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Fisheries and Aquatic Sciences

Original Article
Fish Aquat Sci 17(3), 325-337, 2014

The Functional Relevance of Prepro-melanin Concentrating Hormone (pMCH) to Skin Color Change, Blind-side Malpigmentation and Feeding of Oliver Flounder *Paralichthys olivaceus*

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Abstract

To assess the functional structure of prepro-melanin-concentrating hormone (pMCH), we isolated and cloned pMCH (*of*-pMCH) mRNA from the brain of the olive flounder, *Paralichthys olivaceus*, and compared its amino acid sequence with those from other animals. In addition, to examine whether activation of the brain *of*-pMCH gene is influenced by background color, density, and feeding, we compared pMCH mRNA activities against different background colors (bright and dark) and at different densities (100% PCA and 200% PCA). To examine whether the pMCH gene is related with malpigmentation of blind-side skin and appetite, we compared pMCH gene expression between ordinary and hypermelanic flounders, and between feeding and fasting flounders. The *of*-pMCH cDNA was 405 bp in the open reading frame [ORF] and encoded a protein of 135 amino acids; MCH was 51 bp in length and encoded a protein of 17 amino acids. An obvious single band of the expected size was obtained from the brain and pituitary by RT-PCR. In addition, *of*-pMCH gene activity was significantly higher in the bright background only at low density (< 100% PCA) making the ocular skin of fish whitening, and in ordinary fish. However, the gene activity was significantly decreased in dark background, at high density (>200% PCA), and in hypermelano fish. These results suggest that skin whitening camouflage of the flounder is induced by high MCH gene activity, and the density disturbs the function of background color in the physiological color change. Moreover, our data suggest that a low level of MCH gene activity may be related to malpigmentation of the blind-side skin. In feeding, although pMCH gene activity was significantly increased by feeding in the white background, the pMCH gene activity in the dark background was not influenced by feeding, indicating that the MCH gene activity increased by feeding can be offset by dark background color, or is unaffected by appetite. In conclusion, this study showed that MCH gene expression is related to ocular-skin whitening camouflage and blind-skin hypermelanosis, and is influenced by background color and density.

Key words: Blind-skin malpigmentation, Camouflage, Color change, Feeding, MCH, *Paralichthys olivaceus*

Introduction

The primary structure of melanin-concentrating hormone (MCH), which is produced in neurons in the hypothalamus and released from the neurohypophysis, was first identified in chum salmon, *Oncorhynchus keta* (Kawauchi et al., 1983), and has been investigated mostly in spindle-shaped teleosts: Chinook salmon, *Oncorhynchus tshawytscha* (Minth et al., 1989); coho salmon, *Oncorhynchus kisutch* (Nahon et al., 1991); rainbow trout, *Oncorhynchus mykiss* (Berman et al., 2009);

Received 4 August 2014; **Revised** 12 September 2014
Accepted 15 September 2014

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tilapia, *Oreochromis mossambicus* (Groneveld et al., 1995); and bonito, *Katsuwonus pelamis* (Kawauchi, 1989). MCH acts as a functional antagonist of α-MSH and aggregates pigment-containing melanosomes around the nucleus in pigment cells (Kawauchi et al., 1983), thereby lightening the color of fish scales and skin (Kawauchi and Baker, 2004). Therefore, MCH is also generally accepted as a key regulator of physiological color changes in fish (Kishida et al., 1989; Suzuki et al., 1995; Kawauchi and Baker, 2004; Kawauchi, 2006). The physiological color changes refer to the direct effects of environmental factors, such as light, background color, and social context, on pigment migration (Nery and Castrucci, 1997; Oshima, 2001). The physiological color change is caused by centripetal and centrifugal movements of pigments in satellite chromatophores, which are determined by many environmental factors, including background pattern (Ramachandran et al., 1996), density stress (Doolan et al., 2008), luminosity (Han et al., 2005), and nutrition (Kalinowski et al., 2005).

Other studies have indicated that MCH and α-MSH (antagonist of MCH) are commonly involved in morphological color change, as well as in the rapid color change of fish that results in skin darkening (Höglund et al., 2002; Sugimoto, 2002). Indeed, it was shown that intraperitoneal injection of MCH turned the ocular skin pale in barfin flounder (*Verasper moseri*), and a high level of MCH suppressed blind-side hypermelanosis (Takahashi et al., 2004; Amiya et al., 2005; Yamanome et al., 2005; Takahashi et al., 2007; Kobayashi et al., 2008), suggesting that MCH may participate in the morphological color change in fish. Blind-side hypermelanosis is common in cultured flatfishes and is economically important (Jeong and Jeon, 2008). When flatfishes reared at high density (Seikai, 1992; Takahashi, 1994; Tagawa et al., 2004; Kang et al., 2011), this malpigmentation develops in tanks that lack sandy substrata (Ottesen and Strand, 1996; Iwata and Kikuchi, 1998; Kang and Kim, 2012) and is induced by a dark background color (Yamanome et al., 2007b). However, questions remain regarding whether the MCH in flatfishes is functionally related to the abnormal morphological color change, and little information is available regarding the involvement of MCH in the morphological color changes. Therefore, it is necessary to search for evidence of this in other species.

In mammals, the neurons in which MCH, as a hypothalamo–pituitary peptide hormone, is produced are the most downstream peptidergic neurons involved in the chain of hypothalamic signals that regulate food intake and energy homeostasis (Pritchard et al., 2002; Shi, 2004; Pissios et al., 2006). A series of studies using rodents established the roles of MCH signaling in the regulation of food intake and energy balance in mammals (Presse et al., 1996; Qu et al., 1996; Rossi et al., 1997; Ludwig et al., 1998; Stricker-Krongrad et al., 2001; Della-Zuana et al., 2002; Abbott et al., 2003; Gomori et al., 2003). However, despite the known involvement of MCH in feeding and somatic growth in mammals, little information is available regarding its effects on food intake in fish. Thus, the effects of MCH on feeding behavior in fish remain to be explored. Some studies (Lin et al., 2000; Matsuda et al., 2006; Matsuda et al., 2007; Pérez Sirkin et al., 2012) have suggested that MCH is involved in the regulation of food intake and energy balance in teleosts. Experiments in barfin flounder suggested that teleost MCH is associated with food intake (Takahashi et al., 2004). However, recent studies indicating that fish possess two forms of MCH precursors (Berman et al., 2009; Tuziak and Volkoff, 2012) suggest that the two prohormones likely have different roles in physiological color change and feeding behavior; fish-type MCH (MCH1) expression may be correlated with pigmentation changes, but increased mammalian-type MCH (MCH2) expression may occur in response to chronic food deprivation. However, there is currently insufficient data to determine the functional role of the fish-type MCH gene in feeding. Therefore, it is necessary to clarify the functional relationships between the fish-type MCH genes with respect to appetite.

