Distribution of Carotenoids and Protective Effects of Zeaxanthin on Retina of Ayu Sweetfish (Plecoglossus altivelis)

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Abstract: Ayu sweetfish (Plecoglossus altivelis) is a diurnal freshwater fish that are surface swimmers and active under broad and short wavelength-dominated light. Biochemical analyses have shown that the ayu fish have abundant carotenoids including zeaxanthin in their integuments. Although zeaxanthin plays an important role in the physiological function of the retina, the amount and location of zeaxanthin in the ayu eye have not been accurately determined. In this study, circular dichroism spectral data and chiral high-performance liquid chromatography analysis showed that zeaxanthin was the primary carotenoid in the ayu eye, and the eye had the highest carotenoid content compared to those in the integuments, subcutaneous fat, and digestive tract. Interestingly, zeaxanthin in the ayu eyeball was expressed in the photoreceptor layer and near the retinal pigmented epithelium. In vitro assays showed that zeaxanthin could protect photoreceptors and retinal pigmented epithelial cell lines against the oxidative stress induced by exposure to L-buthionine-(S,R)-sulfoximine/glutamate. These findings indicate that zeaxanthin plays protective roles against oxidative stress in the vision of wild ayu.

Key words: ayu, photoreceptor, retinal pigmented epithelium, sweetfish, zeaxanthin

1 Introduction
The “Ayu of the Nagara River System” was designated as a Globally Important Agricultural Heritage System in December 2015. Since the Nagara River supports the livelihood of 860,000 people along its basin, extensive environmental efforts have been exerted to preserve the river. Ayu (Plecoglossus altivelis), commonly known as sweetfish, are raised and nurtured in these waters, and the economy, history, and culture of this region of Japan are deeply tied to its preservation. In 2015, the total catch of ayu was over 2,400 tons. Ayu are composed of both the amphidromous and landlocked forms. Ayu of the Nagara River are of the former form, spawning in freshwater, and the newly hatched larvae drift downstream to spend the winter in the sea. These facts indicate that ayu can adapt to various environments, and has access to nutrients to support this life cycle. However, the nutritional needs of ayu are not entirely clear.

Among farm-reared fish, salmon and trout are well-known for having high levels of carotenoids¹ ². Yamashita et al. reported that ayu also have high levels of carotenoids including lutein and zeaxanthin in their integuments³. They also reported that zeaxanthin administrated by diet increased the level of zeaxanthin in the integuments of ayus⁴. Thus, ayu can take zeaxanthin from outside and maintain it in tissues. Earlier studies have shown that injections of zeaxanthin into the anterior chamber of the zebrafish eye improve the average visual acuity as determined by optokinetic responses⁵. Carotenoids identified in human macula are lutein, (3R,3R)-zeaxanthin, and meso (3R,3S)-zeaxanthin⁶. In humans, zeaxanthin is accumulated in the macula⁷ and plays a pivotal role in the retina⁸. Although fishes do not have a macula, zeaxanthin has been shown to play a role in the visual acuity of zebrafish⁹. Thus, we reasoned that zeaxanthin in the retina might play an important role in the physiology of ayu retina.

Chucair et al. have reported that exposure of rat photoreceptor cells to zeaxanthin in culture prevented their apoptosis induced by H₂O₂⁰, thereby indicating that zeaxanthin had an anti-oxidative effect in the retina. Reactive
oxygen species (ROS) produced by excessive light induce the oxidation of lipids, and lipid hydroperoxide stored in the retina can cause tissue degeneration\(^9\). Additionally, ROS are used to maintain the homeostasis by energy generation and cell activity. The disruption of the balance between ROS produced and consumed is related to cell damage\(^10, 11\). Glutathione is the major endogenous antioxidant, and it acts by directly neutralizing free radicals and reactive oxygen compounds\(^12, 13\). In short, the reduction of glutathione (GSH) by aging and some stress degrade its role as an anti-oxidative agent. In fact, GSH depletion increase intracellular ROS in ocular diseases such as glaucoma and diabetic retinopathy\(^14, 15\). Although it is expected that zeaxanthin would protect the retinal cells in an in vitro GSH depletion assay, there is no report on this.

Thus, the purpose of this study was to determine the presence and distribution of the carotenoids, more specifically zeaxanthin, in the retina of wild ayu. In addition, we examined whether exposure of cone photoreceptors and retinal pigment epithelium (RPE) cell lines to zeaxanthin had a protective effect on the death induced by the oxidative stress of L-buthionine-(S,R)-sulfoximine (BSO)/GSH depletive treatment.

