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The regulation of melanocyte-stimulating hormone on the pigment granule dispersion in the xanthophores and melanophores of the large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

The large yellow croaker (*Larimichthys crocea*) is one of the most valuable economic mariculture fish in China, and its yellowness in skin is considered as the most important traits that determine the market value. The fish skin colors present naturally silvery white during the daytime and golden yellow during the night-time. In the present study, we found that the xanthosomes of xanthophores aggregated during the daytime and dispersed during the night-time, while the melanosomes of melanophores always dispersed all day long *in vivo*. We further cloned three proopiomelanocortin (POMC) genes of the large yellow croaker. All these POMC genes were expressed in the pituitary, but only POMC-C mRNA was expressed in the skin. The results of single-cell RT-PCR showed that the POMC-C mRNA was expressed in isolated xanthophores but not in melanophores. The xanthophores, but not melanophores, were found in the ventral skin, where only one subtype of melanocortin receptor (MCR), *i.e.* MC5R, was expressed. MC1R, MC4R and MC5R mRNAs were detected in the dorsal skin, where there existed both the xanthophores and melanophores. The results of single-cell RT-PCR showed that only MC5R mRNA was expressed in the xanthophores, while only MC1R mRNA was detected in the melanophores. Thirty min after *in vivo* and *in vitro* treatment with melanocyte-stimulating hormone (MSH) peptides (α-MSH, Des-Ac-α-MSH, α-MSH-C and β-MSH-C), dispersion degrees of the xanthosomes and melanosomes significantly increased, even under light exposure. Interestingly, when we immediately observed the dorsal scales that have been maintained under *in vitro* complete dark condition without MSH peptides treatment for at least 30 min, the melanosomes aggregated as expected, but the xanthosomes dispersed. Both α-MSH and α-MSH-C exhibited similar, but stronger effects than Des-Ac-α-MSH on dispersion of both the xanthosomes and melanosomes. Although the two MSH peptides (α-MSH-C and β-MSH-C) are generated from POMC-C, the former showed stronger effect on xanthosome dispersion than the latter. Forskolin, an Adenylyl cyclase activator, dispersed pigment granules in both the xanthophores and melanophores. H 89 2HCl, a specific inhibitor of protein kinase A blocked α-MSH-induced pigment granule dispersion of xanthophores and melanophores. Taken together, our results indicated that the dispersion of melanosomes is stimulated by MSH, possibly secreted from pituitary, through endocrine pathway. The yellowness of the large yellow croaker skin was mainly due to the dispersion of xanthosomes. The dispersion of xanthosomes was regulated by MSH that is possibly released both from the pituitary through endocrine pathway and from the xanthophores through autocrine pathway.

1. Introduction

The large yellow croaker (*Larimichthys crocea*) is mainly limited to coastal waters of continental East Asia (Kong et al., 1987). This species was once one of the four major marine fisheries of China. Heavily exploited since the 1950s, the maximum catch was about 200,000 t in the middle 1970s, which resulted in severe depletion of wild stocks. By the 1980s, catches of this species declined by over 90% in China. The great majority now on sale are from mariculture. The production of this species in 2016 was 165,496 t (China Fishery Statistical Yearbook 2017), ranking the top of the mariculture fishes in China. As the meaning of its common name, the skin (especial ventral area) of this

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fish exhibits golden yellow color, which means 'fortune' in Chinese culture. Therefore, skin coloration is considered as one of the most important traits that determine the market value. However, this fish colors present silvery white during the daytime and golden yellow during the night-time under culture conditions. Therefore, the farmers usually harvest the large yellow croaker at night for higher benefit. However, this fishing method is inconvenient and dangerous. In order to obtain the golden yellow fish during the daytime, the physiological mechanisms of body color changes need to be investigated.

The chromatophores include melanophores (black or brown), xanthophores (ocher or yellow), erythrophores (red), iridophores (metallic or iridescent), leucophores (whitish) and cyanophores (blue), which are responsible for the revelation of integumentary coloration in fish (Fujii, 2000). The pigment granules of melanophores and xanthophores are called melanosomes and xanthosomes respectively (Fujii, 1993; Hearing, 2000). In teleosts, body color changes—pigment granule aggregation and dispersion in chromatophores—are controlled by endocrine and nervous systems. The hypothalamus-pituitary axis plays a pivotal role in the body color changes employing melanocyte-stimulating hormone (MSH) (Fujii, 2000; Sköld et al., 2013).

The precursor proopiomelanocortin (POMC) can be cleaved into different MSH peptides, *e.g.*, α-melanocyte-stimulating hormone (α-MSH), β-melanocyte-stimulating hormone (β-MSH) and γ-melanocyte-stimulating hormone (γ-MSH) (which has been lost in teleosts), all of which are characterized by a core His-Phe-Arg-Trp (HFRW) sequence and localized in different domains of the precursor: γ-MSH in the N-terminal POMC (N-POMC) domain, α-MSH in the adrenocorticotropic hormone (ACTH) domain, and β-MSH in the C terminal β-lipotropic hormone (β-LPH) domain (Barr, 1991; Bertagna, 1994; Metz et al., 2006). In teleosts, MSH dispersed the pigment granules of melanophores or xanthophores in the zebrafish (*Danio rerio*) (Logan et al., 2006), two spotted goby (*Gobiusculus flavescens*) (Sköld et al., 2008), barfin flounder (*Verasper moseri*) (Kobayashi et al., 2009), goldfish (*Carassius auratus*) (Kobayashi et al., 2011), Japanese flounder (*Paralichthys olivaceus*) (Kobayashi et al., 2012) and African cichlid fish (*Astatotilapia burtoni*) (Dijkstra et al., 2017). Usually, acetylation increases the melanin-dispersing activity of α-MSH in teleosts (Kawauchi et al., 1984) as well as amphibians (Eberle, 1988). However, study in the barfin flounder showed that N-terminal acetylation of α-MSH reduced the activity of α-MSH on melanophores, while it enhanced activity on xanthophores. On the other hand, replacement of the C-terminal residue of α-MSHs may have negligible effects on the pigment dispersing activities (Kobayashi et al., 2009).

The major MSH-producing tissue is the pituitary gland (Smith and Funder, 1988; Castro and Morrison, 1997). It has been generally accepted that information perceived by lateral eyes and other sense organs is transferred *via* the optic nerve to the central nervous system, where it is integrated to affect the expression of pituitary MSH that regulates chromatophores *via* endocrine route (Fujii, 2000). Interestingly, in our preliminary experiment, we covered the large yellow croaker body (except the head) with a light-proof material for 30 min during the daytime and found that the color of covered body changes from the silvery white to the golden yellow, but the color of uncovered head remains the silvery white. This phenomenon suggests that there exist autocrine and/or paracrine regulation systems of the xanthosome dispersion of xanthophores in the skin of the large yellow croaker. Here, we cloned the cDNA sequences of POMC genes, and subsequently investigated expression patterns of POMC genes in the pituitary and skin of the large yellow croaker. We further examined the effects of MSH peptides, which are derived from different subtypes of POMCs, on the pigment granule dispersion of xanthophores and melanophores of the large yellow croaker *in vivo* and *in vitro*. In addition, we also determined the signaling pathway of MSH *via* their melanocortin receptors (MCRs).

2. Materials and methods

2.1. Experimental fish

Adult large yellow croaker (*Larimichthys crocea*) (body length: 34.6 ± 0.9 cm; body weight 695.3 ± 16.0 g) were purchased from a fish farm in Ningde, Fujian province, China. Fish were temporarily maintained in indoor plastic tanks with seawater at salinity 30 and temperature 25 °C. Before sampling, the fish were anaesthetised with 0.01% MS222 (Sigma-Aldrich, St. Louis, MO, USA). All experiment protocols were approved by the Institutional Animal Care and Use Committee of Xiamen University.

2.2. cDNA cloning of POMC and MCR genes

The gene sequences of the four subtypes of the large yellow croaker MCR are available in GenBank: (MC1R, XM_010741507.2; MC2R, XM_010747834.1, MC4R, XM_019264696.1, MC5R, XM_010747833.2). We cloned the full-length of the large yellow croaker POMC genes. Briefly, total RNA were isolated from pituitary using RNAzol® reagent (Molecular Research Center Inc., Cincinnati, OH, USA), and reverse transcribed into first cDNA using SMARTer® RACE cDNA amplification kit (Clontech, Tokyo, Japan) following the manufacturer's instructions. Primers for cloning of partial sequences of the large yellow croaker POMC genes were designed (Table 1) from the reference sequences of the POMC genes reported in GenBank (XM_019270382.1, XM_010756210.2, XM_010756316.2, XM_019276771.1, XM_019276772.1, XM_010734682.2). The PCR amplification was conducted with the following thermocycles: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 30s, 72 °C for 1 min; a final extension 72 °C for 10 min. Based on the partial cDNA sequences obtained, gene-specific primers were designed for 5′- and 3′-RACE. The PCR products were cloned into pMD19-T simple vector (TaKaRa, Dalian, China) and sequenced by Invitrogen Ltd. (Guangzhou, China).

Table 1
Nucleotide sequences of the primers for cloning and RT-PCR of the POMC and MCR genes.

