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The influence of ontogeny and light environment on the expression of visual pigment opsins in the retina of the black bream, *Acanthopagrus butcheri*

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SUMMARY

The correlation between ontogenetic changes in the spectral absorption characteristics of retinal photoreceptors and expression of visual pigment opsins was investigated in the black bream, *Acanthopagrus butcheri*. To establish whether the spectral qualities of environmental light affected the complement of visual pigments during ontogeny, comparisons were made between fishes reared in: (1) broad spectrum aquarium conditions; (2) short wavelength-reduced conditions similar to the natural environment; or (3) the natural environment (wild-caught). Microspectrophotometry was used to determine the wavelengths of spectral sensitivity of the photoreceptors at four developmental stages: larval, post-settlement, juvenile and adult. The molecular sequences of the rod (*Rh1*) and six cone (*SWS1*, *SWS2A* and *B*, *Rh2Aα* and *β*, and *LWS*) opsins were obtained and their expression levels in larval and adult stages examined using quantitative RT-PCR. The changes in spectral sensitivity of the cones were related to the differing levels of opsin expression during ontogeny. During the larval stage the predominantly expressed opsin classes were *SWS1*, *SWS2B* and *Rh2Aα*, contrasting with *SWS2A*, *Rh2Aβ* and *LWS* in the adult. An increased proportion of long wavelength-sensitive double cones was found in fishes reared in the short wavelength-reduced conditions and in wild-caught animals, indicating that the expression of cone opsin genes is also regulated by environmental light.

Key words: photoreceptor, cone, spectral tuning, fish, microspectrophotometry, environmental light.

INTRODUCTION

The neural process of vision begins with the absorption of light by the retinal photoreceptors. It follows, therefore, that the ability of an organism to detect a light stimulus will depend upon the spectral absorption properties of the visual pigments located within the photoreceptors. Most vertebrates possess a number of different photoreceptor classes, each expressing a different visual pigment, and it is the specific absorption of the visual pigment that can be 'tuned' to the spectral qualities of the light environment (for reviews, see Bowmaker and Hunt, 1999; Bowmaker and Hunt, 2006).

Teleost fishes, with their wide range of natural habitats, have become the model for examining the relationship between ambient light and the spectral sensitivity of visual pigments; it has been shown that species from bodies of water with differing spectral irradiance tend to possess visual pigments that are related to the most abundant wavelengths (Bowmaker et al., 1994; Bowmaker and Hunt, 2006; Douglas et al., 2003; Loew and Lythgoe, 1978; Lythgoe et al., 1994; Partridge et al., 1989; Partridge et al., 1992a). Furthermore, the ontogenetic migrations of fishes from one body of water to another or to greater depths is accompanied by changes in the spectral sensitivity of the retina in many species (for reviews, see Beaudet and Hawryshyn, 1999; Collin and Shand, 2003). For example, those that migrate to deeper water during ontogeny exhibit a narrowing in the spectral range of their pigments as the spectral range of the light environment also narrows (Bowmaker and Kunz, 1987; Shand et al., 1988; Shand, 1993; Hope et al., 1998).

The spectral sensitivity of a visual pigment is dependent on the amino acid sequence of the opsin protein component of the molecule. There are four opsin classes in vertebrate cone photoreceptors: short wavelength-sensitive 1 (SWS1) pigments with peak sensitivities (λ_{max}) in the UV–violet region of the spectrum, short wavelength-sensitive 2 (SWS2) pigments with λ_{max} in the blue region, middle wavelength-sensitive rod-like (Rh2) pigments with λ_{max} in the green region, and long wavelength-sensitive (LWS) pigments with λ_{max} in the yellow–red region (for reviews, see Bowmaker and Hunt, 2006; Yokoyama, 2000). The λ_{max} of a visual pigment also depends on whether the chromophore present in the pigment molecule is derived from vitamin A_1 (retinal) or A_2 (3,4-didehydroretinal) giving a rhodopsin or porphyropsin pigment, respectively. The latter pigments are long wavelength shifted compared with the former, with a greater effect at longer wavelengths (Whitmore and Bowmaker, 1989).

It was originally assumed that chromophore changes or the loss of a cone type from the photoreceptor mosaic would be the main way in which changes in spectral sensitivity are facilitated (for reviews, see Bowmaker, 1995; Beaudet and Hawryshyn, 1999). However, changes in opsin expression were subsequently implicated in a number of species such as the pollack (Shand et al., 1988), goatfish (Shand, 1993) and flounder (Evans et al., 1993), and were shown to account for the blue–green sensitivity shift in the rods of the eel (Archer et al., 1995; Hope et al., 1998). More recently, changes in cone opsin expression have been demonstrated in

zebrafish (Chinen et al., 2003; Takechi and Kawamura, 2005), Pacific pink salmon (Cheng and Novales Flamarique, 2004), rainbow trout (Veldhoen et al., 2006; Cheng and Novales Flamarique, 2007) and cichlids (Spady et al., 2006).

A recent microspectrophotometric study of the black bream *Acanthopagrus butcheri* revealed a changing pattern of cone photoreceptors with different λ_{max} values in fish at different developmental ages (Shand et al., 2002). In general terms, larval fish have cones that absorb maximally at shorter wavelengths, while juveniles and adults have longer wavelength sensitivity. However, a degree of variability was noted and animals from the wild were found to have longer wavelength-absorbing pigments than their aquarium-reared counterparts. It was predicted from modelling changes in bandwidth of the visual pigment that a change in opsin expression was taking place rather than a simple switch from an A_1 to an A_2 chromophore. In order, therefore, to establish the basis for the visual pigment changes, we have identified the different rod and cone opsins expressed in the black bream retina and used quantitative RT-PCR (qPCR) to determine their expression levels in larval and adult fishes. In addition, we have sought to establish whether the cone class/visual pigment changes are pre-programmed developmental events or are influenced by environmental light by using microspectrophotometry (MSP) to compare fishes reared in two different light regimens with wild-caught fishes at equivalent ages.