## 2 Materials and Methods

### 2.1 Materials

Wild mature ayu (\textit{P. altivelis}) were purchased from Hoshino tennen-ayu hanbaiten (http://tennenayuhanbai.sakura.ne.jp/). From a nutritional point of view, we selected horse mackerels (\textit{Trachurus japonicus}) as a control group, which are fishes that are typically eaten in Japan. Horse mackerels were purchased from a local fish market in Kyoto City, Japan. Once purchased, both ayu and horse mackerels were frozen quickly to preserve freshness. As the fishes were already dead, we were not required to comply with the relevant guidelines and regulations. Bulk powder of zeaxanthin was provided by JXTG Nippon Oil & Energy (Tokyo, Japan). The Dulbecco’s modified Eagle medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). L-glutamic acid monosodium salt (glutamate) was purchased from Nacalai Tesque (Kyoto, Japan). BSO and N-acetyl cysteine (NAC) were purchased from WAKO (Osaka, Japan). Hoechst 33342 and propidium iodide (PI) were purchased from Thermo Fischer Scientific (Waltham, MA, USA). Penicillin and streptomycin were purchased from Meiji Seika Kaisha Ltd. (Tokyo, Japan).

### 2.2 Extraction and identification of carotenoids

Ayu and horse mackerel were analyzed using five fishes each. Component analysis was carried out in the same way as previously reported\(^16\). The carotenoids were extracted from the eyes, integuments, and viscera with acetone at room temperature between 21-24°C. After filtration, the acetone extract was partitioned with \textit{n}-hexane/diethyl ether (Et\textsubscript{2}O) (1:1) and water. Carotenoids were transferred to the \textit{n}-hexane/Et\textsubscript{2}O (1:1) phase. The total amount of carotenoid was quantified by the absorbance of wavelength (\(\lambda_{max} = 450 \text{ nm}\)) with the coefficient of E1\% cm = 2400 in \textit{n}-hexane/Et\textsubscript{2}O (1:1) solution. Then the \textit{n}-hexane/Et\textsubscript{2}O (1:1) layer was evaporated to dryness. For the integuments, the residue contained a large amount of lipids, and the carotenoids existed as esterified forms of fatty acids. Thus, the residue extract of the integuments was saponified with 5% KOH/MeOH (10 mL) at room temperature for 2 h for the removal of lipids contaminants and hydrolysis of carotenoid fatty acid esters. Then, the carotenoids were extracted with \textit{n}-hexane/Et\textsubscript{2}O (1:1) from the saponified solution and washed with water. After evaporating the extract solution, the carotenoids were analyzed by liquid chromatography–mass spectrometry (LC/MS). The carotenoid composition was calculated from the peak area at 450 nm by the ultraviolet-visible (UV-VIS) and electrospray ionization time-of-flight (TOF) MS spectral data and the retention time of high-performance liquid chromatography (HPLC) was compared to that of an authentic sample. Carotenoids in eyes and viscera were presented as non-esterified forms. Therefore, carotenoids extracted from eyes and viscera were analyzed by LC/MS without saponification. Fucoxanthinol in the viscera were separated by silica gel column chromatography and identified by proton nuclear magnetic resonance (\(\text{\textit{1}}\text{H-NMR}\)) and circular dichroism (CD) spectral data to confirm the identification of zeaxanthin in the integuments and viscera.

### 2.3 Analysis of zeaxanthin stereoisomers

The zeaxanthin fraction was collected from the macula area of the retina by silica gel HPLC using a Cosmosil 5SL-II column with acetone/hexane (2:8, \textit{v/v}) for the mobile phase as described above. This fraction was evaporated to dryness, dissolved in isopropanol/hexane (4:96, \textit{v/v}), and subjected to chiral HPLC. A chiralcel AD-H (250 x 4.6 mm i.d., Daicel Corporation, Japan) was used as the stationary phase for the chiral HPLC, and isopropanol/hexane (4:96, \textit{v/v}) was used as the mobile phase at a flow rate of 1.0 mL/min. Assessments were performed at 450 nm. Stereosomers of zeaxanthin were prepared from \(\beta, \beta\)-carotene-3, 3’-dione by reduction with NaBH\textsubscript{4}\(^17\).

