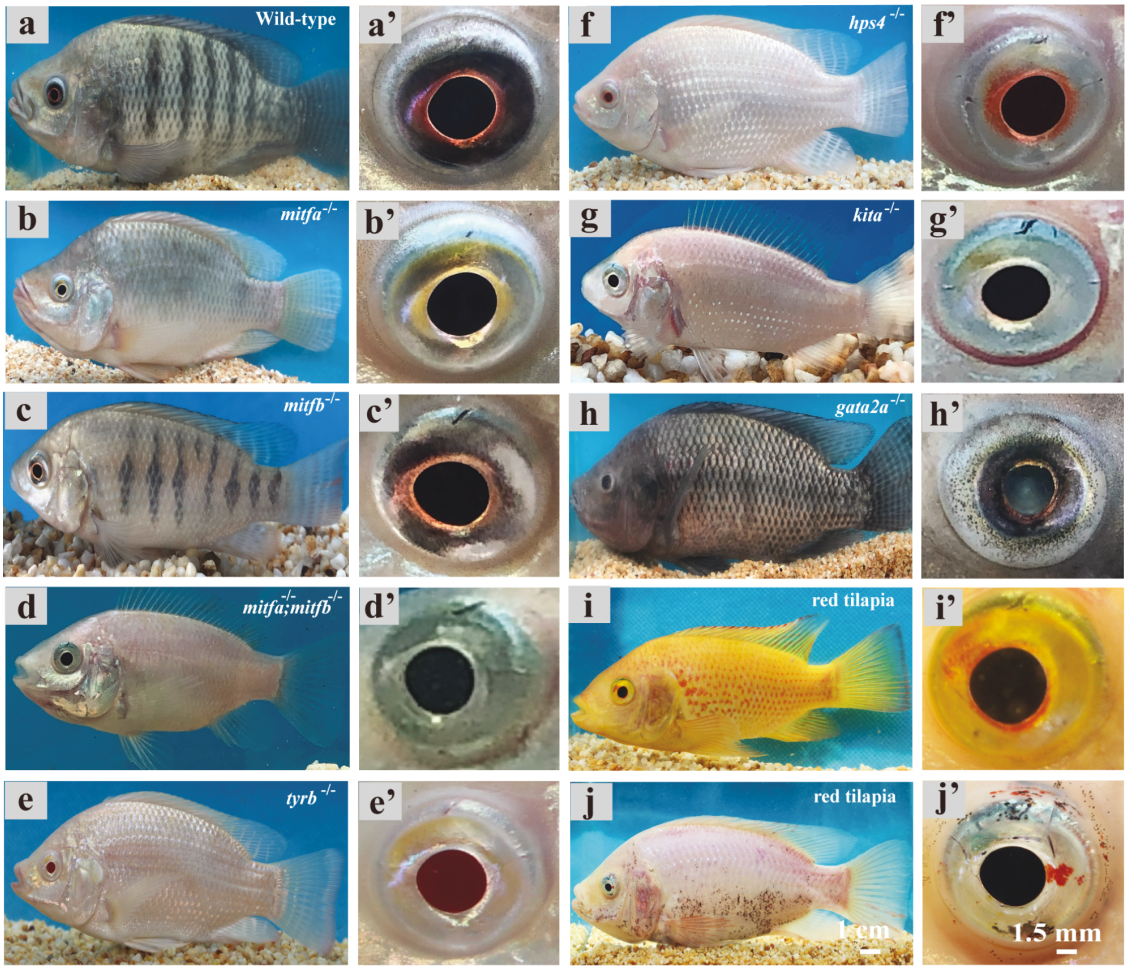


Figure 4. Phenotype mainly in the eyes of the F2 homozygous mutants of some of the specified color genes. (**a and a'**) Wild-type fish showing hyper-pigmentation in RPE and iris, with some carotenoids contributed orange pigmentation in iris. (**b and b'**) *mitfa* homozygous mutant fish showing light pigmented iris with yellow pigmentation, and the RPE color is black. (**c and c'**) *mitfb* homozygous mutant fish showing lightly hyper-pigmentation in iris, which is a signal of under high stress, and the RPE color is black. (**d and d'**) *mitfa;mitfb* double mutants showing even more serious hypopigmentation in iris than *mitfa* homozygous mutants, while the RPE color is still black. (**e and e'**) *tyrb* homozygous mutants showing albinism phenotype in iris and RPE, the iris is a bit yellowish, and the RPE is pink. (**f and f'**) *hps4* homozygous mutants showing albinism phenotype in iris and RPE, orange pigmentation is detected in the iris, and the RPE is wine red. (**g and g'**) *kita* homozygous mutant fish showing pink body color in whole fish, iris is silver white, with iridescent color, and the RPE is black. (**h and h'**) *gata2a* homozygous mutants showing microphthalmia phenotype in eyes together with abnormal pigmentation in RPE. (**i, i', j, and j'**) in the red tilapia strains, the iris color ranged from yellow to silver white, mainly determined by their trunk surface colors. Carotenoids composed color is also detected in some iris. Few melanocytes are detected in iris or surrounding tender surface. However, the RPE color is still black.

in red tilapia. However, the RPE color was still black (Figure 4i,j). Additionally, homozygous mutation of both *tyrb* and *hps4* has led to complete albinism phenotype, but their RPE might not be pigmented on the same level. In *tyrb* mutants, the RPE color was pink, while in the *hps4* mutants, the RPE were either wine red or black.

Body Color and Eye Color Might be Controlled by Different Genes

