



# A low body-color regulating ability of spotted halibut *Verasper variegatus*: Evaluation of the roles of melanin-concentrating hormone and proopiomelanocortin systems

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## Abstract

In teleosts, melanin-concentrating hormone (MCH) induces body brightening. On the other hand,  $\alpha$ -melanophore-stimulating hormone ( $\alpha$ -MSH), derived from the precursor proopiomelanocortin (POMC), leads to body darkening. In many teleosts, the expression level of *pmch* is high in light backgrounds, while that of *pmc* is low in dark backgrounds. In the present study, we investigated the ability of spotted halibut *Verasper variegatus* to regulate body coloration. Intraperitoneal administration of MCH resulted in a dose-dependent brightening of body color. The body color and brain contents of *pmch1* and *pmch2* in spotted halibut acclimated to the black or white background were not almost affected by background color. In addition, pituitary mRNA levels of *pmc-a*, *pmc-b*, and *pmc-c* genes in fish acclimated to the white background often exceed those levels of fish kept under the black background. These results suggest that the weak response of expression levels of *pmch* genes in response to background color is a factor in the blunt ability of spotted halibut to regulate body color. The different profiles of *pmc* gene expression to background color compared to that reported in other teleosts may be another factor for the weak ability of body color change in the spotted halibut.

**Keywords**  $\alpha$ -melanophore-stimulating hormone · Background color · Body color · Green light · Melanin-concentrating hormone · Spotted halibut

## Introduction

Camouflage, the adaptation of body color to blend with background color, is a critical biological process in fish, enabling them to avoid predators or to approach prey. The combination of several chromatophores, such as melanophores, xanthophores, erythrophores, and cyanophores containing light-absorbing pigment granules, provides body color that harmonizes with the respective habitat (Fujii 2000; Leclercq et al. 2010; Sugimoto 2002). Iridophores and leucophores, which reflect and scatter a wide range of light wavelengths,

also contribute to the brilliant colors in some fishes (Oliphant and Hudon 1993).

Flatfish, such as barfin flounder *Verasper moseri*, have been shown to be well adapted to the background, white and black, by changing body color with a combination of melanophores and xanthophores (Amiya et al. 2005; Mizusawa et al. 2011; Takahashi et al. 2011; Yamanome et al. 2007). To adapt to a white background, melanin aggregation is induced in part by melanin-concentrating hormone (MCH), which is biosynthesized in the hypothalamus and released from the pars nervosa of the pituitary (Mizusawa et al. 2011; 2015). Noradrenalin in the sympathetic nervous system is also associated with pigment aggregation (Mizusawa et al. 2013). In contrast to MCH and noradrenalin,  $\alpha$ -melanophore-stimulating hormone ( $\alpha$ -MSH), which is secreted from the pars intermedia of the pituitary, induces pigment dispersion in chromatophores (Kobayashi et al. 2009; Mizusawa et al. 2011; Takahashi et al. 2006). In barfin flounder,  $\alpha$ -MSH induces pigment dispersion

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ex vivo, such as in experiments using dissected skin or scale, but not in vivo, such as intraperitoneal injection (Kobayashi et al. 2009; Yamanome et al. 2007). It is possible that a dominant function of the sympathetic nervous system might mask  $\alpha$ -MSH activity. It has been proposed that  $\alpha$ -MSH exhibits pigment-dispersing activity when the functions of MCH and noradrenalin are weak because the proopiomelanocortin (*pomc*) gene encoding  $\alpha$ -MSH is somewhat constitutively expressed in barfin flounder (Mizusawa et al. 2013).

Melanin-concentrating hormone and  $\alpha$ -MSH are also known to participate in food intake or appetite in mammals with orexigenic and anorexigenic functions, respectively (Baldini and Phelan 2019; Griffond and Risold 2009), while these peptides have been shown to be anorexigenic in goldfish (Matsuda et al. 2009). However, MCH has been suggested to be an orexigenic peptide in barfin flounder because the fish reared under a white background grew faster than those reared in a black background (Takahashi et al. 2004; Yamanome et al. 2005). The association of MCH with growth under a white background has been suggested by augmented expression of *pmch1* gene in the brain (Takahashi et al. 2004), increased number of MCH-producing neurons in the hypothalamus (Yamanome et al. 2005), higher MCH content in the brain, pituitary, and plasma (Amiya et al. 2005; 2008), and facilitated food intake (Sunuma et al. 2009). The involvement of MCH in food intake has also been suggested in barfin flounder, in which growth is promoted by exposure to green light (Takahashi et al. 2016; 2018).