### 2.4 Apparatus

The UV-VIS absorption spectra were recorded with a Hitachi U-2001 spectrophotometer (Hitachi Field Navigator, Tokyo, Japan) in Et\textsubscript{2}O. LC/MS analysis of carotenoids was performed with a Waters Xevo G2S Q TOF mass spectrometer (Waters Corporation, Milford, CT, USA) equipped with an Acquity ultra-performance liquid chromatography system. Electro-spray ionization TOF MS spectra were ac-
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2.5 Retinal localization of zeaxanthin in ayu

2.5.1 Histological analysis

For histological analysis, eyes were enucleated from wild mature ayu, and the eyes were fixed in 4% paraformaldehyde for at least 48 h. The eyes were embedded in paraffin, and 5 μm sections were cut parallel to the maximum circumference, then stained with hematoxylin and eosin. Photographs were taken with the BZ-X710 fluorescence microscope (KEYENCE, Osaka, Japan).

2.5.2 Immunohistochemistry

The paraffin sections were immersed three times in xylene for 15 min each and then, twice in anhydrous ethanol for 10 s each. Next, the sections were placed in 99% ethanol twice for 10 s each, 90% ethanol for 10 s, 70% ethanol for 10 s, and distilled water for 10 s. Each section was treated with citrate buffer (pH 6.2, 100°C) for 15 min and washed with phosphate-buffered saline (PBS). Sections were immersed in 10% goat serum (Vector Laboratories, CA, USA) for blocking for 1 h. Next, the samples were washed twice with PBS for 2 min, and the sections were incubated with rabbit anti-GSH S-transferase pi 1 (GSTP1) polyclonal antibody (1:2000, 4°C, overnight; Aviva systems Biology Corp., CA, USA). Afterward, we washed twice with PBS for 10 min each, and sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:2000, 15°C–25°C, 1 h; Thermo Fisher Scientific, Waltham, MA, USA). Again, after washing twice with PBS for 10 min, sections were incubated with Hoechst 33342 (1:2000, 15°C–25°C, 30 min; Thermo Fisher Scientific) for staining the nuclei. Finally, the samples were washed twice with PBS for 10 min, and sections were covered with fluoromount (Diagnostic Biosystems, CA, USA). Photographs were taken with the BZ-X710 for Figs. 3D–3E and FLUOVIEW FV10i (Olympus, Tokyo, Japan) for Fig. 3F.

2.6 Pharmacometrics of zeaxanthin for photoreceptors

2.6.1 Cell cultures

A cone photoreceptor cell line derived from a murine retina tumor, 661W cells, was provided by Dr. Muayyad R. Al-Ubaidi (University of Houston, TX, USA). The cells were immortalized by the expression of the SV40-T antigen under the control of the human IRBP promoter. The 661W cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere of 5% CO₂ at 37°C. ARPE-19 cell line, a human retinal pigmented epithelium cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in the same way as described above.

2.6.2 Cell death assay

ARPE-19 or 661W cells were plated at a density of 3,000 cells/well in 96 well culture plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA). After 24 h, cells were washed twice with DMEM and then immersed in DMEM supplemented with 1% FBS. After 1 h of pretreatment of zeaxanthin or NAC, 500 μm BSO plus 10 μm glutamate was added to these cultures for 24 h. Cell death was assessed by a combination of fluorescent staining with Hoechst 33342 (Molecular probes, Eugene, OR, USA) PI (Molecular probes). The slides were photographed with the BZ-X710 fluorescence microscope, and the total number of cells was counted in a masked way. The percentage of PI-positive cells was calculated.

2.7 Statistical analysis

Data are presented as the means ± standard error of the means (SEM). Statistical comparisons were performed by the Dunnett’s multiple comparison tests using the SPSS software (version 16.0j; IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA). A p value < 0.05 was considered to be statistically significant.

3 Results

3.1 Identification of carotenoids

HPLC chromatograms of carotenoids were shown in the eye (Fig. 1A), viscera (Fig. 1B) and integument (Fig. 1C) of Nagara River wild ayu. β-Cryptoxanthin, zeaxanthin, diatoxanthin, fucoxanthinol, and myxoxanthophyll were identified in the viscera. Zeaxanthin was identified as the major carotenoid along with β-cryptoxanthin, 3'-hydroxy-echinenone, diatoxanthin, and alloxanthin in the integument. These results are consistent with those of earlier studies. 

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Fig. 1  High-performance liquid chromatographic (HPLC) profile of the eye, viscera, and integument of wild ayus. Typical HPLC chromatogram of carotenoids present in the eye (A), viscera (B) and integument (C). Peak 1, Myxoxanthophyll; Peak 2, Galloxanthin; Peak 3, Fucoxanthinol; Peak 4, Diatoxanthin; Peak 5, Zeaxanthin; Peak 6, 3'-Hydroxyechinenone; Peak 7, β-Cryptoxanthin; and peak 8, β-Carotene. The peaks were detected by UV-VIS at 450 nm.
Zeaxanthin and β-cryptoxanthin were identified as the major carotenoids in the ayu eyes. CD spectral data and chiral HPLC analysis revealed that zeaxanthin in the ayu eyes was the \(3R,3L\) isomer (Supplementary Tables 1 and 2).