In some F2 mutants and red tilapia, we detected hypo or hyper pigmentation in whole fish. Hypopigmentation of body, from trunk to fins, was observed in *mitfa* and *kita* homozygous mutants, *mitfa;mitfb* double mutants and red tilapia, while hyper-pigmentation phenotype was observed in *mitfb* and *gata2a* homozygous mutants. However, these mutants, including red tilapia, displayed normal RPE pigmentation as the wild-type fish, suggesting that these genes were not responsible for RPE pigmentation. In contrast, the iris color were consistent with the hypo- and hyper-pigmentation found in these mutants, but different with the wild-type fish. Complete albinism, including the RPE and iris, were detected in *tyrb* and *hps4* homozygous mutants, as reported in other vertebrates, different from the phenotype of the *mitf*, *kita* and *gata2a* mutants. On the other hand, the *tyrb* and *hps4* homozygous mutants displayed similar background body color but different RPE pigmentation with the red tilapia strains. Black spots were never observed in *tyrb* and *hps4* homozygous mutants, while often observed in red tilapia, especially in winter, induced by low temperature (Wang et al. 2019) and some other unknown factors. Taken together, these results suggested that color patterning in the body and eye is controlled by different genes. Most probably, the locus for red tilapia is a gene responsible for melanophores precursor migration or differentiation, instead of the melanin biosynthesis, such as *tyrb* and *hps4*, as mutation of these genes resulted in complete albinism, including the eye. In zebrafish, melanophores in RPE and trunk are from different multipotent cells. The melanophores in RPE are from optic cup, while

the melanophores in trunk, fins and peritoneum are from neural crest cells (Lister 2002), which indicated that black pigmentation in eyes and trunk can be determined by separate genes. Our results confirmed that melanophores in RPE and trunk were controlled by different genes. In contrast, the iris color is always in consistent with the trunk in tilapia, as it is a part of the skin tender surface.

Xanthophore Differentiation

The differentiation and migration of xanthophore precursors has been related to the function of several genes in fish, including *gch2*, *csf1ra,* and *pax7*. In xanthophores and erythrophores, the yellow/orange pigments like carotenoids and pteridine are important for their color patterning (Grether et al. 2001; Ziegler 2003). Genes like *scarb1*, *bco*, *gch2*, *spra*, *pts*, *xdh*, have been found to be the key members in their metabolism processes (Ziegler 2003; Le Guyader et al. 2005; Sköld et al. 2016; Lister et al. 2019).