The spotted halibut *V. variegatus* is a flatfish of the family Pleuronectidae. It is a demersal fish that lives on sandy, muddy bottoms in the sublittoral coastal zone at depths of up to 100 m (330 ft). Its native habitat is the northwestern Pacific, from Japan to Korea and the East China Sea. The spotted halibut has potential for stock enhancement because of its high commercial value in Japan (Wada et al. 2006). We reported that the growth of the fish was promoted by exposure to green light (Shimizu et al. 2019) as observed in barfin flounder (Takahashi et al. 2016; 2018). Recently, we reported no difference in the growth of this fish between white and black backgrounds, in contrast to the faster growth of barfin flounder on a white background (Shimizu et al. 2021). These results have led us to suggest that the response of spotted halibut to the background color, at least in relation to growth, is different from that of barfin flounder. Despite accumulating evidence on body-color regulation of barfin flounder, no research has been conducted to evaluate the body-color regulating ability and related neuroendocrine and endocrine systems in spotted halibut. Thus, the present study was undertaken to examine the roles of the MCH and POMC systems in background adaptation and response to green light irradiation in spotted halibut.

## Materials and methods

### Fish

The early juvenile, juvenile, and immature spotted halibut (*V. variegatus*) used in the present study were bred at the Miyako Laboratory, Japan Fisheries Research and Education Agency, Iwate, Japan. All experiments were conducted in accordance with the Guidelines for the Care and Use of Animals of Kitasato University.

### Intraperitoneal administration of MCH [Experiment 1]

A peptide corresponding to the spotted halibut MCH1 sequence (GenBank: BBG56857) was synthesized and purchased from PH Japan (Hiroshima, Japan). Before MCH1 administration, juvenile spotted halibut were reared in a black tank (diameter, 96 cm; height, 76 cm) for 17 days from January 4 to January 21, 2020. The water temperature was maintained at approximately 15 °C during the experiment. Fish were irradiated by a white light-emitting diode (LED) lamp (Model LLM0200A; Stanley Electric Co., Tokyo, Japan) with peak wavelengths of 447 nm and 550 nm under a 10:14 h light dark cycle (light phase [07:00]–[05:00]). The photon flux density (PFD) from the LED lamps on the water surface was  $10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . They were fed a satiety amount of Otohime EP-3 (Marubeni Nissin feed Co. Ltd.) diet once a day. These fish,  $12.2 \pm 0.07$  cm total length (TL) and  $20.8 \pm 0.14$  g body weight (BW) on average ( $n=24$ ), were injected intraperitoneally with the synthesized MCH1 (0.01, 0.1, or 1 nmol/g BW [0.021, 0.21, or 2.1  $\mu\text{g/g}$ ]) dissolved in 0.9% NaCl under anesthesia with 0.05% 2-phenoxyethanol from [10:00] to [11:00]. Control fish received 0.9% NaCl. After the injection, the fish were returned to the other four black-colored tanks (diameter, 60 cm; height, 41.5 cm) containing approximately 80 l of running seawater and irradiated with white LED light. The PFD from the LED lamps at the water surface was  $2.3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The eyed-side body of the fish was recorded by scanning 3 h after the injection, and the brightness of a part of the area shown in Online Resource Supplementary Fig. 1 was calculated (see “Evaluation of color brightness”). Moreover, the brain and pituitary gland were collected and frozen on dry ice. The tissues were stored at  $-80$  °C until further analysis.

## Effects of background color

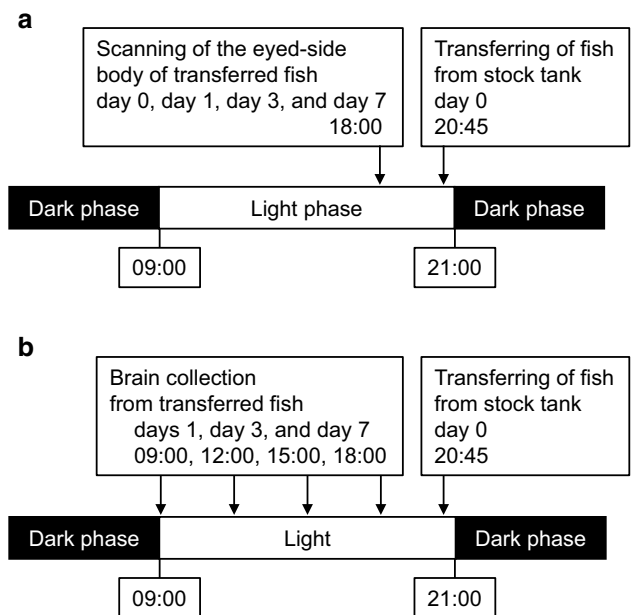
### Rearing under white or black background for terminal evaluation [Experiment 2]