In the present study, we first determined the primary structure of the fish-type prepro-MCH of olive flounder (*of*-pMCH) by cloning and peptide analysis of brain tissue, identified the sequence of *of*-pMCH, and examined the structural features of *of*-pMCH. Then, to examine whether the *of*-pMCH gene is related to physiological color change against background color, and whether density influences the physiological color change and gene activation, we compared pMCH mRNA activities against two background colors (white and dark-green) at two densities (100% PCA and 200% PCA). To determine whether the fish-type MCH gene is related to blind-side malpigmentation and appetite, we compared *of*-pMCH gene expression between ordinary and hypermelanic flounders and between feeding and fasting flounders.

Materials and methods

Identification of the *of*-pMCH gene

To isolate *of*-pMCH, we sampled the whole brain of one ordinary flounder (TL 16.2 ± 0.2 cm, BW 41.9 ± 1.9 g). Total RNA was extracted from the sampled brain using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA). Genomic DNA in extracted tissue was eliminated using recombinant DNase I solution (Promega, Madison, WI) in accordance with the manufacturer's protocol. Then, we measured total RNA content in the extracted tissue with a Nanovue Plus Spectrophotometer (GE Healthcare, Buckinghamshire, UK). The concentration of total RNA was determined by measuring the absorbance at 260 nm (A_{260}). The quality and integrity of the total RNA were assessed based on the ratio of A_{260} to A_{280} and by visualization of 28S and 18S rRNA bands on 1% agarose gels. The average of the A_{260}/A_{280} ratio was 2.12 ± 0.01 (n = 20).

Reverse transcription and PCR (RT-PCR) amplification

were performed to detect partial strand of pMCH mRNA. RT-PCR primers (Table 1) were designed from highly conserved regions of pMCH from barfin flounder (GenBank BAC82350). PCR amplification were performed using AccuPower RT/PCR PreMix (Bioneer, Daejeon, Korea) with MyiQ PCR system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The amplified PCR product was separated on an agarose gel, extracted using the AccuPrep Gel Purification Kit (Bioneer), and sequenced by COSMO Gen Tech. (Seoul, Korea). A single PCR product of the expected size (280 bp) was obtained.

For rapid amplification of the cDNA-ends (RACE) reactions, gene-specific primers (GSP) were selected from the 280 bp PCR product obtained by RT-PCR (Table 1). For RACE, the RACE primers supplied with the CapFishing Full-Length cDNA Premix Kit (Seegene, Seoul, Korea) was used as the sense and anti-sense primers. After preincubation at 94°C for 180 min, PCR was carried out for 35 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 60 s, followed by an extension step of 5 min at 72°C. After RACE, we obtained target cDNA 5′- and 3′-RACE products that shared an overlapping region. The full-length cDNA was generated by direct PCR using 5′- and 3′-RACE fragments.

The full-length cDNA was inserted into a PCR cloning vector using a TOPO TA Cloning Kit (Invitrogen), which was then transformed into *Escherichia coli* cells. The cells were spread on lysogeny broth (LB) plates and incubated overnight at 37°C. The white or light-blue colonies were picked and cultured overnight in LB medium. Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK), and then digested with *Eco*RI at 37°C for 1 h. Finally, the plasmid DNA was sequenced by Macrogen (Seoul, Korea). The amino acid sequence of *of*-pMCH was compared to those deduced from cDNAs of other teleost species and mammals.

pMCH tissue distribution analysis and phylogenetic tree

The brain (B), pituitary (P), liver (L), eye (E), kidney (K), gill (G), heart (H), stomach (S), intestine (I), muscle (M), testis (Te), ocular skin (Os), and blind skin (Bs) were removed from ordinary olive flounder (TL 16.2 ± 0.2 cm, BW 41.9 ± 1.9 g; n = 5) and stored at –70°C until required for investigation of the tissue distribution of pMCH by RT-PCR. First, pMCH-specific RT-PCR primers for tissue distribution analyses were designed as Table 2. Then, Reverse transcription and PCR (RT-PCR) amplification were performed using the sampled tissues. We investigated whether a single band of the expected size (390 bp) was amplified from all sampled tissues. In addition, Multiple-sequence alignment of nucleotides and amino acids were performed using ClustalW at MEGA 4.1 BETA and GeneDoc (version 2.7). Sequences for other animals pMCH genes were retrieved from the NCBI site (www.ncbi.nih.gov) using the GenBank database. Then, we compared the amino acid sequences of *of*-pMCH with the pMCH sequences of other fishes and manmals sequences. Also, we constructed a phylogenetic tree of ray-finned fish and vertebrate pMCH genes via the neighbor-joining method (NJ method; p-distance = 0.05) using the MEGA4.1 program (Tamura et al., 2007) based on amino acid differences (p-distance) and complete deletion. The reliability of the tree was assessed by bootstrapping (1,000 replications), and the tree is rooted from human pMCH (pMCH/GenBank AAH18048).

Table 1. Primers used for the isolation of MCH 1 & 2 cDNAs, the synthesis of full length- first strand cDNA and the 5′-and 3′-RACEs

Gene	Clone	Primer position	Primers	Size
MCH	I(RT-PCR)	sense (152~171) antisense (412~431)	5′-TACCCATGGCCAAGACTGAA-3′ 5′-CTCCTCAGGATGGGGATGTT-3′	280 bp
	II(5′-RACE)	sense (-22~-1; kit) antisense (283~302, GSP)	5′-GTCTACCAGGCATTCGCTTCAT-3′ 5′-GGATCGGCGACAACGATTAT-3′	325 bp
	III(3′-RACE)	sense (223 ~242, GSP) antisense (623~644; kit)	5′-ACGGAAAACAGCCTCAGCGA-3′ 5′-CTGTGAATGCTGCGACTACGAT-3′	422 bp
	IV(end-to-end)	sense (-22~302) antisense (223~644)	5′-end target cDNA 3′-end target cDNA	623 bp

Table 2. Gene specific primers used to analyze tissues distribution and compare mRNA expression between groups

Gene	Primer position	Primers	Product size	MT (°C)	R^2	E (%)
MCH (GenBank EU232720)	Sense (5-25) Antisense (106-125)	5′-GGCAGTCGTTCATGTCCATC-3′ 5′-GAGCAGGAAACTTGGCCTC-3′	121 bp	85.0	0.998	100
18S rRNA (GenBank EF126037)	Sense (1219 ~1229,GSP) Antisense (1320~1339,GSP)	5′-GACTCAACACGGGAAACCTCA-3′ 5′-CAGACAAATCGCTCCACCAA-3′	121 bp	84.0	0.994	92.9

MT, melting temperature; R^2, Pearson correlation coefficient; E, real-time PCR efficiency ($E=10^{[-1/slope]}$).