MATERIALS AND METHODS

Animals

Black bream, *Acanthopagrus butcheri* (Munro), were obtained from local aquaculturists and housed at the University of Western Australia. The marine recirculating system operated at 18°C (±2°C). The larvae were fed rotifers until they were introduced to a diet of cultured branchiopod brine shrimps (*Artemia* spp.) between 22 and 25 days. From 55 days the juveniles were gradually shifted onto a diet of dried pellets (0.2–0.4 mm diameter). Rearing procedures are as detailed previously (Jenkins et al., 1999). Experimental procedures were approved by the University of Western Australia Ethics Committee and followed the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

For MSP, 100 larval fish were obtained at day 4 and transferred to rearing tanks. The cohort was divided between two experimental lighting conditions: (1) broad spectrum fluorescent lighting (Philips Coolwhite, 36 W) and (2) short wavelength-reduced lighting as is typical in estuarine conditions in which chlorophyll and tannins absorb short wavelength light (Lythgoe, 1979). To achieve the tannin-like conditions, yellow acetate filters (no. 764, Lee Filters, Andover, UK) surrounded the tanks and this treatment is referred to as the yellow filter group hereafter. The broad spectrum tanks were enclosed within neutral density acetate filters (optical density 0.2) to equate the intensity (quanta) of the treatments at approximately 70 μW cm^{-2} (400–700 nm). The spectral characteristics of the two rearing conditions and the transmission characteristics of the yellow filter are shown in Fig. 1. For the standard conditions, fishes were examined by MSP during the larval (0–40 days; *N*=6), post-settlement (41–100 days; *N*=8), juvenile (101–210 days; *N*=8) and adult stages (>1 year and 10 cm standard length; *N*=6). Fishes from the yellow filter conditions were sampled during the larval (*N*=4), post-settlement (*N*=6) and juvenile (*N*=6) stages. It was not possible to rear adult fish in the yellow filter group within the time frame of the investigation. In addition, post-settlement (*N*=4), juvenile (*N*=6) and adult fish (*N*=4) were caught from their natural environment, the estuarine section of the Swan River, Western Australia, and the λ_{max} of their photoreceptors determined by MSP within 1 week of capture. All attempts to capture live larval fish from the wild were unsuccessful. For molecular investigation, fishes reared in broad spectrum lighting were sampled at two stages: larval aged 20 days (<5 mm standard length) and adults.

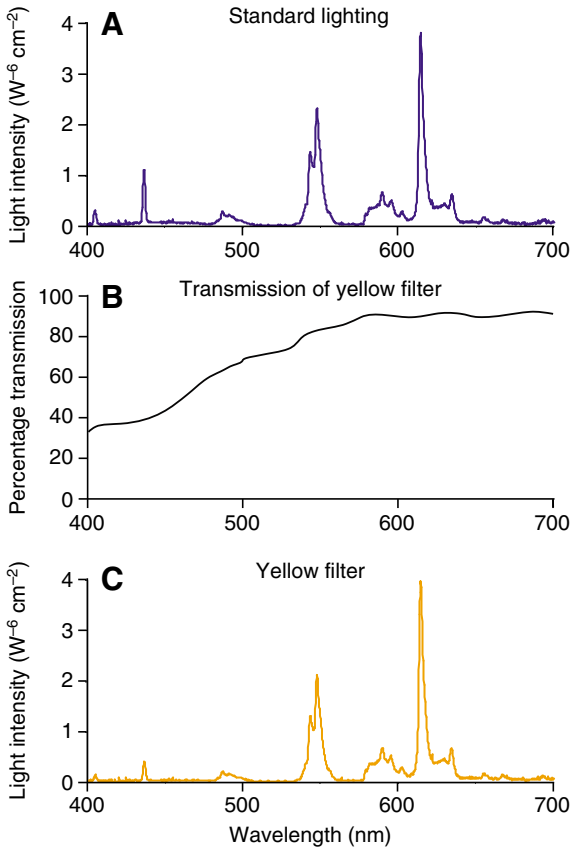

Fig. 1. Spectral characteristics of the lighting used for the two different rearing conditions. (A) Light intensity with broad spectrum fluorescent lights, plus a neutral density filter (0.2 optical density) around the tanks to equate the intensity of light with that of the short wavelength-reduced conditions (shown in C). (B) The transmission characteristics of the yellow filter used to reduce short wavelength light. (C) Light intensity with the tanks surrounded by the yellow filter (*note the reduction in the peaks at the shorter wavelengths).

Microspectrophotometry

Fishes were dark adapted for at least 2 h before being killed by immersion in a lethal dose of methanesulphonate (MS222, Sigma-Aldrich Pty, Castle Hill, NSW, Australia; 1:2000 w/v in seawater). For MSP examination, preparations of unfixed retinal tissue were teased apart in teleost Ringer solution (0.1 mol l^{-1} of the following: NaCl, KCl, Na_2HPO_4, $CaCl_2$) containing 10% dextran (Sigma, 250 000 M_r), on a rectangular 50 mm×22 mm no. 1 coverslip. The preparation was covered with a smaller (19 mm^2) no. 1 coverslip and sealed with nail varnish to prevent dehydration of the sample. All preparations were carried out under infrared illumination and visualised using an infrared image converter (FJW Industries Inc., Chicago, IL, USA).

A single-beam wavelength-scanning microspectrophotometer was used to measure the absorption characteristics of the

Table 1. Primers used for PCR amplification of opsin gene sequences from black bream retinal cDNA

	Forward primer (5′–3′)	Reverse primer (5′–3′)
Rh1	TTYCCNGTCAAYTTYCTCACN	GTTCATGAAGACRTAGATDAYAGGGTTRTA
LWS	AGTGGGGAGATGCAATTTT	GTTCATGAAGACRTAGATDAYAGGGTTRTA
Rh2Aα	GCGCGAATTCCACCATGGTTTGGGACGGC	CGGCGTCGACGCAGACACAGAGGACACTTCCGT
Rh2Aβ1	GGTAAGCGCGAATTCCACCATGGYTTGGGA	TTTCGGCGTCGACGCAGACACAGAGGACAC
Rh2Aβ2	AATATGGCTTGTGCTGCACCCCTCTTTTCG	AATACAGCCGGCAAATGAGGCATATGGGAC
SWS2	CACCTCAACTACATCCTGGT	ACAGAGCAAAGGAGGCGTAA
SWS1	CCACCTGTACGAGAACATCTCC	GTTCATGAAGACRTAGATDAYAGGGTTRTA

photoreceptor outer segments. The microspectrophotometer has been described previously (Partridge et al., 1992b), although recent modifications have been made to improve the optics and hence transmission of short wavelengths to the specimen (Shand et al., 2002). Spectral absorbance measurements were made by placing the outer segment of the photoreceptor in the path of the measuring beam and scanning over the wavelength range 350–750 nm. A single bidirectional scan (750 nm to 350 nm to 750 nm) was made for each outer segment, but this was combined with two separate baseline scans from an area adjacent to the outer segment being scanned. The two absorbance spectra thus obtained were averaged to improve the signal-to-noise ratio of the absorbance spectra used to determine the λ_{max} values. Following these 'pre-bleach' scans, outer segments were bleached with white light from the monochromator for 2–4 min and an identical number of sample and baseline scans subsequently made. The post-bleach average spectrum thus obtained was subtracted from the pre-bleach average to produce a difference spectrum for each outer segment and thereby confirm the presence of visual pigment.