White- and black-colored round indoor tanks (diameter, 96 cm; height, 75.5 cm) containing approximately 400 l of running seawater at controlled temperature (12.8–15.2 °C) were used for fish rearing. Photic conditions were the same as those in Experiment 1. Juvenile spotted halibut was transferred from a stock tank to a white or black tank, where they were reared for 12 days from January 9 to January 21, 2020. Although each tank initially contained 15 fish, the number decreased to ten in the black-colored tank because of an accidental overflow of water on day 10. On the final day of rearing, from [10:00] to [11:00], the eyed-side body of the fish,  $12.3 \pm 0.40$  cm TL and  $22.6 \pm 0.40$  g BW on average ( $n = 25$ ), were recorded by scanning, and then the brain and pituitary were collected and frozen on dry ice. The tissues were stored at  $-80$  °C until further analysis.

### Rearing under white background for time-course evaluation

**Rearing in white-colored tank for body-color evaluation [Experiment 3]** Early juvenile spotted halibut of approximately 4 cm TL and 0.5 g BW were reared in a stock tank, a conventional glass tank, for several days before being transferred to a white tank. The white tank was 45 cm (width) × 30 cm (height) × 30 cm (depth), which was filled with 28 l of artificial seawater (Aquarium Systems, France) and equipped with fluorescent light (FL). The lighting of the FL was performed under a controlled period (12:12-h light–dark cycle; light on [09:00]–[21:00]). The PFD from FL was approximately  $8 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at the surface of the water. Eight to ten fish were transferred from a stock tank to a white tank at 20:45 on day 0. Body color was scanned at [18:00] on day 0 (as an initial control), day 1, day 3, and day 7 (Scheme 1a). Scanning for initial control was done for fish taken from the stock tank, and rearing experiments were conducted between June 18 and 27, 2019.

**Rearing in white tank for brain collection [Experiment 4]** Fish, tank, and photic conditions were the same as those in Experiment 3. Twelve rearing experiments—four sampling times ([09:00], [12:00], [15:00], and [18:00]) of three rearing periods (1 day, 3 days, and 7 days) were conducted. In each rearing experiment, ten fish were transferred from a stock tank to a white tank at [21:00] and subsequently reared for 1, 3, or 7 days. Brains were taken from the fish at [09:00], [12:00], [15:00], and [18:00] in every rearing experiment (Scheme 1b). The pituitary glands were too small to collect.



**Scheme 1** Schedule of Experiment 3 and 4. **a** In Experiment 3, fish were transferred from a stock tank to a white-colored tank at 20:45 on day 0. Experiments were done independently for 1-, 3-, and 7-day rearing. Eyed-side body was scanned at 18:00 after each rearing period. Scanning was also done at 18:00 also for fish in the initial control, which were taken from a stock tank and not reared in a white tank. **b** In Experiment 4, fish were transferred from a stock tank to a white tank at 20:45 on day 0. Experiments were done independently for 1-, 3-, and 7-day rearing. Brain was collected at 09:00, 12:00, 15:00, and 18:00 after each transfer and subsequent rearing.

These rearing experiments were conducted between May 17 and June 5, 2019.

### Rearing under white and green light in white- and black-colored tanks [Experiment 5]

The brain and pituitary were collected from immature spotted halibut reared in a previous experiment from June 15, 2019 (day 0) to July 25, 2019 (Shimizu et al. 2021). In brief, fish ( $n = 8$  in each) were housed in two black and two white round indoor tanks (diameter, 96 cm; height, 75.5 cm) containing approximately 400 l of running seawater. The water temperature increased from 14.2 to 17.8 °C during the rearing period. The PFD of indoor room light at the water surface was  $0.2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  from [07:00] to [17:00] h. One black and one white tank were each exposed to white LED light, and the others were exposed to green LED light. The PFD from the LED lamps on the water surface was  $10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The initial BW and TL were  $123.5 \pm 1.9$ , and  $21.0 \pm 0.1$  cm, respectively ( $n = 32$ ). Fish were fed Otohime EP-3 intermittently ( $40 \text{ g}\cdot\text{tank}^{-1}\cdot\text{day}^{-1}$ ). Tissue sampling was performed under anesthesia from [10:00] to [11:00] on the final day.