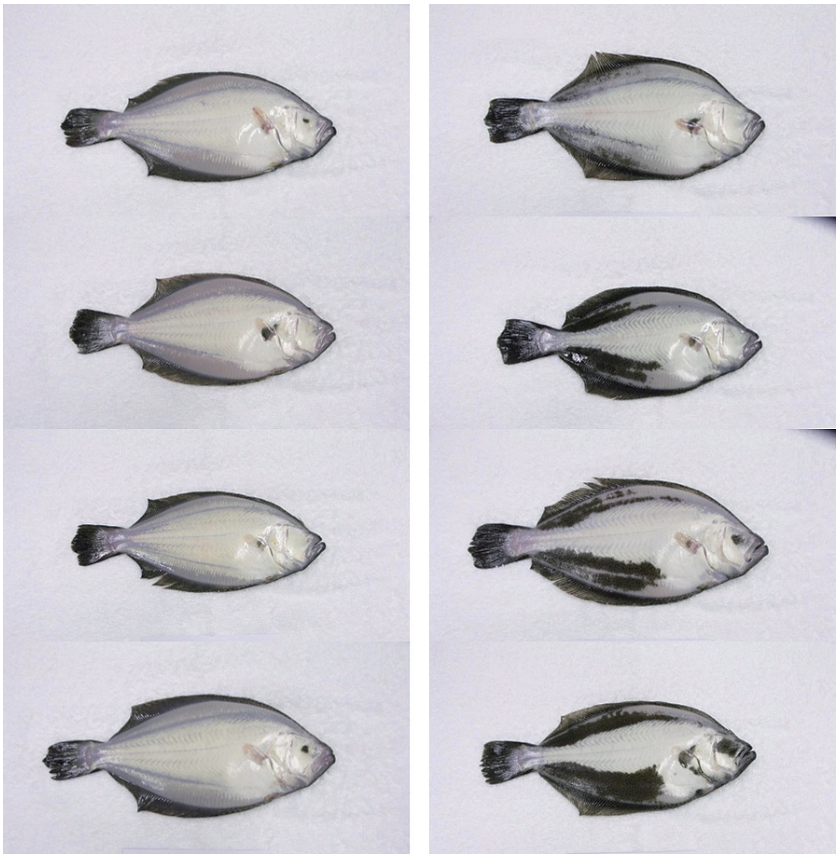

Fig. 1. Photographs of ordinary and hypermelanic olive flounders *Paralichthys olivaceus*.

Influence of background color, density, and appetite on MCH gene expression

Experimental animals and experimental conditions

To evaluate the influence of density and background color on *of*-pMCH gene expression, size-matched ordinary flounders (TL 20.8 ± 0.5 cm, BW 91.7 ± 6.5 g) were randomly divided into two groups and transferred to dark-green and white tanks (1000 L/m^3), where they were reared at a density of 30 fish/m^2 (PCA = 100%) or 60 fish/m^2 (PCA = 200%) for 10 days. Five fish were then sampled from each group reared on white and dark-green backgrounds, and the brains were extracted. In addition, to assess the relevance of MCH to blind-side hypermelanosis, we size-matched ordinary and hypermelanic flounders (TL 21.4 ± 0.1 cm, BW 87.9 ± 5.2 g) that were reared in flat-bottomed and gravel-bottomed dark-green FRP tanks (1000 L/m^3) for 120 days, respectively, and then sampled the brains from five fish of the ordinary and hypermelanic groups (Fig. 1).

To determine whether MCH gene expression is related to food intake, we randomly divided size-matched hypermelanic flounders (TL 33.2 ± 1.31 cm, BW 468.5 ± 72.82 g) into feeding and fasting groups, and transferred the fish from the two groups into dark-green and white tanks (1000 L/m^3) at a density of 10 fish/m^2 (PCA = 50%). The fish were acclimated to each tank supplemented with food for 3 days. After 3 days, we continuously supplied food to fish in one group (feeding group), while those in the other group were deprived of food (fasting group) for 7 days. On the final day, we sampled five fish from the dark-green and white tanks in the feeding and fasting groups and extracted the brains. Sampled fish were euthanized with the general anesthetic 2-phenoxyethanol (1/1000 dilution, 0.3–0.4 mg/L), and then rinsed with distilled water to remove salt. The brain tissues were removed from flounders and stored at –70°C for comparison of pMCH mRNA expression.

Comparison of *of*-pMCH mRNA expression

To measure pMCH mRNA expression levels in the brain, we extracted total RNA from the whole brains of flounders (n = 15) using a Maxwell 16 LEV simplyRNA tissue kit (Promega) and Promega Maxwell 16 instrument, and then elimi-