Baseline and sample data were converted to absorbance values at 1 nm intervals and the upward and downward scans were averaged together by fitting a weighted three-point running average to the absorbance data (Hart et al., 2000). Absorbance spectra were normalised to the peak and long wavelength-offset absorbances, obtained by fitting a variable-point unweighted running average. Following the method of MacNichol (MacNichol, 1986), a regression line was fitted to the normalised absorbance data between 30% and 70% of the normalised maximum absorbance at wavelengths longer than that of the absorbance peak. The regression equation was used to predict the λ_{max} and fit the visual pigment template following the methods of Govardovskii et al. (Govardovskii et al., 2000). Acceptable pre-bleach spectra (Levine and MacNichol, 1985; Partridge et al., 1992b) all had a characteristic 'bell-shaped' curve with a clear alpha peak, low noise and a flat long wavelength tail above the wavelength at which the absorbance had fallen to less than 0.5% normalised maximum absorbance. To establish the cone classes present at different developmental stages, the λ_{max} values of the spectra of individual photoreceptors were presented as frequency histograms. Changes in the frequency of records within each cone class at each stage of development in fishes either reared in the different lighting regimens or wild-caught were compared using contingency chi-square.

Amplification of gene sequences

Fish were anaesthetised by immersion in MS222 (1:2000 w/v in seawater). Due to the small size of the larval fish, whole heads were pooled and used for extraction of mRNA. Three pools of 20 larval heads and four separate adult retinae were used. Prior to the isolation of RNA, eyes were placed in RNAlater (Ambion, Austin, TX, USA) to minimise RNA degradation. All tissue samples were initially stored at 4°C overnight to maximise the diffusion of the RNA preservation solution throughout the whole tissue and then stored at –80°C for further analysis.

RNA samples were treated with DNase to remove any contaminating genomic DNA. Following isolation of total RNA using Epicenter Technologies MasterPure^{TM} complete RNA purification kit (Madison, WI, USA), retinal mRNA was prepared using either the Qiagen Oligotex mRNA purification kit (Doncaster, Vic, Australia) or the Quickprep micro mRNA purification kit (Amersham Biosciences, Little Chalfont, UK). Genomic DNA was isolated from liver tissue using a standard phenol/chloroform method.

cDNA was prepared using Expand reverse transcriptase and oligo dT primers, and rod and cone opsin gene fragments were PCR amplified using the primers listed in Table 1. A full-length copy of the *Rh2Aα* sequence was obtained using 5′- and 3′-RACE with a primer pair specific for *Rh2Aα*. For *Rh2Aβ*, the sequence was obtained as two overlapping fragments, using primer pairs for *Rh2Aβ1* and *2*, which were subcloned into pMT4 to give a full-length sequence.

qPCR analysis of opsin expression

First strand cDNA, prepared from total RNA (1 μg) extracted from whole heads of larvae and the retinae of adult fish as described above, was used for qPCR. Individual visual pigment transcripts were quantified using gene-specific forward and reverse primers designed specifically to amplify *SWS1*, *SWS2*, *Rh1*, *Rh2* and *LWS* opsin transcripts (Table 2). In addition, forward and reverse primers were

Table 2. Primers used for the quantification of visual pigment transcripts from black bream retinal cDNA by qPCR

	Forward primer (5′–3′)	Reverse primer (5′–3′)
SWS1	TTTGAGCAGGTACATCCCTGAGGGTTTAGG	CCTTGTTTTCGTCTGTCGAGTAGGCGAAGT
SWS2A	ATTGGTGGTATGGTCAGCCTGTGGTCTCTT	TGCCGATTTCAGTATGAGGAGCAGCTGTGA
SWS2B	TTTGGCGGTATGGTCAGTCTGTGGTCTCTA	GGCCATTTTCAGTGTGATGAGCAGCTGAGT
Rh2Aα	TTTTTAGCTTGCGCTGCTCCCCCTCTTTTC	AATCCAGCCGGCAAATGTTGCGTATGGAGT
Rh2Aβ	AATATGGCTTGTGCTGCACCCCTCTTTTCG	AATACAGCCGGCAAATGAGGCATATGGGAC
LWS	AACAGGTACTGGCCTCATGGTCTGAAGACT	AAAATGCATATCCCGGGTTAGCCGCAGCAA
Rh1	TTTGTCTGCAAGCCCATCAGCAACTTCCGT	TTTTAATTCCCTCTCAGCCCTCTGGGTGGT

qPCR, quantitative PCR.

designed to amplify transcripts of the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) to use as an internal control to correct for sample-to-sample variation.

In all cases, regions of divergence between the different opsins were identified and 30-mer oligonucleotides were designed manually with non-conserved nucleotides present at the 3′-end of all forward and reverse primers to facilitate amplification of specific transcripts. The specificity of each primer was assessed and the production and validation of qPCR protocols was performed as previously described (Davies et al., 2007). All standard curves (semi-log plot of PCR cycle value for above background threshold value against log of input DNA concentration) gave line gradients very close to –3.32. The efficiency of the reaction is calculated from the line gradient value; in this study, all amplification efficiencies were close to 100% and the standard curve plots showed a very high coefficient of determination (R^2>0.99, P<0.01). In all cases, the triplicate reactions gave very small error bars showing a high reproducibility of amplification at each point. All primer combinations traversed at least one exon–exon boundary and yielded amplicon lengths of 336±8 bp. Assays were performed in triplicate with three independent cDNA templates (40 ng), using 1× Platinum SYBR Green qPCR SuperMix-UDG-Master mix kit (Invitrogen, Paisley, UK), and forward and reverse primers at 200 nmol l^{-1}. A Corbett Life Science Rotor-Gene qPCR detector was used to detect SYBR Green reporter dye fluorescence and data were analysed offline. A typical protocol took 2 h to complete and included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 20 s (denaturation), 56°C for 20 s (annealing) and 72°C for 30 s (extension), with a plate read at the end of the extension phase for each cycle. Melting and standard curves were generated for each amplicon using a 10-fold serial dilution (1 pg to 10 ng) of input template. Fluorescence was quantified as previously described (Davies et al., 2007).

