

The visual pigments of a deep-sea teleost, the pearl eye *Scopelarchus analis*

Marie A. Pointer, Livia S. Carvalho, Jill A. Cowing, James K. Bowmaker and David M. Hunt*

UCL Institute of Ophthalmology, 11–43 Bath Street, London, EC1V 9EL, UK

*Author for correspondence (e-mail: d.hunt@ucl.ac.uk)

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Summary

The eyes of deep-sea fish have evolved to function under vastly reduced light conditions compared to those that inhabit surface waters. This has led to a bathochromatic shift in the spectral location of maximum absorbance (λ_{\max}) of their rod (RH1) pigments and the loss of cone photoreceptors. There are exceptions to this, however, as demonstrated by the deep-sea pearl eye *Scopelarchus analis*. Here we show the presence of two RH1 pigments (termed RH1A and RH1B) and a cone RH2 pigment. This is therefore the first time that the presence of a cone pigment in a deep-sea fish has been confirmed by molecular analysis. The λ_{\max} values of the RH1A and RH1B pigments at 486 and 479 nm, respectively, have been determined by *in vitro* expression of the recombinant opsins

and show the typical short-wave shifts of fish that live in deep water compared to surface dwellers. RH1B, however, is expressed only in more adult fish and lacks key residues for phosphorylation, indicating that it may not be involved in image formation. In contrast, the RH2 pigment has additional residues near the C terminus that may be involved in phosphorylation and does not show temporal changes in expression. The distribution of these pigments within the multiple retinæ of *S. analis* is discussed.

Supplementary material available online at
<http://jeb.biologists.org/cgi/content/full/210/16/2829/DC1>

Key words: visual pigment, deep-sea fish, opsin.

Introduction

The deep-sea teleost *Scopelarchus analis* (Brauer) has intrigued visual scientists for decades. It has a highly modified cylindrical eye that is directed dorsally to provide a binocular image of the water column above (Fig. 1). The lens is spherical, as with most fish, but also acts as a light filter, markedly reducing transmission below 450 nm (Douglas and Thorpe, 1992). The most unusual morphological adaptation, however, is the presence of multiple retinæ; a main retina lies at the base of the cylinder, an accessory retina along the nasal wall, and a retinal diverticulum between the two (Locket, 1977). Matthiessen's ratio confirms that the distance between the main retina and the lens will enable a focussed image to be formed, whereas the focal length between the accessory retina and the lens does not fulfil Matthiessen's ratio and is therefore too short for image forming. This suggests that the role of the accessory retina is not for fine visual acuity but more for gross light perception. A lens pad (sometimes termed the pearl organ) is present that provides an extension of the visual field (Munk, 1966), and it has been proposed (Locket, 1977) that this extends the ventrolateral monocular field of view by wave-guiding light on to the accessory retina. The diverticulum is described as transitional in structure between the main and accessory retinæ (Collin et al., 1998). It receives light stimulation from below the eye and from within the mouth and, in a closely related species, *S. michaelsarsi*, a mixture of rod and cone photoreceptors is present (Collin et al., 1998).

The organisation of the retina has been determined in detail

for *S. michaelsarsi* (Collin et al., 1998). In this species, rod-like photoreceptors are present in all regions of the retina but their organisation is determined by their location. For example, in the centronasal region of the main retina, they are ungrouped with very long outer segments (340 μm), whereas groups of 35 receptors with shorter outer segments (40 μm) that form 'receptive cups' and act as macroreceptors are found in the centrotemporal region. Cone-like photoreceptors are only present in the ventral region of the accessory retina where they are found intermingled with rod-like photoreceptors in a nasotemporal band. In this latter regard, *S. michaelsarsi* differs from *S. analis* since cone-like receptors are only found in the temporal main retina of *S. analis*. In contrast therefore to most deep-sea fish that have a rod-only retina (Douglas et al., 2003), cone photoreceptors are retained in *Scopelarchus* sp.

Microspectrophotometry of photoreceptor outer segments from the main and accessory retinæ of *S. analis* has identified three visual pigments with λ_{\max} values at 444, 479 and 505 nm (Partridge et al., 1992). The main retina contains only the 505 and 444 nm pigments, with both present in the same outer segments. The accessory retina contains all three pigments, with the 505 and 444 nm pigments again found together in the same outer segments. In both cases, sequential scans at 8 μm intervals along the outer segment have demonstrated that the 505 nm pigment is always located at the distal end and the 444 nm pigment at the proximal end (Partridge et al., 1992), with an abrupt transition between the two. The accessory retina also contains receptors that solely express the 444 or 479 nm

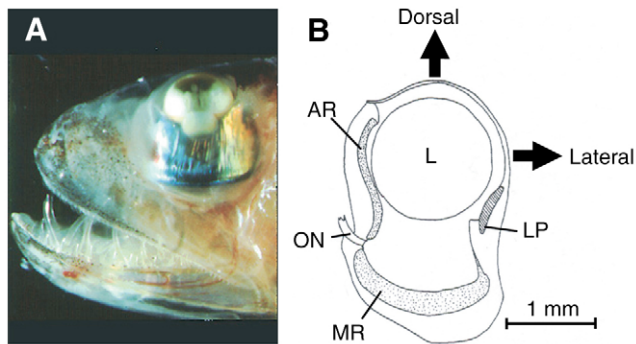


Fig. 1. *Scopelarchus analis*. (A) Photograph of the tubular eye, courtesy of Prof S. Collin. (B) Diagram of a vertical section through the eye. AR, accessory retina; L, lens; LP, lens pad; MR, main retina; ON, optic nerve. Redrawn from Partridge et al. (Partridge et al., 1992).

