Biological Rhythm Research

ISSN: 0929-1016 (Print) 1744-4179 (Online) Journal homepage: http://www.tandfonline.com/loi/nbrr20

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To cite this article: Cheol Young Choi, Tae Hwan Kim, Young-Hwan Oh, Tae-Sun Min, Ji Yong Choi & Jin Ah Song (2018): Effects of various LED light spectra on circadian rhythm during starvation in the olive flounder (Paralichthys olivaceus), Biological Rhythm Research, DOI: 10.1080/09291016.2018.1447331

To link to this article: https://doi.org/10.1080/09291016.2018.1447331

Published online: 05 Mar 2018.

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BIOLOGICAL RHYTHM RESEARCH, 2018
https://doi.org/10.1080/09291016.2018.1447331

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Effects of various LED light spectra on circadian rhythm during starvation in the olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

In aquaculture, feeding is essential for the maintenance of metabolic processes and homoeostasis of fish. However, fasting acts as a stressor. In this study, we investigated the effect of circadian rhythm under various LED wavelengths [blue (460 nm), green (520 nm) and red (630 nm)] and two light intensities (0.3 and 0.6 W m^{-2}) over a 9-days period in the olive flounder (*Paralichthys olivaceus*). We analysed clock genes like period 2 (Per 2) and cryptochrome 1 (Cry 1), and serotonin and arylalkylamine-*N*-acetyltransferase 2 (AANAT 2), which control circadian rhythms. Per 2, Cry 1, serotonin and AANAT 2 were significantly decreased during the starvation period compared to the normal feeding group. Nevertheless, their levels increased in the groups exposed to green- and blue LED light during the experimental period. These results confirmed that green and blue wavelengths are effective in maintaining the circadian rhythm in olive flounder.

ARTICLE HISTORY
Received 27 February 2018
Accepted 1 March 2018

KEYWORDS
Arylalkylamine-*N*-acetyltransferase 2; circadian rhythm; cryptochrome 1; period 2; melatonin; serotonin

1. Introduction

In the fish body, the energy and nutrients obtained through food intake play important roles in various metabolic processes and in homoeostasis (Lall and Tibbetts 2009; Feng et al. 2011; Hixson 2014). Therefore, fasting may have negative effects on fish metabolism and physiology (Shan et al. 2008; Choi et al. 2012).

Starvation is also a stressor and adversely affects the 24-h (h) circadian rhythms of various organisms, including fish (Winberg et al. 1992; Sanchez and Sanchez-Vazquez 2009). Circadian rhythm is controlled by clock genes and governs biochemical, behavioural and physiological functions recurring at regular intervals (King and Takahashi 2000; Simensen et al. 2000), Light is one of the most important factors controlling circadian rhythm (Pierce et al. 2008; Choi et al. 2016). Light regulates pineal gland stimulation and is transmitted to the hypothalamus via retinal photoreceptors (Gothilf et al. 1999). In recent years, light-emitting diodes (LEDs), which only produce specific wavelengths, have been used extensively in research (Villamizar et al. 2009; Kim et al. 2016; Yuan et al. 2017). Certain studies have tested particular light

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wavelengths on fish maturation, immunity, stress reduction and the regulation of circadian rhythms (Kim et al. 2014; Jung et al. 2016; Takahashi et al. 2016).

Period 2 (Per 2) and cryptochrome 1 (Cry 1) are known to be representative clock genes (Klein 2007; Yúfera et al. 2017). The hormone Per 2 is rapidly induced in the suprachiasmatic nucleus (SCN) by retinal optical signals. It regulates circadian rhythm by resetting the biological clock and is highly expressed during the daytime (Delaunay et al. 2000; Vallone et al. 2004). Cry 1, a key regulatory protein in the circadian rhythm system, is rapidly induced by light and decreases at night (Besharse et al. 2004).