The contents and proportions of carotenoids obtained from organ samples such as ayu integument, subcutaneous fat, digestive tract, and eye are shown in Table 1. Among these, the eye had a predominantly large quantity of total carotenoids. Moreover, the most abundant component was zeaxanthin and it was found in the integument, subcutaneous fat, digestive tract and eye. Particularly, zeaxanthin made up about 80\% of all the carotenoid components.

Figure 2A shows the structures of zeaxanthin, β-cryptoxanthin, \(3^-\)hydroxy-echinenone, diatoxanthin, diadinochrome, galloxanthin, fucoxanthinol, and myxoxanthophyll. These were the carotenoids found in the tissues of ayu.

We also determined the amount and types of carotenoids in horse mackerel, \(T.\) japonicus. The amount of carotenoid in the integument was larger than that in the eye (Table 2). The carotenoids in both the integument and eye of the horse mackerel in order of descending concentrations were \(3R,3R,6S\)-lutein, zeaxanthin, \(3S,6S,3R,6S\)-tunaxanthin, \(3S,6S,3S,6S\)-tunaxanthin, and \(3S,3S,6S\)-tunaxanthin, and β-cryptoxanthin (Table 2). The Ayu had only the \(3R,3R\)-zeaxanthin which is derived from blue-green algae\(^{19,20}\). Conversely, horse mackerel had \(3R,3R\)-, meso \(3R,3S\)-, and \(3S,3S\)-zeaxanthin. These isoforms were derived from the astaxanthin of crustaceans. The chemical structures of the carotenoids contained in horse mackerel are listed in Fig. 2B.

### 3.2 Retinal localization of zeaxanthin in ayu

Hematoxylin and eosin staining of the ayu ophthalmic tissues was performed to determine the tissues that expressed zeaxanthin. The ayu has a large lens as observed in other fishes\(^{21}\). Additionally, the retinal laminar structure of the ayu appears similar to that of fishes and mammals. All the highly colored dots represent the nuclei, and the pink color represents either muscle or collagen, while the brown-black areas contain melanin (Figs. 3B–3D). Each layer was identified, with that reported in the zebrafish as a reference\(^{22}\). We confirmed the presence of a ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, rod and cone photoreceptors, and the outer segments in the ayu retina (Fig. 3D).

The ayu retinal sections were immunostained with an antibody against GSTP1, a zeaxanthin-binding protein. GSTP1 was strongly expressed in the photoreceptor layer (Fig. 3E–3F). Higher magnification of confocal microscopic images showed that GSTP1 was localized around the nuclei of the rods and cones and the outer segments of the ayu retina (Fig. 3G).

### 3.3 Protective effect of zeaxanthin on death of 661W cells, photoreceptor cell line, induced by oxidative stress in culture

BSO/glutamate was used as an oxidative stress inducer. BSO is toxic because it drains intracellular GSH which is an intracellular antioxidant to protect the cell against oxidative stress\(^{23}\). Glutamate induces oxidative stress by inhibiting cystine uptake, ultimately resulting in cytotoxicity\(^{24}\). Our results showed that BSO plus glutamate induced the death of 661W cells, a photoreceptor cell line, and NAC, an

| Carotenoid Composition (%) |
|---------------------------|
| Zeaxanthin | 84.3 | 80.6 | 30.5 | 78.3 |
| β-Cryptoxanthin | 3.4 | 6.3 | 5.6 | 15.1 |
| \(3^-\)Hydroxy-echinenone | 1.8 | 4.3 | 3.9 | 1.1 |
| Diatoxanthin | 1.2 | 1.2 | 1.6 |
| Diadinochrome | 3.4 |
| Galloxanthin | 3.4 |
| Fucoxanthinol | 14.5 |
| Myxoxanthophyll | 3.4 |
| Others | 3.4 | 4.2 | 3.9 | 1.9 |

Table 1 The content of carotenoids in the wild ayu.

