General and Comparative Endocrinology 271 (2019) 82–90

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Effects of green light on the growth of spotted halibut, *Verasper variegatus,* and Japanese flounder, *Paralichthys olivaceus*, and on the endocrine system of spotted halibut at different water temperatures

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A B S T R A C T

We have previously shown that the somatic growth of barfin flounder, *Verasper moseri,* was promoted by green light. The present study was undertaken to elucidate whether growth-promoting effect of green light can be observed in other flatfishes and to understand the roles of endocrine systems in green light-induced growth. Herein, we demonstrated facilitation of growth by green light in the spotted halibut, *Verasper variegatus,* and Japanese flounder, *Paralichthys olivaceus*. Blue and blue-green light showed potencies that were similar to that of green light, while the potencies of red and white light were equivalent to that of ambient light (control). We also examined the effects of green light on growth and endocrine systems of *V. variegatus* at various water temperatures. Growth of the fish was facilitated by green light at four different water temperatures examined; the fish were reared for 31 days at 12 and 21 °C, and 30 days at 15 and 18 °C. Increase in condition factor was observed at 15 and 18 °C. Among the genes encoding hypothalamic hormones, expression levels of melanin-concentrating hormone 1 (*mch1*) were enhanced by green light at the four water temperatures. Expression levels of other genes including *mch2* increased at certain water temperatures. No difference was observed in the expression levels of pituitary hormone genes, including those of growth hormone and members of proopiomelanocortin family, and in plasma levels of members of the insulin family. The results suggest that green light may generally stimulate growth of flatfishes. Moreover, it is conceivable that MCH, production of which is stimulated by green light, is a key hormone; it augments food intake, which is intimately coupled with somatic growth.

1. Introduction

Somatic growth of fishes is dependent on environmental factors such as photic conditions and water temperature as shown by accumulating evidence observed in a variety of fishes such as Arctic char *Salvelinus alpinus* (Gunnarsson et al., 2014) and Atlantic salmon *Salmo salar* (Døskeland et al., 2016; Imsland et al., 2014) among Salmoniformes; European sea bass *Dicentrarchus labrax* (Villamizar et al., 2009), Nile tilapia *Oreochromis niloticus* (El-Sayed et al., 2004), and red sea bream *Pagrus major* (Biswas et al., 2005) among Perciformes, and southern flounder *Paralichthys lethostigma* among Pleuronectiformes (Luckenbach et al., 2007). The endocrine system of fishes is intimately linked with the physiological processes of growth that are affected by environmental factors (Boeuf, 1999). Several neuropeptides produced in hypothalamus regulate food intake (Volkoff, 2016). Growth hormone (GH) secreted by pituitary gland promotes growth by its direct effects and by indirect effects through stimulation of insulin-like growth factor-I (IGF-I) release from liver (Björnsson, 1997; Mommsen, 2001; Pérez-Sánchez et al., 2002). Insulin (INS), a member of INS family, together with IGF-I, which is secreted from Brockmann bodies (equivalent to Langerhans islets), enhances anabolic pathways (Andoh, 2007; Navarro, 2006).

We have shown that lighting conditions such as background color and chromatic light, are the factors of photic environments that promote the somatic growth of barfin flounder *Verasper moseri*, a flatfish species. Firstly, white background was shown to promote growth in the fish; its association with melanin-concentrating hormone (MCH) was suggested based on the increase in MCH somata and tissue content of MCH in the brain of fish reared in white background compared to those in black background (Amiya et al., 2005, 2008; Yamanome et al., 2005). Secondly, rearing barfin flounder under a green filter facilitated the growth rate compared to those under blue, red, and white filters (Yamanome et al., 2009). Thirdly, green light from light-emitting diode (LED) was shown to exhibit greater effects than blue and red light (Takahashi et al., 2016). In addition to MCH, involvement of some hormones was suggested in the experiments. Finally, when the effects of

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https://doi.org/10.1016/j.ygcen.2018.11.005
Received 12 October 2018; Accepted 8 November 2018
Available online 09 November 2018
0016-6480/

light intensities on growth and endocrine functions were examined using green light, intensity-dependent growth was observed, which was suggested to be associated with MCH (Takahashi et al., 2018). Thus, we have accumulated a series of evidence on growth-promoting effects of chromatic light, especially green light, and suggested association of endocrine systems with the growth events of barfin flounder.

It is, however, uncertain whether growth promoting effects of green light are specific to barfin flounder or widely observed events also in other fishes. Moreover, the endocrine functions that are linked to physiological processes of growth, which result from stimulation of chromatic lights, remain unknown. Therefore, we first examined the effects of green light in spotted halibut *Verasper variegatus* and Japanese flounder, *Paralichthys olivaceus*. Subsequently, the endocrine factors that link green light to food intake, growth, and anabolic pathways were explored using spotted halibut.

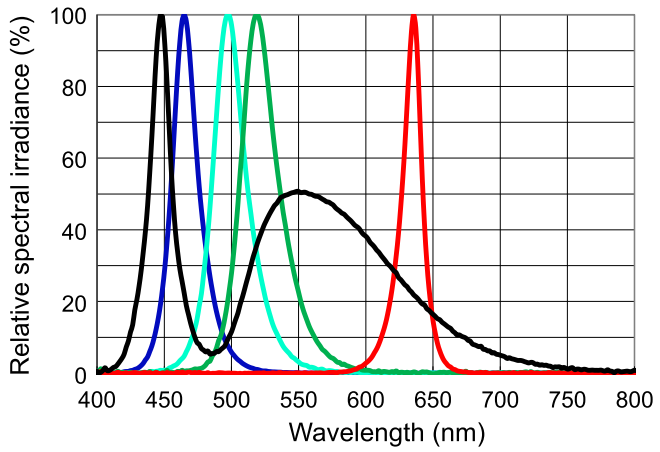
2. Materials and methods

2.1. Flounders

V. variegatus and *P. olivaceus* were bred at the Miyako Laboratory, Tohoku National Fisheries Research Institute, Japan Fisheries and Education Agency, Iwate, Japan. All experiments were conducted according to the Guidelines for the Care and Use of Animals of Kitasato University.