Primer	Primer sequence
Primers for partial fragment/ RT-PCR	
POMC-A Fw1	5′-GGTCGACTCTGAGAGGAGCATGC-3′
POMC-A Rv1	5′-TCACTGCTCTCTCTTCTGCTCCT-3′
POMC-B Fw1	5′-AGAATGTGTCCTGCGTGGCTGT-3′
POMC-B Rv1	5′-TCACTTCTGCTGCTGTCCGTCT-3′
POMC-C Fw1	5′-GGTTGAAGATGGTGTGTCTATG-3′
POMC-C Rv1	5′-TCATCCCATTATCCTCTGCACAT-3′
MC1R Fw1	5′-GTCAGTCATTATATAGAGGTCAGAC-3′
MC1R RV1	5′-TCGTTTTCATCTTAAATGCATCCGG-3′
MC2R Fw1	5′-AGCTGTGAACCAGTCTGACTGC-3′
MC2R RV1	5′-TCCAGTCTGAACAAAGCAGCATC-3′
MC4R Fw1	5′-AAGAAAACTCAACTCCGAGAATT-3′
MC4R RV1	5′-ATTCGCAAATCGCGGCTGACATT-3′
MC5R Fw1	5′-ATGAATGTCTCACATGAGTCCTC-3′
MC5R RV1	5′-TTAATACTTATCTGTCAGGGCAC-3′
β-actin Fw1	5′-CATCACCATCGGCAATGAGAGG-3′
β-actin Rv1	5′-GTACATGGTGGTACCTCCAGAC-3′
Primers for 5′RACE	
POMC-A GSP1	5′-CGACTCCTCCTCCACGCCGTTGGAGGTG-3′
POMC-B GSP1	5′-GCGGACTCCTCCTCCATGCCGTTGGAGG-3′
POMC-C GSP1	5′-ACCTTCACAGGCCGGCGTTTACGGCCAG-3′
Primers for 3′RACE	
POMC-A GSP2	5′-GCCAAGCGCTCCTACTCCATGGAGCACT-3′
POMC-B GSP2	5′-CATCCCAGGCAGAGCCCACCTCCAGCCT-3′
POMC-C GSP2	5′-GAAATCCCACAGCGACCAGCGACGCTCC-3′

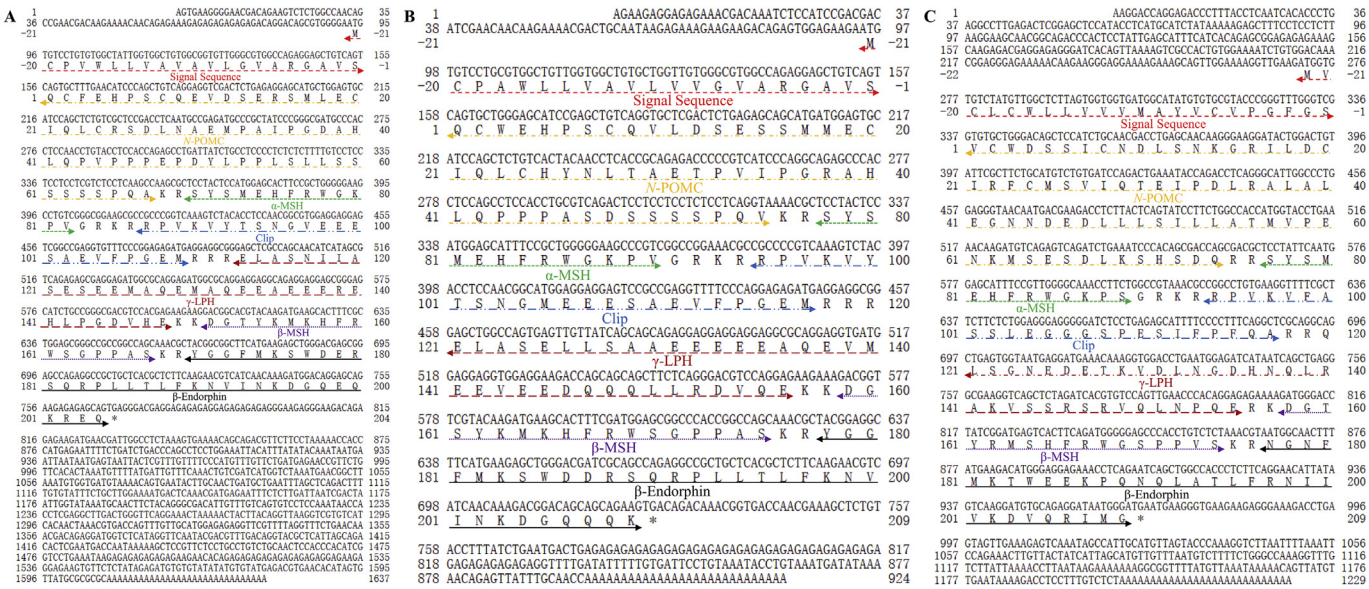


Fig. 1. Full-length cDNA sequences and deduced amino acid sequences of POMC-A cDNA (A), -B cDNA (B) and -C cDNA (C). Signal sequence is indicated by red line; *N*-POMC, orange line; α-MSH, green line; Clip (Corticotropin-like intermediate lobe peptide), blue line; γ-LPH, dull-red line; β-MSH, purple line; β-Endorphin, black line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Homology and phylogenetic analyses

Sequences for other fish POMCs were retrieved from the National Centre for Biotechnological Information (NCBI), using the GenBank database (http://www.ncbi.nlm.nih.gov). The homology analysis of the POMC amino acid sequences was performed using ClustalX. The phylogenetic reconstruction was performed using MEGA software 6.0 (http://www.megasoftware.net) by the Neighbor-joining method, and a bootstrap consensus tree was inferred from 1000 replicates.

2.4. Reverse transcription (RT)-PCR for target genes detection in skin and pituitary

For the tissue specificity study of target genes expression, total RNAs were isolated from the ventral and dorsal skins and pituitary, and further treated with DNase I (Thermo Fisher Scientific, San Jose, CA, USA) for 1 h at 37 °C. For each tissue, an equal amount of total RNA (2 μg) from three individual fish was reverse transcribed to the first-strand DNAs using the RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, San Jose, CA, USA), and then subjected to PCR amplification with specific primers for target genes (Table 1). These PCR products were analyzed by 1.2% agarose gel (Biowest, Barcelona, Spain) electrophoresis. Photographs were taken using a Gene genius Bioimaging System (Syngene, Cambridge, UK).

2.5. Single cell isolation and RT-PCR

The method for isolation of single cell was fundamentally the same as those described in the previous reports (Kobayashi et al., 2009). The ventral and dorsal scales were rinsed in Hanks' balanced salt solution (HBSS) twice. The samples were allowed to stand for 10 min at room temperature in a dissociation medium [DM: 1 mg/mL collagenase type III (Worthington, Freehold, NJ, USA), 1×10^{-4} M epinephrine (Sigma–Aldrich, St. Louis, MO, USA), 2 mg/mL bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA), 0.1 mg/mL soybean trypsin inhibitor (Sigma–Aldrich, St. Louis, MO, USA), and 5 U/mL DNase I (Takara, Dalian, China)] and then gently agitated for 10 min in the same solution. The DM was removed, and the samples were rinsed three times with HBSS. Finally, during the gentle agitation in fresh DM, dispersed single xanthophores and melanophores were isolated using glass capillaries under a microscope. cDNA from an isolated cell was synthesized using the Super-Script III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA obtained from three cells was pooled, and a 6-μL aliquot was analyzed using PCR to detect POMC and MCR mRNAs. As a control, 2 μL was analyzed to detect β-actin mRNA. The primer sets for POMC and MCR mRNAs were showed in Table 1.

2.6. MSH peptides

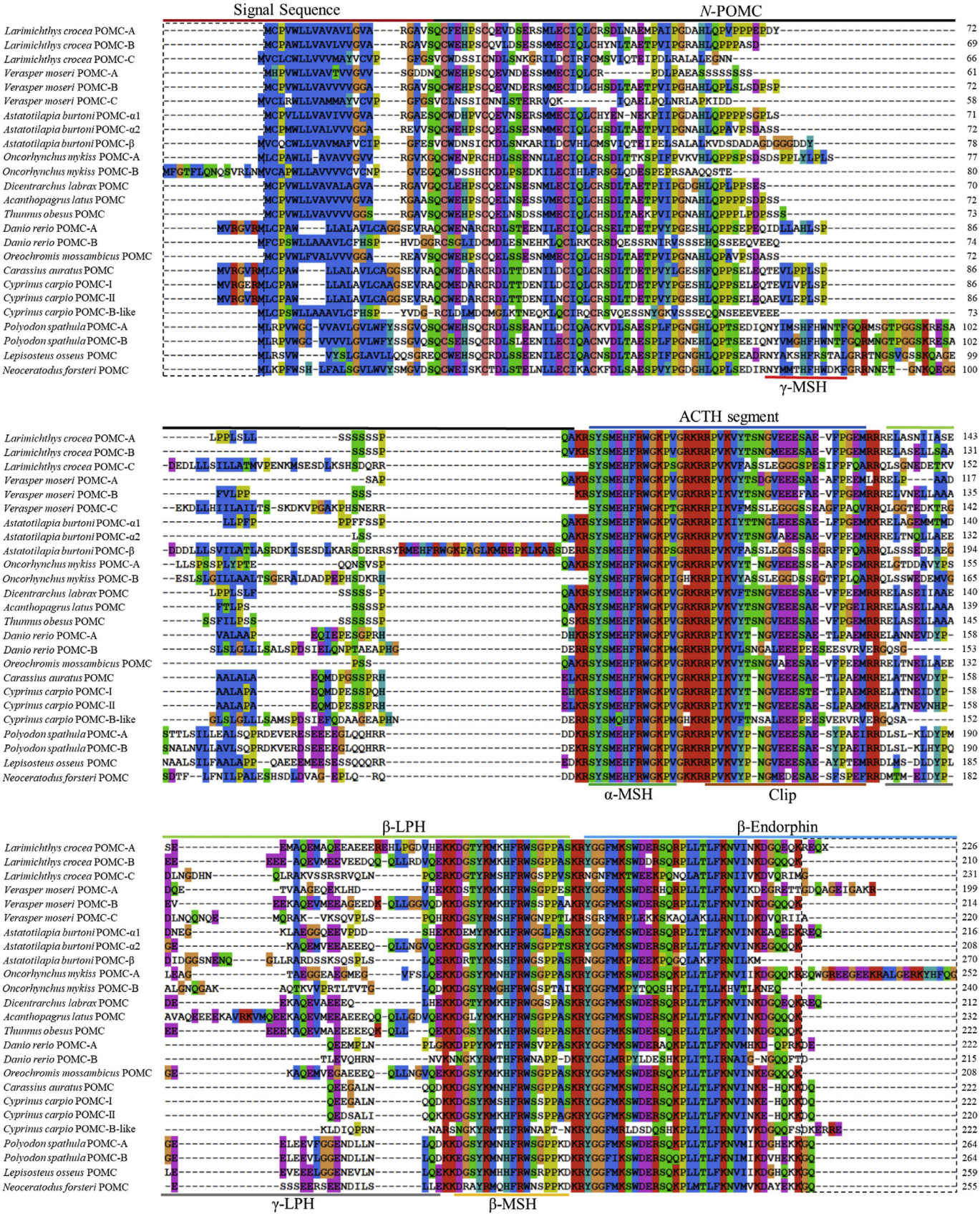
The α-MSH, Des-acetyl (Ac)-α-MSH, α-MSH-C and β-MSH-C were synthesized and purchased from Meilun Biotechnology Co., Ltd. (Dalian, China). Here, α-MSH refers to the peptide derived from POMC-A which is identical to that from POMC-B. α-MSH-C and β-MSH-C indicate the peptides derived from POMC-C which are different from those from POMC-A and POMC-B. Des-Ac-α-MSH has a free N-terminus, while α-MSH, α-MSH-C and β-MSH-C have an acetylated N-terminus respectively. The amino acid sequences of MSHs are as follows:

α-MSH, Ac-SYSMEHFRWGKPV-NH_2; Des-Ac-α-MSH, SYSMEHFRWGKPV-NH_2; α-MSH-C, Ac-SYSMEHFRWGKPS-NH_2; β-MSH-C: Ac-DGTYRMSHFRWGSPPVS-NH_2.

2.7. Effects of MSH peptides on the body coloration of the large yellow croaker in vivo

The solution of MSH peptides (α-MSH, Des-Ac-α-MSH, α-MSH-C, and β-MSH-C) was prepared by dissolving the powder in saline (0.85%). Fish injected intraperitoneally with 1 mL saline (0.85%) containing different MSH peptides at a concentration of 100 μM were used as treated groups, those injected intraperitoneally only with 1 mL saline (0.85%) during the daytime as the daytime control, and those injected intraperitoneally only with 1 mL saline (0.85%) during the night-time as the night-time control. Four fish were used in each group. Body color photographs were taken using a DSC-HX300 camera (Sony, Tokyo, Japan) after 30 min of MSH peptides injection. The chromatophore observation of the effect of α-MSH on body coloration was made using an Olympus DX51 Microscope Microscope (Olympus, Tokyo, Japan).

To quantify skin coloration, we used the color parameters: L^*, a^* and b^* for lightness, redness and yellowness, respectively, in accordance with the recommendation of the Commission Internationale



(caption on next page)

Fig. 2. A comparison of the amino acid sequences of large yellow croaker POMCs with those of other teleosts. Amino termini of POMC-A and -B were determined by the identification of *N*-POMCs while the amino terminus of POMC-C was deduced by analogy with other fish POMCs. Signal sequence is indicated by dull-red line. *N*-POMC, black line; γ-MSH, red line; ACTH, blue line; α-MSH, green line; Clip, yellow line; β-LPH, light-green line; γ-LPH, gray line; β-MSH, orange line; β-Endorphin, light-blue line. The amino acid sequences boxed by broken lines were removed to construct a phylogenetic tree in Fig. 3. Multiple species' amino acid sequences of fish POMCs were aligned using ClustalX. The GenBank accession numbers for sequence data analyzed are: *Verasper moseri* POMC-A, BAB18467.1; *Verasper moseri* POMC-B, BAB18468.1; *Verasper moseri* POMC-C, BAB18469.1; *Astatotilapia burtoni* POMC-α1, AGN30560.1; *Astatotilapia burtoni* POMC-α2, AGN30562.1; *Astatotilapia burtoni* POMC-β, NP_001273211.1; *Oncorhynchus mykiss* POMC-A, CAA49466.1; *Oncorhynchus mykiss* POMC-B, CAA49467.1; *Dicentrarchus labrax* POMC, AAU00742.1; *Acanthopagrus latus* POMC, AAF22342.1; *Thunnus obesus*, BAA35125.1; *Danio rerio* POMC-A, AAI33875.1; *Danio rerio* POMC-B, NP_001076520.1; *Oreochromis mossambicus* POMC, AAD41261.1; *Carassius auratus* POMC, CAD24030.1; *Cyprinus carpio* POMC-I, CAA74968.1; *Cyprinus carpio* POMC-II, CAA74967.1; *Cyprinus carpio* POMC-B-like, XP_018936281.1; *Polyodon spathula* POMC-A, AAD41263.1; *Polyodon spathula* POMC-B, AAD41264.1; *Lepisosteus osseus* POMC, AAB03227.1; *Neoceratodus forsteri* POMC, AAD37347.1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

de L'Eclairage. $L^*a^*b^*$ is a standardized, perceptually uniform and device-independent color space and it has been frequently used in fish color quantifications (Sköld et al., 2008). Color quantifications of the ventral and dorsal skins were performed on the digital images which were converted to L^*, a^* and b^* using Adobe Suite Photoshop version CS6 (Adobe, San Jose, CA, USA).

2.8. Effects of MSH peptides on pigment granule dispersion of chromatophores in vitro

To examine the cellular effects of MSH peptides (α-MSH, Des-Ac-α-MSH, α-MSH-C and β-MSH-C) on the pigment granule dispersion of chromatophores, we conducted a pharmacological experiment on the large yellow croaker scales with xanthophores or melanophores *in vitro*. Briefly, scales with xanthophores or melanophores from 4 fish were isolated from the ventral or dorsal skins and rinsed in PBS (Gibco, Carlsbad, CA, USA) three times, then incubated in Leibovitz's L-15 Medium (no phenol red, Gibco, Carlsbad, CA, USA) for 24 h. After this pretreatment, the pigment granules of both menlanophores and xanthophores were aggregated. Thereafter, scales were treated for 30 min with PBS containing different concentrations of MSH peptides (1, 10, 100 and 1000 nM) or with PBS as the control.

The method of estimating the dispersion degree of pigment granules was fundamentally the same as those described in the previous reports (Chen et al., 2014a; Chen et al., 2014b; Chen et al., 2015). Images were taken using Axio Observer A1 with Axiovision SE64 Rel. 4.9 software (Carl Zeiss Jena GmbH, Jena, Germany) and analyzed using MATLAB software (Mathworks, Natick, MA, USA) for pixel counts of pigment granules-covered area of a chromatophore in a series of images. First, a chromatophore was selected with the rectangular tool in Adobe Suite Photoshop version CS6 (Adobe, San Jose, CA, USA), fitting it as close to the cell dendrites as possible. The selected image was converted to a binary image using a threshold level which defined pixel numbers with the highest correlation to the results obtained using Photoshop software with manual selection. Pixel intensity values greater than the threshold were determined a value of 1. The sum of values in each image was used to represent the pigment-covered area within a xanthophore/melanophore.

The maximum capacity (A_0) of the translocation of pigment granules was calculated as:

$$A_0 = A_{full\ dispersion} - A_{full\ aggregation} \quad (1)$$

where $A_{full\ dispersion}$ and $A_{full\ aggregation}$ denote the pixel counts of each xanthophore/melanophore at full dispersion and aggregation, respectively.

The $A_{full\ dispersion}$ for both xanthophores and melanophores was determined in the large yellow croaker with *in vivo* α-MSH treatment for 30 min (see section 2.7). In order to prevent endogenous effect from MSH, the $A_{full\ aggregation}$ for both xanthophores and melanophores was determined *in vitro*. The full aggregation of melanosomes of melanophores only occurred *in vitro* after rinsed in PBS three times and incubated for 24 h, and the full aggregation of xanthosomes of xanthophores occurred under light treatment for 10 min both *in vitro* and *in vivo*.

Therefore, the dispersion degree of pigment granules after 30 min MSH peptides treatment was estimated as:

$$A/A_0 = (A_t - A_{full\ aggregation})/(A_{full\ dispersion} - A_{full\ aggregation}) \quad (2)$$

where A_t denotes the pixel counts of each xanthophore/melanophore after 30 min MSH peptides treatment. The mean value of six randomly selected xanthophores from ventral scales or six melanophores from dorsal scales was used to estimate the dispersion degree of the pigment granules.

2.9. Effect of an AC activator on pigment granule dispersion of chromatophores in vitro

In order to determine the possible involvement of Adenylyl cyclase (AC) and cyclic adenosine monophosphate (cAMP) in the pigment granule dispersion of xanthophores and melanophores, the effect of forskolin (Selleck Chemicals, Houston, TX, USA), an AC stimulating agent that increases cAMP levels, on the pigment granule dispersion was examined. Forskolin was first dissolved in DMSO (Solarbio, Beijing, China) at a concentration of 10 mM, then diluted in PBS to different concentrations (1 nM, 10 nM, 100 nM, 1000 nM) as treated groups. The control was treated with PBS containing 0.01% DMSO (volume/volume). The dispersion degree of pigment granules was determined as described in section 2.8.