pigments, a situation not present in the main retina. These observations were made on a single fish that was considered to be in the process of maturing from a shallow-living juvenile to a deep-sea adult, and the replacement of the 505 nm with the 444 nm pigment in the same photoreceptors was considered to be a reflection of this maturation (Partridge et al., 1992). A similar situation has been shown to exist in rod photoreceptors during the metamorphosis of the common eel *Anguilla anguilla*, where one rod pigment is replaced by a second with a λ_{\max} that is also shifted to a shorter wavelength (Hope et al., 1998).

Materials and methods

Collection of fish

Specimens of *Scopelarchus analis* (Brauer) were caught from the NERC research ship *RRS Discovery* during cruise D243 by deep-water trawling with a rectangular midwater trawl combination net in the area of the North Eastern Atlantic south of the Canary Islands.

Fish were dead when brought to the surface. Dissected tissue samples or whole fish were either rapidly cooled to -80°C for storage or placed in absolute ethanol prior to storage at -20°C .

Nucleic acid extraction

Retinal mRNA was isolated from deep-frozen eyes with the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Tadworth, Surrey, UK) and complementary DNA (cDNA) was synthesised from this mRNA using the Superscript First-Strand Synthesis System (GibcoBRL, Paisley, UK). Genomic DNA (gDNA) was isolated from a whole body sample minus head using a standard phenol/chloroform method.

PCR, gel electrophoresis and sequencing

The standard PCR reaction contained 0.4 mmol l^{-1} dNTPs, 1.5–3.0 $\mu\text{mol l}^{-1}$ MgCl_2 , 0.4 $\mu\text{mol l}^{-1}$ each of the forward and reverse primers, 2.5 U Biotaq DNA polymerase (Biolone, London, UK) and approximately 30 ng cDNA or 100 ng gDNA in a total volume of 50 μl . Cycling conditions were an initial denaturation at 94°C for 3 min, then 35 cycles of denaturing at 94°C for 30 s, annealing for 45 s and extension at 72°C for 45 s. This was followed by a final extension for 7 min at 72°C . On

completion, 40 μl was run on a 1–2% (w/v) agarose gel containing ethidium bromide at $1 \mu\text{g ml}^{-1}$. A DNA 1 kb plus ladder (GibcoBRL) was also run to allow approximate size determination of DNA fragments.

Gene-walking PCR on gDNA was carried out according to published methods (Dominguez and Lopez-Larrea, 1994). 5' and 3' rapid amplification of cDNA ends (RACE) was achieved using the FirstChoiceTM RLM-RACE Kit (Ambion, Austin, TX, USA). Methods were carried out in accordance with the manufacturer's instructions.

In all cases, PCR products were inserted into the pGEM-T easy cloning vector (Promega, Southampton, Hampshire, UK) and fully sequenced on either an ABI 373a automated DNA sequencer or 3100 Gene Analyser (Foster City, CA, USA) with Big Dye terminator chemistry.

In vitro expression of pigments

Opsin coding sequences were amplified from retinal cDNA using *Pfu* polymerase with primers to the 5' and 3' ends that included *EcoRI* and *SalI* sites, respectively, to provide complementary 'sticky ends' for directional cloning into the expression vector pMT4. After digestion, the resulting fragments were inserted into *EcoRI/SalI* digested pMT4. This plasmid is a derivative of the mammalian expression vector pMT2 and additionally carries the sequence of the bovine 1D4 epitope, including the stop codon, downstream of and in frame with the *SalI* site. In all cases, the opsin coding sequences were then checked using gene specific primers.

HEK 293T cells were then transfected with the recombinant vector using Lipofectamine (Invitrogen, Paisley, UK). Thirty \times 90 mm plates were used per sample. Cells were harvested 48 h post transfection, washed four times with PBS, pH 7.0, and the cell pellets stored at -80°C prior to generation of the pigments. Pigments were generated by suspending cells in PBS, pH 7.0, and incubating with 40 $\mu\text{mol l}^{-1}$ 11-*cis* retinal in the dark. The pigment was solubilised from cell membranes and purified by immunoaffinity chromatography using an anti-1D4 antibody coupled to a CNBr-activated sepharose column following previously published methods (Molday and MacKenzie, 1983). For some pigment regenerations, phosphatidylcholine (0.8 mg ml^{-1}) was sonicated and added to the membrane preparation. Purified pigment was eluted from the column and stored on ice. Absorption spectra were recorded in the dark using a Spectronic Unicam UV500 dual beam spectrophotometer (Cambridge, UK). The sample was then photobleached for 5 min using white light from a fluorescent bulb and the spectrum recorded again. The λ_{\max} value of the pigment was determined by subtracting the photobleached spectrum from the dark spectrum to produce a difference spectrum to which a Govardovskii template was fitted (Govardovskii et al., 2000) using a Solver add-in to Microsoft Excel, which varies the λ_{\max} until the best fit to the template is found.

