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Effects of Green Wavelength Light on Antioxidant Responses in Olive Flounder *Paralichthys olivaceus* Exposed to Starvation and Re-feeding

Cheol Young Choi [1],* iD , Jin Ah Song [1], Young-Ung Choi[2], Tae Ho Lee[1], Han Seok Ryu[1]

[1]Division of Marine BioScience, National Korea Maritime and Ocean University, Busan 49112, Republic of Korea.
[2]Marine Ecosystem and Biological Research Center, KIOST, Busan 49111, Republic of Korea.

Corresponding Author
Tel.: +82514104756
E-mail: choic@kmou.ac.kr

Abstract

An understanding of responses to antioxidant enzymes in different starvation and re-feeding protocols is critical in the aquaculture of fish. This study investigated the effect of green wavelength (520 nm) on the antioxidant response of the olive flounder, *Paralichthys olivaceus*, exposed to different starvation and re-feeding protocols for 4 weeks. We analyzed mRNA expression of peroxisome proliferator-activated receptor alpha (PPARα) and the levels of plasma glucose, superoxide dismutase (SOD), and catalase (CAT), hydrogen peroxide (H_2O_2), and lipid peroxidation (LPO). Furthermore, we performed immunohistochemistry staining (IHC) to observe the SOD expression in the liver visually. We found that expression and activity of antioxidant enzymes (PPARα, SOD, and CAT), as well as plasma H_2O_2 and LPO levels, increased significantly during the starvation period compared to the fed group. However, antioxidant enzymes and oxidative stress increased during the fasting period were significantly decreased during the re-feeding period, especially in the group irradiated with green light. Also, using IHC staining, we found that green light and different feeding protocols affected the expression of SOD in the liver. These findings show that irradiation with green light was effective at alleviating the increased oxidative stress during starvation and re-feeding periods in olive flounder.

Introduction

Recent studies have reported that light of a particular wavelength can act as an important environmental factor in regulating the endocrine signals of fish in captivity, and can also affect the expression of oxidative stress through regulation of reactive oxygen species (ROS) production (Choi *et al*., 2012; Choi *et al*., 2018). Recently, green LED light irradiation has been reported to reduce oxidative stress in starved fish effectively and has attracted much attention from researchers (Choi *et al*., 2018). Furthermore, a green wavelength has a positive effect on growth and immunity in fish exposed to various environmental stresses (Shin, Lee, & Choi, 2012).

The liver is known to be the organ responsible for energy metabolism and homeostasis (Babaei, Kenari, Hedayati, Sadati, & Meton, 2016). It produces glucose through the decomposition of glycogen (catabolism), the first substrate used as an energy metabolite in fish exposed to changes in feeding environment such as fasting and re-feeding (Mohapatra *et al*., 2015). The peroxisome proliferator-activated receptors (PPARs), which are produced in the liver, are the main transcriptional regulators of antioxidant defense as well as energy homeostasis and are affected by the nutritional status of the organism (Picard & Auwerx, 2002; Rakhshandehroo, Knoch, Muller, & Kersten, 2010). In the nucleus, PPARs are hormone receptors with three subtypes (α, β, and γ) that regulate lipid

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metabolism and energy homeostasis (Griffin, 2015). In particular, PPARα expressed in the liver not only regulates glucose homeostasis through the gluconeogenesis process (Li *et al.*, 2017) but also directly affects antioxidant mechanisms by direct transcriptional control of the major antioxidant enzyme, superoxide dismutase (SOD) (Rakhshandehroo *et al.*, 2010).

Additionally, changes in the feeding environment lead to changes in cell activity in fish and increase the production of ROS that cause oxidative stress (Choi, Choi, Choi, & Yoo, 2018). Representative ROS such as superoxide ($O2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen ($^1O_2$) are overproduced in the fish body, increasing lipid peroxidation (LPO), oxidizing cellular components such as lipid, proteins, and DNA, and inducing apoptosis, eventually negatively affecting cell survival (Kim & Yang, 2013; Regoli & Giuliani, 2014). Thus, fish activate the antioxidant defense mechanism to protect themselves from ROS caused by changes in the feeding environment (Pandey *et al.*, 2003; Yang *et al.*, 2019). Antioxidant enzymes involved in such defense mechanisms are SOD, catalase (CAT), and glutathione peroxidase (GPx) (Regoli & Giuliani, 2014). Firstly, SOD converts O_2^- into O_2 and H_2O_2 ($2O_2^- + H^+ \rightarrow H_2O_2 + O_2$), and the produced toxic material H_2O_2 is decomposed to into non-toxic material H_2O and O_2 by CAT and GPx ($2 H_2O_2 \rightarrow 2 H_2O + O_2$) (Choi, Shin, Choi, & Kim, 2012; Regoli & Giuliani, 2014).

Recently, the aquaculture industry has focused on maintaining production efficiency, reducing feed loss, and reducing adverse environmental impacts through feeding regimes appropriate to fish species (Ali, Martínez-Llorens, Moñino, Cerdá, & Tomás-Vidal, 2016). It is known that re-feeding after a certain period of fasting reduces feed loss and costs and achieves faster growth than that in continuously fed fish (Najafi, Salati, Yavari, & Asadi, 2014; Yang *et al.*, 2019).

In general, fish have hyperphagia when they are refed after fasting (Bar, 2014; Ali *et al.*, 2016). Not only is the insufficient nutritional status due to fasting satisfied through the re-feeding, but the fish also show a higher growth rate than those fed continuously (Won & Borski, 2013). In addition, fish are known to regulate metabolic and energy homeostasis through the liver to adapt to unfavorable and irregular feeding environments such as feed deprivation (Pérez-Jiménez, Guedes, Morales, & Oliva-Teles, 2007). Although previous studies based on unfavorable feeding regimes have been widely reported for aquaculture species, most have investigated hematological and physiological responses and focused on growth index analysis and stress response (Antonopoulou *et al.*, 2013). Therefore, further studies on the regulation of energy metabolism and changes in antioxidant enzymes in fish exposed to different feeding environments, such as fasting and re-feeding, need to be carried out.