In addition to clock genes, 5-hydroxytryptamine (serotonin) and arylalkylamine-*N*-acetyltransferase (AANAT) regulate the circadian rhythm (Kordon et al. 1980; Klein 2007; Shin et al. 2014). They act as neuroendocrine transporters (Reiter 1991). Serotonin is a melatonin precursor, expresses strongly at night and regulates circadian rhythm (Coon et al. 1999; Iuvone et al. 2005). Serotonin induces cortisol secretion by regulating the hypothalamic-pituitary-interrenal (HPI) axis. It also plays a role in stress reduction in the fish body (Lim et al. 2013). AANAT is a "rate-limiting enzyme" secreted mainly from the pineal gland and retina. It controls melatonin synthesis (Arendt 1998; Klein et al. 2002). AANAT is synthesised mainly at night. In most teleosts, AANAT 1 is expressed in the retina, and AANAT 2 is expressed in the pineal gland (Isorna et al. 2006). AANAT 2 is a core molecular oscillator and has a strong influence on circadian rhythm (Ben-Moshe et al. 2014). It induces the synthesis of the night hormone melatonin from serotonin (Coon et al. 1999).

The objective of this study was to investigate the effects of specific light wavelengths on the regulation of circadian rhythm in the olive flounder (*Paralichthys olivaceus*) subjected to starvation stress. Fish were starved for 3, 6 or 9 days, and exposed to various light wavelengths (white fluorescent light, 27 W; blue, 450 nm; green, 530 nm; red 630 nm) and intensities (0.3 and 0.6 W m^{-2}). Changes in the expression levels of Per 2, Cry 1, serotonin and AANAT in response to these stimuli were measured.

2. Materials and methods

2.1. Experimental fish and conditions

Olive flounder juveniles ($n = 160$, length 12.3 ± 0.6 cm; mass 16.2 ± 0.5 g) were purchased from a commercial aquarium (Jeju, Korea). They were kept in eight 300-L circulation filter tanks in a laboratory and allowed to acclimate for 2 wks.

The fish were divided into control (normal feeding) and experimental (starvation condition) groups. The fish in the control group were exposed to 20-W white fluorescent light (Philips, Amsterdam, The Netherlands; light intensity at the water surface ~0.96 W m^{-2}). They were fed a commercial feed twice daily. The fish in the experimental groups were exposed to various light spectra [white fluorescent light, 0.96 W m^{-2}; blue (460 nm); green (520 nm) and red (630 nm)] (Figure 1) at two different intensities (0.3 and 0.6 W m^{-2}) for 9 days without feeding (starvation treatment).

The water temperature was 20 ± 1 °C and the photoperiod was 12 h light (L): 12 h dark (D) cycle (lights on at 07:00 h and off at 19:00 h). The light sources were placed 50 cm above the water surface. The water depth was 50 cm. Accounting for external light interception, the irradiance levels at the tank bottoms were maintained at ~0.3 and ~0.6 W m^{-2} using a spectrometer (MR-16; Rainbow Light Technology Co. Ltd., Taoyuan, Taiwan) and the HD

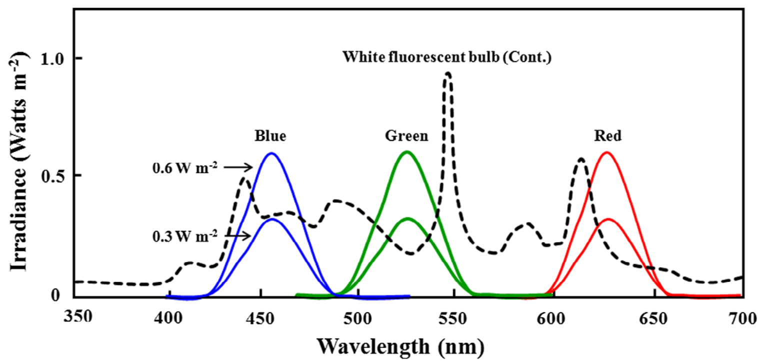

Figure 1. Spectral profiles of light-emitting diodes (LEDs; blue, 450 nm; green, 520 nm; red, 640 nm) and a white fluorescent bulb (Cont.) used in the present study. Two different intensities were used (0.3 and 0.6 W m^{-2}) in each LED treatment.

2102.1 Photo-Radiometer (Delta OMH Co., Caselle di Selvazzano, Italy). Five fish were sampled from each experimental group at 14:00 h on 0, 3, 6 and 9 days. The fish were anaesthetised to minimise stress during the collection of brain and blood samples and these samples were stored at −80 °C until analysis.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from each sample using TRI Reagent® (Molecular Research Center Inc., Cincinnati, OH, U.S.A) according to the manufacturer's instructions. Two micrograms of total RNA were reverse-transcribed in a total reaction volume of 20 µL using an oligo-$(dT)_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A) according to the manufacturer's protocol. The cDNA product was diluted and stored at 4 °C for use in PCR- and quantitative real-time PCR (qRT-PCR) analyses.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

A qRT-PCR analysis was conducted to determine the relative expression levels of Per 2 and Cry 1 associated with the circadian rhythm gene using the total RNA extracted from the olive flounder brain.