In some cases (as noted in the Results), the pigments were exposed to a pressure of 10 MPa during regeneration. All manipulations prior to regeneration were carried out at atmospheric pressure but immediately after the addition of 11-*cis*-retinal, the samples were transferred into a high-pressure system. This consisted of a cell with sapphire windows, which

fitted into a dual-beam absorption spectrometer. The sample was contained in a sealed cuvette that sits between the windows in the beam-path. Pressure was supplied by a capillary feed from a manual screw pump, with either ethanol or water as the pressure fluid. The pump is capable of reaching 700 MPa in less than 1 min.

Phylogenetic analysis

Neighbour-joining (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the opsin gene sequences. The degree of support for internal branching was assessed by bootstrapping with 1000 replicates. All computations were carried out with either the MEGA3 computer package (Kumar et al., 2001) or PAUP 4.0b10 (Swofford, 1991).

Results

Identification of rod opsin sequences

A PCR with gDNA as a template and with the degenerate rod (RH1) opsin primers Rod+ and Rod- (supplementary material Table S1) generated a 650 bp fragment, which was cloned into the pGEM-T-Easy vector. Thirty clones were sequenced and two distinct RH1 opsin sequences identified. Neither contained introns, which is typical of teleost RH1 opsin genes (Fitzgibbon et al., 1995). The two opsins have been termed RH1A and RH1B. The coding sequences of both genes were completed using a combination of gene walking with genomic DNA and

3' RACE using cDNA synthesised from mRNA isolated from intact eyes of three fish, two caught at 300 m and one at 950 m. The deduced amino acid sequences plus the 5' upstream sequences for RH1A and RH1B are shown in Fig. 2A,B. Note the deletion of amino acid residues 335–341 in the RH1B sequence.

The identity between the two sequences is 77% and 81% at the nucleotide and amino acid levels, respectively. Both show highest identity to other RH1 opsins, as determined by nucleotide and protein BLAST (BLAST database at <http://www.ncbi.nlm.nih.gov/BLAST>), and their identity as RH1 opsin genes is confirmed by neighbour-joining (Saitou and Nei, 1987) phylogenetic analysis using pairwise deletion (Fig. 2C). Both sequences clade with the other visual RH1 sequences and separate from the non-visual brain and pineal extra-retinal rod-like sequences found in *Takifugu rubripes* and *Salmo salar*, respectively. RH1A is most closely related to the RH1 sequences of *Takifugu rubripes* and *Metriaclima zebra*, whereas RH1B is placed in a separate branch with an origin at the base of the Euteleost lineage. The unusual position of RH1B in the gene tree is not, however, a consequence of the loss of codons 335–341, as its position is unaltered when this region is totally excluded from the analysis. It would appear therefore that RH1B has arisen from a very early duplication event deep in the evolution of the euteleosts. The 5' upstream sequences for RH1A and RH1B are shown in Fig. 2B. These sequences were