Therefore, in this study, we investigated the effect of green wavelength on the expression of antioxidant responses induced by starvation and re-feeding in olive flounder *Paralichthys olivaceus*. Firstly, the flounders were exposed to each experimental environment for 4 weeks, and the PPARα mRNA expression, SOD and CAT plasma levels, as well as H_2O_2, LPO, and glucose concentration in plasma were measured. In addition, SOD molecules were stained in liver cells by immunohistochemical staining (IHC) to observe the expression changes of the antioxidant enzyme SOD visually.

Materials and Methods

Experimental Fish and Sampling

The handling of all experimental samples complied with the Animal Protection Act guidelines issued by the Institutional Animal Care Use Committee of Korea. Juvenile olive flounder *Paralichthys olivaceus* (n = 160, length 11.2 ± 0.6 cm; weight 11.7 ± 1.2 g) were purchased from a commercial aquarium (Gochang, Korea), and fish were subsequently allowed to acclimate in eight 300-L circulation filter tanks prior to laboratory experiments. For the experiment, the tanks were divided into four groups, with two tanks in each group and 25 fish per tank. All experimental fish were exposed to 20°C and 35 psu salinity. The photoperiod consisted of a 12-h light:12-h dark cycle (lights were turned on at 07:00 and turned off at 19:00 each day). During the feeding periods of the experiment, fish were fed twice daily at 09:00 and 17:00 with floating commercial feed containing 50% crude protein (KOFEC Feed, Korea). Five fish from each experimental group were sampled at 1-week intervals over 4 weeks. Prior to blood and tissue collection, fish were anesthetized with 200 μg L^{-1} 2-phenoxyethanol (Daejung Co., Korea) to minimize stress. Blood was collected rapidly from the caudal vein using a 1-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 10,000 × *g*, for 10 min) and stored at -80°C until analysis.

Experimental Conditions

The experimental groups were irradiated with green (520 nm) light-emitting diode (LED) (1.0 W/m^2 intensity, Daesin, LED Co., Korea) or white fluorescent bulb (0.96 W/m^2 intensity, Philips, Netherlands) during 4 weeks at different feeding protocols (Figure 1). Different feeding groups were designed as follows; first group (4F) fed for 4 weeks; the second group (1S-3F) starved 1 week and then refed for 3 weeks; the third group (2S-2F) starved 2 weeks and refed 2 weeks; the fourth group (4S) starved for 4 weeks. Of the above experimental groups, we used 4F as the control group and irradiated with a green LED or white fluorescent bulb. The light sources were placed 50 cm above the water surface, and the depth of the water column was 50 cm. The irradiance levels at the bottom of the tanks were maintained at approximately 1.0 W/m^2 using a spectrometer (MR-16; Rainbow Light Technology,

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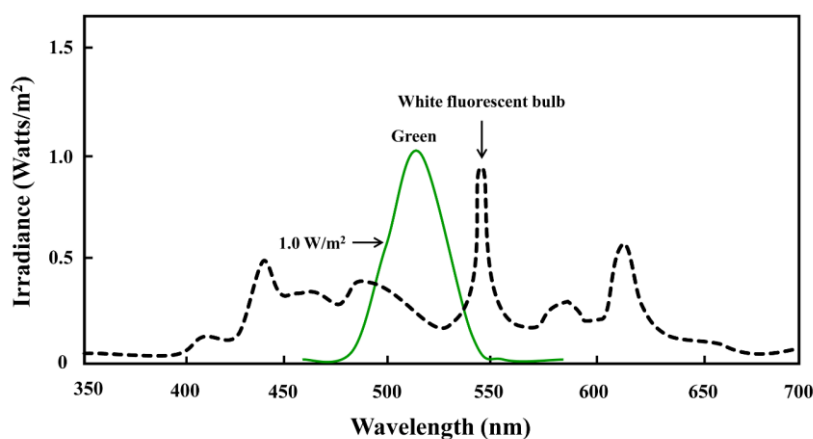*Turk. J. Fish.& Aquat. Sci. 20(9), 659-669*

Figure 1. Spectral profiles of the green (520 nm) LED and the white fluorescent bulb (dotted line) with light intensity of 1.0 W/m².

Taiwan) and a photoradiometer (HD 2102.1; Delta OMH Co., Italy).

Survival Rate and Weight Changes

Survival rates (%) were determined at daily intervals for 28 days and calculated as follows: survival rate (%) = number of fish at the time of evaluation / number of fish at the beginning of the experiment × 100. Weight gain (g/fish) was measured weekly for 28 days calculated as follows: weight gain = final weight-initial weight.

Real-time Quantitative Polymerase Chain Reaction (qPCR)

From each liver sample, total RNA (treated with DNase to remove genomic DNA) was extracted using the TRI Reagent® (Molecular Research Center, Inc., USA) according to the manufacturer's instructions. Then, a 2 μg sample of total RNA was reverse-transcribed in a total reaction volume of 20 μl using an oligo-(dT)$_{15}$ anchor and M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. The relative expression levels of PPARα (GenBank Accession No. HM147767) and β-actin (GenBank Accession No. HQ386788) were determined using qPCR. The primers for qPCR were based on known sequences of olive flounder: PPARα: forward (5'-ACA ATG ATG CCC TCA GCT CT-3') and reverse (5'-AGA ACT CCT CTT GGC CCA TT-3'); and β-actin: forward (5'-GGA CCT GTA TGC CAA CAC-3') and reverse (5'-TGA TCT CCT TCT GCA TCC TG-3'). qPCR amplification was performed using a Bio-Rad CFX96™ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) with the following conditions: 0.5 μl of cDNA, 0.26 μM of each primer, 0.2 mM dNTP, SYBR Green, and Taq polymerase in buffer (10 mM Tris–HCl [pH 9.0], 50 mM KCl, 1.4 mM MgCl$_2$, and 20 nM fluorescein) to a total volume of 25 μl. The qPCR process was carried out as follows: 1 cycle of initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s and annealing at 55°C for

20 s, followed by 5 min at 72°C for the final extension. All analyses were based on the Ct values of PCR products. The calibrated ΔCt value (ΔΔCt) for each sample and internal controls (β-actin) was calculated as $\Delta\Delta Ct = 2^{-(\Delta Ctsample - \Delta Ctinternal\ control)}$.