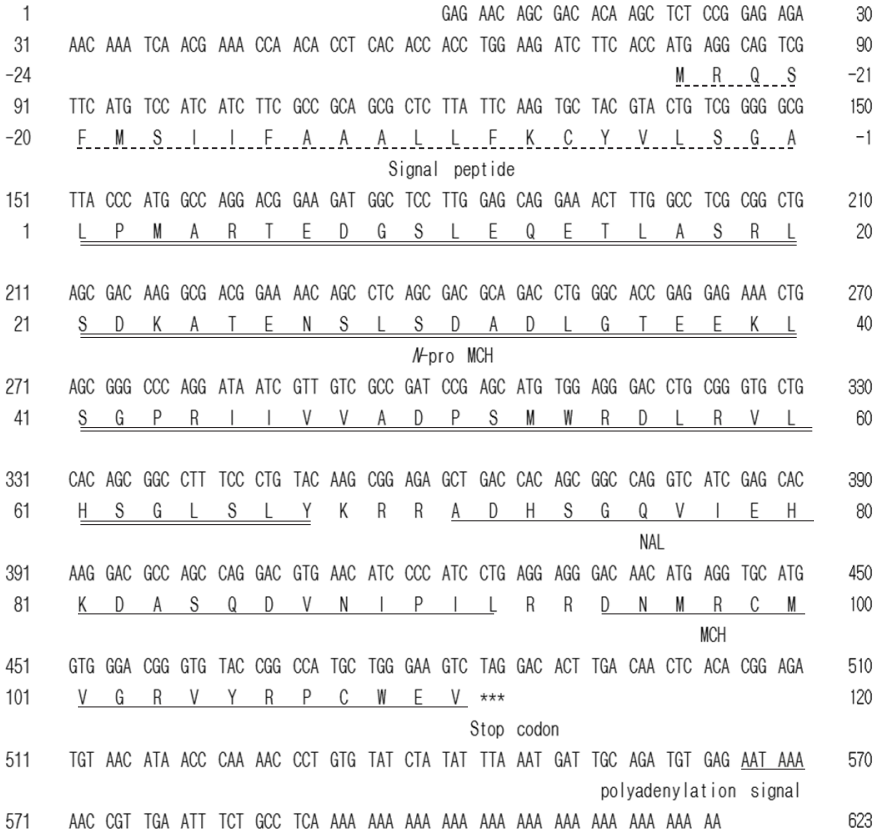

Fig. 2. Structure of *of*-pMCH cDNA. The nucleotide sequence of *of*-pMCH cDNA (not including the poly-A tail) and deduced amino acid sequence. Positions of nucleotides and amino acid residues are indicated on both sides. The N-terminus of pro-MCH in the deduced amino acid sequence of pMCH is designated position 1. The signal peptide is indicated by a broken line. N-pro MCH, NAL, and MCH are distinguished by underlining. The underline in the 3'-untranslated region shows the polyadenylation signal. (***) Stop codon.

nated genomic DNA from the extracted tissue using DNase I (Promega). In addition, samples were subjected to treatment with DNAse I to remove contaminating genomic DNA in the total RNA samples.

Aliquots of 1 μg of total RNA were reverse-transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad). We then performed real-time PCR using the IQ SYBR Green Supermix (Bio-Rad).

To compare mRNA expression between groups, the qPCR gene-specific primers (GSP) of *of*-pMCH used in the tissue distribution analyses were used again, and primers for 18S ribosomal RNA (rRNA) (GenBank EF126037) were newly designed to serve as a housekeeping gene control (Table 2). The housekeeping gene was unaffected by feeding regimen and background color in the brain as determined by examination of Ct values. The real-time PCR amplification efficiencies and R2 for each primer pair were calculated from the given slopes using the CFX-96 Manager software. The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/slope]}$. The transcripts investigated showed high real-time PCR efficiency rates and linearity (Table 2).

These data were used to produce real-time amplification curves for pMCH by plotting the Δ normalized reporter against the cycle number. The cycle threshold (Ct) value, which is the PCR cycle at which a statistically significant increase in reported fluorescence above the baseline could be detected (set at Δ Rn = 0.2), was determined for pMCH. The Ct values were used in the comparative Ct method (Livak and Schmittgen, 2001) in accordance with the manufacturer's protocol (Bio-Rad) for relative quantification. We evaluated the expression of 18S rRNA mRNA in each reaction as a control. The expression levels in the two tissues were normalized with respect to the 18S rRNA signal and expressed as the relative expression level.

Statistical analysis

The data were analyzed by Mann–Whitney U-test using SPSS (Korean ver. 7.50; SPSS Inc., Chicago, IL). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