| Total carotenoid contents (μg) | Integument | Subcutaneous fat | Digestive tract | Eye |
|-------------------------------|------------|-----------------|-----------------|-----|
| μg/g (each tissue)            | 36.5       | 0.69            | 24.3            | 1840 |
| μg/fish                       | 328.5      | 12.5            | 291.6           | 552 |

| Carotenoid Composition (%) |
|---------------------------|
| Zeaxanthin | 84.3 | 80.6 | 30.5 | 78.3 |
| β-Cryptoxanthin | 3.4 | 6.3 | 5.6 | 15.1 |
| \(3^-\)Hydroxy-echinenone | 1.8 | 4.3 | 3.9 | 1.1 |
| Diatoxanthin | 1.2 | 1.2 | 1.6 |
| Diadinochrome | 3.4 |
| Galloxanthin | 3.4 |
| Fucoxanthinol | 14.5 |
| Myxoxanthophyll | 3.4 |
| Others | 3.4 | 4.2 | 3.9 | 1.9 |

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Fig. 2  Chemical structure of carotenoids obtained in the ayus (A) and horse mackerel (B).
antioxidant, reduced the number of dead 661W cells (Fig. 4). These results showed that this experimental system could determine the degree of cell death induced by oxidative stress. Our results indicated that exposure of 661W cells to zeaxanthin significantly reduced the number of BSO plus glutamate-induced cell deaths (Fig. 4A). In Fig. 4B, the efficacy of zeaxanthin on the same cell death model with BSO plus glutamate was evaluated in ARPE-19 cells. Both zeaxanthin and NAC significantly inhibited the cell death rate.

4 Discussion

The Nagara River is a favorable habitat for the ayu with an ample supply of algae upon which the ayu feed. Myxoxanthophyll, fucoxanthinol, diadinochrome, diatoxanthin, zeaxanthin, and β-cryptoxanthin are present in the viscera of the Nagara River wild ayu. Fucoxanthinol derived from fucoxanthin by hydroxylation, diadinochrome derived from diadinoxanthin under acidic conditions, and diatoxanthin are known to be characteristic carotenoids in diatoms. The presence of these carotenoids in ayu indicates that ayu feed not only on the diatoms but also on blue-green algae. Zeaxanthin was found to be a major carotenoid along with β-cryptoxanthin in the ayu eyes. CD spectral data and chiral HPLC analysis revealed that the zeaxanthin in ayus had the 3R,3’R stereochemistry. It has been reported that zeaxanthin in blue-green algae also had the 3R,3’R stereochemistry, indicating that ayu ingest and store zeaxanthin from blue-green algae without modification. Our results also confirmed the presence of carotenoids in the horse mackerel eyes. Although the horse mackerels have carotenoids in the eye, the amount of total carotenoids was extremely low compared with that in the wild ayus.

Table 1 showed that zeaxanthin existed in the integument, the subcutaneous fat, digestive tract, and the eye. We found that zeaxanthin was the highest component in each tissue in the mature wild ayu (Table 1). Interestingly, we found high carotenoid concentrations in other organs of the ayu, and zeaxanthin was the primary carotenoid (Table 1). This result suggests that zeaxanthin plays a pivotal role in enabling the wild ayu to see things under the various light conditions in the environment. Additionally, ayu have extremely high concentrations of carotenoids in the eye compared to that in the horse mackerel (Table 2). Thus, ayu may use carotenoids, especially zeaxanthin, to maintain the physiological function of their eyes.