Phylogenetic analysis

Neighbour joining (Saitou and Nei, 1987) was used to construct phylogenetic trees from opsin amino acid sequences after alignment with ClustalW (Higgins et al., 1996). The degree of support for internal branching was assessed by bootstrapping with 1000 replicates using the MEGA2 computer package (Kumar et al., 2001).

In vitro expression and analysis of recombinant pigments

The *Rh2Aα* and *Rh2Aβ* sequences were cloned into the pMT4 expression vector using forward and reverse primer pairs for *Rh2Aα* and *Rh2Aβ1* (Table 1). Human embryonic kidney (HEK-293T) cells were transiently transfected with 7 μg per plate of opsin-pMT4 recombinant expression vector by GeneJuice (Merck, Hoddesdon, UK), using thirty 90 mm plates per experiment. After 48 h, transfected cells were harvested and washed four times with phosphate-buffered saline (PBS, pH 7.0) and stored at –80°C until required. The recombinant visual pigments were generated by suspending the cells in PBS, followed by incubation with 40 μmol l^{-1} 11-*cis*-retinal in the dark. The membrane-bound pigments were solubilised and purified by immunoaffinity chromatography using the anti-Rho1D4 antibody coupled to a CNBr-activated Sepharose column as previously described (Molday and MacKenzie, 1983), eluted, and stored on ice.

Chilled reconstituted visual pigment samples were subjected to spectrophotometric analysis and absorbance spectra were recorded in the dark using a Spectronic Unicam UV500 dual-beam spectrophotometer. Subsequently, the samples were bleached by exposure to fluorescent light for 10 min. Spectrophotometric recordings were repeated three times per sample and the bleached spectra were subtracted from the dark absorbance spectra to produce difference spectra for the calculation of λ_{max} for each expressed black bream visual pigment. The resultant visual spectra were overlaid with visual pigment templates (Govardovskii et al., 2000) and best-fit spectral curves were obtained using the Solver add-in function in Microsoft Excel to vary the λ_{max}. As absorbance spectra are distorted by the underlying absorbance and scatter of the protein, difference spectra were used as the more accurate estimation of λ_{max} values.

RESULTS

Spectral absorption characteristics of the photoreceptors

Spectra obtained from cone outer segments of fish reared in standard aquarium lighting at the four different developmental stages are shown in Fig. 2. During the larval stage, the majority of cones could be assigned to one of two classes with λ_{max} values ranging from 418 to 430 nm (425 nm class) and from 520 to 545 nm (535 nm class). A single cone with λ_{max} at 457 nm was also identified. By the post-settlement stage, the number of cones with λ_{max} values between 430 and 475 nm had increased, indicating a transition to

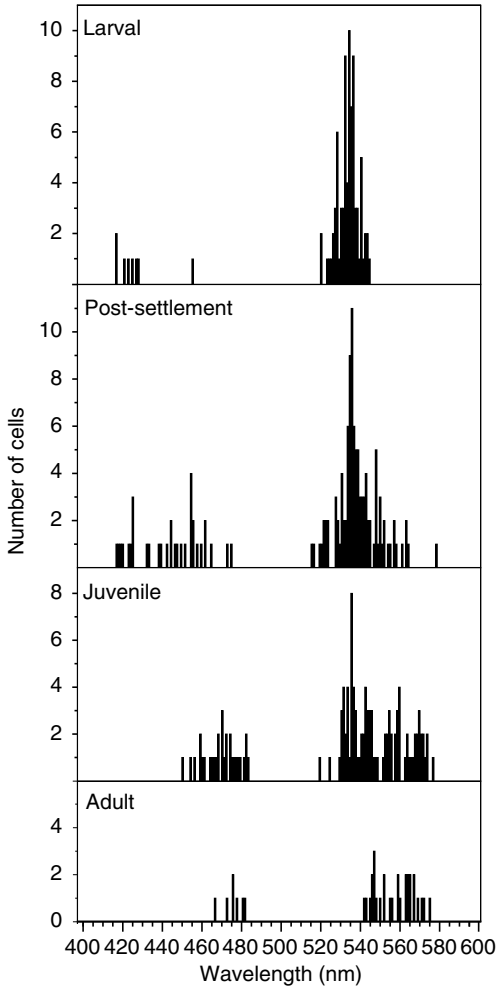

Fig. 2. Changes in the frequency of cones during development in fish reared in standard lighting conditions. The individual λ_{max} values from all cones scanned, as determined by curve fitting, are plotted at 1 nm intervals.

longer wavelength sensitivity. The majority of the long wavelength-sensitive cones were still centred on 535 nm, but a few cones with longer λ_{max} values were present. By the juvenile stage, the 425 nm class was absent and the short wavelength class had λ_{max} values centred on 475 nm. In the long wavelength cones, there was a range of λ_{max} values between 520 and 576 nm but with classes centred on 535, 555 and 570 nm. In the adult, the majority of longer wavelength-sensitive cones had λ_{max} values between 540 and 575 nm. Overall, therefore, there was a clear temporal shift in sensitivity of the cone photoreceptors to longer wavelengths.