## Evaluation of color brightness

The eyed-side body was scanned using a CanoScan LiDE 220 image scanner (Canon, Tokyo, Japan). The data were processed according to previously described methods (Kasagi et al. 2020a, b). In brief, images were imported to ImageJ 1.46r software (Schneider et al. 2012). The entire area of the eyed-side body picture was chosen as the ROI (region of interest, typically 700,000 pixels in Experiment 2 and 5700 pixels in Experiment 3) from each flounder individual (Online Resource Supplementary Fig. 1). For each ROI, non-weighted RGB intensity scores were determined using the ImageJ “measure” tool. The average intensity scores from each ROI were calculated to determine the average group scores.

## Measurement of hormone mRNA content in brain and pituitary

Total RNA was extracted from the brain and pituitary of spotted halibut using ISOGEN II (FUJIFILM Wako) and treated with RNase-free DNase (TaKaRa, Otsu, Japan) to eliminate genomic DNA contamination and then purified by phenol–chloroform extraction followed by isopropanol precipitation. The mRNA contents of *pmch*, *pmch2*, *pmc-a*, *pmc-b*, and *pmc-c* were estimated by quantitative real-time polymerase chain reaction (qRT-PCR) as described previously (Shimizu et al. 2019). Briefly, the qRT-PCR was conducted using a One Step PrimeScript RT-PCR kit (TaKaRa, Kusatsu, Shiga, Japan) and Light Cycler 96 (Roche Diagnostics, Basel, Switzerland). The reaction was started with reverse transcription at 42 °C for 5 min, followed by the amplification protocol: 95 °C for 1 min, 60 cycles at 95 °C for 15 s, 55–61 °C for 20 s, and 72 °C for 15 s.

## Statistics

R version 3.6.1 (R Core Team, Vienna, Austria) was used for the statistical analyses. All data are presented as the mean  $\pm$  standard error. The significance of differences between the values was assessed using one-way analysis of variance (ANOVA) for Experiments 1, 3, and 4, or two-way ANOVA for Experiment 2. Tukey’s honestly significant difference (HSD) test was used as a post hoc test. For Experiment 2, significant differences between the values were assessed using *t* tests. Differences were considered statistically significant at *P* values less than 0.05.

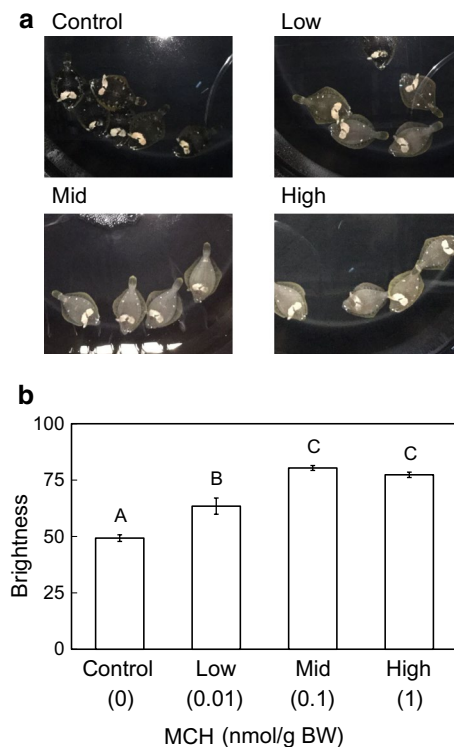
## Results

### In vivo effects of MCH on body color [Experiment 1]

The responsiveness of spotted flounder body color change against MCH was examined in vivo. The administration of three different doses of MCH (0.01, 0.1, and 1 nmol/g) resulted in paler body colors in the fish receiving MCH compared to those of control fish at approximately 3 h after treatment (Fig. 1a). The body color-paling effect of MCH was confirmed by a statistically significant increase in brightness in a dose-dependent manner (Fig. 1b). The lowest dose of MCH (0.01 nmol/g) significantly increased brightness, and the higher doses of MCH (0.1 and 1 nmol/g) were more potent than the lowest dose.