2.2. Rearing of spotted halibut under several LED light colors (Experiment 1)

Black colored indoor round tanks (diameter, 100 cm; height, 80 cm) containing approximately 400 L of running seawater at natural water temperature were used for fish rearing under natural photoperiod. These tanks were equipped with LEDs (Model LLM0200A, Stanley Electric Co., Tokyo. Japan, Scheme 1) with peak wavelengths of 447 nm and 550 nm (white), 464 nm (blue), 497 nm (blue-green), 518 nm (green), and 635 nm (red), and were exposed to a controlled photoperiod (10 h:14 h, light–dark cycle; 07:00–17:00, light). Photon flux density (PFD) from LEDs was 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the water surface. Thus, fish received both controlled LED lighting and ambient light under natural photoperiod. As a control, fish were reared in an indoor tank under ambient light. Each tank contained 40 fish. Fish were reared for 60 days from May 7, 2015 to July 6, 2015. Water temperature was increased from 10.9 °C to 18.7 °C during the rearing period. Fish were fed commercial pellets (Otohime EP-3, Marubeni Nisshin Feed, Tokyo, Japan) six times/day from 09:00 to 16:00 until satiety using automatic feeders (Fiap Clockwork Belt Feeder, Sterner AquaTech UK, Inverness-Shire, UK). Residual feed was siphoned from the tanks every day before feeding (08:00 to 09:00). The fish were not fed on the final day. Total length (TL) and body weight (BW) were measured on days 0 (initial), 20, 40, and 60 without anesthesia. On day 0, TL and BW of 40 control fish (rearing under ambient light) were measured as representatives of all the fish before transferring them to an experimental tank. On days 20 and 40, fish were returned to respective original tanks after recording the measurement, where they were further reared.



Scheme 1. The spectra of LED lamps with the following peak wavelengths: 447 nm and 550 nm (white), 464 nm (blue), 497 nm (blue-green), 518 nm (green), and 635 nm (red).

2.3. Rearing of Japanese flounder under several LED light colors (Experiment 2)

Japanese flounders were reared in running seawater at natural water temperature under the conditions described in Section 2.2 with reference to tanks, lighting, feeding, and cleaning. Fish were reared for 60 days from Aug. 7, 2015 to Oct. 27, 2015. Water temperature was decreased from 20.7 °C to 15.7 °C during the rearing period. TL and BW were measured at days 0 (initial), 30, and 60. Each tank contained 30 fish. On day 0, fish were transferred from a stock tank to experimental tanks after measuring TL and BW. On day 30, fish were returned to respective original tanks after recording the measurement, where they were further reared.

2.4. Rearing of spotted halibut under green light at different water temperatures (Experiment 3)

Spotted halibuts were reared in running seawater at controlled water temperature under conditions described in Section 2.2 with reference to tanks, feeding, and cleaning condition. Tanks were equipped with green LED lights with a peak wavelength of 518 nm (Scheme 1) under a controlled photoperiod (10 h:14 h, light–dark cycle; 07:00–17:00, light). Photon flux density from LEDs was 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the water surface. Average water temperatures were 12 °C, 15 °C, 18 °C, and 21 °C (rounded off to the closest whole number). Each tank contained eight fish that were implanted with an electric tag (Pit Tag, Biomark, Boise, ID). Duration of rearing period was 31 days for tanks maintained at 12 °C and 18 °C, and 30 days for those at 15 °C and 21 °C. TL and BW were measured on the initial and final days under anesthesia, which was achieved by administering 0.05% 2-phenoxyethanol. Subsequently, brains and pituitary glands were immediately dissected and snap-frozen on dry ice. Blood was collected using heparinized needles and syringes, and then plasma was separated by centrifugation (3000*g* for 15 min at 4 °C). The samples were stored at −80 °C until analysis. Specific growth rate (SGR) and condition factor (CF) were calculated as follows:

$$SGR(\%\,day^{-1}) = [(\ln W_f - \ln Wi)/t] \times 100$$

$$CF(\%) = (W_f/TL_f^{3}) \times 100$$

where, W_i, W_f, t, and TL_f are the initial weight (g), final weight (g), time period (days), and final total length (cm), respectively. When SGR was calculated for total length, L_i (initial length) and L_f (final length) were used instead of W_i and W_f respectively.

2.5. cDNA for quantification

The cDNA that was used as a template for synthesizing cRNA for quantitative reverse transcription polymerase chain reaction (qRT-PCR) was prepared as described previously (Kasagi et al., 2015). Custom oligonucleotides for PCR were synthesized at Eurofins genomics (Tokyo, Japan). Primers used for amplifying the cDNAs of *mch1*, *mch2*, neuropeptide Y (*npy*), orexine (*orx*), proopiomelanocortin-a (*pomc-a*), *pomc-b*, *pomc-c*, and *gh* were the same as those described in a previous report (Takahashi et al., 2016), while those for pituitary adenylate cyclase-activating polypeptide (*pacap*), agouti-related proteins 1 and 2

Table 1
List of oligonucleotide PCR primers used for cDNA cloning.

Gene		Nucleotide sequences	Amplicon size (bp)
pacap	forward	GCGACCTTAGCCTTACTCATCTATGGAATC	372
	reverse	GTGTCGCTTGGACAGGGGCTCTG	
sl	forward	AGTACATGGGGGCTAGTAGGAAGACCA	783
	reverse	ATATATTAGTAGGGTTTGAATGCGGTGGGAATA	
prl	forward	GTTCGGACCAACTGCACTCGCTCAG	450
	reverse	GGAAGCAGGACAACAGGAAGTGGAAGTTGA	
agrp1	forward	ATGTTTGGCTCTGTGCTGCTCTG	359
	reverse	AAGAAGCGACAGTAGCAGGTGTCA	
agrp2	forward	ATGAGGAAGATCGCCGGCAAACA	345
	reverse	CTATGTCCTCTTTTGGCACACGTGGT	

All nucleotides sequences are denoted in 5′-3′ direction. The nucleotide sequences were designed on the basis of Japanese flounder *Paralichthys olivaceus pacap* (JX152586), Atlantic halibut *Hippoglossus hippoglossus sl* (L02117) as well as Japanese flounder *sl* (M33695), Japanese flounder *prl* (XM_020110606), starry flounder *Platichthys stellatus agrp1* (KX279353) and *agrp2* (KX279354).