2.10. Effect of a PKA inhibitor on α-MSH-induced pigment granule dispersion of chromatophores in vitro

H 89 2HCl (ApexBio, Houston, TX, USA), a specific inhibitor of protein kinase A (PKA), was first dissolved in DMSO at a concentration of 50 mM, then diluted in PBS to different concentrations (1 μM, 10 μM, 50 μM, 100 μM) as treated groups. The control was treated with PBS containing 0.2% DMSO (volume/volume).

After the scales with chromatophores had been treated with H 89 2HCl for 30 min *in vitro*, the culture medium were replaced by PBS containing 1 μM α-MSH for another 30 min treatment. Images were taken to estimate the dispersion degree of pigment granules. The dispersion degree of pigment granules was determined as described in section 2.8.

2.11. Statistical analysis

Data were presented as means ± standard error of the mean (SEM). Depending on the experimental design, data were analyzed using either Student's *t*-test or one-way ANOVA followed by Fisher's PLSD *post hoc* test to assess statistical differences among the individual groups using the SPSS (version 22.0) statistical software package (IBM, Armonk, NY, USA).

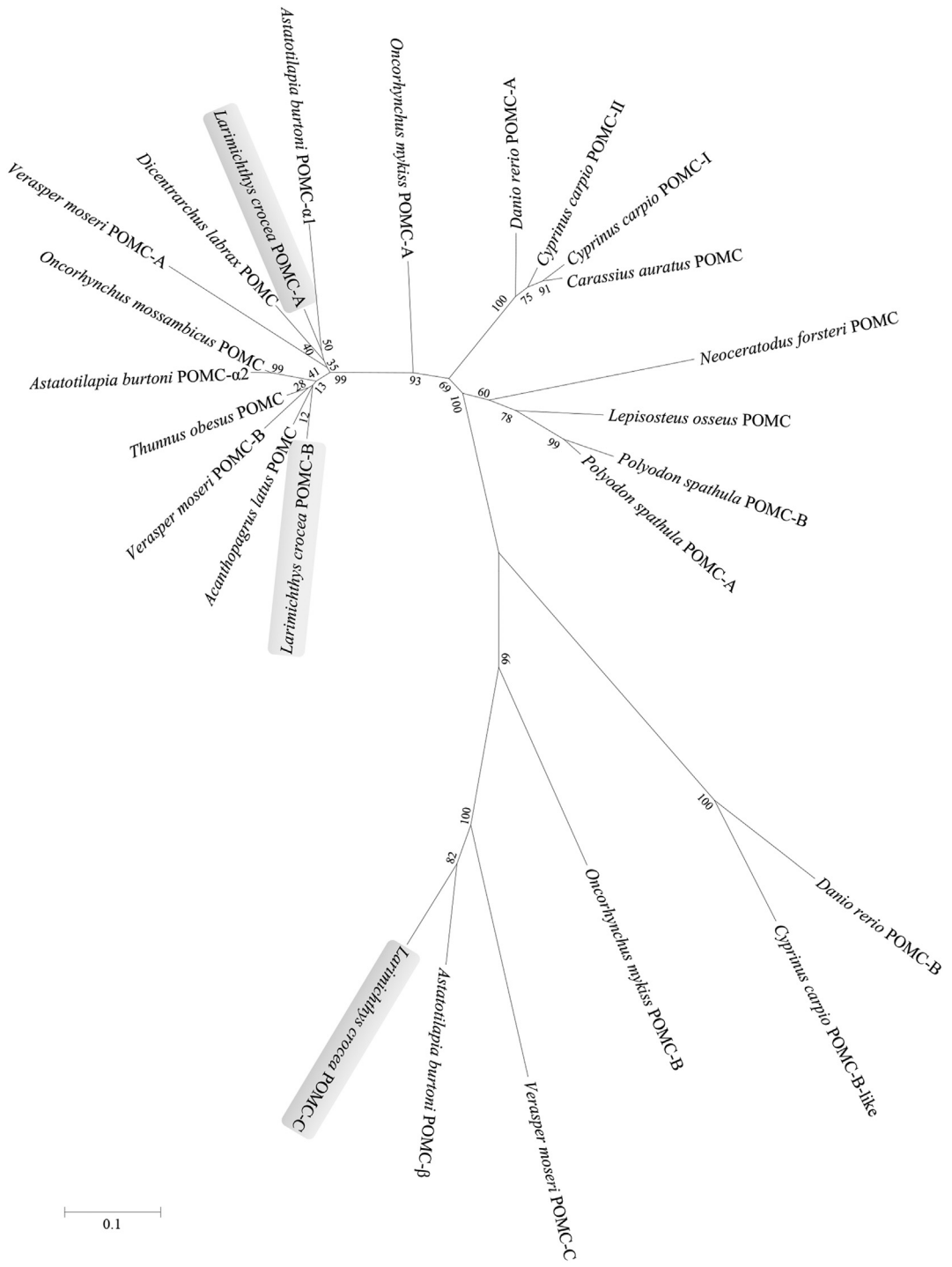


Fig. 3. Phylogenetic tree of POMCs between the large yellow croaker and other teleosts. The evolutionary tree was formed using the neighbor-joining method and a bootstrap analysis with 1000 replicates was used to assess the strength of nodes in the tree. Light gray indicates the large yellow croaker POMCs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Sequence analyses of POMCs cDNA

The full-length sequences of the obtained POMC-A, -B and -C genes of the large yellow croaker (accession number: POMC-A, MH823737; POMC-B, MH823738; POMC-C, MH823739) were 1643 bp, 930 bp and 1235 bp, including an ORF which is consisted of 678 bp, 633 bp and 696 bp, respectively. The deduced precursor proteins of POMC-A, -B, and -C are composed of 225 aa, 211 aa and 232 aa, respectively (Fig. 1). Protein sequence alignment of fish POMCs reveals that the large yellow croaker POMC-A, -B and -C are composed of a putative signal peptide region (POMC-A, POMC-B: M^{1}-S^{21}; POMC-C: M^{1}-S^{22}), an N-terminal (N-POMC) segment (POMC-A: Q^{22}-A^{88}; POMC-B: Q^{22}-V^{76}; POMC-C: V^{23}-Q^{96}), an ACTH segment containing an α-MSH segment (POMC-A: S^{91}-V^{103}; POMC-B: S^{79}-V^{91}; POMC-C: S^{99}-S^{111}) and Clip segment (POMC-A: R^{108}-M^{130}; POMC-B: R^{96}-M^{118}; POMC-C: R^{116}-A^{139}), a β-lipotropic hormone (LPH) segment containing γ-LPH (POMC-A: E^{134}-E^{167}; POMC-B: E^{122}-E^{157}; POMC-C: L^{143}-E^{177}) and β-MSH (POMC-A: D^{170}-S^{188}; POMC-B: D^{160}-S^{176}; POMC-C: D^{180}-S^{196}), and a β-Endorphin (POMC-A:

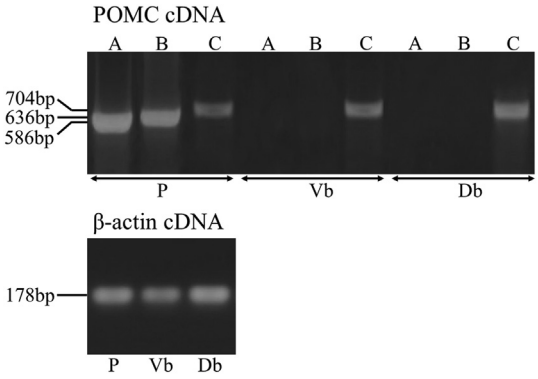


Fig. 4. Expression of POMC mRNAs in the pituitary and skins. Total RNAs prepared from three individuals were combined. A, B, C show the molecular type of the POMC cDNA. β-actin is an internal control. P, pituitary; Vb, ventral skin; Db, dorsal skin.

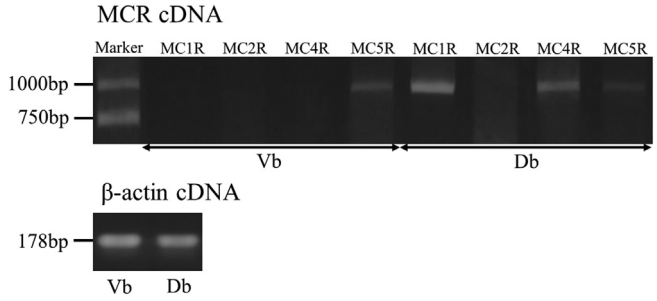


Fig. 5. Expression of MCR mRNAs in the ventral and dorsal skins. Total RNAs prepared from the three individuals were combined. β-actin is an internal control. Vb, ventral skin; Db, dorsal skin.

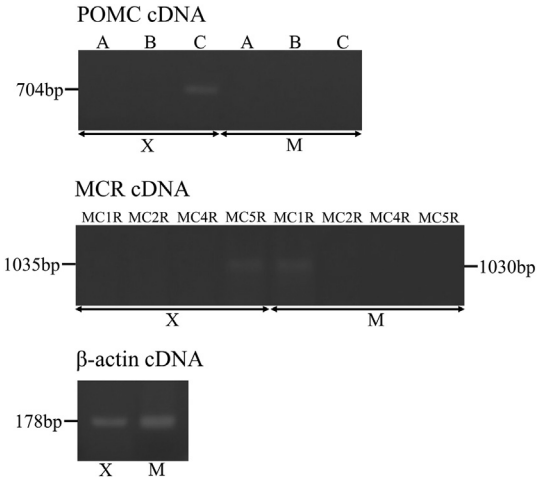


Fig. 6. Expression of POMC and MCR mRNAs in the xanthophores and melanophores. A, B, C indicated the molecular type of the POMC cDNA. β-actin is an internal control. X, xanthophores; M, melanophores.