### Effects of white and black background colors on body color and the expression of *pmch/pmch* genes (terminally evaluation) [Experiment 2]

The effects of background color on body color and gene expression in *pmch* and *pmc* of spotted halibut, kept in a



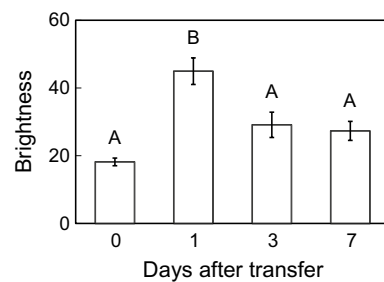
**Fig. 1** Body color of barfin flounder approximately 3 h after receiving MCH intraperitoneally. **a** Pictures taken from directly above the tank. Note that the photo shows only some of the individuals in the tank. **b** Body color brightness. Different letters indicate statistically significant differences ( $P < 0.05$ ) estimated by Tukey’s HSD test following one-way ANOVA ( $n = 6$ ,  $F = 46.09$ ,  $P < 0.0001$ )

white tank or black tank for 12 days, are shown in (Fig. 2). No difference was observed in the brightness of the eyed-side body between the fish kept in the white and black tanks (Fig. 2a, Online Resource Supplementary Fig. 2). No difference between the fish was observed in *pmch1* and *pmch2* mRNA levels in the brain (Fig. 2b, c). Regarding *pomc* gene expression, no difference between the fish was observed for *pomc-a* and *pomc-b* mRNA, while the *pomc-c* mRNA content was higher in the pituitaries taken from the fish kept in a white tank than those kept in a black tank (Fig. 2d, e, f).

**Effects of white background color on body color and the expression of *pmch* genes (time-course evaluation) [Experiments 3 and 4]**

Results of Experiment 3: body-color changes of spotted halibut in response to white background after transferring from a stock tank are shown in Fig. 3 and Online Resource Supplementary Fig. 3. The fish kept against a white background for 1 day exhibited higher brightness in the eyed-side body than in the initial control (day 0). The fish kept against a white background for 3 and 7 days showed no difference in brightness against the initial control.

The results of Experiment 4: the expression profiles of *pmch1* and *pmch2* genes on days 1, 3, and 7 are shown in (Fig. 4). No difference was observed in the *pmch1* mRNA levels in the brains of the fish kept against a white background and exposed to fluorescent lighting for 0, 3, 6, and

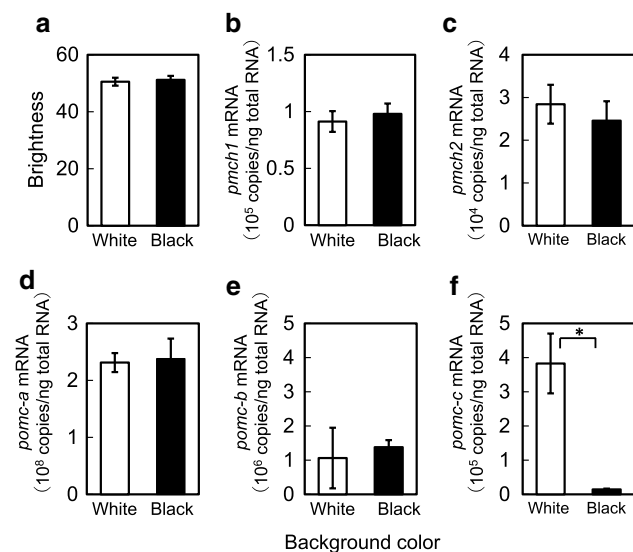


**Fig. 3** Body-color brightness of spotted halibut in a white-colored tank for 1, 3, and 7 days after transfer from a stock tank. Day 0 is an initial control. Different letters indicate statistically significant differences ( $P < 0.05$ ) estimated by Tukey’s HSD test following one-way ANOVA ( $F = 13.10$ ,  $P < 0.0001$ ). Sample sizes were 10 for 0-, 1-, and 3-day rearing, and 8- for 7-day rearing. See Online Resource Supplementary Fig. 3 for the picture