(*agrp1* and *agrp2*), prolactin (*prl*), and somatolactin (*sl*) were newly prepared in the current study (Table 1). Amplified cDNA fragments containing the reading frame of each gene product were subcloned into the pGEM-T Easy vector (Promega, Fitchburg, WI, USA), followed by the standard alkaline-SDS plasmid preparation method. Nucleotide sequences were confirmed by DNA sequence analyses carried out for the cloned DNA using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3130 xl genetic analyzer (Applied Biosystems).

2.6. Quantitative reverse transcription polymerase chain reaction

qRT-PCR was performed as described previously (Takahashi et al., 2016). Briefly, a series of mRNA concentrations was prepared by *in vitro* transcription of the pGEM-T plasmid clones using the cDNAs corresponding to hormone sequences containing the entire coding region, which were used as standard references for quantifying mRNA concentrations. The plasmid clones were digested with *Sal* I at the 3′-terminal of the inserts. The sense-strand RNA was synthesized using a MAXIscript T7 kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions and quantified using serial dilutions. LightCycler 96 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) and OneStep PrimeScript RT-PCR kits (TaKaRa, Kusatsu, Japan) were employed for qRT-PCR. The TaqMan probes and primers were the same as those used in a previous report (Takahashi et al., 2016) and those shown in Table 2. The PCR mixture (10 μl) contained Takara OneStep RT-PCR buffer, RT Enzyme Mix, Ex Taq Mix, 400 μM of the dNTP mix, 2.5 pM each of forward and reverse primers, and 2.5 pM of the TaqMan probe. The reaction was initiated with reverse transcription at 42 °C for 5 min, followed by the amplification protocol: 95 °C for 1 min (activation of HotStarTaq DNA polymerase), 60 cycles at 94 °C for 20 s (denaturation), 55–61 °C for 20 s (annealing), and 72 °C for 20 s (extension). The levels of mRNA of each hormone gene in the total RNA were quantified based on the amplitude scale using the serially diluted reference RNA (10 ag–100 pg) for each gene. The quantity of mRNA was converted to copies per nanogram of total RNA, as previously described (Takahashi et al., 2016). An identical mRNA sample prepared from the spotted halibut brain was used as an internal standard to correct any intra-assay variance. The number of samples in each experiment was 8.

2.7. Time-resolved fluoroimmunoassay

IGF-I was extracted from plasma according to the procedure described by Shimizu et al. (2000). IGF-I concentration was measured using the anti-barramundi IGF-I antiserum and biotinylated recombinant tuna/flounder IGF-I (GroPep Bioreagents Pty Ltd, Thebarton, SA, Australia) by time-resolved fluoroimmunoassay according to Andoh (2005). Furthermore, INS was measured by a time-resolved fluoroimmunoassay using the anti-barfin flounder INS antiserum and biotinylated barfin flounder INS according to Andoh (2007). A multimode microplate reader (Spark 10M, Tecan Trading AG, Switzerland) was used for the measurements. The number of samples was 8.

Table 2
List of oligonucleotide PCR primers and TaqMan probes used for quantitative RT-PCR.

Gene		Nucleotide sequences	Amplicon size (bp)
pacap	forward	TACGACAGAGACCAAATGGATGTG	100
	reverse	CTGCGTGCCTTTCCGTTCT	
	TaqMan probe	CCGCCTGTCGATGGCGACGTC	
sl	forward	GCTCCAAAAGTGAAATCCAACAG	75
	reverse	TCCACGACTGGACCAGCAA	
	TaqMan probe	TATCTGATACATGGCTGCTCCACTCCGTG	
prl	forward	CAGAGCAGCATATTCAACAAGGT	92
	reverse	GCACCCATTTTCCCAGAGA	
	TaqMan probe	CGCGAGCTGCAGGAGCACTCC	
agrp1	forward	GCCCCTGAGCCCATAAATG	69
	reverse	GTCCATCAGGAAGTGATCATCCA	
	TaqMan probe	CCGCCCTCCTGCCTGTCGACT	
agrp2	forward	GCAAACACTTGTTGTGCTTCCT	87
	reverse	CATTCTCAGTCTTCTTCGCATCCT	
	TaqMan probe	CCTCCCGCTGTCCTGGGCTGA	

All nucleotides sequences are denoted in 5′-3′ direction. TaqMan probes were labelled with 5′-6 FAM, 3′-TAMRA. The nucleotide sequences were designed as described in Table 1.

2.8. Statistics

All data are presented as mean ± standard error. Differences between control and green light tanks were assessed by Student's *t*-test in each temperature group. Differences among control and green light groups were assessed by one-way ANOVA. Subsequently, the significance of difference between the mean values was tested using the Tukey-Kramer test. Statistical significance was accepted at $P < 0.05$ level of significance.

3. Results

3.1. Various wavelengths of light and flounder growth

Fig. 1 shows the effects of particular wavelengths of light on the growth of spotted halibut. While TL and BW were not different among the experimental groups on day 20, their increase was statistically significant in blue, blue-green, and green LED light treatment groups for TL, and blue-green and green LED light treatment groups for BW on day 40, compared to ambient light treatment groups. The highest average increase in TL and BW was observed in the green light treatment groups on day 60. Japanese flounder also showed similar responses to LED lights of different colors with reference to somatic growth (Fig. 2).