The qRT-PCR primers were designed using known olive flounder sequences (Table 1). The qRT-PCR amplification was run using a Bio-Rad iCycler iQ multicolour real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A) and the iQ SYBR green supermix

Table 1. Primers used for qRT-PCR amplification.

Gene (accession no.)	Primer	DNA sequences
Per 2 (**AB729074**)	Forward	5′-GTA CCT AGA GAG TTG TAA CAC C-3′
	Reverse	5′-GTC ATC GTT GGA GGA GTT-3′
Cry 1 (**LC145811**)	Forward	5′-CTA CAC CGA TGC CAC CAA-3′
	Reverse	5′-CGG TCC CAG GGT ATC ATG-3′
β-actin (**HQ386788**)	Forward	5′-GGA CCT GTA TGC CAA CAC TG-3′
	Reverse	5′-TGA TCT CCT TCT GCA TCC TG-3′

(Bio-Rad Laboratories Inc., Hercules, CA, U.S.A) following the manufacturer's instructions. As a control, the β-actin gene was also amplified for each sample. All data were expressed as the difference from the corresponding calculated β-actin threshold cycle (Ct) levels. The Ct values of the PCR products formed the basis for all analyses. They were defined as the PCR cycle in which the fluorescence signal crossed a threshold during the exponential phase of the amplification curve. The calibrated ΔCt value (ΔΔCt) per sample and the internal control (β-actin) were calculated as follows: [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{sample} - \Delta Ct_{internal\ control})$]. After the PCRs were completed, the qRT-PCR data from three replicate samples were analysed using the Bio-Rad system to estimate the transcript copy numbers for each sample.

2.4. Western blot analysis

The total protein content of the olive flounder brain and livers was extracted using a T-PER® tissue protein extraction reagent (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A) following the manufacturer's instructions. Thirty micrograms of protein were loaded per lane into Mini-PROTEAN® TGX™ gels (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A) and a protein ladder (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A) was used as a reference. The samples were electrophoresed at 180 V and immediately transferred to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A) at 85 V for 3 min using the Trans-Blot® Turbo™ transfer system (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A). The membranes were blocked with 5% milk in Tris-buffered saline buffer (pH 7.4) for 45 min then washed in pure Tris-buffered saline buffer. Membranes were incubated with AANAT 2 antibodies (dilution 1:4000, AP32681SU-N, Acris Antibodies Inc., San Diego, CA, U.S.A) then incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (AANAT 2; dilution 1:4000, Bio-Rad Laboratories Inc., Hercules, CA, U.S.A) for 60 min. β-tubulin (dilution 1:4000, ab6046, Abcam, Cambridge, U.K.) was used as an internal control. Bands were detected using WesternBright™ ECL (Advansta, Menlo Park, CA, U.S.A) and 30 s exposure with a Molecular Imager® from ChemiDoc™ XRS Systems (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A). The membrane images were scanned using a high-resolution scanner and the band densities were estimated using Image Lab™ v. 3.0 (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A).

2.5. Plasma parameter analysis

Plasma was separated from whole blood by centrifugation (4 °C, 1000 ×*g*, 20 min). The plasma was collected by coagulation with heparin. Samples were immediately stored at −20 °C. The levels of Per 2, Cry 1, serotonin and AANAT 2 were determined by immunoassay with an ELISA kit (Per 2, MBS108495; Cry 1, MBS041774; serotonin, MBS9368536; AANAT 2, MBS021281) from MyBioSource Inc., San Diego, CA, U.S.A).

2.6. Statistical analysis

All data were analysed using SPSS v. 19.0 (IBM Corp., Armonk, NY, U.S.A). A one-way ANOVA and Tukey's *post hoc* test were used to compare differences in the data ($p < 0.05$). Values are expressed as mean ± standard error (SE).