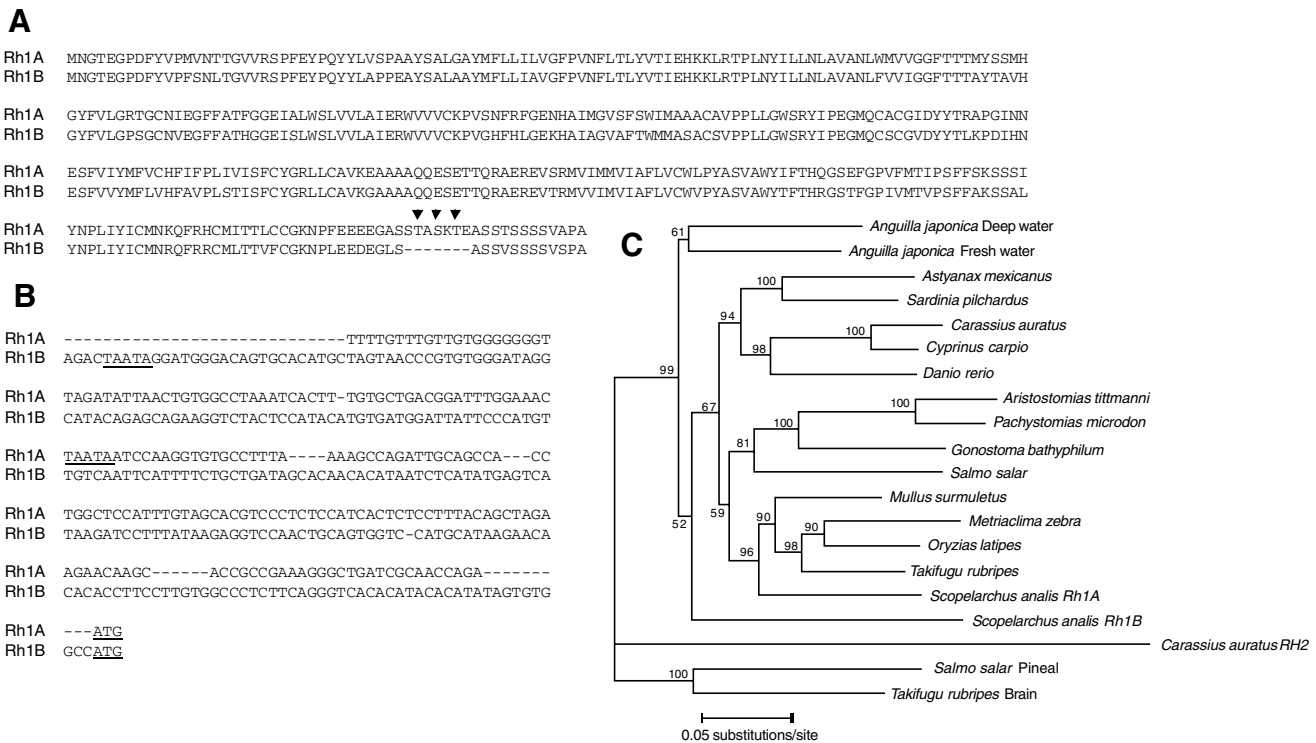


Fig. 2. Identity of RH1 opsins. (A) Alignment of RH1A and RH1B amino acid sequences. The deleted residues in RH1B that are involved in phosphorylation are indicated by arrowheads. (B) Alignment of 5' upstream region of RH1A and RH1B. The putative TATA boxes and start codons are underlined. (C) Phylogenetic tree of RH1 opsin nucleotide sequences generated by the neighbour-joining method. The RH2 sequences from *Carassius auratus* and extra-retinal sequences from *Takifugu rubripes* and *Salmo salar* were used as outgroups. All sequences were aligned with ClustalV. GenBank accession numbers for the sequences (in order from the top of the tree) are as follows: AJ249203, AJ249202, U12328, Y18677, L11863, S74449, AF105152, EF517407, EF517408, AY141256, AF201470, Y18666, AB185235, AB180742, NM_001078631, EF517404, EF517405, L11865, AF201469, NM_001033849.

generated by a gene-walking method from genomic DNA, so it is not possible to define the 5' UTR region. Both sequences have a putative TAATA box but otherwise share only 13% identity, consistent with the early origin of this gene duplication and indicating that the regulation of expression of the two genes may be quite different.

Other visual opsin genes

Degenerate forward primers, RH2 224+ and RH2 599+ (supplementary material Table S1), were designed to conserved regions of teleost RH2 cone opsins found on GenBank and used for 3' RACE with the eye cDNA sample from the 300 m fish. The inner RACE PCR amplified three products of approximately 500, 650 and 850 bp in size as determined by gel electrophoresis, and sequencing showed that all three were from the same RH2 opsin. The gene sequence was completed by a combination of degenerate PCR and gene walking. A reverse primer (RH2 596-) was designed to the novel sequence and used with a degenerate primer, Greenstart, designed to the first seven codons of teleost RH2 sequences on GenBank. A PCR using this primer with the cDNA from the 300 m caught fish as template amplified a product of 650 bp. This product from two

different PCRs was directly sequenced to complete the 5' end of the RH2 coding sequence. The final step was to obtain the first 20 bp of sequence at the 5' end and this was achieved with a gene walk using outer and inner primers, RH2 WKO and RH2 WKI. The resulting product extended the sequence into the 5' untranslated region of the gene. The walk also identified an intron within the RH2 gene sequence, thereby confirming that the gene is not another RH1 gene copy. Further confirmation that this opsin gene is a member of the RH2 cone class is shown in the phylogenetic tree in Fig. 3 where it clades with the B class of euteleost RH2 sequences (Parry et al., 2005). The presence of the RH2 transcript in eye cDNAs from both the 950 m and 300 m fish was then confirmed by the generation and sequencing of a fragment with primers RH2+ and RH2-. The complete deduced amino acid sequence of *S. analis* RH2, aligned with RH2 from the cichlid, *Metriaclima zebra*, is shown in Fig. 3A. Note that the *S. analis* RH2 opsin has a number of extra Ser residues at the C terminus, which may provide additional targets for phosphorylation by rhodopsin kinase.

A number of degenerate primers were designed to teleost SWS2 cone opsin sequences found in GenBank (SWS2 F (1–12) and SWS2 R (1–8) in supplementary material Table S1).