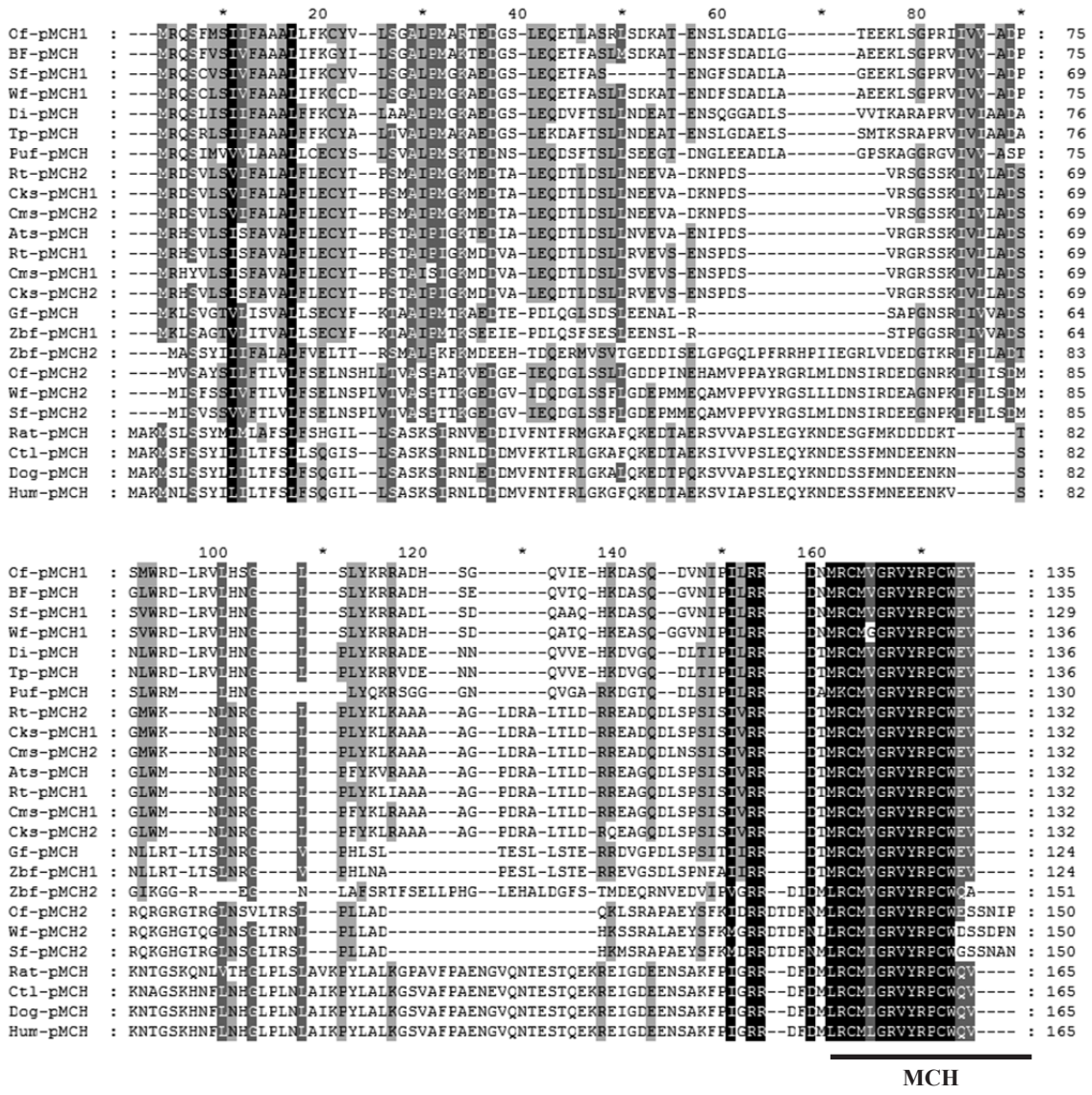

Fig. 3. Comparison of the *of*-pMCH amino acid sequence (*of*-pMCH1, GenBank ABY73341; *of*-pMCH2, GenBank AAF67166) with those of barfin flounder (*bf*-pMCH, GenBank BAC82350.1), starry flounder (*sf*-pMCH1, GenBank KF621304; *sf*-pMCH2, GenBank KF621305), winter flounder (*wf*-pMCH1, GenBank AEE36642; *wf*-pMCH2, GenBank AEE36640), dimerus (*di*-pMCH, GenBank ACT33940), Mozambique tilapia (*tp*-pMCH, GenBank CAA57050), green spotted puffer, (*puf*-pMCH, GenBank CAF93560), rainbow trout (*rt*-pMCH1, GenBank CAA52059; *rt*-pMCH2, GenBank CAA52060), Chinook salmon (*cks*-pMCH1, GenBank AAA49422; *cks*-pMCH2, GenBank AAA49423), chum salmon (*cms*-pMCH1, GenBank AAA49418; *cms*-pMCH2, GenBank AAA49419), Atlantic salmon (*ats*-pMCH, GenBank ACI70019), goldfish, *Carassius auratus* (*gf*-pMCH, GenBank CAL48577), zebrafish (*zbf*-pMCH1, GenBank ACO35933; *zbf*-pMCH2, GenBank ACO35934), cattle (*ctl*-pMCH, GenBank ABF59972), dog (*dog*-pMCH, GenBank AAU43637), rat (*rat*-pMCH, GenBank AAA41580), and human (*hum*-pMCH, GenBank AAH18048).

Results

Identification of *of*-pMCH cDNA

A PCR-based cloning strategy (RT-PCR followed by 3′- and 5′-RACE) was used to clone a cDNA encoding a putative pMCH from the olive flounder brain (Table 1). The first PCR using stage-I sense and antisense primers amplified the middle region (nt 152–431; clone I). Based on this nucleotide sequence, a stage-II antisense primer was synthesized for 5'-RACE. PCR using the stage-II antisense primer and a 5'-RACE kit primer (abridged universal amplification primer) amplified the *of*-pMCH cDNA (nt 1–302; clone II). Based on clones I and II, a stage-III sense primer was synthesized for 3'-RACE. Clone III encoding *of*-pMCH cDNA (nt 223–590) was amplified using the stage-III sense primer C and 3'-RACE kit

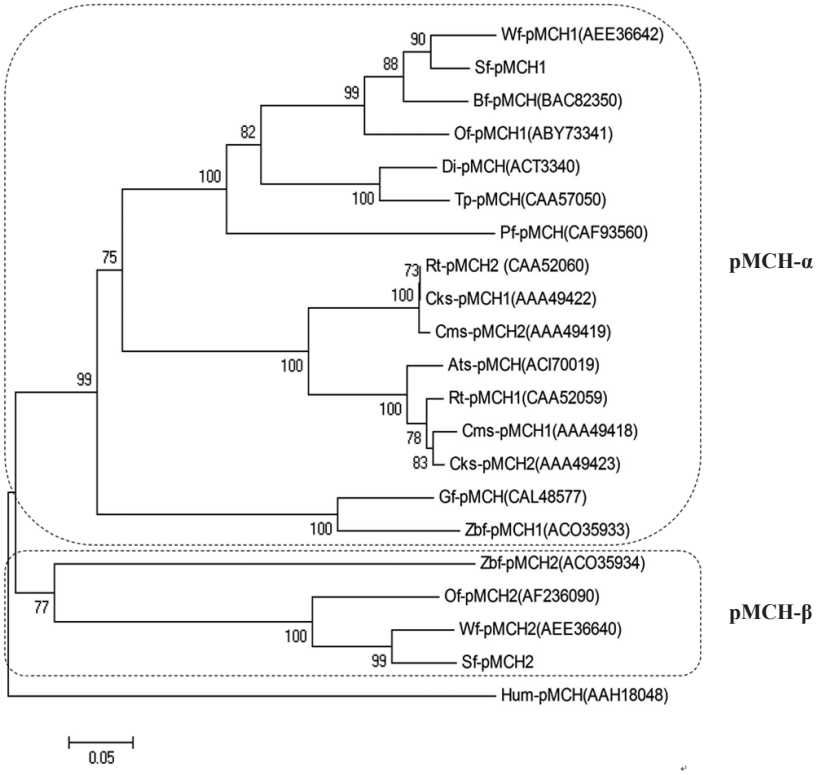

Fig. 4. Consensus phylogenetic tree constructed with the fish pMCH amino acid sequences by neighbor-joining analysis based on amino acid differences (p-distance). The tree is rooted from human pMCH (GenBank AAH18048). Reliability of the tree was assessed by bootstrapping (1,000 replications).

primer (clone III). The three PCR-amplified cDNAs were then merged to provide the entire sequence of *of*-pMCH cDNA. Based on our data, the *of*-pMCH cDNA (GenBank accession no. ABY73341) has a 405 bp ORF, which starts with an ATG codon at nt 79 and ends with a TAA stop codon at nt 483 (Fig. 2). The *of*-pMCH cDNA encodes 135 amino acids and consists of a signal peptide, N-terminal peptide (N-pro MCH), neuropeptide AL (NAL), and MCH. The amino acid sequence (aa) –24 to –1 of pMCH is referred to as the signal peptide, and aa 1–67 of pMCH is referred to as N-pro MCH. The NAL is located at aa 71–92, and MCH is located at the C-terminal end (aa 95–111) of *of*-pMCH. The *of*-MCH cDNA amino acid sequence encoded a deduced product of 17 amino acid residues (DNMRCMVGRVYRPCWEV) (Fig. 2).