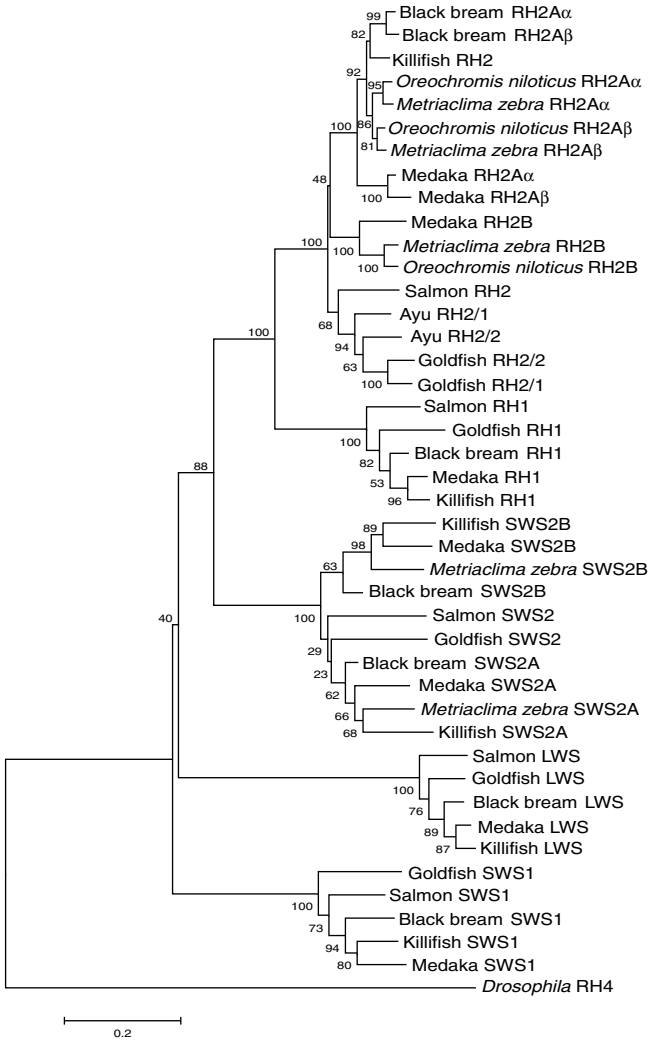

Fig. 3. Phylogenetic tree for visual opsin gene sequences. The tree was generated by the neighbour-joining method (Saitou and Nei, 1987) using amino acid sequences aligned by ClustalW (Higgins et al., 1996). The degree of support for internal branching was assessed by bootstrapping with 1000 replicates using the MEGA2 computer package (Kumar et al., 2001). GenBank accession numbers for the sequences (from top to bottom) are EU090913, EU090914, AY296739, DQ235683, DQ088651, DQ235682, DQ088650, AB223053, AB223054, AB223055, DQ0088652, DQ235681, AY214132, AB098703, AB098704, L11865, L11866, AF201470, L11863, DQ354577, AB180742, AY296738, AY296736, AB223057, AF247118, DQ354581, AY214134, L11864, DQ354580, AB223056, AF247114, AY296737, AY214131, L11867, DQ354578, AB223051, AY296739, D85863, AY214133, DQ354579, AY296735, AB223058, NM_057353.

Rod and cone opsin genes

In order to relate the different visual pigments present to visual opsin genes expressed in the retina, PCR, with retinal cDNA as the template and with the primer pairs listed in Table 1, was used to amplify the expressed visual opsins. The amplified fragments revealed seven different sequences that were identified by phylogenetic analysis (Fig. 3) as a single rod *Rh1* opsin, and six cone opsins comprising a single *LWS* opsin, two *Rh2* opsins, two *SWS2* opsins and a single *SWS1* opsin.

The two *Rh2* genes were found to be closely related, with 96% identity of the predicted amino acid sequence. Phylogenetic analysis indicated that they belonged to the *Rh2A* group (Parry et al., 2005) and their proximity in the tree indicates that they had arisen from a recent gene duplication within the black bream lineage. The finding is similar therefore to the *Rh2Aα* and *Rh2Aβ* genes in cichlids, which also derive from a duplication within their own lineage (Parry et al., 2005). The black bream genes have accordingly been designated *Rh2Aα* and *Rh2Aβ*.

The two *SWS2* genes were found to be more divergent with only 84% identity of the predicted amino acid sequence and fell into the separate *SWS2A* and *SWS2B* clades. Similar variants were also found in cichlids, killifish and medaka (Spady et al., 2006). The gene duplication is more ancient therefore than the *Rh2A* gene duplication and probably occurred near the base of the Paracanthoptergian/Acantopterygian radiation. The black bream sequences have been designated *SWS2A* and *SWS2B* based on their phylogenetic placement.

A *SWS1* opsin was also identified even though no UV-sensitive cones were found by MSP in this or our previous study (Shand et al., 2002). The discrepancy is, however, not unexpected as sampling by MSP of UV-absorbing cones presents technical difficulties. From the coding sequence, the opsin would be expected to generate a UV-sensitive pigment (see below).

The opsin sequences from black bream have been deposited in GenBank under the following accession numbers: *Rh1*, DQ354577; *LWS*, DQ354578; *Rh2Aα*, EU090913; *Rh2Aβ*, EU090914; *SWS2A*, DQ354580; *SWS2B*, DQ354581; *SWS1*, DQ354579.

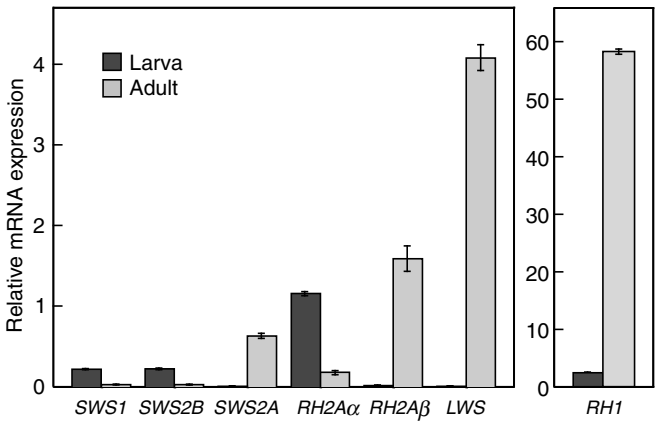

Fig. 4. Quantification of opsin gene transcript levels in the larval and adult retina. Each bar shows the mean (±s.e.m.) relative expression of each opsin in either the larva or adult. The values were obtained from separate RNA preparations from four adult retinae and three pools of 20 larval heads. For each opsin, the difference in relative expression between larva and adult was statistically significant (Student's *t*-test) at the 1% probability level.