Plasma Parameter Analysis

Plasma samples were centrifuged at 5,000 × g for 5 min at 4°C. The supernatants were collected carefully, and the remained plasma was used for the analyses. The levels of SOD, CAT, and LPO activities were analyzed using an immunoassay from an ELISA kit (SOD, MBS282055; CAT, MBS705697; LPO, MBS013426; MyBioSource Inc., San Diego, USA), respectively. The absorbance was read at 450 nm using a plate reader. Plasma H$_2$O$_2$ levels were determined using a PeroxiDetect kit (Sigma, USA). Absorbance was read at 560 nm, and H$_2$O$_2$ concentration was interpolated from a standard curve. Plasma glucose levels were analyzed by a dry multiplayer analytic slide method using an automatic biochemical analyzer (Fuji Dri-Chem 4000, Fujifilm, Japan).

Immunohistochemistry Staining

For identification of SOD immunoreactive expression, liver tissues were removed after 4 weeks in each experimental group. Tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature, dehydrated in ethanol, and then xylene. Tissue was embedded in molten paraffin and, after the paraffin solidified, paraffin blocks were kept at 4°C until sectioning. Embedded tissues were sectioned to 6-μm thickness and floated in a 50°C water bath containing distilled water. Sections were mounted onto gelatin-coated slides and incubated overnight at room temperature. The slides were deparaffinized and rehydrated by immersing them in xylene, ethanol, and distilled water. Primary mouse anti-SOD1 antibody (NBP1-47443; 1/1,000 dilution; Novus Biologicals®; USA) diluted in 1% animal serum 1X phosphate-buffered

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saline with Tween® detergent (PBS-T) was added, and slides were incubated at room temperature for 1–2 hours. Afterward, sections were washed twice with 1% serum PBS-T for 10 min each. A biotinylated secondary antibody was added, and slides were incubated at room temperature for 1 hour. After washing each section twice with 1% serum PBS-T for 10 min, ABC-HRP reagent was added and sections were incubated at room temperature for 1 hour. 3,3'-Diaminobenzidine (DAB) working solution was applied to the tissue sections, and the resulting chromogenic reaction was monitored (i.e., epitope sites turned brown). Slides were counterstained with hematoxylin, dehydrated, and mounted with Canada balsam for observation under a light microscope.

Statistical Analysis

All data were analyzed using SPSS version 25.0 (IMB SPSS Inc., USA). All parameters analyzed (i.e., exposure to different concentrations and sampling after different exposure times) were compared using two-way ANOVA. Where significance was indicated, the Tukey post hoc test for multiple comparisons was used. ANOVA assumptions (Levene's test for homogeneity of variances and the Kolmogorov-Smirnov test for data normality) were previously checked. The significance level adopted was 95% ($P < 0.05$). Values are expressed as the means ± standard deviation (SD).

Results

Changes in Body Weight

Survival rate (%) and weight gain (g/fish) of olive flounder during the different feeding regimes are presented in Table 1. The survival rate was not significantly affected by the feeding regime, ranging from 95.3% to 99.1%. Comparison of weight gain showed that 4F (white; 21.7 ± 2.63 g, green; 22.9 ± 2.57 g) and irradiation of green light to 1S-3F (21.8 ± 1.56 g) had significantly higher weight gain compared to the other treatments. After this, the weight gain for IS-3F

with white light was greater than that for 2S-2F, which in turn was greater than 4S.

Changes in Plasma Glucose Levels

Plasma glucose level responses to different feeding regimes and light sources were assessed (Figure 2). There were significant interactive effects between factors ($P < 0.05$). Plasma glucose levels remained constant in 4F, while they decreased in the other groups (1S-3F, 2S-2F, and 4S). In 1S-3F and 2S-2F, plasma glucose levels significantly increased after re-feeding. Moreover, the groups exposed to green light irradiation had higher plasma glucose levels than those exposed to white light.

Changes in PPARα Expression and SOD and CAT Activities

PPARα mRNA expressions in liver tissues, and plasma SOD and CAT activities in response to the starvation and re-feeding periods are shown in Figures 3 to 5. There were significant interactive effects between factors ($P < 0.05$). The 4F group showed no significant differences in mRNA expression of PPARα, or activities of SOD and CAT throughout the experiment. However, the other groups showed significantly increased activity of these enzymes during starvation but significantly decreased activity during re-feeding; also, enzyme activity significantly decreased under green LED light irradiation. In addition, PPARα expression, and the activity of SOD and CAT at 4 weeks, showed that 1S-3F was not significantly different from the 4F, while the other groups were significantly higher.

Immunohistochemistry Staining of SOD During the Experimental Period

We observed the expression of SOD evaluated by immunohistochemistry during the starvation and re-feeding periods (Figure 6). These results showed similar trends to those of plasma SOD activities (Figure 4). The immunohistochemical expression of SOD did not differ

Table 1. Changes in weight gain and survival rate of the olive flounder exposed to 4 different feeding conditions under 2 different light sources for 4 weeks

Treatments	Survival rate (%)	Initial weight (g/fish)	Final weight (g/fish)	Weight gain (g/fish)
4F white	98.4 ± 1.53	11.4 ± 0.91	32.2 ± 3.1	21.7 ± 2.63[a]
4F green	99.1 ± 0.82	11.7 ± 1.13	34.4 ± 3.7	22.9 ± 2.57[a]
1S-3F white	97.8 ± 2.23	11.6 ± 1.01	30.5 ± 3.3	19.2 ± 1.87[b]
1S-3F green	98.1 ± 1.94	11.5 ± 1.07	32.6 ± 2.9	21.8 ± 1.56[a]
2S-2F white	96.4 ± 2.51	11.4 ± 1.05	20.2 ± 2.7	9.8 ± 1.35[c]
2S-2F green	96.8 ± 2.62	11.6 ± 0.98	22.7 ± 3.1	11.1 ± 1.53[c]
4S white	95.3 ± 2.55	11.7 ± 1.17	12.1 ± 1.6	0.5 ± 0.32[d]
4S green	95.7 ± 2.41	11.4 ± 1.03	12.3 ± 1.8	0.7 ± 0.57[d]

The values are mean ± SD (*n*=5). The different lower-case letters indicate significant differences based on exposure to the different light and period ($P < 0.05$).