We focused on zeaxanthin which is abundant in the eye and reasoned that zeaxanthin is involved in the visual function of ayu. We used GSTP1 as a zeaxanthin marker, and the results indicated that zeaxanthin was expressed in the photoreceptor cell layer especially in the area surrounding the rods and cones (Fig. 3F, 3G). Our results also showed that zeaxanthin protected 661W cells, a mouse cone photoreceptor cell line, and ARPE-19, a human retinal pigment epithelial cell line, against oxidative stress induced by BSO/glutamate (Fig. 4). Zeaxanthin seems to have exerted more protective effects on the RPE cells than those exerted on the photoreceptor cells. This may be related to the fact that photoreceptor cells are more vulnerable to oxidative stress than RPE cells. In fact the rate of induced cell death was significantly higher in photoreceptor cells. The rate of photoreceptor cell death was approximately 50% (Fig. 4A), while the rate of RPE cell death was approximately 10% (Fig. 4B). A characteristic of RPE cells is the intracellular biosynthesis and storage of melanin, which has a strong antioxidant effect. In other words, RPE cells contain melanin, which may have protected them from BSO/glutamate induced oxidative stress. However, the protective effect of zeaxanthin may be stronger in RPE cells than in photoreceptor cells, because zeaxanthin protects photoreceptors at 10 μM and RPE cells at 0.1 μM. In short, it is thought that both the difference in the effect of zeaxanthin itself on each cell and the homeostasis of each cell against oxidative stress are responsible for the difference in the effect of zeaxanthin. These findings suggest that the high concentration of zeaxanthin in the outer retinal layer may be a self-defense process against cell damage induced by light exposure. Despite the obvious genetic differences between fish and humans, the presence of high concentrations of zeaxanthin in the eyes is common to both. There are limitations to this study. We could not administer zeaxanthin to the wild ayu and examine accumulations of zeaxanthin in ayu eyes in the dark. In the future, whether zeaxanthin directly plays a protective role in photoreceptor cell death and whether light provides the accumulation of...
Fig. 3  The localization of zeaxanthin in the retina of ayus. (A) Nagara river wild ayu sweetfish (*Plecoglossus altivelis*). (B) Representative photomicrographs showing hematoxylin and eosin-stained sections of retina from mature ayu. (C) Enlarged figure of the portion marked with line in (B). (D) Enlarged figure of the portion marked with dotted line in (C). (E, F) Immunofluorescent staining of glutathione S-transferase P1 (GSTP1), a zeaxanthin marker, in the retina of mature ayu. (F) Enlarged figure of the portion marked with line in (E). (G) Confocal laser scanning microscopic analysis in part of the corresponding image of (F). Scale bars, 1 cm (A), 1,000 μm (B), 100 μm (C), 50 μm (D), 1,000 μm (E), 100 μm (F) and 30 μm (G). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; OS, outer segments.
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Fig. 4 Effects of zeaxanthin on 661W cells, a photoreceptor cell line (A), and ARPE-19 cells, a retinal pigment epithelium cell line, on the damage induced by oxidative stress from buthionine sulfoximine (BSO)/glutamate. Representative fluorescence microscopic images of retinal sections stained with Hoechst 33342 and PI staining (added 24 h after BSO/Glutamate) on 661W cells (photoreceptor cells) (A) and ARPE-19 (retinal pigment epithelium) (B). Apoptotic 661W (A) and ARPE-19 (B) cell death was quantitatively evaluated by the number of PI positive cells at 24 h after BSO/Glutamate exposure. NAC, N-Acetyl-L-cysteine; BSO, buthionine sulfoximine. Data are shown as mean ± standard error of the means (SEMs; n = 3-10 **p < 0.01 versus BSO/Glutamate plus the vehicle-treated group). Scale bar, 100 μm.

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\begin{array}{cccc}
\text{Zeaxanthin (μM)} & - & 10 & 1 & 10 & - \\
\text{BSO/glutamate} & - & + & + & + & + \\
\end{array}
\]

\[
\begin{array}{cccc}
\text{Zeaxanthin (μM)} & - & 0.1 & - & 0.1 & - \\
\text{BSO/glutamate} & - & - & + & + & - \\
\end{array}
\]
zeaxanthin in wild ayu should be examined.

5 Conclusion
High concentrations of zeaxanthin are present in the photoreceptor layer of ayu retina, suggesting that zeaxanthin may play important roles in maintaining the physiological function of photoreceptors.

Competing financial interests
There is no potential conflict of interest to disclose.

Supporting Information
This material is available free of charge via the Internet at http://dx.doi.org/10.5650/jos.ess20108

Author Contributions
S. Nakamura and H. Hara designed the experiments. S. Nakamura, T. Maoka, Y. Kuse, A. Muramatsu, and Y. Yoshino performed the experiments. S. Nakamura, T. Maoka, M. Shimazawa, and H. Hara performed the analyses. S. Nakamura and H. Hara wrote the paper. All authors contributed to the editing of the paper and to scientific discussions.