Quantitative analysis of opsin expression levels in larval and adult retinae

The developmental changes in opsin gene expression were examined at two stages, larval and adult. Values were obtained from four separate adult retinae and three pools of 20 larval heads. As shown in Fig. 4, the predominantly expressed opsins at the larval stage were *SWS1*, *SWS2B* and *Rh2Aα*. Only very low levels of *SWS2A*, *Rh2Aβ* and *LWS* transcripts were detected. In adult samples, however, the predominantly expressed opsins switched to *SWS2A*, *Rh2Aβ* and *LWS*, consistent therefore with the overall shift shown in photoreceptor sensitivity to longer wavelengths evident in Fig. 2. Note also the 25-fold rise in the relative level of *Rh1* expression from the larval stage to adult. In the larval stage, the combined expression of cone opsins represented 40% of the total (rod + cone) expressed opsin but the value fell to only 10% in the adult, as the result of a substantial increase in the expression of *Rh1* opsin.

Since whole heads were used to obtain RNA at the larval stage, opsins expressed in the pineal gland will be included in the sample. Relevant to this is the report that SWS1 opsin is expressed in the embryonic pineal gland of the halibut (Forsell et al., 2001; Forsell et al., 2002), although retinal expression was also seen. It is possible therefore that the absence of UV tracings amongst the MSP records reflects a true absence of UV cones and that the SWS1 opsin is expressed only in the pineal gland. This is, however, unlikely, especially since the relative level of SWS1 transcript is similar to that for SWS2B, with the latter correlating with one of the two cone classes found at relatively high frequency by MSP in the larval retina. However, the possibility that a small amount of SWS1 expression may be attributable to the pineal gland cannot be excluded.

Correlation of cone opsins and visual pigment classes

Based on the spectral ranges already known for different opsin classes, it was possible to relate cone class with visual pigment gene. The expression of the different cone opsin genes was compared with the frequency of the different cone classes in larval and adult fish. As shown in Fig. 5, the predominant transcripts in the larval retina were from the *SWS2B* and *Rh2Aα* genes and these correlate with the 425 and 535 nm cone classes, whereas the transcripts in the adult retina arose largely from the *SWS2A*, *Rh2Aβ* and *LWS* genes, which correlate with the 475, 555 and 570 nm cone classes. On this basis, the pigment expressed in each cone class can be identified as follows: 425 nm class, SWS2B; 475 nm class, SWS2A; 535 nm class, Rh2Aα; 555 nm class, Rh2Aβ; 570 nm class, LWS.

In vitro expression and regeneration of the Rh2 pigments

Since the λ_{max} value of the Rh2Aβ pigment at 555 nm is very long wavelength shifted compared with orthologous pigments from other species, the spectral characteristics of both Rh2A pigments were examined by *in vitro* expression of a recombinant opsin, followed by pigment regeneration with 11-*cis*-retinal and spectral analysis. As shown in Fig. 6, the fitted Govardovskii opsin template gave a λ_{max} of 527 nm for Rh2Aα, consistent therefore with the average λ_{max} of the 535 nm cone class. Surprisingly, however, the Rh2Aβ pigment gave a similar λ_{max} at 534 nm, which is significantly shorter than the average value of 555 nm obtained by MSP. A possible explanation for the discrepancy is that the adult pigment has a mixed A_1/A_2 chromophore.

Effects of rearing conditions

The relative frequencies of the different cone classes identified by MSP at four developmental stages are summarised in Fig. 7, together with similar data obtained from wild-caught fishes and from fishes reared under yellow filters. No wild-caught larvae or adults reared under yellow filters were available for analysis. At the post-settlement stage, the spectral peaks of the individual cones showed a wide variation consistent with the presence of 425, 475, 535, 555 and 570 nm classes in both aquarium-reared and wild-caught fish. What is striking at this stage is the substantial increase in the frequency of the 555 and 570 nm classes in the yellow filter fish and the 570 nm class in the wild-caught fish. The trend continues into the adult in the wild-caught fish, where the 570 nm class is present at a much higher frequency than the 555 nm class. Except for the wild-caught and yellow filter fish at the post-settlement stage (P=0.02), all the differences in the distribution of cone classes are statistically

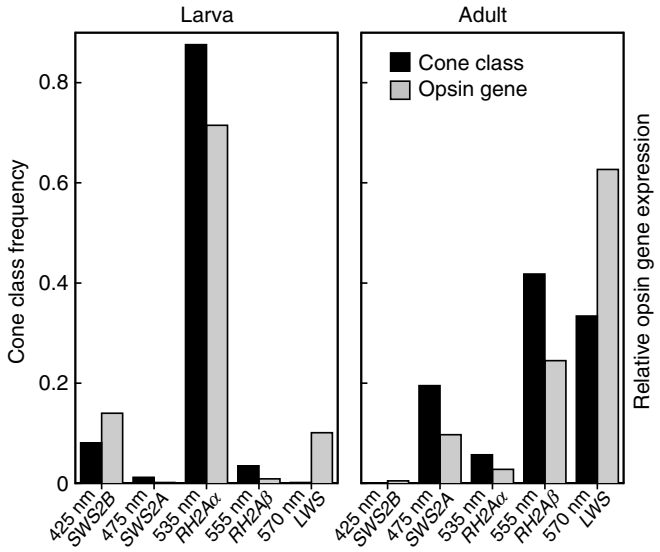

Fig. 5. Comparison of the frequency of cone classes measured by microspectrophotometry and opsin gene expression relative to total cone opsin expression in the larval and adult retina.

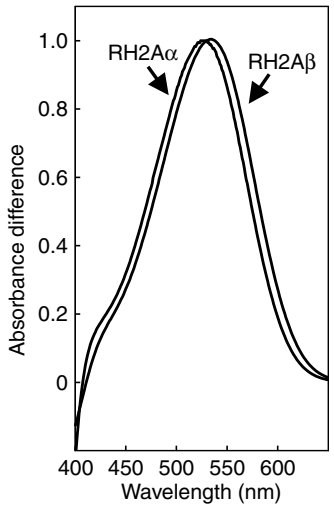

Fig. 6. Absorption spectra for Rh2Aα and Rh2Aβ pigments generated *in vitro*. The difference spectrum (dark minus bleached) for each pigment is shown as a fitted Govardovskii template (Govardovskii et al., 2000). The λ_{max} values for Rh2Aα and Rh2Aβ are 527 and 534 nm, respectively.