A

<i>S. analis</i>	-----MEN GTEGKNFYIP MNNRSLVRS PYEYPQYYLA DPILYKFQAA YMLFLMFTGG PINILTLVVT AQNKKLRQPL NFILVNLALA GAIMVFGGFV
<i>M. zebra</i>	MAWDGGLPEP GTEGKNFYIP LNNKTGLVRS PFEYPQYYLA DPWFPRLLAF YIFSLVITGF PINFLTLVVT AQNKKLRQPL NFILVNLAVA GLIMVIFGPT
<i>S. analis</i>	ITFYTSMNGY FLLGPTSCAV EGFMATLGGQ ISLWSLVVLA VERYIVVCKP MGSFKFSATH AGIGCGITWF MAMACAAPPL VGWSRYLPEG LQCSGCPDYY
<i>M. zebra</i>	VTIFSCVNGY PALGPLSCAI EGFMATIGGQ VSLWSLVVLA VERYIVVCKP MGSFKFTATH AGVGCFTWI MALACAAPPL FGWSRYIPEG IQVSCGPDYY
<i>S. analis</i>	TMAPGYNNES YVIYMFTCHF CFPVFTIFPT YGSLVLTVKA AAAQQQESSES TQKAEREVTR MCVLMVGLFL TAWVPYASLA AWIFFNKGAA FSAVGMVAVPA
<i>M. zebra</i>	TLAPGYNNES YVIYMFTCHF CVPVFTIFPT YGNLVTVKA AASQQQDSAS TQKAEREVTR MCILMVFGFL LAWTPYASFA AWIFFNKGAA FTATAMAIPA
<i>S. analis</i>	FFAKSASLTN PVIYVLLNKQ FRNCMLTTIG MGGMVDESS ASGSSSSSKT EVSSVSQG
<i>M. zebra</i>	FFSKSSALFN PIIYILMNKQ FRNCMLSTVG MGGMVEDETS VS-----SKT EVSSV---

B

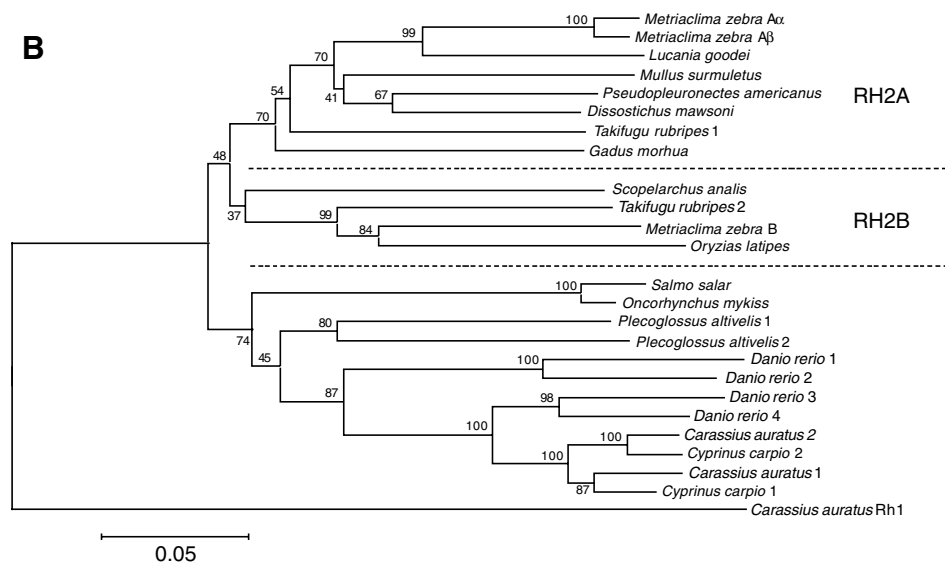


Fig. 3. Identification of RH2 opsin. (A) Amino acid sequence of *S. analis* RH2 opsin aligned with the orthologous sequence from *Metriaclima zebra*. Note the additional Ser residues near the C terminus of the *S. analis* sequence. (B) Phylogenetic tree of RH2 opsin nucleotide sequences generated by the neighbour-joining method. The RH1 sequence from *Carassius auratus* was used as an outgroup. All sequences were aligned with ClustalV. Accession numbers for the sequences (in order from the top of the tree) are as follows: DQ088651, DQ088650, AY296739, Y18680, AY631037, AY771352, NM_001033712, AF38524, EF517406, AY599603, DQ088652, AB223053, AY214132, AF425076, AB098703, AB098704, AB087805, AB087806, AB087807, AB087808, L11865, AB110603, L11866, AB110602, L11863.

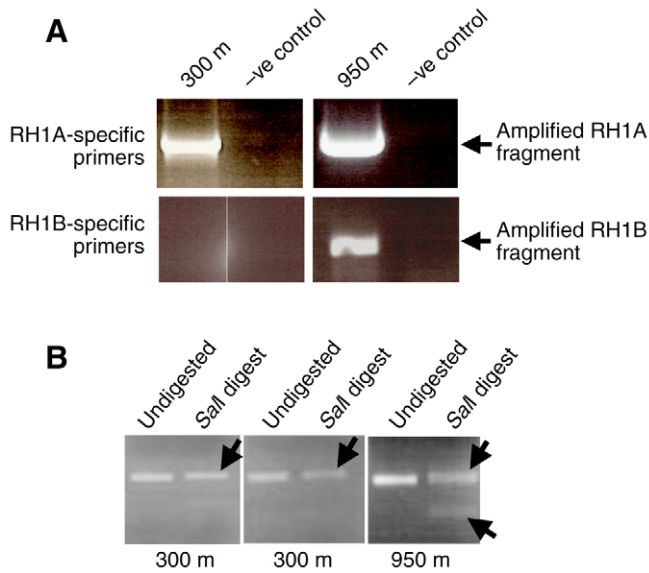


Fig. 4. Differential expression of RH1A and RH1B opsins. (A) Agarose gel electrophoresis separation of PCR products using RH1A- or RH1B-specific primers and cDNA from 300 m and 950 m caught fish. Note presence of RH1A transcripts in fish caught at both depths but RH1B transcripts only in fish caught at 950 m. (B) Relative levels of expression of RH1A and RH1B in two fish caught at 300 m and one at 950 m. Agarose gel electrophoresis separation of PCR products amplified using forward and reverse primers to identical sequences in RH1A and RH1B sequences. Amplified fragments are arrowed. Note smaller digested *SalI* fragments identifying RH1B transcripts only in cDNA from 950 m fish.