References
1) Matsumoto, Y.; Fukamachi, S.; Mitani, H.; Kawamura, S. Functional characterization of visual opsin repertoire in Medaka (Oryzias latipes). Gene 371, 268-278 (2006).
2) Matsuno, T.; Katsuyama, M.; Nagata, S. Comparative biochemical studies of carotenoids in fishes, 19: Carotenoids of chum salmon, coho salmon, biwa trout, red-spotted masu salmon, masu salmon, and kokanee [Onchorhynchus spp.]. Bull. Japan Soc. Sci. Fish. 46, 879-884 (1980).
3) Yamashita, E.; Maruyama, Y.; Katsuyama, M.; Tsushima, M.; Arai, S.; Matsuno, T. The presence and origin of an apocarotenoid, galloxanthin in ayu Plecoglossus altivelis. Fish. Sci. 64, 826-830 (1998).
4) Saidi, E.A.; Davey, P.G.; Cameron, D.J. The effect of zeaxanthin on the visual acuity of zebrafish. PLoS One 10, e0135211 (2015).
5) Bone, R.A.; Landrum, J.T.; Hime, G.W.; Cains, A.; Zamor, J. Stereochemistry of the human macular carotenoids. Invest. Ophthalmol. Vis. Sci. 34, 2033-2040 (1993).
6) Bone, R.A.; Landrum, J.T.; Tarsis, S.L. Preliminary identification of the human macular pigment. Vis. Res. 25, 1531-1535 (1985).
7) Akuffo, K.O.; Nolan, J.M.; Howard, A.N.; Moran, R.; Stack, J.; Klein, R.; Klein, B.E.; Meuer, S.M.; Sabour-Pickett, S.; Thornham, D.I.; Beatty, S. Sustained supplementation and monitored response with differing carotenoid formulations in early age-related macular degeneration. Eye 29, 902-912 (2015).
8) Chucair, A.J.; Rotstein, N.P.; Sangiovanni, J.P.; During, A.; Chew, E.Y.; Politi, L.E. Lutein and zeaxanthin protect photoreceptors from apoptosis induced by oxidative stress: Relation with docosahexaenoic acid. Invest. Ophthalmol. Vis. Sci. 48, 5168-5177 (2007).
9) Wiegand, R.D.; Giusto, N.M.; Rapp, L.M.; Anderson, R.E. Evidence for rod outer segment lipid peroxidation following constant illumination of the rat retina. Invest. Ophthalmol. Vis. Sci. 24, 1433-1435 (1983).
10) Bonne, C.; Muller, A.; Villain, M. Free radicals in retinal ischaemia. Gen. Pharmacol. 30, 275-280 (1998).
11) Finkel, T.; Holbrook, N.J. Oxidants, oxidative stress and the biology of aging. Nature 408, 239-247 (2000).
12) Hughes, R.E. Reduction of dehydroascorbic acid by animal tissues. Nature 203, 1068-1069 (1964).
13) Dringen, R. Metabolism and functions of glutathione in brain. Prog. Neurobiol. 62, 649-671 (2000).
14) Maher, P.; Hanneken, A. Flavonoids protect retinal ganglion cells from oxidative stress-induced death. Invest. Ophthalmol. Vis. Sci. 46, 4796-4803 (2005).
15) Tezel, G. Oxidative stress in glaucomatous neurodegeneration: mechanisms and consequences. Prog. Retin. Eye Res. 25, 490-513 (2006).
16) Maoka, T.; Arai, A.; Shimizu, M.; Matsuno, T. The first isolation of enantiomeric and meso-zeaxanthin in na -
17) Yamashita, E.; Maruyama, Y.; Katsuyama, M.; Tsushima, M.; Arai, S.; Matsuno, T. The presence and origin of an apocarotenoid, galloxanthin in ayu Plecoglossus altivelis. Fish. Sci. 64, 826-830 (1998).
18) Saidi, E.A.; Davey, P.G.; Cameron, D.J. The effect of zeaxanthin on the visual acuity of zebrafish. PLoS One 10, e0135211 (2015).
19) Bone, R.A.; Landrum, J.T.; Hime, G.W.; Cains, A.; Zamor, J. Stereochemistry of the human macular carotenoids. Invest. Ophthalmol. Vis. Sci. 34, 2033-2040 (1993).
20) Bone, R.A.; Landrum, J.T.; Tarsis, S.L. Preliminary identification of the human macular pigment. Vis. Res. 25, 1531-1535 (1985).
21) Akuffo, K.O.; Nolan, J.M.; Howard, A.N.; Moran, R.; Stack, J.; Klein, R.; Klein, B.E.; Meuer, S.M.; Sabour-Pickett, S.; Thornham, D.I.; Beatty, S. Sustained supplementation and monitored response with differing carotenoid formulations in early age-related macular degeneration. Eye 29, 902-912 (2015).
22) Chucair, A.J.; Rotstein, N.P.; Sangiovanni, J.P.; During, A.; Chew, E.Y.; Politi, L.E. Lutein and zeaxanthin protect photoreceptors from apoptosis induced by oxidative stress: Relation with docosahexaenoic acid. Invest. Ophthalmol. Vis. Sci. 48, 5168-5177 (2007).
23) Wiegand, R.D.; Giusto, N.M.; Rapp, L.M.; Anderson, R.E. Evidence for rod outer segment lipid peroxidation following constant illumination of the rat retina. Invest. Ophthalmol. Vis. Sci. 24, 1433-1435 (1983).
24) Bonne, C.; Muller, A.; Villain, M. Free radicals in retinal ischemia. Gen. Pharmacol. 30, 275-280 (1998).
25) Finkel, T.; Holbrook, N.J. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239-247 (2000).
26) Hughes, R.E. Reduction of dehydroascorbic acid by animal tissues. Nature 203, 1068-1069 (1964).
27) Dringen, R. Metabolism and functions of glutathione in brain. Prog. Neurobiol. 62, 649-671 (2000).
28) Maher, P.; Hanneken, A. Flavonoids protect retinal ganglion cells from oxidative stress-induced death. Invest. Ophthalmol. Vis. Sci. 46, 4796-4803 (2005).
29) Tezel, G. Oxidative stress in glaucomatous neurodegeneration: mechanisms and consequences. Prog. Retin. Eye Res. 25, 490-513 (2006).
30) Maoka, T.; Arai, A.; Shimizu, M.; Matsuno, T. The first isolation of enantiomeric and meso-zeaxanthin in nature. Comp. Biochem. Physiol. B 83, 121-124 (1986).
31) Maoka, T. New acetylenic carotenoid 6-epimonadozeaxanthin from the rosary goby Gymnogobius castaneus. J. Oleo Sci. 67, 1259-1263 (2018).
32) al-Ubaydi, M.R.; Hollyfield, J.G.; Overbeek, P.A.; Baehr, W. Photoreceptor degeneration induced by the expression of simian virus 40 large tumor antigen in the retina of transgenic mice. Proc. Natl. Acad. Sci. U S A 89, 1194-1198 (1992).
33) Abe, S.; Katano, O.; Nagumo, T.; Tanaka, J. Grazing effects of ayu, Plecoglossus altivelis, on the species composition of benthic algal communities in the Kiso River. Diatom 16, 37-43 (2000).
34) Healey, F.P. The carotenoids of four blue-green algae (1). J. Phycol. 4, 126-129 (1968).
35) Chhetri, J.; Jacobson, G.; Gueven, N. Zebrafish–on the move towards ophthalmological research. Eye 28, 367-380 (2014).
22) Iribarne, M.; Nishiwaki, Y.; Nakamura, S.; Araragi, M.; Oguri, E.; Masai, I. AipII is required for cone photoreceptor function and survival through the stability of Pde6c and Gc3 in zebrafish. Sci. Rep. 7, 45962 (2017).