Mutation of key genetic factors for xanthophore precursor differentiation and carotenoids metabolism led to disrupted body color and color patterns in tilapia. We mutated 10 genes in the xanthophore differentiation pathway. Four of these (*gch2*, *csf1ra*, *pax7b,* and *bco2b*) showed obvious phenotypes in the F0/F2. We found no obvious phenotype for mutations in *pts* and *xdh*, both of which are known to be involved in synthesis of pteridine.

Gch2

GTP cyclohydrolase is the first enzyme in the pathway for synthesis of pteridine. In zebrafish, *gch2* is required for development of pteridine pigmentation in embryo and larval xanthophores, but has no influence on pigmentation of adult fish (Lister 2019). As a gene encoding an important enzyme in pteridine synthesis, it has also been identified as a shared marker of melanoblasts and xanthoblasts (Parichy et al. 2000).

In the *gch2* F0 mutant tilapia, disrupted color pattern in the trunk was detected, which suggested the core enzyme in pteridine metabolism matters in tilapia body color or color pattern formation. In *gch2* F0 mutant fish, disrupted color patterns were detected in the adult trunk, with irregularly spread melanophores and xanthophores in those pattern disrupted area (Figure 3l). However, we did not detect obvious phenotype of xanthophore pigmentation in embryonic stage so far. We suspect that pteridine metabolism was heavily disrupted in the F0 mutants at later stage, thus we detected obvious phenotype in adult stage. In contrast, this gene was demonstrated to be responsible for xanthophore pigmentation in early developmental stage in zebrafish, and the xanthophores were gray without pteridine contributed color in the *camembert*/*gch2* mutants, which was rescued by injection of *gch2* BAC (Lister 2019). Right now, we do not have any explanation for this discrepancy.

Csf1ra

Colony-stimulating factor 1 receptor is another receptor tyrosine kinase important for pigment cell development (Parichy et al. 2000). In zebrafish, *csf1ra* is fundamental for the migration and differentiation of xanthophore precursors. It is crucial for stripe formation, by recruiting neighboring melanophores and limiting the spreading of iridophores. However, even though mutation of this gene led to complete absence of xanthophores, the numbers of melanophores did not drop significantly, which is different from the phenotype of *pax7* mutants (Parichy and Turner 2003; Patterson et al. 2014; Nord et al. 2016). In the vertically barred cichlid *Haplochromis latifasciatus*, there was no difference in xanthophore numbers or the expression level of *csf1ra* between the bars and light-colored inter-bars (Liang et al. 2020).

In our *csf1ra* mutants, disrupted color patterns in the trunk and partial bar loss were observed, indicating the critical roles of this gene for color pattern formation in tilapia. In *csf1ra* mutants, disrupted yellow pigmentation in the trunk surface and fins were detected. Xanthophores were heavily reduced in the trunk. Color patterns were disrupted, similar to those observed in melanophore gene disruption. We also detected displacement of black pigmentation in the mutant fish (Figure 3m,n). Mutations in *csf1rb* showed no obvious phenotype.

Pax7b

Paired box genes encode transcription factors essential for a variety of developmental processes (Nord et al. 2016; Roberts et al. 2017; Santos 2017). In zebrafish, the *pax7* paralogs are necessary for xanthophore-directed horizontal stripe formation. Double mutation of *pax7a/b* resulted in the absence of both xanthophores and stripes (Nord et al. 2016). Variation in *pax7* has also been found to be linked with the orange-blotch color pattern in some East African lake cichlids, however, loss or gain of function studies have not been conducted so far (Roberts et al. 2017; Santos 2017).