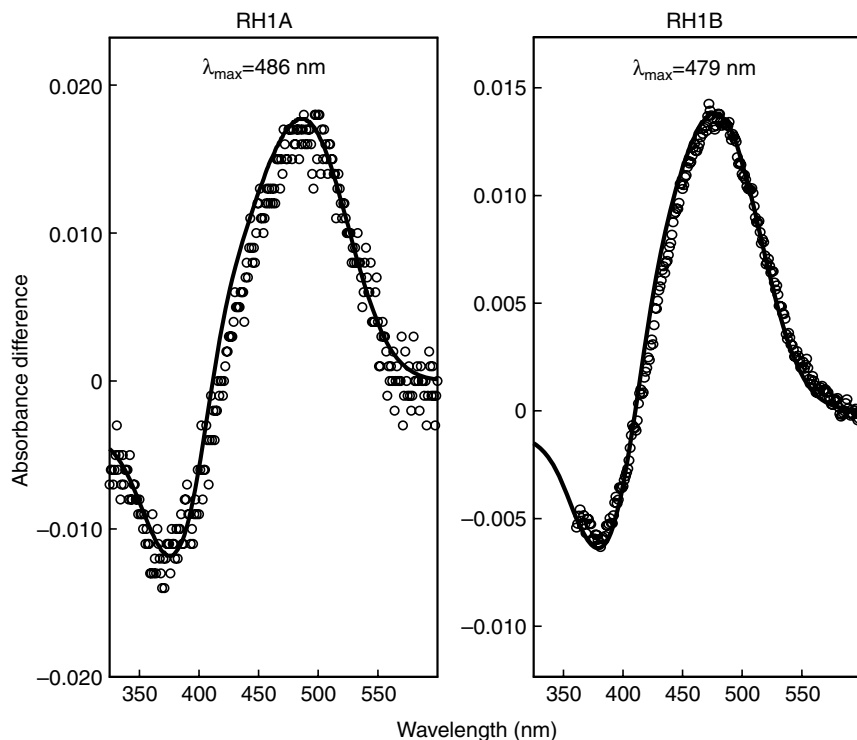


Fig. 5. Spectra of *in vitro* expressed RH1A and RH1B pigments. In both cases, a difference spectrum obtained by subtracting the bleached spectrum from the dark spectrum is shown. λ_{\max} values were obtained by fitting a visual pigment template to the difference spectrum (Govardovskii et al., 2000).

These were used in many different combinations in a number of PCR and RACE experiments with eye cDNAs as template, but no SWS2 opsin fragments were generated.

Differential expression of RH1A and RH1B

In order to determine whether the two RH1 opsin isoforms showed differential expression, gene-specific primers to RH1A or RH1B (supplementary material Table S1) were used in PCRs with the cDNA samples from the 300 m and 950 m fish as templates. As shown in Fig. 4A, the RH1A specific primers amplified a product from both cDNA samples whereas the RH1B specific primers amplified a product only from the 950 m cDNA. Both RH1 opsins are expressed therefore in the fish caught at 950 m but only RH1A is expressed in the smaller and therefore presumably more juvenile fish caught at 300 m.

To examine the relative expression of the two genes, forward (ScopRodsF) and reverse (ScopRodsR) primers (supplementary material Table S1) were designed to regions of RH1A and RH1B sequences that are 100% identical, thereby avoiding any bias in annealing or amplification from cDNA during the PCR. The relative quantity of each amplified product should be an accurate reflection therefore of the relative amount of each transcript in the 300 m and 950 m cDNAs used as templates. The amplified 641 bp fragment was digested with *SalI*. This cuts the RH1B but not the RH1A fragment into two fragments of approximately equal size, which appear as a single band on an electrophoresis gel. The results of this experiment are shown in Fig. 4B. A DNA hyperladder was run alongside so that adjustments could be made for the effects of fragment size on ethidium bromide-stained fluorescence. With cDNA from the 950 m fish as template, digestion with *SalI* revealed two fragments, as expected. Quantification of fluorescence showed that RH1A is expressed at a threefold higher level than RH1B. In contrast, when cDNA isolated from either of the 300 m fish was used as a template, only a single undigested product was present, confirming that only RH1A is expressed in the more juvenile form.

Spectral analysis and tuning of pigments

The full-length coding regions for RH1A and RH1B were amplified from the 950 m cDNA using the primer pairs RH1A F/RH1A R and RH1B F/RH1B R, respectively (supplementary material Table S1). The amplified fragments were digested with restriction enzymes *EcoRI* and *SalI* for RH1A and *EcoRI* and *XhoI* for RH1B. This created complementary ends that were used to ligate into the *EcoRI/SalI* digested pMT4 expression vector. The inserts were sequenced to check for PCR incorporation errors.