Y^{191}-Q^{225}; POMC-B: Y^{179}-K^{210}; POMC-C: N^{199}-G^{231}) segments (Fig. 2). All of the functional domains are flanked by paired-basic amino acids (potential proteolytic cleavage sites). Among the large yellow croaker POMCs, there are mutations from Val to Ser take place at the last residue of the α-MSH segment in POMC-C compared with POMC-A and −B from Ala to Val take place at the penultimate residues of β-MSH segment in POMC-C compared with POMC-A and −B, and form Ser to Thr take place at the third residues residues of β-MSH segment in POMC-C compared with POMC-B. Compared to the ancient fish, γ-MSH lacks in the large yellow croaker and other teleosts (Fig. 2).

3.2. Phylogenetic analysis

The phylogenetic tree showed that the large yellow croaker POMC-A and −B are included in a clade composed of Pleuronectiformes, Perciformes, and are most related to the African cichlid fish (*Astatotilapia burtoni*) POMC-α1 and yellowfin bream (*Acanthopagrus latus*) POMC, respectively (Fig. 3). The large yellow croaker POMC-C is included in a clade together with the African cichlid fish POMC-β, barfin flounder (*Verasper moseri*) POMC-C, rainbow trout (*Oncorhynchus mykiss*) POMC-B, Common carp (*Cyprinus carpio*) POMC-B-like *and* zebrafish (*Danio rerio*) POMC-B (Fig. 3).

3.3. Expression of POMC mRNAs in pituitary and skin

Expression of POMC mRNAs in the large yellow croaker pituitary and skin is shown in Fig. 4. Among the three POMC mRNAs, POMC-A, -B and -C mRNAs were detected in the pituitary, while only POMC-C mRNA was detected in both the ventral and dorsal skins.

3.4. MCR mRNAs expression in the skin

The present study aims to investigate the effect of MSH on skin coloration. Therefore, we further examined the receptor types of MSH expressing in the large yellow croaker skin. The results of RT-PCR showed that only MC5R mRNA was expressed in the ventral skin, while MC1R, MC4R and MC5R mRNAs were detected in the dorsal skin (Fig. 5).

3.5. POMC and MCR mRNAs expression in dispersed pigment cells

RT-PCR amplifications of POMC and MCR mRNAs were performed using total RNA prepared from single xanthophores and melanophores isolated from the large yellow croaker scales. The results of single-cell RT-PCR showed that a cDNA fragment of POMC-C was amplified from total RNA derived from xanthophores, but no POMC transcripts were amplified from total RNA derived from melanophores. Besides, only MC5R mRNA was expressed in the xanthophores, while only MC1R mRNA was detected in the melanophores (Fig. 6).

3.6. Effects of MSH peptides on body coloration in vivo

The large yellow croaker body color, especially in the ventral skin, is silvery white during the daytime, and becomes golden yellow during the night-time. In the present study, we observed that xanthophores were presented in both the dorsal and ventral skins, and the xanthosomes aggregated during the daytime (Fig. 7a, d) and dispersed during the night-time (Fig. 7b, e). The sizes of xanthophores in ventral skin were bigger than those in dorsal skin. The melanophores were only expressed in the dorsal skin, and the melanosomes of melanophores dispersed all day long *in vivo* (Fig. 7a, b).

During the daytime, after *in vivo* α-MSH treatment, the body color changed from the silvery white to the golden yellow (Fig. 7A, B), and the xanthosomes of xanthophores in both dorsal and ventral skins were dispersed (Fig. 7c, f). In addition, *in vivo* α-MSH treatment slightly enhanced the dispersion degree of melanosomes of melanophores (Fig. 7c). There results indicated that α-MSH had a distinct effect on yellowness enhancement of the large yellow croaker skin *via* the xanthosome dispersion in xanthophores (Fig. 7).

Table 2 shows the effects of MSH peptides (α-MSH, Des-Ac-α-MSH, α-MSH-C, and β-MSH-C) on the dorsal and ventral skin colors of the large yellow croaker during the daytime when the fish color is silver white. There were no significant differences of the L^* or a^* values between α-MSH, Des-Ac-α-MSH, α-MSH-C, β-MSH-C and the control in both the dorsal and ventral skins. However, the b^* values (indicating yellowness) with MSH peptides treatments were significantly higher than those of the daytime control (Student's *t*-test, $p < .05$). α-MSH-C

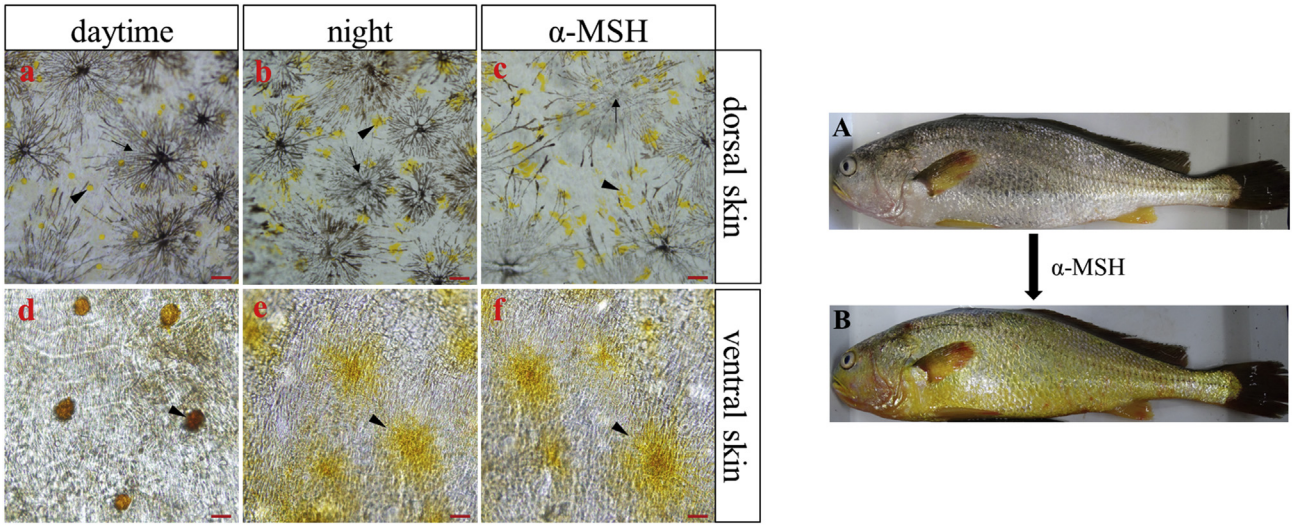


Fig. 7. The dispersion and aggregation status of pigment granules of the chromatophores in dorsal and ventral skins (a-f) and the body color changes with α-MSH injection for 30 min (A, B) *in vivo*.
a and d, saline injection during the daytime; b and e, saline injection during the night-time; c and f, α-MSH injection during the daytime. A, saline injection during the daytime; B, α-MSH injection during the daytime. Arrowhead, xanthophores; Arrow, melanophores. Scale bars: 50 μm.

Table 2
Effects of MSH peptides on the skin colors of the large yellow croaker.

Intraperitoneal injection	Dorsal skin			Ventral skin		
	*L**	*a**	*b**	*L**	*a**	*b**
α-MSH (1 nmol/g)	37.83 ± 2.50^{a}	−1.67 ± 0.33^{a}	21.33 ± 1.73^{b}	75.33 ± 1.86^{a}	1.83 ± 0.48^{a}	52.83 ± 3.39^{b}
Des-Ac-α-MSH (1 nmol/g)	38.76 ± 2.23^{a}	−1.85 ± 0.51^{a}	8.96 ± 2.11^{c}	76.32 ± 1.74^{a}	1.26 ± 0.53^{a}	24.83 ± 3.56^{c}
α-MSH-C (1 nmol/g)	40.33 ± 2.03^{a}	−1.50 ± 0.43^{a}	23.83 ± 1.65^{b}	74.83 ± 1.28^{a}	1.50 ± 0.43^{a}	51.83 ± 3.57^{b}
β-MSH-C (1 nmol/g)	39.34 ± 2.42^{a}	−1.47 ± 0.54^{a}	23.57 ± 1.76^{b}	76.74 ± 1.67^{a}	1.49 ± 0.43^{a}	49.75 ± 3.47^{b}
Saline (night-time)	40.12 ± 2.15^{a}	−1.59 ± 0.38^{a}	23.67 ± 1.89^{b}	74.63 ± 1.72^{a}	1.74 ± 0.51^{a}	50.47 ± 3.61^{b}
Saline (daytime)	39.67 ± 3.12^{a}	−2.17 ± 0.48^{a}	3.83 ± 0.70^{a}	75.83 ± 1.68^{a}	1.00 ± 0.37^{a}	5.17 ± 0.60^{a}

Note: The data were shown as the mean ± SEM. In the same column, values with different superscript letters represent significant differences between each other (Student's t-test, $p < .05$). *L**, *a** and *b** indicating the lightness, redness and yellowness, respectively; the greater the *L** value, the higher the lightness of the body coloration; the higher the *a** value, the closer to the red of the body coloration; the higher the *b** value, the closer to the yellow of the body coloration.