Comparison of *of*-pMCH with vertebrate pMCHs, and phylogenetic analysis

The amino acid sequence of *of*-pMCH was compared with those deduced from the pMCH cDNAs of other vertebrate species (Fig. 3). The levels of amino acid sequence identity of *of*-pMCH were 85% with pMCH of barfin flounder, 80% with *sf*-pMCH1 and *wf*-pMCH1, 68% with *di*-pMCH and *tp*-pMCH, 42–43% with salmonid species pMCHs, 32% with *gf*-pMCH, 29% with *zbf*-pMCH1, 25% with *zbf*-pMCH2, 26% with *of*-pMCH2, 24% with *sf*-pMCH2 and *wf*-pMCH2, and 19–20% with mammalian pMCHs, and 19% with Hum-pMCH.

The relationships among a number of pMCHs are shown in a phylogenetic tree constructed by the NJ method (Fig. 4). We found that there are two principal teleost pMCH clades (pMCH-α and pMCH-β) in the phylogenetic tree. *of*-pMCH1 is included in pMCH-α clade, a single large clade, which contains the majority of the teleost pMCH transcripts reported to date. In the POMC-α clade, there are duplicate genes from several flatfishes, such as, chinook salmon (*cks*-pMCH1, *cks*-pMCH2), rainbow trout (*rt*-pMCH1, *rt*-pMCH2), and chum salmon (*cms*-pMCH1, *cms*-pMCH2). This duplication have occurred later than the event which gave rise to the two principal teleost pMCH clades. *of*- pMCH2 is included in pMCH-β clade with the pMCH2 of winther flounder (*wf*-pMCH2), zebrafish (*zbf*-pMCH2), and starry flounder (*sf*-pMCH2).

pMCH mRNA expression according to tissue and environmental factors

Expression of the *of*-pMCH gene was detected by RT-PCR. An obvious single band of the expected size was obtained from the brain and pituitary, but a blurred single band was

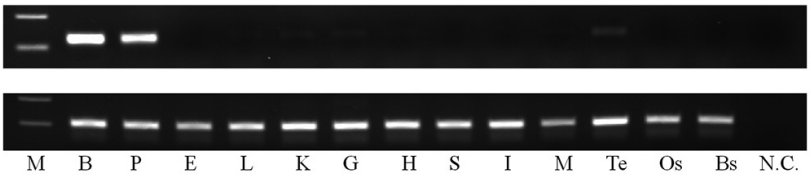

Fig. 5. Tissue distribution of *of*-pMCH in olive flounder *Paralichthys olivaceus* by RT-PCR. N.C. represents the negative control. Arrow indicates the expected size of pMCH (390 bp) (B: brain, P: pituitary, E: eye, L: liver, K: kidney, G: gill, H: heart, S: stomach, I: intestine, M: muscle, Te: testis, Os: ocular skin, Bs: blind skin, N.C.: negative control).

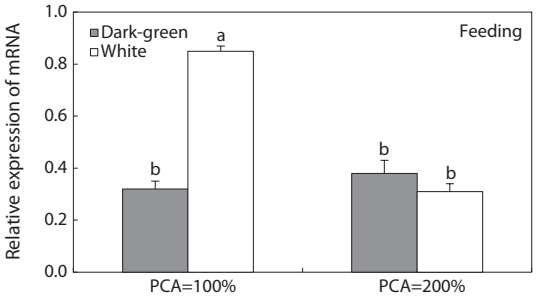

Fig. 6. Relative expressions of *of*-pMCH mRNA of olive flounders *Paralichthys olivaceus* cultured with a dark-green or white background at a density of 30 fish/m^2 (PCA = 100%) or 60 fish/m^2 (PCA = 200%). Each value represents the mean ± SEM (n = 5).

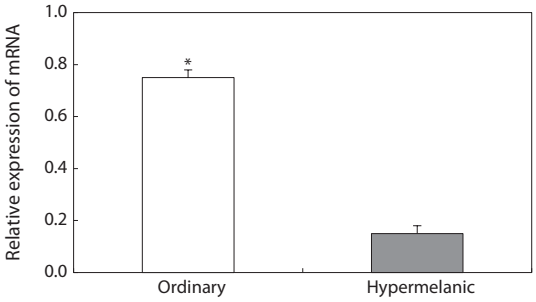

Fig. 7. Relative expressions of *of*-pMCH mRNA in ordinary and hypermelanic olive flounders *Paralichthys olivaceus*. Each value represents the mean ± SEM (n = 5).

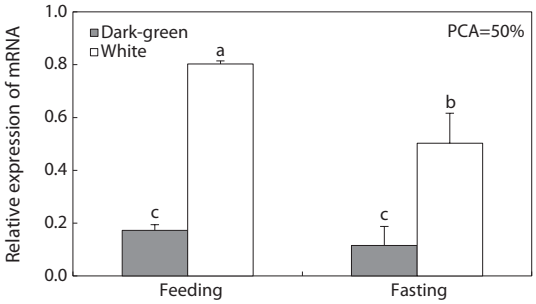

Fig. 8. Relative expressions of *of*-pMCH mRNA of olive flounders that had been feeding or fasting with a dark-green or white background at a density of 10 fish/m^2 (PCA = 50%). Each value represents the mean ± SEM (n = 5).

detected in the testis, gill, and kidney. No amplification of the pMCH gene transcript was detected in other tissues (Fig. 5). To examine the influence of environmental factors in pMCH mRNA activity in flatfish, we measured the relative activities of *of*-pMCH mRNA according to background color, density, and food intake. The level of gene expression was significantly higher in the white background group than the dark-green background group at 100% PCA ($P < 0.05$), but was not different between the two background colors at 200% PCA (Fig. 6). The pMCH gene expression level was significantly lower in hypermelanic fish than in ordinary fish ($P < 0.05$; Fig. 7). With respect to feeding, feeding fish reared at 50% PCA showed significantly higher *of*-pMCH gene expression than fasted fish with the white background ($P < 0.05$), but there was no significant difference between the two groups with the dark-green background ($P > 0.05$; Fig. 8).