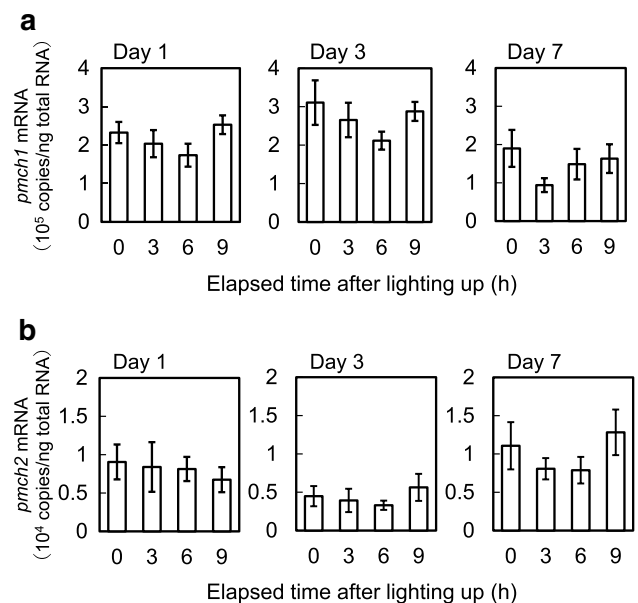
9 h on days 1, 3, and 7 (Fig. 4a). No difference in *pmch2* mRNA content was observed in the same fish used for the measurement of *pmch1* mRNA (Fig. 4b).

**Effects of tank color and light color on the expression of *pmch* genes in brain and *pomc* genes in pituitary [Experiment 5]**

The effects of tank color (white and black) on *pmch* gene expression in the spotted halibut brain under white and green



**Fig. 2** Effects of background color, white and black, on the a brightness of eyed-side body, the brain contents of mRNA for b *pmch1* and c *pmch2*, and the pituitary contents of d *pomc-a*, e *pomc-b*, and f *pomc-c* of spotted halibut after 12 days rearing in Experiment 2. The asterisk shows statistical difference ( $P < 0.05$ ) estimated by *t* test. Sample sizes were 15 and 10 for white and black backgrounds, respectively



**Fig. 4** The expression profiles of a *pmch1* and b *pmch2* in a white-colored tank on days 1, 3, and 7 after transfer from a stock tank. Fish were exposed to fluorescent light with duration indicated under horizontal bar. No statistical difference was observed by one-way ANOVA ( $n = 8$ ; *pmch1*:  $F = 1.155$ ,  $P = 0.3443$ ; *pmch2*:  $F = 0.2010$ ,  $P = 0.8947$ )



LED lighting are shown in (Fig. 5a, b). No difference was observed in *pmch1* mRNA content between the fish kept in white- and black-colored tanks under both white and green LED light (Fig. 5a; effect of background:  $F=0.1901$ ,  $P=0.1789$ ; effect of light:  $F=0.9226$ ,  $P=0.3450$ ; interaction effect:  $F=0.2152$ ,  $P=0.1536$ ). No difference was also observed in *pmch2* mRNA content between the fish kept in white- and black-colored tanks (effect of background:  $F=0.9126$ ,  $P=0.3476$ ; effect of light:  $F=1.257$ ,  $P=0.2718$ ; interaction effect:  $F=0.05079$ ,  $P=0.8233$ ).

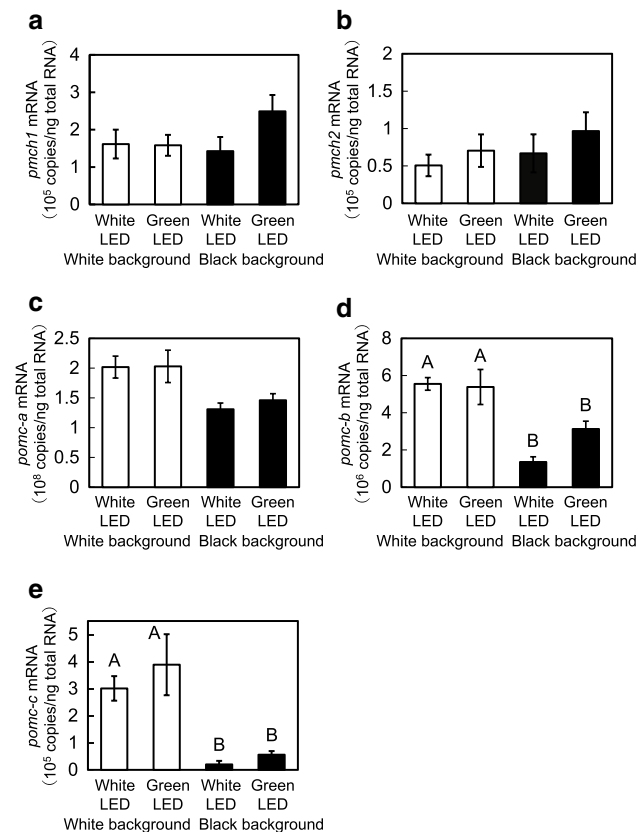
The effects of tank color (white and black) on the expression of *pomc* genes in the spotted halibut pituitary under white and green LED lighting are shown in Fig. 5c, d, e. The effect of background color on *pomc-a* mRNA content was significant, whereas that of light color and interaction effect were not ( $n=8$ ; effect of background:  $F=11.80$ ,  $P=0.0019$ ; effect of light:  $F=0.1959$ ,  $P=0.6616$ ; interaction effect:  $F=0.1413$ ,  $P=0.7099$ ), although Tukey's HSD test showed no significant difference in *pomc-a* mRNA content between