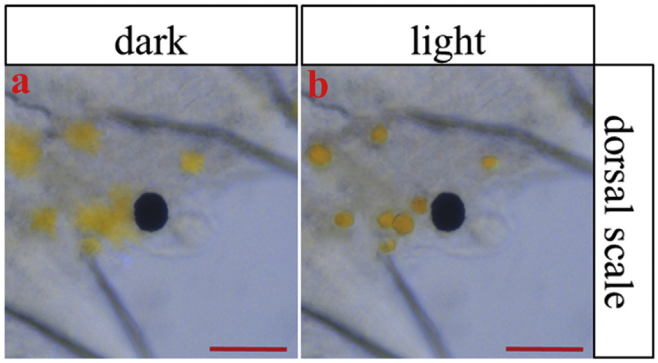


Fig. 8. The dispersion status of the xanthophores and melanophore from one dorsal scale under the dark (a) and light (b) condition *in vitro*. Scale bar: 50 μm.

and β-MSH-C treatments and the night-time control showed similar effects on the *b** values. The *b** value with Des-Ac-α-MSH treatment was significantly less than those with α-MSH, α-MSH-C and β-MSH-C treatments and the night-time control (Student's t-test, $p < .05$), but significantly higher than that of the daytime control (Student's t-test, $p < .05$).

3.7. MSH peptides dispersed pigment granules of both the xanthophores and melanophores in vitro

Since the POMC-C expressed in both the pituitary and skin of the large yellow croaker, we further examined whether the skin color changes are regulated *via* autocrine and/or paracrine pathways. When we immediately observed the dorsal scales that had been maintained under complete dark *in vitro* condition for at least 30 min, the melanosomes of melanophores were completely aggregated (Fig. 8a), however, the xanthosomes of xanthophores close to the melanophores were dispersed (Fig. 8a). Interestingly, as soon as the dorsal scales were exposed to the light, the melanosomes remained aggregation (Fig. 8b), but the xanthosomes started aggregating and became complete aggregation within 5 min (Fig. 8b). These results suggest that there is a specific autocrine regulation mechanism for pigment translocation of xanthophores in the skin of the large yellow croaker.

We further found that α-MSH had a clear time (Fig. 9a-h) and a dose (Fig. 9A, E) dependent stimulatory effects on pigment granule dispersion in both the xanthophores and melanophores, and other three MSH peptides (Des-Ac-α-MSH, α-MSH-C, and β-MSH-C) also showed a time and a dose (Fig. 9B-D, F-H) dependent stimulatory effects as α-MSH *in vitro*. α-MSH-C showed a similar effect on pigment granule dispersion to α-MSH (Fig. 9A, C, E, G), with a minimum significantly effective concentration at 10 nM, and the effects of these two peptides on pigment granule dispersion were greater than those of Des-Ac-α-MSH (Fig. 9B, F) in both the xanthophores and melanophores. Des-Ac-α-MSH showed

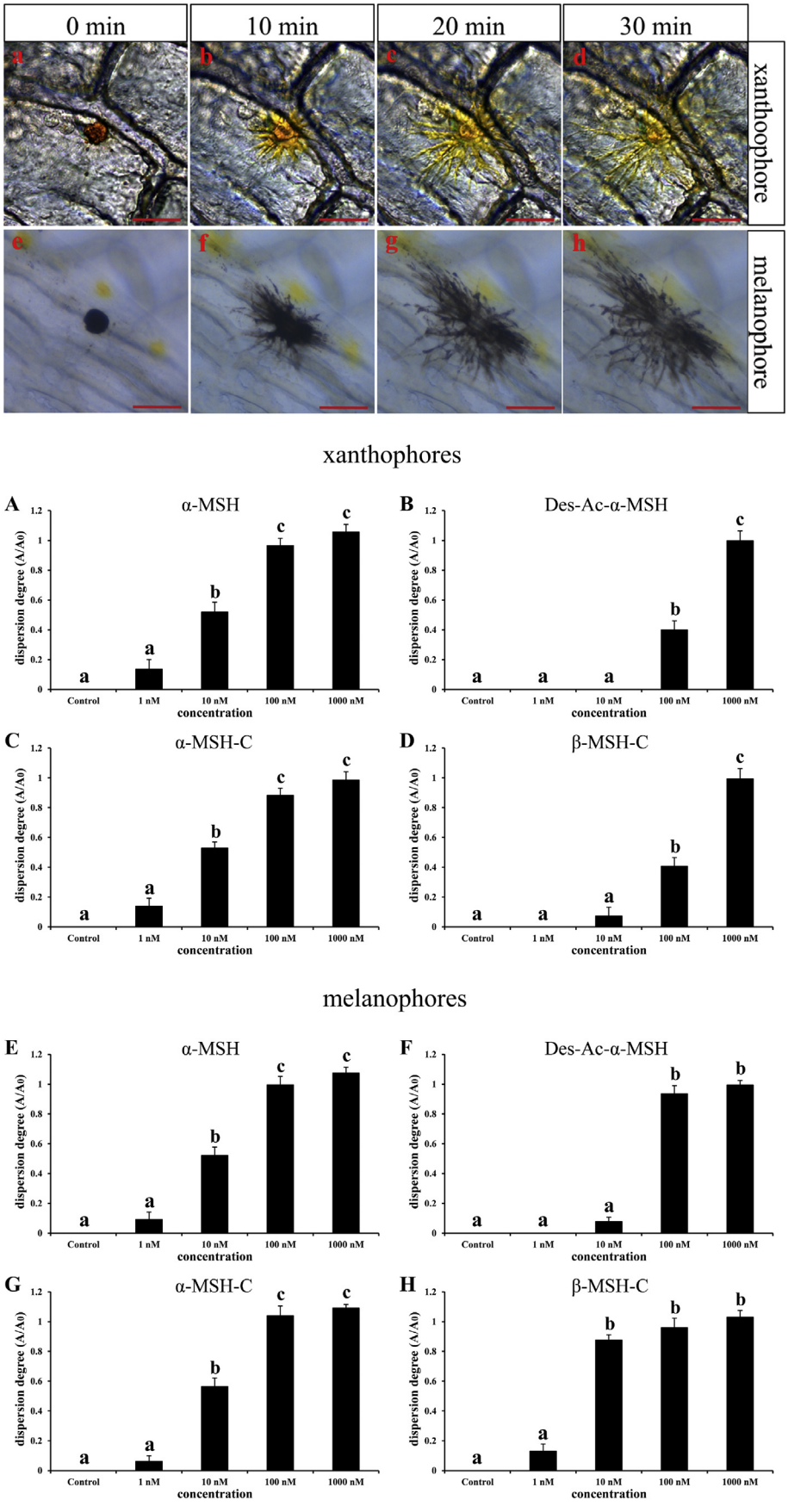


Fig. 9. Effects of MSH peptides on the pigment granule dispersion of xanthophores and melanophores in the large yellow croaker *in vitro*.
a-h, microphotographs showing time-dependent pigment granule dispersion in the xanthophore and melanophore exposed to 1000 nM α-MSH for 30 min. Scale bar: 50 μm.
A-H, the pigment granule dispersion degrees of xanthophores and melanophores after 30 min treatment with α-MSH, Des-Ac-α-MSH, α-MSH-C and β-MSH-C at different concentrations. The data were shown as the mean ± SEM; mean values marked with different letters are significantly different from each other (one way ANOVA, post-hoc Tukey test, $p < .05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