23) Martensson, J.; Jain, A.; Stole, E.; Frayer, W.; Auld, P.A.; Meister, A. Inhibition of glutathione synthesis in the newborn rat: A model for endogenously produced oxidative stress. Proc. Natl. Acad. Sci. U S A 88, 9360-9364 (1991).

24) Murphy, T.H.; Miyamoto, M.; Sastre, A.; Schnaar, R.L.; Coyle, J.T. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2, 1547-1558 (1989).

25) Stancher, B.; Zonta, F.; Favretto, L.G. High-performance liquid chromatography of carotenoids from some marine shellfish. J. Chromatogr. 440, 37-46 (1988).

26) Frank, H.A.; Cua, A.; Chynwat, V.; Young, A.; Gosztola, D.; Wasielewski, M.R. The lifetimes and energies of the first excited singlet states of diadinoxanthin and diatoxanthin: the role of these molecules in excess energy dissipation in algae. Biochim. Biophys. Acta 1277, 243-252 (1996).

27) Withers, N.W.; Alberte, R.S.; Lewin, R.A.; Thornber, J.P.; Britton, G.; Goodwin, T.W. Photosynthetic unit size, carotenoids, and chlorophyll-protein composition of Prochloron sp., a prokaryotic green alga. Proc. Natl. Acad. Sci. U S A 75, 2301-2305 (1978).

28) Samuel, W.; Jaworski, C.; Postnikova, O.A.; Kutty, R.K.; Duncan, T.; Tan, L.X.; Poliakov, E.; Lakkaraju, A.; Redmond, T.M. Appropriately differentiated ARPE-19 cells regain phenotype and gene expression profiles similar to those of native RPE cells. Mol. Vis. 23, 60-89 (2017).

29) Feeney, L. Lipofuscin and melanin of human retinal pigment epithelium. Fluorescence, enzyme cytochemical, and ultrastructural studies. Invest. Ophthalmol. Vis. Sci. 17, 583-600 (1978).

30) Sundelin, S.P.; Nilsson, S.E.; Brunk, U.T. Lipofuscin formation in cultured retinal pigment epithelial cells is related to their melanin content. Free Radic. Biol. Med. 30, 74-81 (2001).