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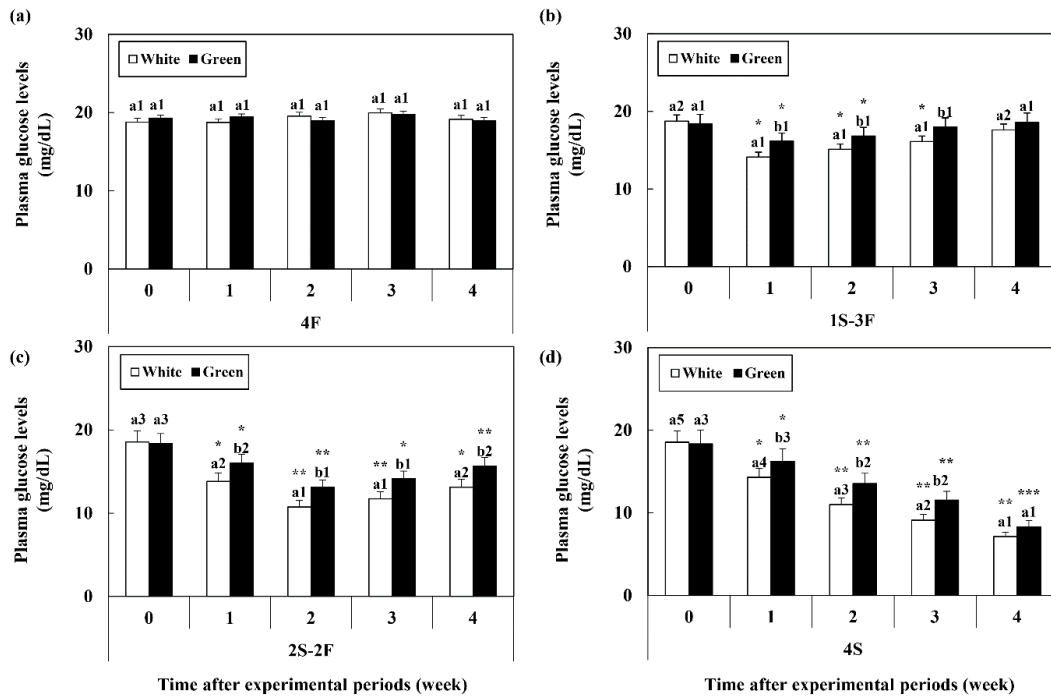*Turk. J. Fish.& Aquat. Sci. 20(9), 659-669*

Figure 2. Plasma glucose levels at different starvation/re-feeding protocols under different types of light. a: 4 weeks fed (4F), b: 1 week starved and 3 weeks fed (1S-3F), c: 2 weeks starved, and 2 weeks fed (2S-2F), d: 4 weeks starved (4S), white: white fluorescent bulb, green: green wavelength LED. Different lower-case letters indicate significant differences between types of light in fish within the same period (P < 0.05). Different numbers indicate significant difference between periods with the same light type (P < 0.05). Asterisk symbols (*) indicate significant differences (P < 0.05) between different starvation/re-feeding periods within the same light type and sampling point. All values are means ± SD (*n* = 5).
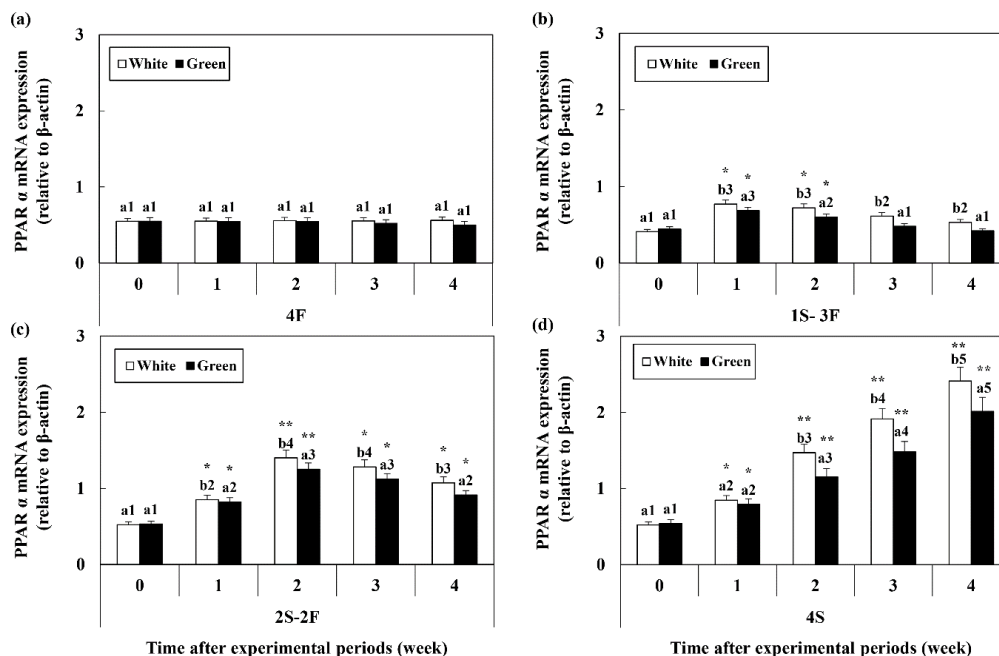

Figure 3. PPARα mRNA expression levels in the liver of olive flounders exposed to different starvation/re-feeding protocols under different types of light. a: 4 weeks fed (4F), b: 1 week starved and 3 weeks fed (1S-3F), c: 2 weeks starved, and 2 weeks fed (2S-2F), d: 4 weeks starved (4S), white: white fluorescent bulb, green: green wavelength LED. Different lower-case letters indicate significant differences between types of light in fish within the same period (P < 0.05). Different numbers indicate significant differences between periods with the same light type (P < 0.05). Asterisk symbols (*) indicate significant differences (P < 0.05) between different starvation/re-feeding periods within the same light type and sampling point. All values are means ± SD (*n* = 5).