In tilapia, *pax7b* mutants showed reduced xanthophore and irregularly spread pigment cells (melanophore and xanthophore) in the trunk surface and fins (Figure 3o). Mutation of *pax7b* resulted in disrupted vertical bars similar to the *csf1ra* mutants, confirming the role of *pax7* in color patterning of cichlids. Mutations in *pax3a*, *pax3b* and *pax7a* showed no obvious phenotypes.

Bco2

Beta-carotene oxygenase 2 is responsible for the asymmetric cleavage of B-carotene (Kiefer et al. 2001). In zebrafish, disruption of *bco2* was found to increase oxidate stress in mitochondria, leading to anemia (Lobo et al. 2012). Overexpression of *bco2,* or morpholino knockdown of *bcox,* led to a reduction in retinal levels, resulting in numerous developmental defects, and a reduction in iridophores during embryogenesis (Lampert et al. 2003). In salmon, variation in expression at *bco2l* locus was firmly related to carotenoid contributed red coloration in muscle, skin, and eggs (Lehnert et al. 2019). In a bird with difference in coloration between males and females, *bco2* expression was founded to be important to turn the orange carotenoid into colorless carotenoid, thus influenced the sexual selection for ornamentation and mate choice (Gazda et al. 2020).

Tilapia *bco2b* mutants displayed hyper-pigmentation in the trunk and fins, but the bars and inter bars were still distinctive, when compared with the wild-type fish. *bco2b* mutants exhibited hyper-pigmentation of melanophores, and absence of red nuptial coloration in trunk and fins. Mutation of this gene also resulted in pericardial edema during embryonic development (Supplementary Figure 8). As a key gene responsible for carotenoids metabolism (the major factor for fish body color), *bco2b* was further confirmed to be necessary for carotenoids contributed body color in tilapia. Mutation of *bco2a* showed no obvious phenotype.

We confirmed the roles of *gch2*, *csf1ra,* and *pax7b* in color patterning in tilapia, as all the mutants displayed different levels of pigmentation disruption in the trunk and fins, together with different levels of bar loss. However, we are not sure whether the number of melanophores was reduced in those mutant fish, but we are sure xanthophore is also necessary for the body color and color pattern formation of tilapia from our observation in those mutants. It should be not ignored that the 3 genetic factors are fundamental

for migration and fate determination of xanthophore, and the latter 2 genes were not responsible for xanthophore color patterning. These results again suggested that genes specific for pigment cell precursors' differentiation and migration are also responsible for color patterning in tilapia, as revealed in melanophore gene mutants.

Iridophore Differentiation

We mutated 3 genes in the iridophore differentiation pathway (Supplementary Figure 3). We detected no obvious phenotypes for mutations in *ltk/shdb*, *sox5*, or *sox10a* mutants.

Unique Interactions Among Cichlid Pigment Cells

In teleost fish, 2 mechanisms for color patterning were the most widely accepted so far (Ceinos et al. 2015). One is the dorsal-ventral counter shading, a classic model, which highlighted the role of Agouti and MCRs family members in teleost body color formation. In the Agouti members, *agrp2*, *asip1,* and *asip2* have been acknowledged to be fundamental for dorsal-ventral counter shading in cichlids, zebrafish, and even carp (Kratochwil et al. 2018; Chen et al. 2018; Cal et al. 2019b). Among the MCRs, *mc1r* was the most widely reported to influence dorsal-ventral counter shading (Suzuki 2013). Studies of melanophore spreading in zebrafish and carp are largely consistent with the results from mammals (Cal et al. 2019a; Mandal et al. 2019). The other MCRs family members, *mc5r*, has been found to be important for xanthophore function, but no gene functional studies by gene editing have been conducted so far (Cal et al. 2017). The other is pigment cell interactions inspired from Turing models. Turing first presented the idea that the periodic structures generated by the reaction–diffusion mechanism may provide correct positional information that is used in the course of animal development (Turing 1952). Animal coat pattern is assumed to be the most typical example of the Turing pattern, on the basis of some mathematical studies. In zebrafish, the local activation and long-range inhibition influence the development, survival and cell adhesion among pigment cells to determine the stripes formation (Nakamasu et al. 2009; Watanabe and Kondo 2015).