white- and black-colored tanks under each light color condition (Fig. 5c). The effect of background color on the mRNA levels of *pomc-b* and *pomc-c* was significant, whereas that of light color and interaction effect were not ( $n=8$ ; effect of background on *pomc-b*:  $F=31.30$ ,  $P<0.0001$ ; effect of light on *pomc-b*:  $F=1.940$ ,  $P=0.1750$ ; interaction effect on *pomc-b*:  $F=2.821$ ,  $P=0.1046$ ; effect of background on *pomc-c*:  $F=23.20$ ,  $P<0.0001$ ; effect of light on *pomc-c*:  $F=0.9417$ ,  $P=0.3405$ ; interaction effect on *pomc-c*:  $F=0.1644$ ,  $P=0.6884$ ). The mRNA levels of *pomc-b* and *pomc-c* were higher in the fish kept in white-colored tanks than those kept in black-colored tanks irrespective of light color (Figs. 5d, e).

## Discussion

Many fish can change body color in response to the background color (McLean 2021). We found that the body-color-regulating ability of spotted halibut (*V. variegatus*) was lower than that of barfin flounder (*V. moseri*) in the same genus. Indeed, barfin flounder changed their body color according to the background color (white and black) of tanks in which they were housed (Amiya et al. 2005; Mizusawa et al. 2011; Takahashi et al. 2011; Yamanome et al. 2005; 2007). In particular, Takahashi et al. (2011) demonstrated that the body color of barfin flounder, acclimated to white, yellow, or black backgrounds for 5 days, changed to match the color of each tank. On the other hand, in the present study, we could not detect significant differences in body color in spotted halibut acclimated white or black flounder for up to 17 days. These results suggest that the ability of spotted halibut to change body coloration is lower than that of barfin flounder, at least within the acclimation period examined in this study. However, it should be noted that the previous studies on barfin flounder were conducted under natural day length and natural water temperature. The possibility that these differences in environmental conditions affected the ability of these flounders to adjust their body coloration will require further investigation.

Here, we showed the in vivo dose-dependent function of MCH in pigment aggregation by intraperitoneal administration between 0.01 and 1 nmol/g BW in spotted halibut. In barfin flounder, an intraperitoneal injection of MCH (0.1 nmol/g BW; Yamanome et al. 2007) and 1  $\mu\text{g}$  (Ca. 2 nmol)/g BW (Takahashi et al. 2004; Yamanome et al. 2005) was sufficient to generate a paler body color for both the eyed and non-eyed sides, while a lower dose of MCH was not examined for either report. Thus, as in the case of barfin flounder, MCH seemed to be effective, and 0.1 nmol/g BW of MCH was the maximum dose required to achieve a physiological body-color change in spotted halibut. Meanwhile, the result that halibut did not change to a pale color