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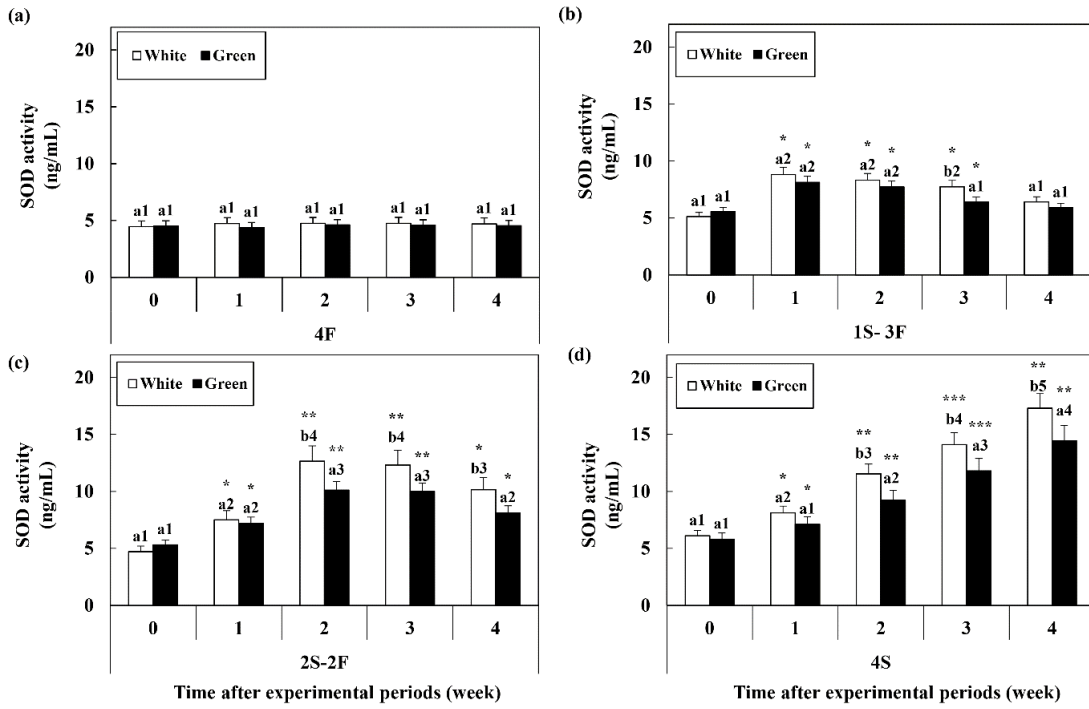*Turk. J. Fish.& Aquat. Sci. 20(9), 659-669*

Figure 4. Plasma SOD levels at different starvation/re-feeding protocols under different types of light. a: 4 weeks fed (4F), b: 1 week starved and 3 weeks fed (1S-3F), c: 2 weeks starved, and 2 weeks fed (2S-2F), d: 4 weeks starved (4S), white: white fluorescent bulb, green: green wavelength LED. Different lower-case letters indicate significant differences between types of light in fish within the same period (P < 0.05). Different numbers indicate significant differences between periods with the same light type (P < 0.05). Asterisk symbols (*) indicate significant differences (P < 0.05) between different starvation/re-feeding periods within the same light type and sampling point. All values are means ± SD (*n* = 5).
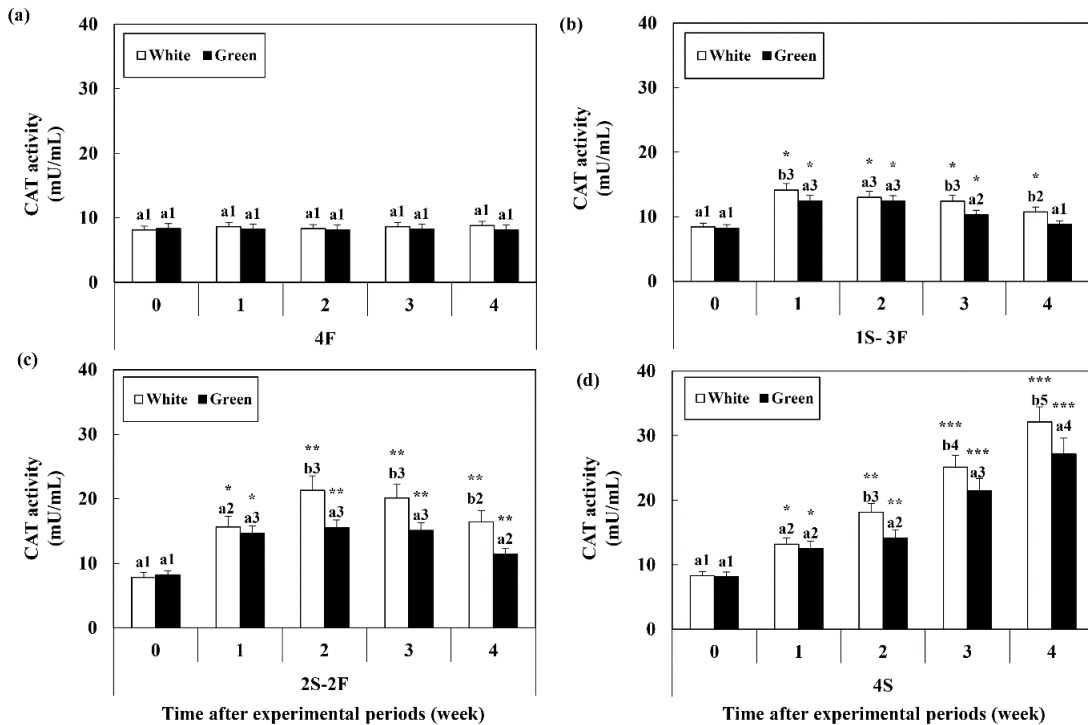

Figure 5. Plasma CAT levels at different starvation/re-feeding protocols under different types of light. a: 4 weeks fed (4F), b: 1 week starved and 3 weeks fed (1S-3F), c: 2 weeks starved, and 2 weeks fed (2S-2F), d: 4 weeks starved (4S), white: white fluorescent bulb, green: green wavelength LED. Different lower-case letters indicate significant differences between types of light in fish within the same period (P < 0.05). Different numbers indicate significant differences between periods with the same light type (P < 0.05). Asterisk symbols (*) indicate significant differences (P < 0.05) between different starvation/re-feeding periods within the same light type and sampling point. All values are means ± SD (*n* = 5).