3.2. Effects of green light on somatic growth of spotted halibut in various water temperature

Effects of green light on TL and BW of spotted flounder at four different temperatures after rearing for approximately four weeks are shown in Fig. 3 and Table 3. In the green light treatment group, significant increase in TL was observed at 21 °C, while significantly higher SGR was observed at 12 °C. In the case of BW, green light treatment

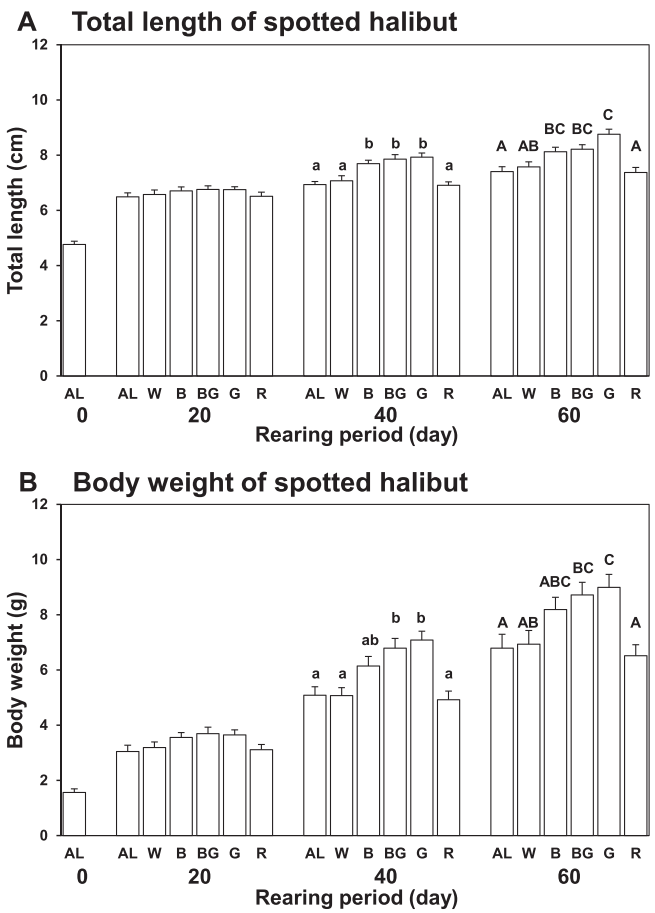


Fig. 1. Somatic growth of spotted halibut exposed to the chromatic light from LED. (A) Total length. (B) Body weight. Spotted halibuts were reared for 60 days in indoor tanks under LED lights. LED light were added to ambient light (AL, control). W, white LED light; B, blue LED light; BG, blue-green LED light; G, green LED light; R, and red LED light. Different alphabets indicate statistically significant differences (n = 40, $P < 0.05$) as determined by one-way ANOVA: Tukey-Kramer test.

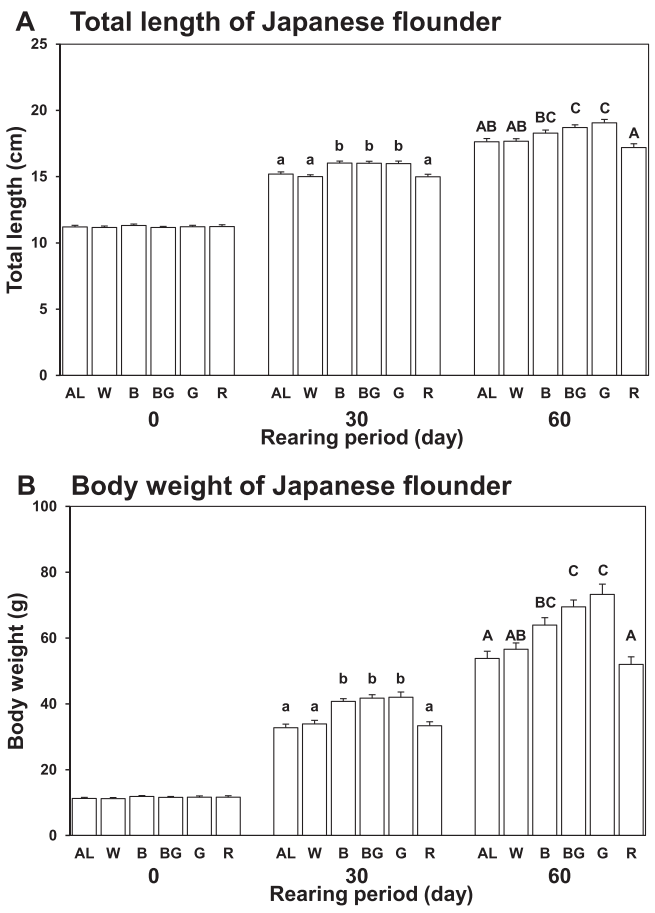


Fig. 2. Somatic growth of Japanese flounder exposed to the chromatic light from LED. (A) Total length. (B) Body weight. Japanese flounders were reared for 60 days in indoor tanks as described in Fig. 1. Different alphabets indicate statistically significant differences (n = 30 for day 0, 26–30 for day 30, 20–25 for day 60. $P < 0.05$) as determined by one-way ANOVA: Tukey-Kramer test.

group showed a significant increase, compared to the control group at all temperatures. Similar trend was observed in the case for SGR. Increase in CF was observed in the fish reared at 15 and 18 °C.

3.3. Effects of green light on the expression of neuropeptide genes in spotted halibut brain at various water temperature

The expression of *mch1* gene in the brain of spotted halibut exposed to green light increased at all the rearing temperatures, compared to respective controls (Fig. 4A). For other neuropeptide genes, the temperatures at which statistically significant increase was observed under green light were as follows: 21 °C for *mch2*, 12 °C for *pomc-c* gene, 15 and 21 °C for *npy* gene, and 12, 18, and 21 °C for *pacap* gene (Fig. 4B, E, F, and H). No difference was observed in the expression levels of *agrp1* and *agrp2* as well as *orx* gene between control and green light groups at any temperatures (Fig. 4C, D, and G). In the control group, differential expression of *mch1*, *mch2*, *npy*, and *pacap* genes was observed at different water temperatures. No difference was observed in the expression of *agrp1*, *agrp2*, *pomc-c*, and *orx*.