Studies of pigmentation in mice and zebrafish have established a strong foundation for understanding the genetic and developmental basis of vertebrate pigmentation generally (Kelsh et al. 2009; Patterson and Parichy 2019). Nevertheless, these model systems are not sufficient to understand the diversity of pigment patterns found in nature. Cichlids especially exhibit a greater number of pigment cell types and a greater variety of patterning, than either of mice or zebrafish. We argue that studies of cichlid fish can expand our understanding of pigmentation in teleosts generally. Tilapia represents a foundational model within the vast radiation of more than 2000 species of cichlid fish.

In tilapia, although the results above confirmed the importance of melanophores and xanthophores in the process of dynamic body color and color pattern formation in tilapia, we are still not sure of the detailed relationship among them. Disruption of genes responsible for melanoblast differentiation, migration, and melanin biosynthesis, like *mitf*, *kita/kitlga*, *pmel*, *tyrb* and *hps4*, have led to different levels of bar loss in F0 mutants and complete absence of melanophores in the homozygous mutants, which confirmed the role of melanophores in bar formation. Similarly, in xanthophore gene mutants, we detected different levels of bars loss, together with irregularly spread xanthophores and melanophores in F0, indicating that xanthophores in tilapia were also necessary for color pattern formation in tilapia. Mutation of *gch2*, a gene acknowledged to be the shared marker of the differentiation of both melanophore and xanthophore precursors (Parichy et al. 2000; Lister 2019), resulted in a phenotype similar to both mutants of melanophore- and xanthophore-specific genes.

The roles of iridophores and erythrophores in color pattern formation remain to be illustrated.

There is much to learn about the role of every type of pigment cell in the color patterning, of skin, scales, fins, or other pigmented tissues like the eyes and peritoneum. The gene mutants we have developed should be useful tools for future studies of color patterning in tilapia. Detailed analysis of the relationship between the 3 types of fundamental pigment cells (melanophore, xanthophore, and iridophores) in zebrafish revealed that they pursue or repel each other over both short and long spatial scales. The same types of pigment cells can interact with each other in different ways through their synaptic junctions and gap junction proteins (Watanabe and Kondo 2012; Frohnhöfer et al. 2013; Usui et al. 2019; Owen et al. 2020). The specific temporal and spatial appearance, and the interactions among these cells, is controlled by fate-determining genes expressed during both early development and metamorphosis (Quigley et al. 2004; Parichy 2006; Kelsh et al. 2009). Thus we can often see changes in body color and color pattern across different life stages. Studying the detailed mechanism of NCCs migration and color patterning can be of great significance for understanding individual development, social behavior, and even the evolutionary history of teleosts. It also provides knowledge to support the artificial creation of commercial and ornamental fish, and may contribute to skin disease research. The mutants we produced in tilapia can serve as models for study of the relationship between the pigment cell types, by combining cross-breeding with cell transplantation, and studies of cell interactions with mathematical modeling.

Red Tilapia

Red tilapia as a natural mutant has drawn the attention of both fish farmers and scholars, for economic trait and fundamental research. Scholars worldwide are struggling to find the key factors that contribute to the pleasant body color of them. Most vertebrates, including birds, reptiles, and fishes, have sexual selection based on attractive coloration, in which the dominant male always has bright body color or color patterns based on carotenoids accumulation (Ahi et al. 2020). And the male with brighter red coloration was easier to attract the female to mate and raise their next generation, as large amount of carotenoids composed body coloration can also be a sign of outstanding ability in catching food, a survival strategy. Additionally, the genetic factor responsible for the body color of red tilapia has long been a hot issue in aquaculture.