3. Results

3.1. Changes in Per 2 mRNA expressions and plasma levels

The mRNA levels and the expression of the circadian rhythm gene Per 2 were measured in olive flounder subjected to starvation and various light spectra over a 9-days period (Figure 2). Over time, Per 2 mRNA expression (Figure 2(A)) and plasma levels (Figure 2(B)) significantly decreased in the experimental group. Those exposed to green LED had significantly higher mRNA expression- and plasma levels than the other LED groups. In contrast, those subjected to red LED had significantly lower mRNA expression-, plasma- and Per 2 levels than the control groups. However, the Per 2 levels increased with light intensity.

3.2. Changes in Cry 1 mRNA, protein expression and plasma level

This study investigated changes in hepatic Cry 1 mRNA expression and levels following starvation (Figure 3). Cry 1 mRNA expression (Figure 3(A)) and levels (Figure 3(B)) were

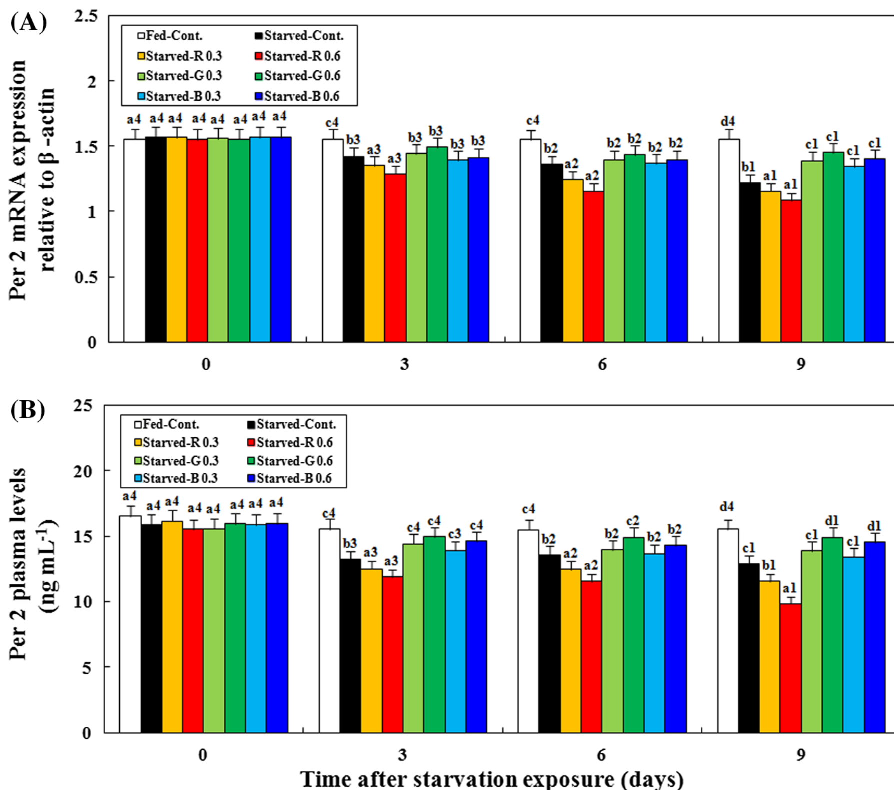

Figure 2. Changes in the mRNA expression (A) and level (B) of Per 2 during a 9-days starvation period under different light wavelengths [red LED (R), green LED (G) and blue LED (B)] at two different intensities (0.3 and 0.6 W m^{-2}) and a white fluorescent bulb (Cont.) as measured by an enzyme-linked immunoassay (ELISA) kit and quantitative real-time PCR (qRT-PCR).

Notes: Total liver RNA (2 μg) was reverse-transcribed and amplified. Results are expressed as normalised fold expression levels with respect to the β-actin levels in each sample. Lowercase letters indicate significant differences between LED spectra within the same starvation period ($p < 0.05$). Values with numbers are significantly different within the same LED spectra during the starvation period ($p < 0.05$). All values are means ± SE ($n = 5$).