3.4. Effects of green light on the expression of pituitary hormone genes in spotted halibut

No difference was observed in the expression levels of *gh*, *prl*, *sl*, *pomc-a*, *pomc-b* and *pomc-c* genes in the fish exposed to green light, compared to the control group (Fig. 5). Contrarily, the expression level of *prl* gene increased with an increase in rearing temperature, in the green light treatment group (Fig. 5B). In the control group, differential expression of *prl* and *pomc-b* genes was observed at different water temperatures. The expression of *prl* gene gradually increased with an increase in water temperature (Fig. 5B). No difference was observed in the expression of *gh*, *sl*, *pomc*-a, and *pomc-c*.

3.5. Effects of green light on the plasma profiles of INS and IGF-I in spotted halibut

No difference was observed in the plasma profiles of INS and IGF-I in the green light treatment group, compared to control group (Fig. 6).

4. Discussion

4.1. Green light and growth of flounders

4.1.1. Effects of light color and temperature

In a previous study, green light was shown to be the most effective chromatic light that promotes somatic growth in barfin flounder among blue (464 nm), green (518 nm), and red (635 nm) lights in white tanks, although the efficacy of these lights varied at different water temperatures; green and blue light showed similar growth-enhancement effect at 14.9 °C and 8.6 °C, while green light showed the highest efficacy at 6.6 °C (Takahashi et al., 2016). Herein, we obtained further evidence showing that the green light was the most effective facilitator

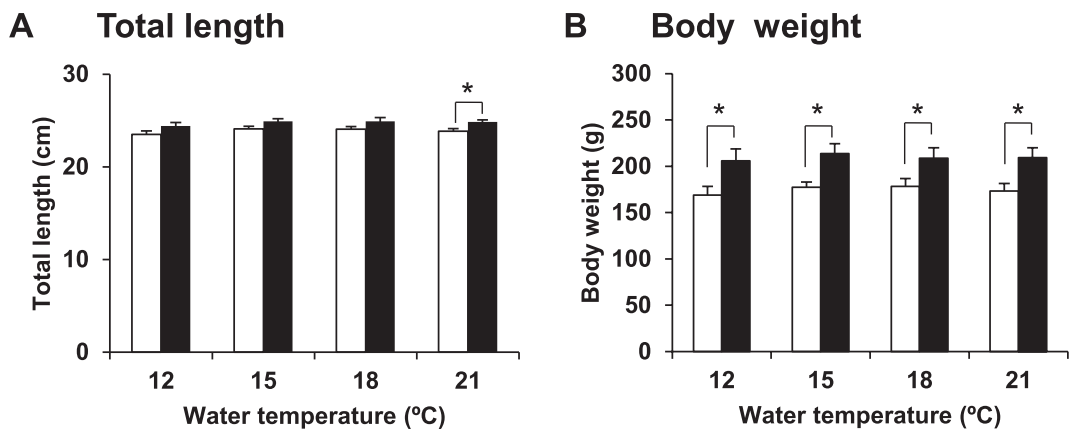


Fig. 3. Somatic growth of spotted halibut exposed to LED-derived green light at four different water temperatures (12 °C, 15 °C, 18 °C, and 21 °C) in indoor tanks for 31 days (at 12 °C and 18 °C) and 30 days (at 15 °C, 21 °C). White bar and black bar represent control group (ambient light) and green light group, respectively. (A) Total length. (B) Body weight. Asterisks indicate statistically significant difference (n = 8, $P < 0.05$) between control and green light group as determined by Student's *t*-test.

of somatic growth in flounders using spotted halibut and Japanese flounder. The effects of blue-green and blue light were comparable to those of green light, suggesting that the lights of shorter wavelengths similarly stimulate the growth of spotted halibut, Japanese flounder, and barfin flounder. No growth-promoting effects of red light, similar to those of the control group, suggesting that lights of longer wavelengths produce weak stimulation in the three flounder species.

White LED light consisting of two peaks of wavelength (447 and 550 nm) covers a wide range of wavelengths (Scheme 1). Although the spectrum contains blue (464 nm), blue-green (497 nm), and green light (518 nm) that promoted growth of the fish, the white LED light did not promote growth in spotted halibut and Japanese flounder. There may be at least two possible explanations for this. Firstly, the absence of growth promoting effects would be caused by the absence of majority of blue, blue-green, and green light spectra in the white LED light as shown in the Scheme 1. Secondly, a mixture of different chromatic lights might attenuate the effects of the green light component of the white LED. The latter interpretation is supported by our previous findings, which show that the growth rate of barfin flounders reared in an indoor tank under ambient light was lower than that of those reared in an indoor tank covered with green filter (Yamanome et al., 2009).

The growth promoting effects of green light were examined in spotted halibut at four different water temperatures. No water temperature-dependent differences were observed in TL and BW. These results indicate that green light effectively stimulates the growth of spotted halibut at least between 12 °C and 21 °C. In these experiments, CF significantly increased at 15 °C and 18 °C under green light. These results suggest that the anabolic pathway enhanced by green light to facilitate BW gain would be maximized around these water temperatures. Increase in CF may be a common effect of green light in flounders, because similar increase was also observed in barfin flounder (Takahashi et al., 2016, 2018).