Discussion

The results of the present study indicated that *of*-pMCH cDNA has a 405-bp ORF encoding a product of 135 amino acids, which consists of a signal peptide, N-pro MCH, NAL, and MCH. In terms of amino acid sequence, the signal peptide, N-pro MCH, and NAL have undergone significant evolutionary changes, while MCH has been relatively well preserved. When the amino acid sequence of *of*-pMCH was compared with those deduced from the cDNAs of other teleost species, in terms of structure, *of*-pMCH has a high degree of similarity to the Pleuronectiformes and Perciformes pMCHs, but a low level of similarity to mammalian pMCHs. Similar to other fishes, including the barfin flounder (Takahashi et al., 2004), trout (Baker et al., 1995), salmon (Kawauchi et al., 1983; Nahon et al., 1991), tilapia (Groneveld et al., 1995), tuna (Kawauchi, 1989), and eel (Kawauchi, 2006), MCH is located at the C-terminus of pMCH and consists of 17 amino acids (DNMRCMVGRVYRPCWEV; GenBank accession no. ABY73341). In comparison with other fishes, *of*-MCH is identical or similar to fish-type MCH (MCH 1) in length and sequence. That is, it is structurally 100% identical to those of barfin flounder and starry flounder, and is 94.1% identical to those of the winter flounder and the spindle-shaped teleosts (dimerus, tilapia, salmon, puffer, etc.), showing outer N-

terminal (N) or one middle (V) amino acid difference from those of other fishes. In addition, *of*-MCH has two fewer amino acids in the N-terminal than those of mammals, which are composed of 19 amino acids, and is 76.5% identical to mammalian homologs. With regard to length and sequence, *of*-MCH is significantly different from olive flounder MCH paralog (MCH-like peptide or MCH2; GenBank AF236090). The MCH paralog, consists of 25 amino acids (DTDFNML-RCMIGRVYRPCWESSNIP), and is structurally more similar to the Pleuronectiformes MCH2 and mammalian MCHs.

In phylogenetic trees (1,000 bootstrap replicates) of fish pMCHs, although a single pMCH was reported in many fish, duplicated pMCHs have also been described in several fish, such as salmon, zebrafish, and flounder species. Also, the phylogenetic analysis in the present study reveals that there are two main clades (pMCH-α and pMCH-β) and that, in some teleost species, the loss of pMCH-β gene was occurred under different selective pressures during their evolution. Here, we confirmed that olive flounder has two pMCHs, indicating that flounder species may have two types of pMCH. Based on aa comparisons and phylogenetic trees, *of*-pMCH is considered invariant copies because they have typical MCH (DN[T]MRCMVGRVYRPCWEV) . This gene is included in pMCH-αclade with the majority of the teleost pMCH genes. Also, in pMCH α-clade, the *of*-pMCH was not included in the salmoniformes clade, but was instead in a separate clade containing Pleuronectiformes and Perciformes. Interestingly, the *of*-pMCH was included in the same clade as dimerus and tilapia, which are neither marine fish nor pleuronectiforms. This supported the suggestion that Pleuronectiformes evolved from Zeiformes or Perciformes, related to Percoidei (Friedman, 2008). Our phylogenetic data also indicated that the *of*-pMCH is far from the clade of *of*-pMCH2, close to the clade of human pMCH. The *of*-pMCH2 is included in POMC-β clade with the pMCH2 of winther flounder (*wf*-pMCH2, GenBank AEE36640), zebrafish (*zbf*-pMCH2, GenBank ACO35934), and starry flounder (*sf*-pMCH2, GenBank KF621305). This gene retains structural characteristics of variant copies because mutations are present in its MCH (DFN[ID]M[L]LRCMI[V] GRVYRPCE). This suggested that fish pMCH2 and human pMCH may have evolved from fish pMCH of α-clade. Fish pMCH2 and human MCH may have become specialized and diverged from a common ancestor (fish MCH1) during evolution. Thus, other bony fishes may also possess a different paralog MCH precursor (or mature MCH) similar to that of mammals. Recently, it was discovered that the genomes of zebrafish, winter flounder, and other teleosts encode two distinct MCH peptides: one (MCH1) similar or identical to the salmonid MCH, and one (MCH2) that bears a striking resemblance to mammalian MCH. Chinook trout (Minth et al., 1989), chum salmon (Takayama et al., 1989), and rainbow trout (Baker et al., 1995) possess two precursors with the same type of MCH sequence, while zebrafish (Berman et al., 2009), winter flounder (Tuziak and Volkoff, 2012), and starry flounder (Kang and Kim, 2013) possess two precursors with another type of MCH sequence, indicating that fishes also have two types of pMCH. These results suggest that olive flounder has two types of MCH with different functions, as Tuziak and Volkoff (2012) reported that MCH1 participates in skin pigmentation and MCH2 activates appetite. Indeed, it has been reported that the fish MCH (MCH1) regulates physiological color change (Kishida et al., 1989; Suzuki et al., 1995; Kawauchi, 2006), and that the mammalian MCH (MCH2) is involved in food intake behavior and energy homeostasis in mammals (Pritchard et al., 2002; Shi, 2004; Pissios et al., 2006) and fishes (Mizusawa et al., 2009; Kang and Kim, 2013).

In this study, we examined the expression of pMCH mRNA in cranial and extracranial organs. Obvious expression of pMCH mRNA was detected in the brain and pituitary. In contrast, a week expression was observed in testis, kidney, and gill, and no expression was observed in the other tissues examined. These observations are consistent with previous reports in other teleost fishes (Naito et al., 1985; Minth et al., 1989; Amano et al., 2003; Pandolfi et al., 2003). In addition, although it is not clear whether MCH expression in extracranial organs is of physiological significance, the MCH mRNA expression in the testis, kidney, and gill suggests that MCHs play a peripheral role in spermatogenesis and osmoregulation. Therefore, further studies are needed to determine the peripheral physiological roles of MCH transcripts in extracranial organs.