Each construct was separately transfected into HEK 293T cells, the expressed opsins were isolated and the corresponding pigment

regenerated with 11-*cis*-retinal. Absorbance spectra were recorded between 250 and 700 nm before and after light bleaching. Difference spectra are shown in Fig. 5, fitted to a template (Govardovskii et al., 2000) to give λ_{\max} values of 486 nm and 479 nm for RH1A and RH1B, respectively.

The coding sequence for the RH2 opsin was amplified with the primers RH2 F and RH2 R and cloned into pMT4. This was used as above for *in vitro* expression but no pigment was produced. Two repeat experiments gave the same result and a third experiment in which sonicated phosphatidylcholine (0.8 mg ml⁻¹) was added to the membrane preparation in order to stabilise the RH2 opsin by mimicking the plasma membrane environment, also failed to yield a pigment.

Finally, since *S. analis* is a deep-sea fish, we examined the possibility that the pigment is only stable when exposed to elevated pressure. The RH2 opsin sample was placed immediately after the addition of 11-*cis* retinal into a high pressure chamber at 10 MPa but again failed to produce a pigment. As a positive control for the protocol, RH1B was also regenerated under pressure and this produced a pigment with a λ_{\max} at 478 nm (data not shown), essentially identical to the λ_{\max} obtained previously for the pigment regenerated at atmospheric pressure.

Discussion

The majority of deep-sea fish have rod-only retinæ with a single RH1 pigment (Hunt et al., 2001). The pearl eye *S. analis* is unusual, therefore, in possessing multiple visual pigments that are based on different opsin proteins. By using degenerate PCR primers and cDNA samples generated from mRNA isolated from whole eyes of *S. analis*, we have been able to identify three of these pigments: a cone RH2 pigment and two rod RH1 pigments.

Expression *in vitro* of the RH1A and RH1B pigments shows that they have very similar λ_{\max} values at 486 nm and 479 nm, respectively, although they show only 81% amino acid identity and are phylogenetically quite distinct, with RH1B falling into a separate branch at the base of the euteleost lineage. RH1B is not, however, an exo-rhodopsin as it lacks introns. It would appear therefore to be an early duplication that was subsequently lost in other euteleost lineages, and the very low identity of the 5' upstream sequence of these two genes is consistent with this interpretation and with the different temporal pattern of expression that these two genes show. RH1A expression is present in fish recovered from two different depths, 300 and 950 m, but RH1B is expressed only in the eyes of fish recovered from 950 m, where its expression level relative to RH1A is about 30%. In addition, RH1B is unique among rod pigments so far sequenced in lacking a stretch of 7 amino acids at the C terminus of the opsin protein that includes three residues, Thr336, Ser338 and Thr340 (Mendez et al., 2000), which are targets for phosphorylation by rhodopsin kinase (Adams et al., 2003; McDowell et al., 1993; Ohguro et al., 1994; Ohguro et al., 1993; Ohguro et al., 1996; Papac et al., 1993) in the deactivation of metarhodopsin II. The loss of one or more of these sites has been shown in transgenic mice to result in a greatly prolonged period of activation (Mendez et al., 2000). This would provide for a greater amplification of the signal than is typical, with a commensurate increase in sensitivity to light,

but the trade-off would be a substantial reduction in the rate of recovery of photoreceptors.

Microspectrophotometry (MSP) identified three visual pigments with λ_{\max} values at 444 nm, 479 nm and 505 nm in the retinæ of *S. analis* (Partridge et al., 1992). However, only the 444 nm and 505 nm pigments were found in the main retina, with the 479 nm pigment confined to the accessory retina where the other two pigments were also found. Since the RH1B pigment gave an *in vitro* λ_{\max} value of 479 nm, this would seem to imply that the *in situ* 479 nm pigment is encoded by the *RH1B* gene. This may be an over-simplification, however, since small differences between *in situ* and *in vitro* values are not uncommon and the *RH1A* gene is the predominant RH1 transcript. The 479 nm *in situ* pigment may correspond therefore to the RH1A or the RH1B pigment, or to a mixture of both. These two pigments do differ in another significant way. Whereas the RH1A pigment possesses a normal complement of phosphorylation sites at the C terminus, the RH1B pigment is deleted for a number of the residues involved in this process. As mentioned above, a consequence of this may be to confer an increase in sensitivity of the animal to light. Only the RH1A pigment is present in more juvenile individuals, with RH1B accounting for about 30% of total RH1 gene expression in the older fish studied. This change would appear to be associated with maturation and migration to greater depths, where light levels are more severely attenuated. Since expression of both pigments is confined to the accessory retina (and diverticulum), it is unlikely that either is involved in image formation. Their role would appear to be as light sensors; the increased sensitivity but poorer temporal resolution that the RH1B isoform would confer may be an adaptation therefore to the reduction in light levels as the animal migrates to deeper water.