4.1.2. *Effects of tank color and ambient light on green light*

In the present study, green light stimulated the growth of spotted halibut reared in an indoor tank with black walls and floor (experiment 1). As black color essentially absorbs light, these fish seemed to receive direct light mainly originating from the top, and minor indirect light reflected from the wall and bottom. Growth rate of spotted halibut exposed to green light was higher than that of control fish also in indoor white tanks (experiment 3). Taken together, growth of spotted halibut was stimulated by green light, irrespective of the tank color—black or white. Unlike black color, white color effectively reflects the light coming from an illuminant. The results from experiments 1 and 3 suggested that tank color may not be so critical factor to modulate the effects of green light under ambient light. On the other hand, our previous study on barfin flounder indicated that presence of ambient light modulated the effects of green light (Takahashi et al., 2018). Specifically, there was no difference among the growth rates of fish reared under different intensities of green light in white tanks under ambient light, while light intensity-dependent growth of barfin flounder was observed in the experiment performed in a dark room. Green light may differentially affect physiological processes related to flounders' growth depending on the conditions of photic environment surrounding the source of green light. Further studies are necessary to elucidate the role of background color in modifying growth-promoting effects of green light, because experiments in the present study were not originally designed to compare the effects of tank color.

Table 3
Effects of green light on specific growth rate and condition factor of spotted halibut shown in Fig. 3.

Temperature	Treatment	SGR of TL (% day^{-1})	SGR of BW (% day^{-1})	CF
12 °C	Control	0.184 ± 0.032	0.46 ± 0.17	1.30 ± 0.04
	Green light	0.318 ± 0.032**	1.10 ± 0.18*	1.40 ± 0.03
15 °C	Control	0.292 ± 0.028	0.74 ± 0.09	1.27 ± 0.01
	Green light	0.395 ± 0.060	1.25 ± 0.20*	1.38 ± 0.03**
18 °C	Control	0.290 ± 0.029	0.67 ± 0.12	1.27 ± 0.02
	Green light	0.361 ± 0.047	1.14 ± 0.15*	1.34 ± 0.02*
21 °C	Control	0.276 ± 0.046	0.61 ± 0.19	1.27 ± 0.03
	Green light	0.386 ± 0.050	1.25 ± 0.21*	1.36 ± 0.05

$^{*}(P < 0.05)$ and $^{**}(P < 0.01)$ indicate significant differences assessed by *t*-test (n = 8).

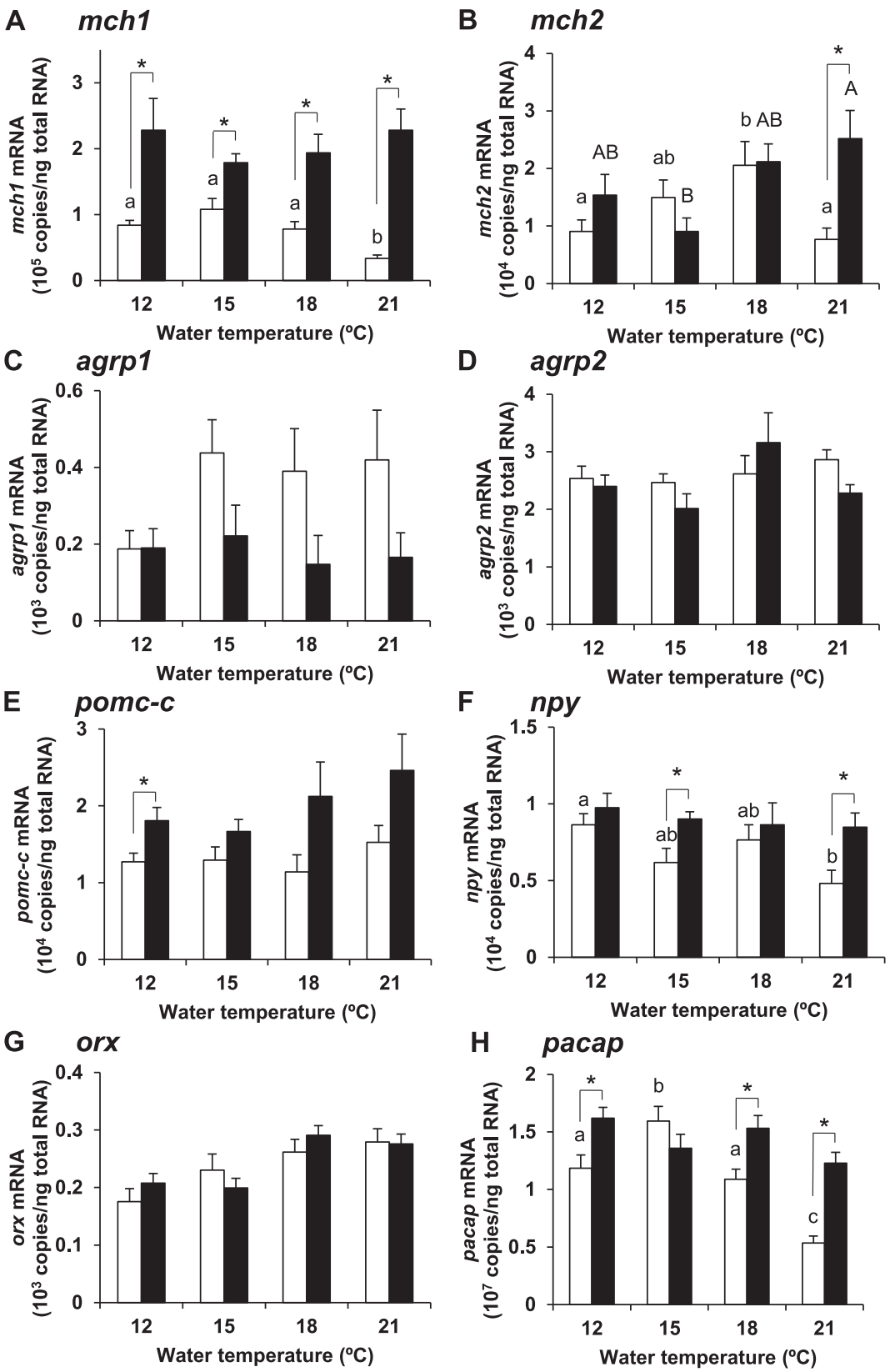


Fig. 4. Brain mRNA contents of *mch1* (A), *mch2* (B), *agrp1* (C), *agrp2* (D), *pomc-c* (E), *npy* (F), *orx* (G), and *pacap* (H) in spotted halibut shown in Fig. 3. White bar and black bar represent control group (ambient light) and green light group, respectively. Asterisks indicate statistically significant difference (n = 8, $P < 0.05$) between control and green light group as determined by Student's *t*-test. Different alphabets indicate statistically significant differences (n = 8, $P < 0.05$) as determined by one-way ANOVA: Tukey-Kramer test for intra-control group (lower case letters) and intra-green light group (capital letters).