A series of studies on the pigment cells and chromosome mapping of the red locus has been conducted, but the key gene that controls this fantastic characteristic is still a mystery. Previous studies revealed that the red locus lies on LG3, a relatively small region close to, but not including *tyrp1a,* a gene fundamental for melanin biosynthesis (Lee et al. 2005). However, none of the usual candidate gene suspects have been identified in the region so far. Red tilapia with black spots is very popular, especially in the winter, when low temperature greatly increases the spots and influences the value of this fish (Wang et al. 2019). The breeding of pure red tilapia is always wanted in aquaculture practice, thus finding the locus for red body color can be of great significance in the future.

Cichlids as a Model for Teleost Pigment Pattern Diversity

Many unique color patterns have been observed in the vast radiation of more than 2,000 species of cichlid fish. The patterns range from

horizontal stripes to vertical bars, traveling waves, or even spots. Even species the same group can have very different body color or color patterns. These patterns can include many different colors, from white to black, yellow to orange. Therefore, cichlids color patterns may be structured by unique mechanisms that do not have counterparts in model fish. The vast number of color patterns found among cichlid species can serve as a model system for the color patterns of teleosts more generally.

Studies of color patterning in zebrafish have deepened our understanding of the molecular mechanism of vertebrates' body color formation. However, studies were lagged far behind in other teleost fish. Cichlids have been acknowledged to be an idea model for scientific research, and a few studies conducted on them have demonstrated that they might also function as an excellent model for color patterning research.

It has been reported that 3 pigment cell types are responsible for the bar, stripe, and spot development in 2 species of sand-dwelling cichlids from Lake Malawi, and that the color patterning, and formation of bars has a chronological order (Hendrick et al. 2019). In another East African cichlid, *Haplochromis latifasciatus*, the density of melanin and melanophores, together with their dispersal are fundamental for the vertical bar and yellow inter-bars formation (Liang et al. 2020). Thus detailed analysis of pigment cell development and the dynamics of cell-cell interactions in tilapia will help us better understand the mechanisms of color patterning in cichlids, and teleosts more generally.

Tilapia, with short regular breeding cycle, has been used as model in our lab to study sexual plasticity for quite a long time. Based on the indoor culture systems and mature CRISPR/Cas9 gene-editing techniques established (Li et al. 2014), we were able to mutate dozens of color genes in a short time. About half of the 38 mutated genes displayed phenotype related to melanophore and xanthophore reduction, absence, and pigment disruption in them. The ability of making these mutants will allow the analysis of new candidate genes in regions identified as QTL in previous studies of cichlid pigment patterns. And it should be highlighted that most of the genes studied in zebrafish are not found within the QTL so far identified in cichlids. Studies in tilapia will reveal the wider network of genes involved in teleost pigment patterns.

Conclusions

In this study, we characterized 4 types of pigment cells and defined their first appearance of tilapia. We also mutated 25 melanophore, xanthophore, and iridophore specific genes and detected obvious body color or color pattern phenotypes in 13 mutants. By comparing the pigmentation in both natural and induced mutants, we founded that color patterning of body and eyes is determined by different genes. Melanophores, xanthophores, and iridophores depend with each other, and they are fundamental for bars formation, while the detailed relationship remains to be illustrated. Color genes like *mitf*, *kita/kitlga*, *pmel*, *tyrb*, *hps4*, *gch2*, *csf1ra*, *pax7b*, and *bco2b* are of great significance for color patterning in tilapia. Our results suggested that tilapia, a fish with 4 types of pigment cells and vertical bars in wild-type, together with various natural and artificially induced color gene mutants, can act as an excellent model for study color patterning in vertebrates.

Supplementary Material

Supplementary material can be found at *Journal of Heredity* online.

Funding

This work was supported by grants 31872556, 31861123001 and 31630082 from the National Natural Science Foundation of China; grants CYS17080 from the Chongqing Municipal Education Commission.

Data Availability

The required links or identifiers for our data are present in the manuscript as described.

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