The other two pigments identified by Partridge et al. (Partridge et al., 1992) were both found by MSP in the main and accessory retina, but with the 505 nm pigment only present at the distal end of outer segments that had the 444 nm pigment present at the proximal end, although outer segments with only the 444 nm pigment were also found. This indicates that these photoreceptors were either undergoing or had completed a temporal transition in pigment production from the 505 nm to the 444 nm pigment. A λ_{\max} of 505 nm is not atypical for a teleost RH2 pigment. Goldfish express two RH2 opsins, one of which regenerates with 11-*cis* retinal to give a λ_{\max} of 505 nm (Johnson et al., 1993), and one of the four RH2 opsins expressed in zebrafish also has a λ_{\max} of 505 nm (Chinen et al., 2003). The *S. analis* RH2 pigment, however, failed to form a pigment *in vitro*, even when placed under high hydrostatic pressure. Such failures are not unknown; the LWS pigment of the lamprey, for example, also fails to form a pigment *in vitro* but the expressed sequence is undoubtedly correct (Davies et al., 2007). HEK 293 cells must provide a very different cellular environment to a photoreceptor cell and it would appear that certain pigments may be unstable when produced in such cells.

The pigment with a λ_{\max} at 444 nm most probably belongs to the SWS2 cone class. We have been unable to amplify an SWS2 pigment from our eye cDNA samples, however, and conclude either that our degenerate primers failed to target the SWS2 transcript or that the fish sampled had yet to enter the pigment transition reported by Partridge et al. (Partridge et al., 1992).

In summary, therefore, the main retina of *S. analis* would appear to be populated with rod-like cones expressing a 505 nm RH2 cone pigment in younger individuals. This is then replaced in older individuals as they move to greater depths by a 444 nm SWS2 cone pigment within the same photoreceptors (Partridge et al., 1992). Such a switch-over between two cone pigments has not been reported before. If these photoreceptors remain functionally cone-like, it means that the upward vision of the dorsally directed cylindrical eyes is entirely photopic. Only the accessory retina would provide scotopic sensitivity via the RH1A and RH1B pigments.

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Table S1. Sequences for PCR primers

Forward primers		Reverse primers	
	Primer sequence (5'–3')		Primer sequence (5'–3')
Rod+	CTTCCYRTCAACTTCCTCAC	Rod–	TGCCTTGTTCAWGCAGATGTAG
RH1A 295+	CATGGCTACTTTGTCTTAGG	RH1A 850–	GGATGGTCATGAAGACGG
RH1B 170+	CTGGTTGTAACGTCGAGG	RH1B 714–	CGAAGAAGGACGGCACC
ScopRodsF	CCGTCAACTTCCTCACTC	ScopRodsR	CGCTGGCGTAGGG
RH1A F	<u>GCGCGAATTCC</u> ACCATGAACAAGCACCGCCGA	RH1A R	CGGCGTCGACGCTGCAGGAGCCACACTGG
RH1B F	<u>GCGCGAATTCC</u> ACCATGAACGGCACAGAAGGAC	RH1B R	CGCGGCCTCGAGGCTGCAGGAGAGAGACAGAGCT
RH2 224+	CAACTWTATKTTGGTCAACC	RH2 596–	CATATGATTTCATTGTTGTAGCC
RH2 599+	TACAACAATGAATCATATGTC	RH2 WKO	TCTGACTCCTGCTGCTGAGCTG
RH2+	CAAACAGTTCGTAACTGC	RH2 WKI	TGAAGAAGATGGTGAAGACG
Greenstart	ATGAAYGGCACTGARGMAA	RH2–	GGAAACTGAGGAGACTTCT
RH2 F	<u>GCGCGAATTCC</u> ACCATGGAGAACGGCACAGAGG	RH2 R	CGGCGTCGACGCGCTCCTTGGGAACTGAGGAG
SWS2 F1	CCATTTTTGGTTCCCCGTTATC	SWS2 R1	AGAGAAAAGCTAGCATATGGCGC
SWS2 F2	CAACGCTCGGAGGAATGG	SWS2 R2	CCAGCATAACCAAGAAGCC
SWS2 F3	ACAACCTCTCAGCCTACAGC	SWS2 R3	CGTCACCTCCCTCTCTGCCT
SWS2 F4	TGGCTGGTCATTTGCAAACC	SWS2 R4	CATAGCATGGTCGGGCTTG
SWS2 F5	TCTCATAGAATGTGTCTC	SWS2 R5	TGCAGGCCTCTGGGATG
SWS2 F6	GTCCTCCTCTGTTTGATG	SWS2 R6	CACCAANAYCACCACCATC
SWS2 F7	CTNAACTAYATYCTKSTGAAC	SWS2 R7	GCAAAGCARAAGCAGAA
SWS2 F8	GYTGTGGTCTCTKGC	SWS2 R8	GCCTTCTGGGTNGARGC
SWS2 F9	TCTGCTTYTGCTTTGCBGT		
SWS2 F10	YTGTTGGTCTCTKGTGT		
SWS2 F11	TGTGGMCCDGACTIONGTTAYAC		
SWS2 F12	CAATGAAWCCTACGTSAT		

The restriction enzyme sites for cloning into the expression vector pmT4 are underlined.