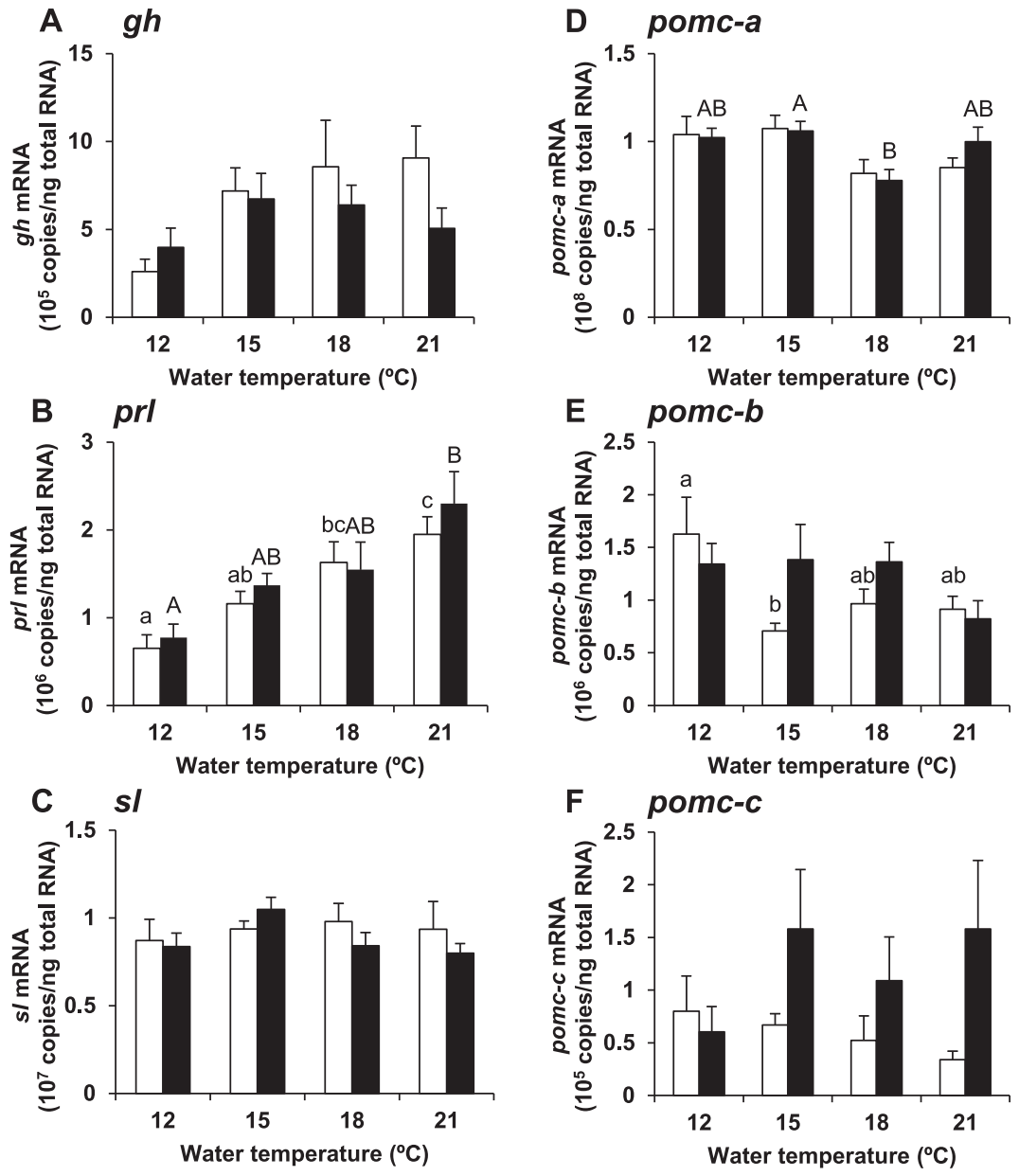


Fig. 5. Pituitary mRNA contents of *gh* (A), *prl* (B), *sl* (C), *pomc-a* (D), *pomc-b* (E), and *pomc-c* (F) in spotted halibut shown in Fig. 3. White bar and black bar represent control group (ambient light) and green light group, respectively. For statistical significance, see the legend of Fig. 4.

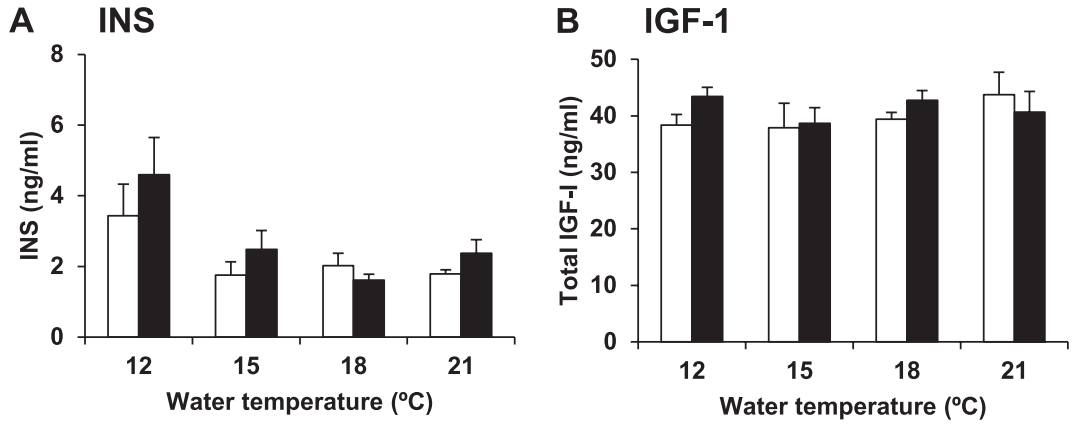


Fig. 6. Plasma levels of INS (A) and IGF-I in spotted halibut shown in Fig. 3. White bar and black bar represent control group (ambient light) and green light group, respectively. No statistical significance was observed.