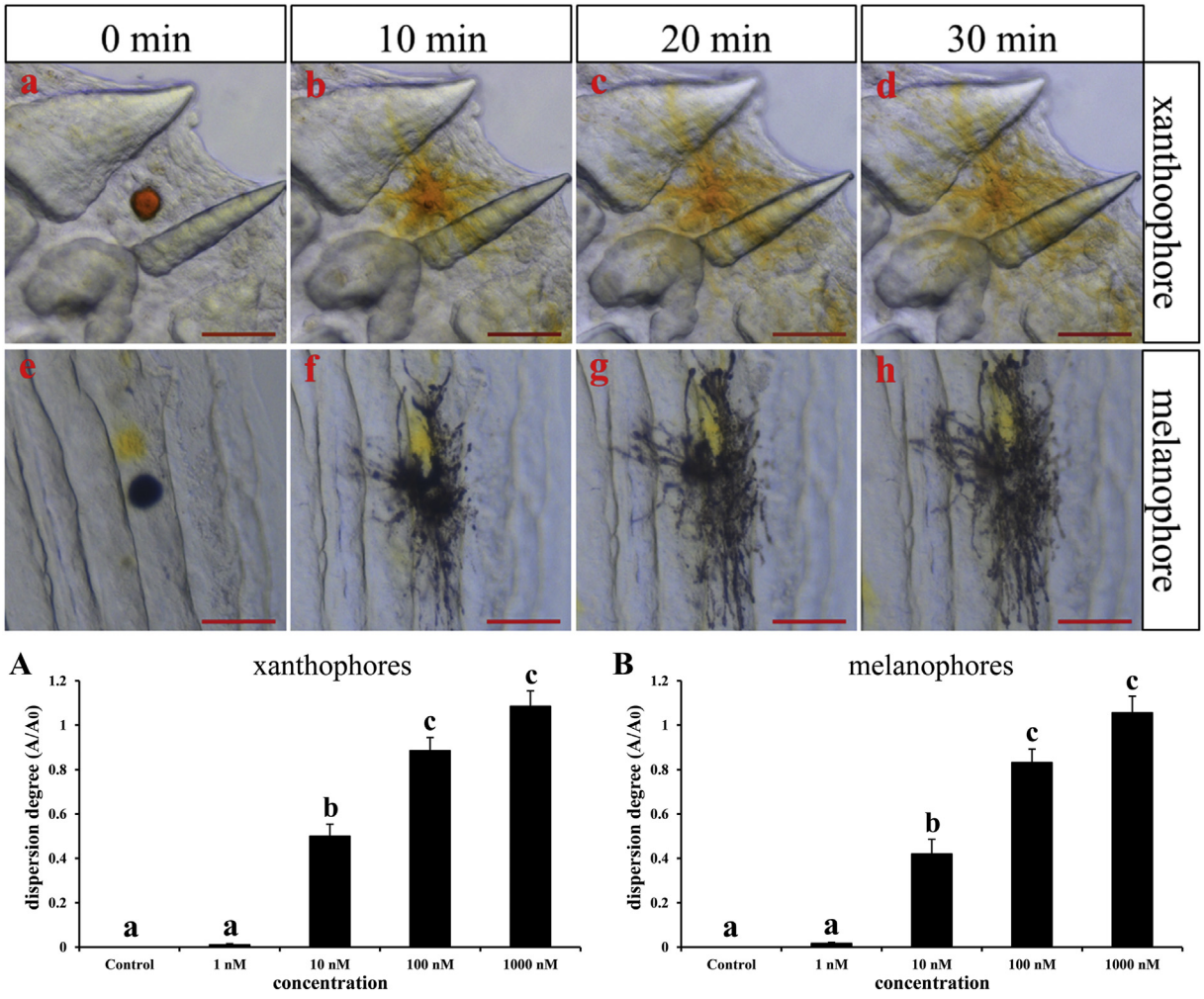


Fig. 10. Effects of forskolin on the pigment granule dispersion of xanthophores and melanophores in the large yellow croaker *in vitro*.
a-h, microphotographs showing time-dependent pigment granule dispersion in the xanthophore and melanophore exposed to 1000 nM forskolin for 30 min. Scale bar: 50 μm.
A and B, the pigment granule dispersion degrees of the xanthophores and melanophores after 30 min treatment with forskolin at different concentrations. The data were shown as the mean ± SEM, mean values marked with different letters are significantly different from each other (one way ANOVA, post-hoc Tukey test, $p < .05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

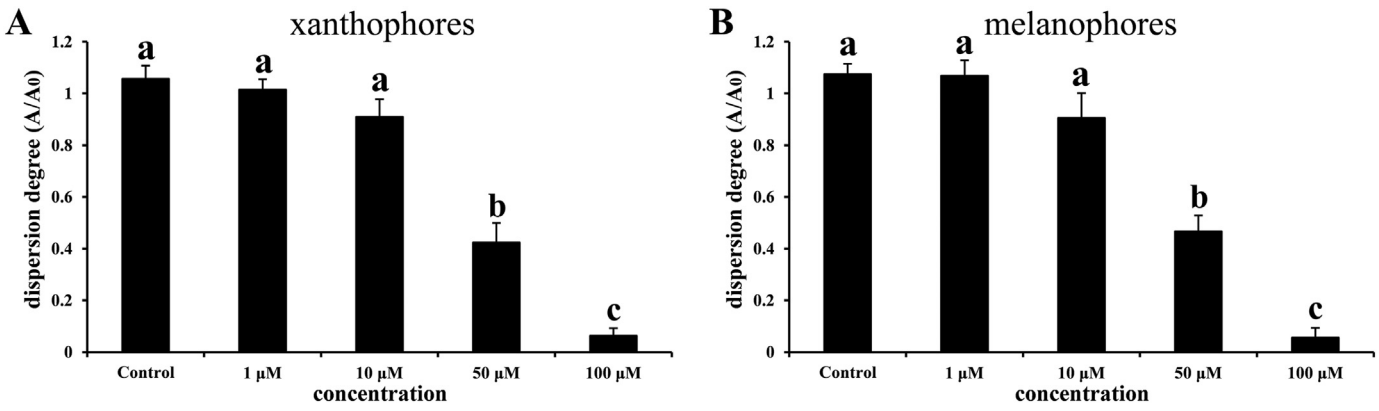


Fig. 11. Inhibitory effects of H 89 2HCl on the pigment granule dispersion of xanthophores and melanophores in the large yellow croaker *in vitro*.
A and B, the pigment granule dispersion degrees of the xanthophores and melanophores pretreated with H 89 2HCl at different concentrations for 30 min, followed by 1 μM α-MSH treatment for another 30 min. The data were shown as the mean ± SEM, mean values marked with different letters are significantly different from each other (one way ANOVA, post-hoc Tukey test, $p < .05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

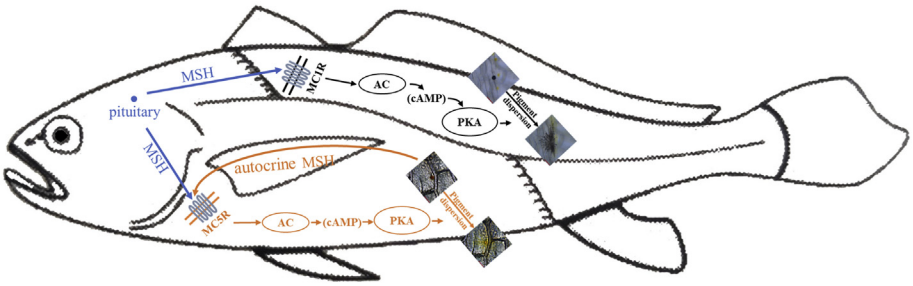


Fig. 12. Diagram showing the possible autocrine function of MSH on the pigment granule dispersion in the xanthophores (ventral), and the possible signaling pathways in the direct action of MSH on the pigment granule dispersion in the xanthophores (ventral) and melanophores (dorsal) of the large yellow croaker. AC, Adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A. (↑), activation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a stronger effect on pigment granule dispersion of the melanophores than of the xanthophores (Fig. 9B, F). β-MSH-C showed a weaker effect than α-MSH in the xanthophores (Fig. 9A, D), but a stronger effect than α-MSH in the melanophores (Fig. 9E. H) on pigment granule dispersion.

3.8. An AC activator dispersed pigment granules of both the xanthophores and melanophores in vitro

Forskolin, an AC activator, had a time (Fig. 10a-h) and a dose (Fig. 10A, B) dependent stimulatory effects on pigment granule dispersion within 30 min of exposure, and the minimum significant effects were observed at the concentration of 10 nM in both the xanthophores and melanophores (one way ANOVA, post-hoc Tukey test, $p < .01$) (Fig. 10A, B).

3.9. A PKA inhibitor blocked α-MSH-induced pigment granule dispersion of both the xanthophores and melanophores in vitro

Fig. 11 shows H 89 2HCl blocked the effects of α-MSH on pigment granule dispersion in both xanthophores (Fig. 11A) and melanophores (Fig. 11B). H 89 2HCl that had been pretreated for 30 min showed a dose-dependent inhibitory effect on 1 μM α-MSH-induced pigment granule dispersion, and the significantly inhibitory effects were observed at 50 μM and 100 μM in both the xanthophores and melanophores (Fig. 11).

4. Discussion

In order to enhance the yellowness of the large yellow croaker skin, several studies have been conducted and the results indicated that dietary supplementation of carotenoids, shrimp, astaxanthin and vitamin E can improve the skin yellow coloration (Yi et al., 2014a; Yi et al., 2014b; Yi et al., 2015; Yi et al., 2016; Yi et al., 2018). However, it takes a few months to enhance the skin pigmentation *via* feeding and the cost of feeds is higher. It is worth noting that the large yellow croaker always becomes golden yellow at night under culture condition. In the present study, for the first time, we revealed that the yellowness of the large yellow croaker skin is mainly related to the xanthosome dispersion degree of xanthophores. The results from the present study suggest that the dispersion of xanthosomes should be controlled by hormones through endocrine and autocrine pathways. So far, the most widely known hormone to control fish chromatophores is melanocyte-stimulating hormone (MSH), which is derived from its precursor protein POMC (Fujii, 2000).

Three subtypes of POMC genes have been reported in vertebrates. Unlike mammalian species that have only one subtype of POMC, most of fish have at least two subtypes of POMC (Salbert et al., 1992; Okuta et al., 1996; Arends et al., 1998; Alrubaian et al., 1999; Danielson et al., 1999; Takahashi et al., 2000), which indicates that the POMC gene has duplicated frequently during the course of evolution in teleosts. In the present study, three subtypes of the POMC genes were cloned in the large yellow croaker, which is consistent with the reports in the barfin flounder (*Verasper moseri*) (Takahashi et al., 2005), African cichlid fish (*Astatotilapia burtoni*) (Harris et al., 2014) and Japanese flounder (*Paralichthys olivaceus*) (Kobayashi et al., 2012). Previous study in African cichlid fish suggested that POMC-α and-β arose during the teleost-specific whole genome duplication event (Harris et al., 2014). In the present study, phylogenetic tree analysis indicated that the large yellow croaker POMC-A and -B are included in a clade together with the African cichlid fish POMC-α1 and -α2. In this clade, only fish belonging to the Perciformes has duplicated POMC-α genes, which suggests that the large yellow croaker POMC-A and -B are co-orthologs to the POMC-α genes of the Perciformes. The large yellow croaker POMC-C, African cichlid fish POMC-β, barfin flounder POMC-C, rainbow trout (*Oncorhynchus mykiss*) POMC-B, common carp (*Cyprinus carpio*) POMC-B-like and zebrafish (*Danio rerio*) POMC-B may have diverged as a variant copy, because they have extreme mutations in N-POMC and β-END segments, and comprise an independent clade. Therefore, as same as POMC-C of other teleost species (Braasch and Salzburger, 2009), the large yellow croaker POMC-C arose during the teleost-specific whole genome duplication. Taken together, the phylogenetic tree analysis suggests that the lineage leading to an ancestor of the large yellow croaker POMC-A and -B and that leading to the large yellow croaker POMC-C may have diverged from a common ancestor at the teleost-specific whole genome duplication, and then the lineage of the large yellow croaker POMC-A may have diverged from that of the large yellow croaker POMC-B at an independent genome duplication event.