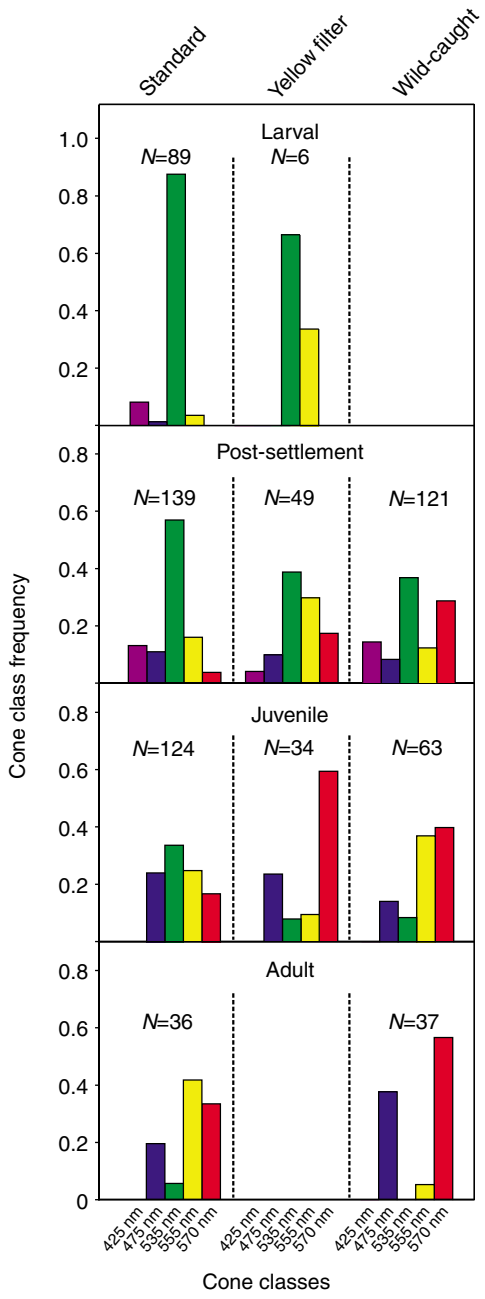

Fig. 7. Frequency of cone classes at different developmental stages. Data from all four stages for fish reared under standard conditions are shown. No data were available for wild-caught larval fish or for adult yellow filter fish.

significant (P<0.001). Overall, therefore, there is a substantial shift in the frequency of long wavelength-sensitive cones in wild-caught adult fish and in older fish reared in aquaria under yellow filters, compared with fish reared under standard laboratory lighting.

DISCUSSION

Changes in the expression of opsins

We have demonstrated that six different cone opsin genes are differentially expressed in the retina of black bream during ontogeny. Our findings confirm the original prediction, derived from modelling changes in visual pigment bandwidths (Shand et al., 2002), that changes in opsin expression underlie the changes in spectral sensitivity of the cone photoreceptors in black bream.

The MSP data obtained at four developmental stages spanning the period from the larval stage to adult identified five cone classes with λ_{max} values around 425, 475, 535, 555 and 570 nm. In the larval retina, the two most abundant cone classes were the 535 nm and the 425 nm classes. The 535 nm class progressively falls in frequency through the post-settlement and juvenile stages and the 425 nm class disappears by the adult stage. Coincidental with these reductions, the 475, 555 and 570 nm classes increase in frequency through these stages. The λ_{max} changes of the visual pigments are closely mirrored by changes in the expression of the six cone opsin genes and these data have enabled the different visual pigments to be assigned to a particular opsin gene.

Phylogenetic analysis identified the cone opsins as members of the *SWS1*, *SWS2*, *Rh2* and *LWS* gene classes. Two copies of *SWS2* (*A* and *B*) and two copies of *Rh2*, both belonging to the *Rh2A* clade (Parry et al., 2005), were also found. The 425 nm cone class expresses the *SWS2B* gene and the 535 nm cone class expresses the *Rh2Aα* class. These two genes represent the 'larval' variants as expression in the adult switches to the *SWS2A* gene, which encodes the 475 nm pigment, and the *Rh2Aβ* gene, which encodes the 555 nm pigment. In addition, the adult retina expresses the *LWS* gene, which encodes the 570 nm pigment at a substantially higher level than in the larval retina. The *SWS1* gene that was amplified from retinal cDNA is not expressed in the adult retina but represents approximately 14% of total cone opsin gene expression in the larval stage. The *SWS1* opsin gene most probably specifies a UV-sensitive pigment since it possesses Phe86. This residue is generally sufficient to specify a UV-sensitive pigment (Hunt et al., 2007). MSP, however, failed to find a corresponding class of cones, most probably due to technical reasons.

Amino acid substitution at nine different sites has been linked to spectral shifts in the SWS2 pigments of Teleostei (Cowing et al., 2002) and Amphibia (Takahashi and Ebrey, 2003). In the black bream, however, substitution is found at only one of these sites, with non-polar Cys at site 94 in 'larval' SWS2B replaced by non-polar Ala in 'adult' SWS2A. Substitution at this site is responsible for the spectral difference between SWS2 pigments of the newt and bullfrog (Takahashi and Ebrey, 2003), but in contrast to the black bream pigments, polar Ser in the newt is replaced by non-polar Ala in the bullfrog to give a 14 nm short wavelength shift. It is unlikely therefore that a Cys to Ala change would produce a similar spectral shift. The molecular basis for the 50 nm difference between the SWS2A and SWS2B pigments remains to be established.

The λ_{max} values obtained for *in vitro* expressed Rh2Aα and Rh2Aβ pigments are very similar at 527 and 534 nm, respectively. The *Rh2Aα* gene, with its strong bias towards larval expression, links the 527 nm pigment with the 535 nm cone class, and expression of the *Rh2Aβ* gene is coincident with the appearance of the 555 nm cone class in the adult. The 534 nm value obtained for the *in vitro* expressed pigment from the *Rh2Aβ* gene is therefore short wavelength shifted by 21 nm compared with the MSP value. Such a discrepancy could arise either from an undefined post-translational change that occurs only in the intact photoreceptor or from the presence of some A_2 chromophore that long wavelength shifts the λ_{max} *in situ*. The spectral analysis carried out previously (Shand et al., 2002) does not rule out the presence of some A_2-based pigment.