4.2. Green light and endocrine functions associated with growth

MCH has been suggested to be associated with somatic growth of barfin flounder based on the enhanced food intake, and because high MCH production together with high growth was observed under white background (Amiya et al., 2005, 2008; Takahashi et al., 2004,; Yamanome et al., 2005). Spotted halibut possesses two types of *mch* genes (Supplementary Fig. 1) as in the case of barfin flounder (Mizusawa et al., 2015, Takahashi et al., 2004). The genes *mch1* and *mch2* code for teleost type MCH1 and mammalian type MCH2, respectively. These genes showed differential expression profiles in response to green light at different water temperatures. Specifically, green light stimulated the increase in *mch1* gene expression at all water temperature examined, while the increase in the expression of *mch2* gene under green light was observed only at 21 °C. It is, therefore, conceivable that the roles of these peptides in mediating the effects of green light are different from each other. MCH1 and MCH2 would be differentially associated with food intake under green light, based on their transcription levels, and the association of MCH1 would be much greater than MCH2. Increase in the gene expression levels in response to green light was also observed in *orx*, *npy*, *pacap,* and *pomc-c* at some but not all water temperatures. Taken together, MCH1 is suggested to be a major hormone that transmits the green light stimulus to fish system to enhance food intake and subsequent somatic growth.

Contrary to the neuropeptide gene expression in brain, no difference was observed in the expression of pituitary hormone genes and plasma profiles of INS and IGF-I under green light. These hormones mediate a variety of functions associated with somatic growth. Firstly, among the members of GH family, GH is the prominent pituitary hormone stimulating growth by direct effect and indirect effect via IGF-I (Björnsson, 1997; Mommsen, 2001; Pérez-Sánchez et al., 2002). PRL has also been shown to stimulate body growth and to be associated with GH-like functions (Nicol, 1993). SL has been shown to be responsible for lipid metabolism (Fukada et al., 2005; Sasano et al., 2012). INS is crucial for anabolic pathway (Andoh, 2007; Navarro, 2006). Melanocortin peptides, derived from a common precursor POMC, inhibit food intake and exhibit lipolytic activities, and endorphin—the other POMC-derived peptide—stimulates food intake (Takahashi and Kawauchi, 2006). However, absence of difference in gene expression under green light suggest that these pituitary hormones and members of INS family may contribute less to the mediation of green light stimulus to somatic growth, compared to the hormones produced by hypothalamus.

With regard to water temperature and PFD of green light, the effects at 12 °C and 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively, are comparable to that observed at 10.2 and 7 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a previous experiment using barfin flounder (Takahashi et al., 2018), when the fish were reared in a dark room. Apparent increase in the expression of *mch1* gene among several brain and pituitary hormone genes was also observed in the barfin flounder under similar green light and water temperature conditions. In the barfin flounder, increase in *mch2* gene expression was also observed. These results support the importance of MCH with regard to growth promoting effects of green light in both spotted halibut and barfin flounder.

4.3. Effects of water temperature on hormone gene expression

The profiles of mRNA content observed in the control groups in experiment 3 indicate that the expression of hormone genes does not change uniformly in response to water temperature in spotted halibut, an ectotherm. Among several genes examined, the expression levels of *prl* constantly increased with the increase in water temperature. Changes in PRL levels have been reported in several ectotherms. In the common carp, *Cyprinus carpio*, *prl* gene expression is enhanced at higher water temperature, which is related to adjustment of fish to seasonal changes (Figueroa et al., 1997). Plasma PRL levels increase in a newt, *Cynops pyrrhogaster*, as the ambient temperature drops; this change is related to spermatogenesis arrest (Yazawa et al., 1999). Plasma PRL concentrations in another newt species, *Triturus carnifex,* rise when the environmental temperature decreases; this change seemed to be associated with the organism's reproductive biology (Mosconi et al., 2002). The most prominent role of PRL in fishes is osmoregulatory adaptation of euryhaline fish to low salinity (Manzon, 2002). Many flounders are euryhaline fish that can survive in low-salinity water or even in freshwater (Hart et al., 1996; Hiroi et al., 1997; Sampail and Bianchini, 2002). Spotted halibut has also been shown to survive in low-salinity water and the percentage of PRL-cell volume to pituitary volume is higher in fish maintained at 4 ppt salinity than control fish (Wada et al., 2004). Therefore, the enhanced *prl* gene expression at higher water temperatures suggests that the low salinity tolerance of spotted halibut was improved via increased PRL production at relatively high temperatures.

In conclusion, we demonstrated that green light promotes somatic growth in the spotted halibut and Japanese flounder, in addition to barfin flounder as we reported previously (Takahashi et al., 2016, 2018). Detailed studies on spotted halibut showed that the growth promoting effects of green light could be observed at a variety of water temperatures. Among the genes of hypothalamo-pituitary system, *mch1* gene emerged as an important endocrine factor associated with increased food intake.

Acknowledgements

The authors thank Mr. Takao Azuhata, Mr. Kazushi Koremura, and Mr. Masamune Endo, Kitasato University, for their technical assistance. This study was supported by grants from the Japan Society for the Promotion of Science to AT (Grant No. 17H01489) and the Project of the NARO Bioriented Technology Research Advancement Institution (the special scheme project on regional developing strategy), Japan to AT (Grant No. 16781428), and from the Sanriku Foundation, Japan to DS (Grant No. SRK-H28-RS002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.11.005.

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