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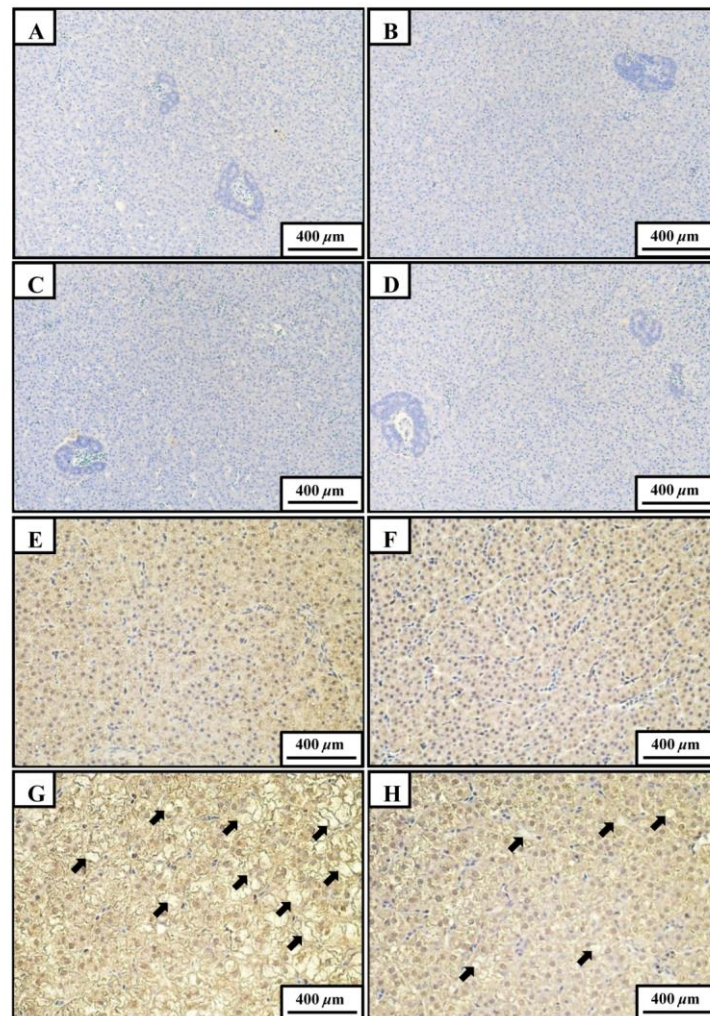
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Figure 6. Localization of superoxide dismutase (SOD) in the liver of olive flounders exposed to different starvation/re-feeding protocols under different types of light. A: After 4 weeks fed under white; B: After 4 weeks fed under green LEDs (green); C: 1 week starved and 3 weeks fed under white; D: 1 week starved and 3 weeks fed under green LEDs (green); E: 2 weeks starved and 2 weeks fed under white; F: 2 weeks starved and 2 weeks fed under green LEDs (green); G: After 4 weeks starved under white; H: After 4 weeks starved under green LEDs (green). The stained cells showing brown color and arrows indicate abnormal hepatic vacuoles. Scale bars (A–H) are 400 μm.

between 4F and 1S-3F, but 2S-2F and 4S showed more immunohistochemical expression of SOD in the liver. Among them, 4S showed the most immunohistochemical expression of SOD, and hepatic vacuoles were also found. The difference due to light source was not observed in 4F and 1S-3F, but the SOD expression in 2S-2F and 4S was lower in the green LED light than in the white light.

Changes in H_2O_2 and LPO Plasma Levels

The levels of H_2O_2 and LPO did not change significantly during experimental periods in the steadily feeding group (4F), while they increased during the starvation period and then decreased during the re-feeding period (Figures 7 and 8). There were significant interactive effects between factors (P < 0.05). However, H_2O_2 and LPO concentrations only returned to initial conditions in 1S-3F and tended to be lower under green LED light than under white light irradiation.

Discussion

In the present study, we investigated the effect of irradiation with white and green light on the plasma glucose and antioxidant responses during starvation and re-feeding periods in olive flounder.

During the experimental period, the weight change of 1S-3F irradiated green light was not significantly different from that of 4F, whereas 1S-3F irradiated with white light and 2S-2F were significantly different from 4F. These results suggest that the 1S-3F group has a weight change depending on the light source even if it has the same 1-week starvation period. In particular, the irradiation with green light meant that, compared with the 4F group, the IS-3F group had recovered from the 1-week starvation after 3-weeks' re-feeding.