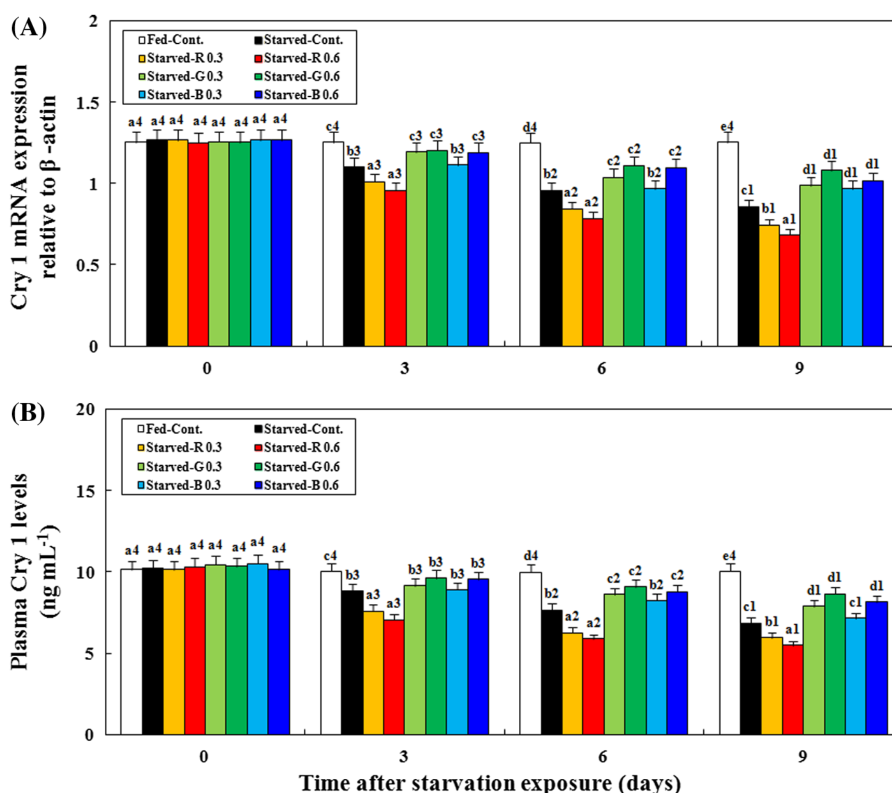

Figure 3. Changes in the Cry 1 mRNA expression (A) and plasma levels in olive flounder exposed to various light sources [red LED (R), green LED (G) and blue LED (B)] at different light intensities (0.3 and 0.6 W m^{-2}) and a white fluorescent bulb (Cont.) as measured by an enzyme-linked immunoassay (ELISA) kit.
Notes: Lowercase letters indicate significant differences between LED spectra within the same starvation period ($p < 0.05$). Values with numbers are significantly different within the same LED spectra during the starvation period ($p < 0.05$). All values are means ± SE ($n = 5$).

significantly lower in the groups subjected to 9 days starvation that in the feeding groups. In addition, the Cry 1 mRNA expression and levels were significantly higher in the group exposed to green LED than the control.

3.3. Changes in plasma serotonin levels

The plasma serotonin levels in the starved fish groups were significantly lower than those of the feeding fish (Figure 4). The serotonin levels in starved fish exposed to green or blue LED were significantly higher than those of the control, and this difference increased with light intensity. However, compared to the controls, the plasma serotonin levels significantly decreased in the groups irradiated with red LED.

3.4. Changes in AANAT of protein expression and plasma levels

The AANAT protein expression and plasma levels in the starvation groups were significantly lower than those in the feeding groups (Figure 5). Western blot analysis revealed a protein

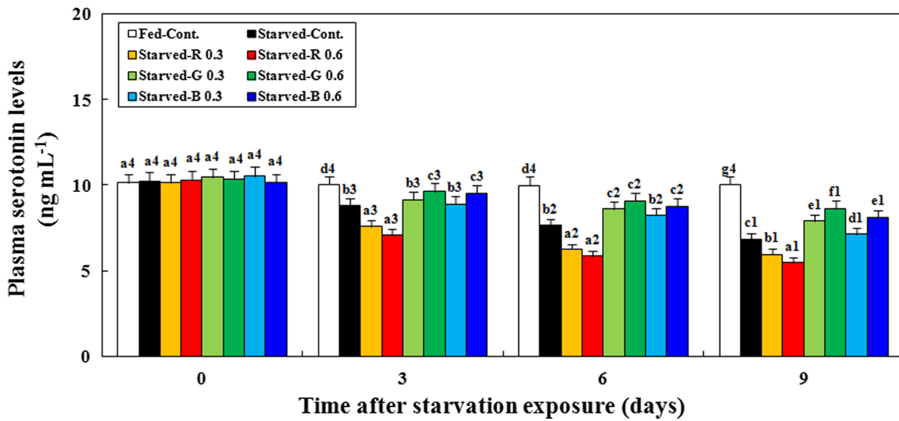

Figure 4. Changes in serotonin levels during a 9-days starvation period under different light wavelengths [red LED (R), green LED (G) and blue LED (B)] at two different intensities (0.3 and 0.6 W m^{-2}) and a white fluorescent bulb (Cont.) as measured by an enzyme-linked immunoassay (ELISA) kit.