Although the POMC gene is expressed in many cell types and tissues, the predominant site of expression and processing lies in the corticotrope and melanotrope cells of the pituitary gland (Metz et al., 2006). In the present study, we demonstrated that all three POMC subtypes were expressed in pituitary, but only POMC-C mRNA was expressed in the ventral and dorsal skins of the large yellow croaker. POMC-derived peptides, such as adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), β-endorphin (β-END) have been identified in cultured mammalian skin cells (Theoharides et al., 2016). In the barfin flounder, mass spectrometry analyses of fractions of skin extract separated by HPLC revealed the presence of a peptide with a molecular mass corresponding to Des-Ac-α-MSH-C derived from POMC-C (Kobayashi et al., 2009). Interestingly, across all five cichlid species, POMC-C (also known as POMC-β) was the only paralog expressed in skin tissue (Harris et al., 2014). To sum up, it is possible that the skin pigmentation of the large yellow croaker is regulated by autocrine and/or paracrine pathways as well as endocrine pathway.

In teleosts, the action of α-MSH is not only restricted to melanophores, but also to bright-colored chromatophores: xanthophores, erythrophores, and leucophores (Jiro et al., 1978; Negishi and Obika, 1980; Fujii and Oshima, 1986; Fujii, 2000; Kobayashi et al., 2011). In the present study, under *in vivo* condition, the melanosomes of melanophores kept dispersion, and the dispersion degree of melanosomes was enhanced after intraperitoneal injection with MSH peptides. However, under *in vitro* condition, the melanosomes of melanophores were aggregated, which suggests melanosome dispersion relies on other endocrine factors. When melanophores were treated with MSH peptides *in vitro*, the aggregated melanosome started to disperse. These results indicate that the melanosome dispersion of melanophores is stimulated

by MSH peptides, possibly secreted from pituitary, through endocrine pathway.

We observed that the xanthosomes were completely aggregated during the daytime, but completely dispersed during the night-time *in vivo*. Moreover, MSH peptides treatment dispersed xanthosomes within 30 min *in vivo* and *in vitro*, resulting in a significant increase of yellow body coloration of the large yellow croaker, which suggests that the xanthosomes of xanthophores may be stimulated by MSH through endocrine system in the large yellow croaker. Interestingly, in contrast to the aggregation of melanosomes of melanophores, xanthosomes of xanthophores nearby melanophores showed dispersion under complete dark condition *in vitro*. Besides, the results of single-cell RT-PCR further showed that POMC-C transcripts were expressed in isolated xanthophores but not in melanophores. All in all, our results suggest that xanthophore itself produces MSH to disperse xanthosomes, namely, the dispersion of xanthosomes might be also regulated by MSH through autocrine pathway. However, when the xanthophores were exposed to the light without exogenous MSH treatment, xanthosomes started to aggregate, which indicates the light can specifically make xanthosomes aggregated. It is presumably that the aggregation effect of the light on xanthosomes suppresses the dispersion effect of endogenous MSH on xanthosomes, which may explain the mechanism that the fish skin is silver white during the daytime and become golden yellow during the night-time under aquaculture conditions. Xanthophores in the adult Japanese medaka (*Oryzias latipes*) were also found to respond to the light by xanthosome aggregation (Kawai, 1989). Opsins have been suggested to be involved in chromatophore photoresponses, as putative opsins have been detected in skin tissues of several teleost species (Lythgoe et al., 1984; Kasai and Oshima, 2006; Ban et al., 2010; Chen et al., 2013). Further study is necessary to investigate the mechanism of light-induced xanthosome aggregation in the large yellow croaker.

The MSH peptides exert their multiple functions *via* MCRs that belong to the superfamily of G-protein coupled receptors (GPCRs) and comprise five members (MC1R to MC5R). In the present study, only MC5R was expressed in xanthophores. Previous studies in the barfin flounder (Kobayashi et al., 2010) and Japanese flounder (Kobayashi et al., 2012) have reported that xanthophores exclusively express MC5R. From the above, we conjecture that the regulatory effect of MSH on xanthophores is mediated *via* MC5R in the large yellow croaker. All MSH peptides tested had dispersion effects on xanthosomes, and the effect of Des-Ac-α-MSH was the weakest among them. Pervious study in the barfin flounder also showed that acetylation of N-terminal enhanced dispersion activity of pigments in xanthophores (Kobayashi et al., 2009). Acetylation-mediated augmentation of the binding affinity of α-MSH was observed during pharmacological studies with human MC5R (Schiöth et al., 1996). Pharmacological studies on the sea bass (*Dicentrarchus labrax*) MC5R have revealed a higher efficacy of α-MSH than of Des-Ac-α-MSH to stimulate cellular activities (Sánchez et al., 2009a). Overall, these results suggest that acetylation enhances the binding affinity of MSH peptides to MC5R in the large yellow croaker, and thus leads to increase pigment granule-dispersing activity in xanthophores. On the other hand, replacement of the C-terminal residue of α-MSHs may have negligible effects on the binding affinity to MC5R, because α-MSH-C and α-MSH exhibit similar effects on the xanthosome dispersion. The β-MSH-C showed weaker effects on xanthosome dispersion than α-MSH and α-MSH-C in the large yellow croaker. Therefore, it is possible that the binding affinity of β-MSH-C to MC5R is weaker than that of α-MSH in this species, which may be due to steric hindrance through the long N-terminal tail (Metz et al., 2006). Although the sizes of xanthophores in the dorsal skin were smaller than those in the ventral skin, the responses of xanthophores to MSH peptides in the dorsal skin (data not shown) were similar to those in the ventral skin. Therefore, the MC5R of the large yellow croaker xanthophores plays a specific role in mediating the dispersion effects of MSH on xanthosomes.

In the present study, MC4R mRNAs were detected in the dorsal skin, however, the results of single-cell RT-PCR showed there were no MC4R transcripts in both xanthophores and melanophores. In mammals, MC4R is an exclusive brain receptor (Mountjoy et al., 1994). Similarly, localization studies in zebrafish, spiny dogfish (*tualus acrchias*), fugu (*Takifugu rubripes*), rainbow trout and barfin flounder unambiguously showed that MC4R abundantly expressed in brain; and was also detected in the peripheral tissues, such as liver, testis, head kidney, ovary, intestine and skin. It must be noted, however, that in all fish species examined, none of the peripheral tissues were consistently reported to harbour MC4R expression (Ringholm et al., 2002; Ringholm et al., 2003; Klovins et al., 2004; Haitina et al., 2004; Kobayashi et al., 2008). Functionally, the MC4R has been shown to be involved in regulating food intake in goldfish (*Carassius auratus*) (Cerdá-Reverter et al., 2003) and sea bass (Sánchez et al., 2009b), and the similar role of MC4R was reported in mammals (Fan et al., 1997; Huszar et al., 1997; Vaisse et al., 1998). Mutations in fish *mc4r* have also been reported and are related to body sizes, reproduction, and adaptation to energy availability (Lampert et al., 2010; Aspiras et al., 2015). So far, little is known about the role of MC4R in skin color changes. The function of MC4R in the large yellow croaker remains to be illuminated in the future study.

The MC1R has been shown as the main receptor to mediate the pigmentary effects of MSH peptides in a number of mammalian, avian and fish species (Jackson, 1997; Van Der Salm et al., 2005; Gross et al., 2009; Sánchez et al., 2010). Acetylation-mediated augmentation of the binding affinity of α-MSH-related peptides was observed during pharmacological studies with mouse, human and fish MC1R (Schiöth et al., 1997; Mountjoy, 2000; Sánchez et al., 2010). However, studies in the barfin flounder (Kobayashi et al., 2010) and Japanese flounder (Kobayashi et al., 2012) showed that α-MSH with N-terminal acetylation has no dispersion effect on the melanosomes, because the melanophores expressing two different subtypes of MCRs result in the formation of MCR heterodimers. In the large yellow croaker, the effect of α-MSH on melanosome dispersion was stronger than that of the Des-Ac-α-MSH in melanophores, suggesting that the large yellow croaker melanophores may only express one subtype of MCR. The results of single-cell RT-PCR confirmed that the dispersion effects of MSH peptides on the melanosomes were mediated by MC1R in the large yellow croaker.

As a member of GPCR, upon stimulation by its ligand, MCRs will regulate the activity of AC to affect cytosolic levels of cAMP (Sammak et al., 1992; Oshima et al., 1998; Fujii, 2000; Logan et al., 2006; Ban et al., 2010). In the present study, after forskolin treatment, pigment granules dispersed in both the xanthophores and melanophores, which were similar to the results after MSH peptides treatment, indicating that AC-cAMP signal pathway mediates the action of MSH on pigment dispersion in the large yellow croaker. Moreover, H 89 2HCl, a specific inhibitor of PKA, inhibited the α-MSH-induced pigment granule dispersion in both the xanthophores and melanophores. In conclusion, our results suggest that MCRs are activated by MSH, followed by increase of intracellular cAMP levels through mediation of AC activated, which further activates PKA, and finally leading to the pigment granule dispersion of chromophores. Further studies are necessary to examine the target proteins of PKA.

In summary, the dispersion of melanosomes was stimulated by MSH peptides, possibly secreted from pituitary through endocrine pathway (Fig. 12). We first revealed that the yellowness of the large yellow croaker skin is mainly caused by the dispersion of xanthosomes. Besides through endocrine pathway, our results indicated that the xanthosome dispersion of xanthophores in skin was possibly regulated through autocrine pathway. Namely, α-MSH-C activates MC5R, which further triggers AC-cAMP-PKA signal pathway (Fig. 12). MSH is a potential feed additive for the large yellow croaker to enhance its yellowness of skin.

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