Plasma glucose levels, the energy metabolism substrate in the feeding environment, were not significantly different during the experimental period in 4F, but significantly decreased in 4S. Furthermore, the
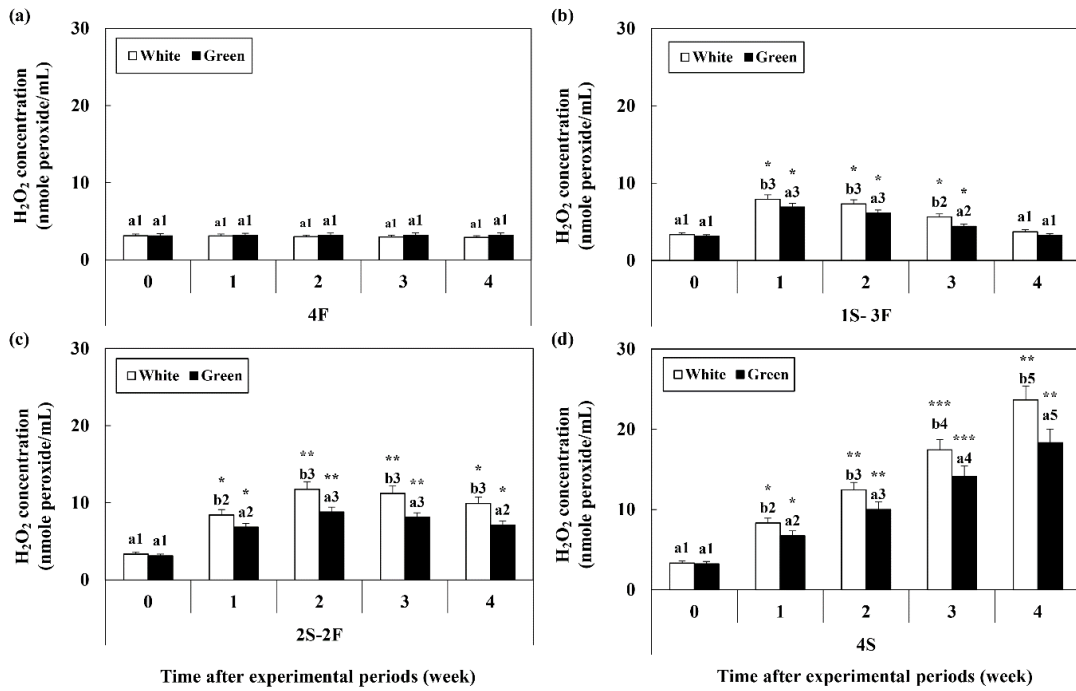
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Figure 7. Concentration of plasma H_2O_2 at different starvation/re-feeding protocols under different types of light. a: 4 weeks fed (4F), b: 1 week starved and 3 weeks fed (1S-3F), c: 2 weeks starved, and 2 weeks fed (2S-2F), d: 4 weeks starved (4S), white: white fluorescent bulb, green: green wavelength LED. The different lower-case letters indicate significant differences based on exposure to the different types of light in fish within the same period (P < 0.05). Different numbers indicate significant differences between periods with the same light type (P < 0.05). Asterisk symbols (*) indicate significant differences (P < 0.05) between different starvation / re-feeding periods within the same light type and sampling point. All values are means ± SD (*n* = 5).
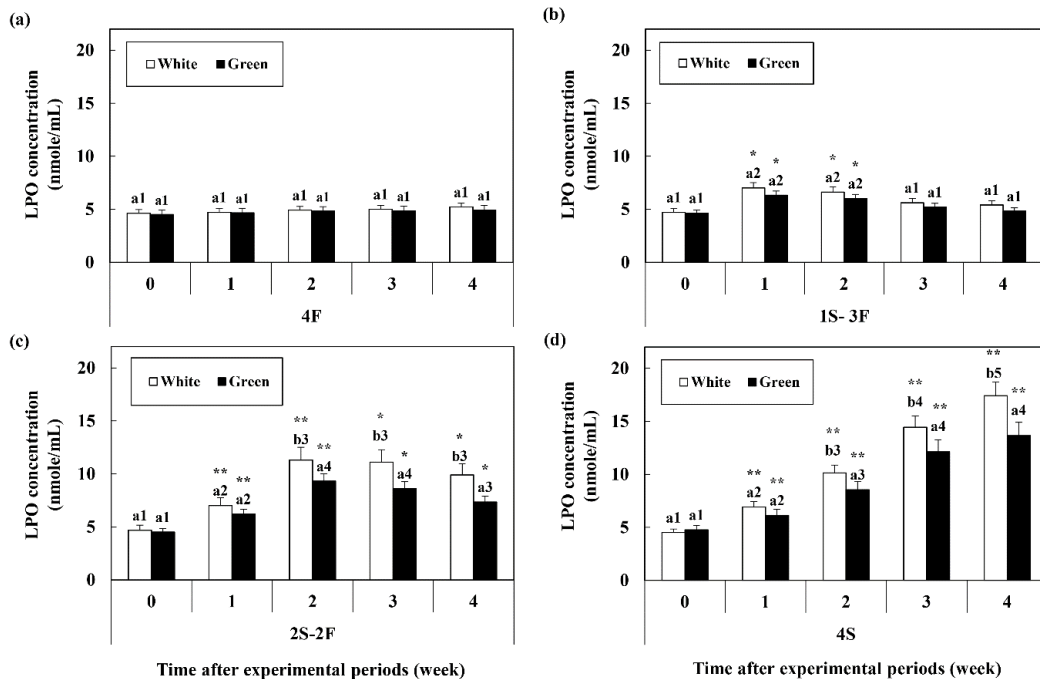

Figure 8. Concentration of plasma lipid peroxidation (LPO) at different starvation / re-feeding protocols under different types of light. a: 4 weeks fed (4F), b: 1 week starved and 3 weeks fed (1S-3F), c: 2 weeks starved, and 2 weeks fed (2S-2F), d: 4 weeks starved (4S), white: white fluorescent bulb, green: green wavelength LED. The different lower-case letters indicate significant differences based on exposure to the different types of light in fish within the same period (P < 0.05). Different numbers indicate significant differences between periods with the same light type (P < 0.05). Asterisk symbols (*) indicate significant differences (P < 0.05) between different starvation/re-feeding periods within the same light type and sampling point. All values are means ± SD (*n* = 5).

glucose concentration significantly decreased in 1S-3F and 2S-2F throughout the starvation period but increased again after re-feeding. The decrease in plasma glucose levels during the starvation period is consistent with most studies in fish (Pérez-Jiménez *et al.*, 2007; Furné *et al.*, 2012). Furné *et al.* (2012) found that for sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss* exposed to 60 days starvation and 72 days re-feeding periods, plasma glucose levels dropped during the starvation period (1 day to 40 days) but increased during the 10-day re-feeding period.