Notes: Lowercase letters indicate significant differences between LED spectra within the same starvation period ($p < 0.05$). Values with numbers are significantly different within the same LED spectra during the starvation period ($p < 0.05$). All values are means ± SE ($n = 5$).

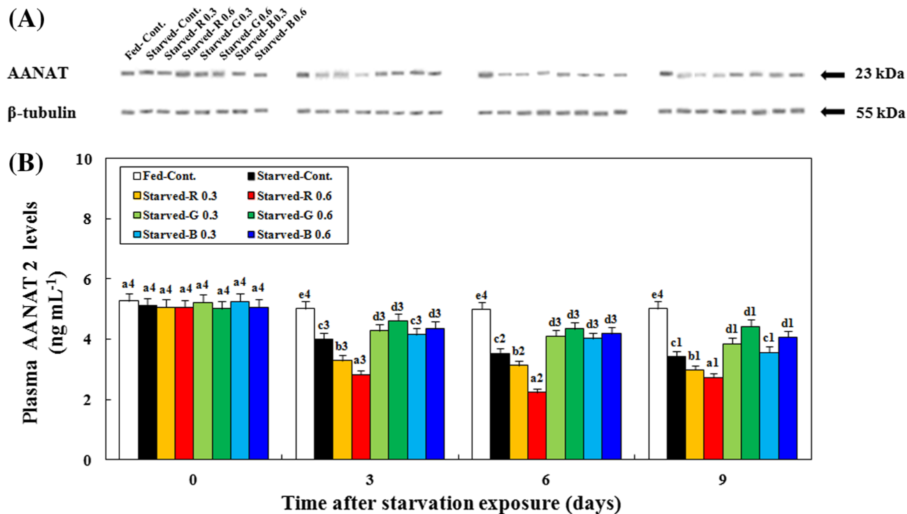

Figure 5. Changes in AANAT 2 protein expression (A) and levels (B) in the liver tissue of olive flounder exposed to various light sources [red LED (R), green LED (G) and blue LED (B)] at different light intensities (0.3 and 0.6 W m^{-2}) and to a white fluorescent bulb (Cont.) as measured by a microplate reader.

Notes: Lowercase letters indicate significant differences between LED spectra within the same starvation period ($p < 0.05$). Values with numbers are significantly different within the same LED spectra during the starvation period ($p < 0.05$). All values are means ± SE ($n = 5$).

with AANAT enzyme-specific immunoreactivity and a mass corresponding to that predicted for olive flounder AANAT (23 kDa; Figure 5(A)). The AANAT protein expression and plasma levels in the groups exposed to green- or blue LEDs were significantly higher than those of the control groups. Specifically, they were significantly higher in the groups exposed to

green LED than in those irradiated with blue- or red LED. However, during the starvation period, the AANAT levels and mRNA expression in the groups irradiated with red LEDs were lower than those in the groups irradiated with white fluorescent light.

4. Discussion

In this study, the effects of light wavelength on the regulation of olive flounder circadian rhythm were determined by subjecting the fish to various light wavelengths (white fluorescent light, red LED, green LED and blue LED) and intensity (0.3 and 0.6 W m^{-2}).

Per 2 mRNA expression and plasma concentration levels were measured to identify changes in the circadian rhythm of olive flounder subjected to starvation. The expression of Per 2 mRNA and plasma concentration in the starvation groups was significantly lower than those in the feeding groups. Per 2 mRNA expression and plasma concentrations decreased with starvation time. The Per 2 mRNA expression and plasma concentrations were significantly higher in fish exposed to green and blue LED than in those exposed to white fluorescent light. In addition, higher Per 2 mRNA expression and plasma concentrations were observed at 0.6 W m^{-2} compared to 0.3 W m^{-2}.