The amino acid sequences of the Rh2Aα and Rh2Aβ opsins in the black bream differ at only 13 sites. Of these, only five are in transmembrane regions, with one of these latter sites involving a

change from a non-polar (Val in Rh2Aα) to a polar (Thr in Rh2Aβ) residue. However, none of the 13 residue differences are at sites previously shown to be involved in spectral tuning, so it is perhaps not surprising that the *in vitro* λ_{max} values of the A_1 pigments are very similar at 527 and 534 nm, respectively.

Spectral shifts between different LWS pigments are generally due to the residues at five sites, 164, 181, 261, 269 and 292 (Yokoyama and Radlwimmer, 2001). At each site, the bream LWS opsin sequence had the amino acid (Ser164, His181, Tyr261, Thr269 and Ala292) associated with long wavelength-shifted pigments. The combination of residues at these key tuning sites would be expected therefore to generate a pigment with a λ_{max} around 565 nm, which is consistent with the average λ_{max} value of 570 nm for the black bream LWS pigment as determined by MSP.

The developmental changes in opsin gene expression in black bream show some similarities to those described in the cichlid *Oreochromis niloticus* [(Nile tilapia (Spady et al., 2006)]. In this species, expression of the *SWS1* gene is also confined to larval and juvenile stages and the *LWS* gene also shows a very substantial rise from the larval stage to adult. However, three *Rh2* genes are present in the Nile tilapia, a pair of recently diverged genes, *Rh2Aα* and *Rh2Aβ* as found in black bream, and an *Rh2B* gene, which arose from a more ancient duplication. The expression of the *Rh2* genes in Nile tilapia differs from that in the black bream with *Rh2B* expressed in the larvae, whereas *Rh2Aα* and *β* are expressed throughout development. The *SWS2A* and *B* genes in the Nile tilapia also show developmental changes, with both forms showing an increase in expression from larva to juvenile but only *SWS2A* continuing at a high level into the adult. In black bream, the *SWS2B* variant is clearly the 'larval' form and *SWS2A* is the 'adult' form.

At the larval stage, *Rh1* opsin gene expression represents about 60% of total opsin gene expression in black bream. The percentage rises, however, to reach about 90% in the adult, which reflects the substantial increase in the frequency of rod *versus* cone photoreceptors during development (Shand et al., 1999). MSP data show that the Rh1 pigment has a λ_{max} at 508 nm (Shand et al., 2002). The major tuning sites for teleost Rh1 pigments identified previously (Hunt et al., 2001) are at residues 83, 122, 261 and 292 with Asp, Glu, Phe and Ala, respectively, at these sites in the black bream pigment. A similar combination of residues was also found in the goldfish with a λ_{max} of 492 nm (Johnson et al., 1993) and in a deep-sea fish, *Phycis blennoides,* with a λ_{max} of 494 nm (Hunt et al., 2001). It remains uncertain therefore which other sites are responsible for the 16–18 nm long wavelength shift present in the black bream pigment.

Effects of environmental light

The effect of rearing black bream under short wavelength-reduced conditions is to increase the frequency of longer wavelength-sensitive cone classes; such changes would be expected to generate a substantial increase in the sensitivity of the fish to long wavelength light. In contrast, studies on the blue acara, *Aequiodens pulcher*, reared under monochromatic blue light revealed a reduction in the frequency of the single cone class maximally sensitive to blue light (Kroger et al., 1999; Kroger et al., 2003; Wagner and Kroger, 2000; Wagner and Kroger, 2005). In adult black bream, the frequency ratios between 550 and 570 nm cone classes rose from 1:0.8 in the standard condition fish to 1:10.5 in the wild-caught fish. This result suggests that the double cones in adult fish switch from an approximately equal frequency of outer segments with the 550 and 570 nm pigments in the standard condition fish to a majority with only the 570 nm pigment in wild-caught fish. The effect of exposure to light reduced in short wavelengths would appear therefore not to be on the production of double cones but on the type of pigment produced in their outer segments.

There are two possible mechanisms by which the opsin changes could be taking place. In the Pacific salmon and rainbow trout (Cheng and Novales Flamarique, 2004; Cheng and Novales Flamarique, 2007), individual single cones have been shown to switch from expressing SWS1 to SWS2 during development, whereas in the zebrafish new opsins are expressed in newly differentiated photoreceptors as the retina progressively grows (Takechi and Kawamura, 2005). Our results imply both mechanisms may be in operation. In black bream, the visual pigment changes are not abrupt and the MSP results give intermediate λ_{max} values during the shift to longer wavelengths, indicating that both the original and new visual pigment are present within the individual outer segments during the transition phase. Such intermediate λ_{max} values would be expected because new opsins expressed in cone outer segments become mixed with those already present within an outer segment (Bok and Young, 1972) and are consistent with the findings in salmonids. It should also be noted, however, that during the larval and juvenile stages the black bream retina undergoes rapid growth by addition of cells at the retinal margins (Shand et al., 1999) and the observations of the differing proportions of double cone λ_{max} values between the post-settlement and juvenile fish in the different light environments (Fig. 7 this study) indicate that the environmental light is also fine-tuning the expression of opsins in the newly developed cells. *In situ* hybridisation studies are needed to confirm how the two mechanisms may be interacting to bring about changes in opsin expression across the retina of black bream.

Intraspecific variation in spectral sensitivity has been observed using compound action potentials from ganglion cells in the three-spine stickleback from lakes with differing spectral light qualities (McDonald and Hawryshyn, 1995). In addition, differences in the levels of cone opsin expression have been related to variation in the frequency of cone classes in bluefin killifish from different habitats (Fuller et al., 2004). Seasonal changes in spectral sensitivity, attributed to changes in chromophore, have also been reported in a number of freshwater species (for review, see Bowmaker, 1995). The black bream investigated here were reared under a regimen that shifts the ambient light to longer wavelengths, which, while designed to be similar to the natural environment, did not reproduce the seasonal changes in light quality in which black bream are found. The ability to respond to changes in environmental light by altering opsin expression in the long wavelength-sensitive double cones, both during growth and in established populations of cells, is likely to be an advantage for fishes in a variable estuarine environment. Our study also has implications for fisheries' restocking programmes in which hatchery-reared fish are released as juveniles to replenish wild stocks. Survival of juveniles, released into a light environment that differs from that of the rearing conditions, may be affected by inappropriate spectral sensitivity for visual tasks such as feeding and predator avoidance.

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