The results of this study and previous studies suggest that fish exposed to fasting have a limited glycogenolysis process owing to insufficient glycogen in the body (Pérez-Jiménez *et al.*, 2007) and that this also causes a decrease in glucose and an imbalance of energy homeostasis. However, during re-feeding after the starvation process, the energy source is recovered by increasing glucose levels. Choi *et al.* (2018) reported that, after 9-day fasting, stress increase in flounder was alleviated by green wavelength irradiation compared to blue and red wavelengths. It is established that fish generally perceive light through the eyes and pineal organ and can recognize wavelengths or light colors. Song *et al.* (2016) reported that the retina of fish recognizes green spectra as a stable environment that can reduce stress in the body. Therefore, green wavelength irradiation reduces the stress caused by fasting and the body glucose requirement, causing glucose concentration in the blood to be higher than under other wavelength lights.

PPARαg mRNA expression was analyzed in each group. Expression levels significantly increased throughout the starvation period and decreased again after re-feeding. Furthermore, PPARα mRNA expression was significantly lower under green light irradiation than under white light irradiation. Morash and McClelland (2011) analyzed PPARα mRNA expression in the liver of rainbow trout after starvation for 5 weeks. They found that PPARα mRNA expression was significantly higher in the starvation group than in the feeding group. The high expression of PPARα in the fasting state appeared to be caused by the role of PPARα in regulating gluconeogenesis to control insufficient energy homeostasis in the body (Morash & McClelland, 2011). Therefore, considering that PPARα is increased in order to increase the insufficient glucose concentration caused by the starvation environment, PPARα is a key sensor for metabolic adaptation and maintenance of energy homeostasis. However, PPARα expression was significantly lower under green wavelength irradiation, which is considered to be caused by a stress reduction.

Antioxidant enzymes, SOD and CAT mRNA, were analyzed to assess their activity in response to starvation stress. It was found that the expression levels of these antioxidant enzymes significantly increased throughout the starvation period and decreased again after re-feeding. Moreover, antioxidant enzyme expression was significantly lower under the green wavelength than under the other light source.

Babaei *et al.* (2016) showed significantly higher SOD and CAT activities in the fasting experimental group than in the feeding experimental group of Siberian sturgeon *Acipenser baerii* after a 2-week fasting period, but SOD and CAT expression levels significantly decreased on re-feeding. Choi *et al.* (2018) reported that the expression level of antioxidant enzymes in flounder decreased only under green wavelength irradiation after fasting for 9 days.

In this study, IHC staining was performed to determine differences in the expression of SOD in the liver. During the starvation period, a large amount of SOD stained with brown colors was observed. However, brown stained expressions of SOD were remarkably lower in the experimental group irradiated with green wavelength than in the group under fluorescent lamps. In particular, in the liver cells of 4S, the formation of abnormal hepatic vacuoles was observed when the nutrients and growth factors were insufficient. However, the size and number of vacuoles and the expression of SOD decreased significantly in the liver of the 4S experimental group under green wavelength irradiation.

Mohapatra *et al.* (2015) observed through IHC staining that red sea bream *Pagrus major* starved for 10 days presented a larger amount of SOD than the control. These results suggest that starvation not only induced abnormal vacuoles in liver cells but also induces apoptosis. Subsequently, the SOD mRNA expression and plasma level increased are consistent with the increase of immunoreactive cells, suggesting that SOD increases in the liver are part of the antioxidant coping mechanism. In this study, the expression level of antioxidant enzymes in the liver changed depending on the feeding environment. Additionally, green wavelengths seemed to reduce the expression of SOD, the antioxidant enzyme in the liver, by alleviating the stress of fish exposed to the starvation environment. Therefore, if green wavelength irradiation is administered to fish exposed to the starvation environment, it will be possible to reduce levels of antioxidant enzymes and stress caused by the starvation environment.

Like the antioxidant enzymes, the concentration of H_2O_2, which is one type of ROS, and LPO, a type of oxidative stress, increased during the starvation period. However, H_2O_2 and LPO concentrations decreased under green wavelength irradiation. Robinson *et al.* (1997) reported that the starvation environment induced reactive oxygen in the body, which not only damaged the nucleic acid but also caused many physiological disorders. Choi *et al.* (2012) confirmed the effect of various light sources on ROS and LPO produced by starvation. In their study, cinnamon clownfish increased the LPO concentration by increased H_2O_2 production during a 12-day starvation period, however, green wavelength irradiation caused a decrease in H_2O_2

and LPO concentration compared to other wavelengths. Therefore, starvation seems to act as a pro-oxidative effect by increasing H_2O_2 and LPO concentration in the body and increases reactive oxygen species and oxidative stress, which can then be reduced by re-feeding. Green wavelengths can positively control starvation-induced antioxidant responses, and this suggests that starvation-induced oxidative stress can be suppressed, and ROS production can be reduced.

The results of this study suggest that starvation caused oxidative stress in the juvenile flounder, but this oxidative stress was reduced by re-feeding. Additionally, green wavelength irradiation inhibited starvation-induced oxidative stress. Moreover, it promoted fish recovery from lowered antioxidant ability by inducing a faster reduction of oxidative stress after re-feeding than occurred in fish under a white light source. In conclusion, the fish experience extreme stress as the starvation period increases. Although the stress increased during long-term starvation, it was confirmed that the oxidative stress and ROS were reduced by the green wavelength and that this effect was enhanced as the exposure period for the green wavelength increased. Further studies are needed to assess the effects of longer starvation and re-feeding periods in various developmental stages, so the changes in the physiological responses of this species can be assessed in more detail.

Conclusion

In this study, when starvation and re-feeding in four different feeding regimes were performed with irradiation by white or green light, it was confirmed that oxidative stress was induced due to fasting in all experimental groups. However, the experimental group irradiated with a green wavelength expressed less oxidative stress than the group irradiated with white light. In addition, it suggests the possibility of being able to use green light irradiation to reduce stress generated through an unintended fasting period due to external changes in the aquaculture environment. This result suggests that green light it will help to produce healthier fish.

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