Jung et al. (2016) reported that the expression of Per 2 mRNA decreased when goldfish (*Carassius auratus*) were exposed to high concentrations (0.5 mg L^{-1}) of ammonia. Nevertheless, the Per 2 mRNA expression level significantly increased in the experimental group exposed to green light. The expression of Per 1 mRNA, a circadian rhythm-related gene, was significantly reduced by 24 h in zebrafish (*Danio rerio*) stressed by a high-calorie diet (Stankiewicz et al. 2017).

The present study corroborated the conclusion of previous reports that starvation significantly decreased Per 2 mRNA expression and plasma concentration in olive flounder. In other words, the starvation state was a stressor in the olive flounder and had a negative effect on maintaining a constant circadian rhythm. Per 2 mRNA expression and plasma concentrations were significantly higher in the short-wavelength experimental groups (green and blue LED) than in the longer wavelength groups (red LED). Therefore, shorter light wavelengths may compensate for the disturbances in biorhythm caused by starvation stress. One reason that shorter wavelengths are more effective at restoring circadian rhythms than longer ones is the light intensities of the former are higher than the latter.

In this study, Cry 1 mRNA expression and plasma concentrations significantly decreased over 9 days starvation. Relative to the control, Cry 1 mRNA and plasma levels significantly increased in starving olive flounder exposed to green- and blue LED but were significantly lower in those exposed to red LED than in those under white fluorescent illumination.

Sanchez and Sanchez-Vazquez (2009) fasted zebrafish for 5 weeks and analysed changes in their 24-h biorhythms. They found that the Cry 1 mRNA expression levels were significantly lower in the starvation group than the control.

In the present study, it was found that starvation in olive flounder was a stressor with a negative effect on the maintenance of a constant circadian rhythm. However, irradiation with short wavelengths (green and blue) restored the olive flounder circadian rhythm and increased Cry 1 mRNA expression and plasma concentrations.

Serotonin, AANAT 2, Per 2 and Cry 1 are well-known as circadian rhythm control substances. Plasma serotonin levels significantly decreased in the experimental group subjected to starvation. Serotonin concentration significantly increased in the fasting group exposed

to green and blue light. However, it significantly decreased in the fasting group exposed to red LED.

According to Winberg et al. (1992), when arctic char (*Salvelinus alpines*) fasted for 4 weeks, their plasma serotonin concentrations significantly decreased relative to those of the feeding group. In addition, the serotonin concentrations in goldfish (*Carassius auratus*) were significantly higher in the experimental group irradiated with green LED wavelength than in those exposed to white, red or blue light (Song et al. 2015).

In the present study, plasma serotonin concentrations significantly decreased in olive flounder subjected to starvation. In the fasting group irradiated with short-wavelength light (green and blue), the plasma serotonin concentrations slightly increased and the circadian rhythms were partially recovered.

The effects of starvation on the expression and plasma concentrations of AANAT 2 protein in olive flounder were also investigated. Plasma AANAT 2 in starving olive flounder decreased with time. However, green and blue wavelengths significantly increased plasma AANAT 2 in the starvation groups, and this increase was significantly greater at 0.3 W m^{-2} than at 0.6 W m^{-2}.

Kim et al. (2017) reported that when red seabream (*Pagrus major*) was subjected to stress from exposures to high copper concentrations (>30 μg L^{-1}) for 120 h, their plasma AANAT 2 concentrations significantly decreased relative to those in untreated controls.

In the present and previous studies, starvation stress significantly reduced AANAT 2 protein expression and plasma concentration in olive flounder compared with feeding controls. However, green and blue light irradiation mitigated these decreases. These results suggest that shorter wavelengths ameliorate the effects of starvation stress and maintain a constant circadian rhythm.

The results of this study showed that starvation was a stress factor in olive flounder and significantly lowered their levels of hormones and proteins related to circadian rhythm. Therefore, starvation adversely affects physiological functions like stress control and homoeostasis in olive flounder. However, short-wavelength irradiation mitigated the effects of starvation stress and helped restore a steady circadian rhythm.

Disclosure statement

The authors report no conflicts of interest.

Funding

This research was supported by the project titled "Development and commercialization of high density low temperature plasma based seawater sterilization pulification system", and "Long-term change of structure and function in marine ecosystems of Korea", funded by the Ministry of Oceans and Fisheries, Korea.

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