**Fig. 5** The brain contents of mRNA for **a** *pmch1* and **b** *pmch2*, and the pituitary contents of mRNA for **c** *pomc-a*, **d** *pomc-b*, and **e** *pomc-c* in the spotted halibut reared in white-colored or black-colored tanks under white or green LED light. Different letters indicate statistically significant differences ( $P<0.05$ ) estimated by Tukey's HSD test following two-way ANOVA ( $n=8$ , see "Effects of tank color and light color on the expression of *pmch* genes in brain and *pomc* genes in pituitary [Experiment 5]" )

on a white background suggests that MCH was not secreted sufficiently to lead to pale body color. Despite the presence of functional MCH systems demonstrated by the *in vivo* study, the expression of *pmch* genes showed little change against different background colors (see Figs. 2b, c, 5a, b). This is in contrast to the barfin flounder, in which expression levels of *pmch* genes are high on a white background and low on a black background (Mizusawa et al. 2015; Takahashi et al. 2004; 2007). Considering the synchronized changes in body color and *pmch* gene expression in barfin flounder, the fact there was no difference in *pmch* expression between the spotted halibut against white and black backgrounds could be a reason for the weak body-color changes. Although the sympathetic nervous system, in which noradrenalin turns the body color paler by aggregating pigments in chromatophores (Mizusawa et al. 2013), is an alternative way of regulating body color, the weak changes in body color suggest a low function of the sympathetic nervous system.

The  $\alpha$ -MSH encoded on the *pomc* is associated with pigmentation in chromatophores. Generally, the expression of *pomc* genes in teleosts, such as goldfish, and rainbow trout, is high and low on a black background and white background, respectively (Mizusawa et al. 2018; Kasagi et al. 2020a, b). Increased production of  $\alpha$ -MSH stimulates the generation of pigments by morphological body color change (Leclercq et al. 2009; Sugimoto 2002). In barfin flounder, however, intraperitoneal injection of  $\alpha$ -MSH does not induce pigment dispersion, although  $\alpha$ -MSH stimulates pigment dispersion on dissected scales, which are free from noradrenalin (Mizusawa et al. 2013; Yamanome et al. 2007). Therefore, the function of the sympathetic nervous system may be dominant over  $\alpha$ -MSH. Barfin flounder is somewhat different from the fish mentioned above because *pomc* expression does not always increase in response to the black background color. We hypothesized that  $\alpha$ -MSH might work irrespective of background color. According to our model, the body color of barfin flounder is regulated by a combination of functions of the MCH and sympathetic nervous systems and constitutive  $\alpha$ -MSH function (Mizusawa et al. 2013). In addition to the *pmch* gene, the present results suggest that the expression profiles of *pomc* genes encoding MSHs in spotted halibut were also distinct from those of barfin flounders and other fishes mentioned above. The expression of all *pomc* genes were higher on a white background than those on a black background in both larger-sized fish in Experiment 5 and *pomc-c* gene in smaller-sized fish in Experiment 2. The cause of these inverse expression profiles of *pomc* genes, which may be associated with the weak body-color changes of spotted halibut, is unknown.

The results of Experiment 5, shown in (Fig. 5), were obtained using immature spotted halibut in our previous study, in which the fish were housed in white-colored or black-colored tanks and received white, or green LED light

(Shimizu et al. 2021). The previous study was conducted to evaluate the effects of the photic environment on somatic growth of spotted halibut because we found that barfin flounder in a white-colored tank grew faster than those in a black tank (Amiya et al. 2005; 2008; Sunuma et al. 2009; Takahashi et al. 2004; Yamanome et al. 2005) and green light also promoted the growth of barfin flounder (Takahashi et al. 2016; 2018), spotted halibut, Japanese flounder (Shimizu et al. 2019), and marbled sole (Sato et al. 2020). In the previous study, no difference was observed in growth between halibut in white and black tanks under both white and green LED lights (Shimizu et al. 2021). In the present study, we used the brains and pituitaries of the immature spotted halibut to estimate mRNA levels of *pmch* and *pomc* genes since we did not measure any mRNA expression levels in the previous study. The measurements showed no difference in the brain contents of *pmch1* and *pmch2* mRNA between the fish housed in white and black tanks, which were exposed to green and white LED light. In the case of *pomc* genes, higher contents of all three *pomc* mRNAs were observed in fish housed in a white tank. Therefore, it is probable that background color, white and black, might not be involved in the *pmch* gene expression regulation, and *pomc* gene expression might be inversely modulated by the background color, high under white background and low under black background, compared to the response in goldfish (Kasagi et al. 2020b).

In conclusion, spotted halibut was found to change their body color little in response to a background color, white or black, in contrast to other species of the genus *Verasper*, barfin flounder. The mechanisms underlying this spotted halibut-specific weak body-color regulation might be related to different response patterns of *pmch* and *pomc* genes compared to other fish such as barfin flounder and goldfish.

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