In the wild, fish often use camouflage to hide from predators. One camouflage tactic is to change the body color to match that of the current background. MCH, a functional antagonist of α-MSH, plays a key role in this type of color change (Baker, 1993; Suzuki et al., 1995). Previous studies demonstrated that many fishes physiologically change their body color to match the background color with changes in MCH gene expression (Ramachandran et al., 1996; Nery and Castrucci, 1997; Nilsson Sköld et al., 2013). In pleuronectiforms, Amiya et al. (2005), Amano and Takahashi (2009), and Yamanome et al. (2005) demonstrated that a bright background color increases MCH gene expression, and then sequentially brightens body color, finally suppressing the blind-side malpigmentation of cultivated flounders. However, in the present study, we found that bright background did not whiten ocular skin, and MCHs do not play a role in the whitening of ocular skin in the olive flounder. The function of MCH in the whitening of ocular skin and bright background was found only at a fish density of 100% PCA, and not at the higher density of 200% PCA. These unexpected results indicated that overcrowding may suppress MCH action in the whitening of skin color, and that MCH is hindered by high density (200% PCA or higher).

In the present study, we investigated whether MCH was related to blind-side hypermelanosis by comparing the expression of *of*-pMCH mRNA between ordinary and malpigmented flounders. The *of*-pMCH expression level was significantly lower in hypermelanic flounders, indicating that low

MCH mRNA expression in hypermelanic fish may be related to blind-side hypermelanosis. A dark background color was shown to depress MCH gene expression (Kobayashi et al., 2008), and then sequentially increase the blind-side malpigmentation of cultivated flounders (Yamanome et al., 2007b; Yamanome et al., 2007a). Therefore, hypermelanosis on the blind side could be prevented by increasing endogenous MCH. Indeed, although these tests were performed at a low density, Takahashi et al. (2004), Yamanome et al. (2005), and Amiya et al. (2005, 2008) reported that hypermelanosis on the blind side of flatfish could be inhibited by increased endogenous MCH activity in the white background tanks. However, it remains unknown whether MCH is related to the morphological color change on the blind side in other pleuronectiforms. It is not yet clear why the morphological color change occurs with decreasing MCH activity when flatfish are unable to burrow in a normal manner at high density (PCA > 200%). To determine why blind-side malpigmentation occurs mostly under artificial rearing conditions without a burrowing substratum at high density, it will be necessary to investigate the relationships between physiological color hormones and abnormal pigmentation from an epigenetic standpoint.

Several studies (Groneveld et al., 1995; Suzuki et al., 1995; Amiya et al., 2005; Yamanome et al., 2005; Takahashi et al., 2007) have indicated that a bright background color increases appetite and growth in fishes through markedly increasing hypothalamic MCH neurons and triggering MCH production in the brain, suggesting that an MCH-related signaling system activated by white background stimulates food intake, which in turn accelerates growth in fishes. Previous studies (Takahashi et al., 2004; Yamanome et al., 2005; Matsuda et al., 2006; Matsuda et al., 2009) have reported that this hormone is also involved in improving appetite in teleosts. Recently, this was also supported by Berman et al. (2009) who found that fasted fishes had greater numbers of detectable MCH-immunoreactive cell bodies in the brain than fed fish, and by Takahashi et al. (2004), Tuziak and Volkoff (2012), and Kang and Kim (2013) who found that MCH gene expression in the brain of starved fish was two-fold greater than that in fed fish. The relevance of MCH to appetite in fish may follow a similar pattern as in mammals; i.e., mammalian-like MCH in combination with neuropeptide circuits (neuropeptide Y, hypocretin, and agouti-related protein) regulates food intake (Lin et al., 2000; Kawauchi, 2006; Matsuda and Maruyama, 2007). However, our data did not indicate that MCH is related to food intake. In the present feeding test, when flounders were reared at 50% PCA, pMCH gene activity was significantly higher with a bright background color than a dark background color, regardless of feeding or fasting. However, the effects of food intake on pMCH gene activity were dependent on background color. Although pMCH gene activity was significantly two-fold higher in the feeding group than the fasting group with a white background, the gene activity with a dark-green background was not significantly different between the two groups. Therefore, our results indicate that the MCH system is under the control of background color, but is not under the control of food intake. However, in previous studies using other flounder species (Takahashi et al., 2004; Tuziak and Volkoff, 2012; Kang and Kim, 2013), brain MCH mRNA levels were increased markedly under fasting conditions. In these studies, ordinary barfin flounder (Takahashi et al., 2004), winter flounder (Tuziak and Volkoff, 2012), and starry flounder (Kang and Kim, 2013) were reared in flat-bottomed black tanks, sandy-bottomed white tanks, and gravel-bottomed dark-green tanks, respectively. However, in the present study, we did not find any increase in MCH gene expression under fasting conditions regardless of background color or tank bottom type. Rather, in the white tank, MCH was significantly increased in the feeding group. These discrepancies in the results were likely caused by interference of background color; i.e., our contradictory results may have been due to the different background color and type of tank bottom employed to evaluate the role of MCH in food intake. Recently, Berman et al. (2009) and Tuziak and Volkoff (2012) reported the existence of two functionally distinct MCH peptides, and disagreed over the relationship of the fish-type MCH gene with food intake. Although Tuziak and Volkoff (2012) suggested that the fish-type MCH gene plays a role in regulation of appetite in flounder, Berman et al. (2009) suggested that the fish-type MCH gene is not principally involved in pigmentation and that the mammalian-type MCH gene is responsive to changes in appetite. Therefore, the relevance of fish-type MCH to the availability of food in fish must be examined carefully, and further experiments are necessary to determine whether the fish-type MCH is functionally related to feeding.

In conclusion, the primary structure of *of*-pMCH is similar to those of other fish species, but differs from mammalian pMCH. In addition, the *of*-pMCH mRNA levels were higher in the brain and pituitary than in other organs, indicating that the cranial organs are the main areas of MCH production. In addition, *of*-pMCH gene expression level was increased with a white background color at low density (<100% PCA), but was suppressed by a dark background color and at high density (>200% PCA), indicating that MCH is primarily related to white color camouflage in flounders against a white color background, and that density is an important factor in MCH gene expression. Moreover, pMCH mRNA expression is weak in hypermelanic flounders malpigmented on their blind-side skin but is strong in ordinary flounders, suggesting that 1 week of MCH expression could induce abnormal morphological color changes (pigment cell differentiation). However, we did not find a direct relationship between MCH and appetite.

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