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Adaptations to an extreme environment: retinal organisation and spectral properties of photoreceptors in Antarctic notothenioid fish

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Summary

The Notothenioid suborder of teleosts comprises a number of species that live below the sea ice of the Antarctic. The presence of 'antifreeze' glycoproteins in these fish as an adaptation to freezing temperature has been well documented but little is known about the adaptations of the visual system of these fish to a light environment in which both the quantity and spectral composition of downwelling sunlight has been reduced by passage through ice and snow. In this study, we show that the red/long-wave sensitive (LWS) opsin gene is not present in these fish but a UV-sensitive short-wave sensitive (SWS1) pigment is expressed along with blue-sensitive (SWS2) and green/middle-wave sensitive (Rh2) pigments. The identity and spectral location of maximal absorbance of the SWS1 and Rh2 pigments was confirmed by *in vitro* expression of the recombinant opsins followed by regeneration with 11-*cis* retinal. Only the SWS2 pigment showed interspecific variations in peak absorbance. Expression of the Rh2 opsin is localised to double cone receptors in both the central and peripheral retina, whereas SWS2 opsin expression is present only in the peripheral retina. SWS1 cones could not be identified by either microspectrophotometry or *in situ* hybridisation, presumably reflecting their low number and/or uneven distribution across the retina. A study of photoreceptor organisation in the retina of two species, the shallower dwelling *Trematomus hansoni* and the deeper dwelling *Dissostichus mawsoni*, identified a square mosaic in the former, and a row mosaic in the latter species; the row mosaic in *Dissostichus mawsoni* with less tightly packed cone photoreceptors allows for a higher rod photoreceptor density.

Key words: icefish, visual pigment, retina, photoreceptor.

Introduction

The Notothenioidei is a suborder of teleosts largely endemic to the Antarctic region of the Southern Ocean, which dominate the Antarctic fish fauna in the number of species (45%) and biomass (>90%; Eastman, 2005). Members of this suborder inhabit a variety of depths under pack and fast ice, and a major adaptation that enables survival at icy, freezing seawater temperatures is the production of 'antifreeze' glycoproteins that prevent the growth of environmental ice crystals that enter the body fluids, preserving their liquid state (DeVries et al., 1970; DeVries et al., 1971; Raymond and DeVries, 1977; for reviews see Cheng, 1998; Fletcher et al., 2001). The visual environment is also affected by this severe habitat; not only is the amount of light reduced by snow and ice cover (Littlepage, 1965; Pankhurst and Montgomery, 1989), but short- and long-wave light is unable to penetrate thick sheets of ice. Therefore, notothenioid fish are exposed to a light environment that is quite different from other aquatic species. A recent study has detailed the cold adaptation of the eye lens of these fish (Kiss et al., 2004), but so far very little information is available on the visual system of these fish in terms of the spectral sensitivity of their photoreceptors.

Both rod and cone photoreceptors are present in the retinae of notothenioid fishes (Meyer-Rochow and Klyne, 1982). Single and double cones have been reported in a number of species (Eastman and Lannoo, 2003; Meyer-Rochow and Klyne, 1982; Miyazaki et al., 2002), and Miyazaki et al. (2001) identified a square mosaic that is formed with double cones at the sides and single cones in the centre and at each of the four corners. Cones in corner positions have been shown to contain a UV-sensitive visual pigment in the retinae of a number of teleost species (Avery et al., 1983; Bowmaker et al., 1991b; Hárosi and Hashimoto, 1983; Hisatomi et al., 1996, 1997; Whitmore and Bowmaker, 1989), so it is possible that UV sensitivity is also present in notothenioid fishes. Meyer-Rochow and Klyne (1982) reported an increase in the overall proportion of rods and a decrease in cones in three species of notothenioid fishes as depth of habitat increased; the deeper dwelling species, *Dissostichus mawsoni*, has a higher concentration of rods with longer outer

segments as compared to the two shallower living species, *Trematomus borchgrevinki* and *T. bernacchii*.

In the present study, we have examined the organisation of the retina in a number of notothenioid species that live in different depth habitats. We have focused on the classes of photoreceptors present, and the spectral characteristics of the associated visual pigments have been determined by microspectrophotometry (MSP). The corresponding visual pigment genes have been sequenced and the peak absorbance (λ_{max}) of encoded cone pigments confirmed by *in vitro* expression of the opsin protein and regeneration with 11-*cis*-retinal. Finally, the organisation of the retina has been examined by conventional histology and *in situ* hybridisation with opsin cRNA probes.

Materials and methods

Fish and tissue collection

Specimens of Antarctic notothenioid fish representing three of the five endemic families were utilised in this study. Many of the nototheniid (family Nototheniidae) species were collected from McMurdo Sound, Antarctica (77°51.0′S, 166°40.0′E) through ice holes. The giant nototheniid *Dissostichus mawsoni* (Norman) was captured at about 350–500 m with baited hooks on a vertical set line, and the shallow water (0–10 m) cryopelagic *Pagothenia borchgrevinki* (Boulenger) by hook and line. The shallow benthic (30–60 m) nototheniid species *Trematomus bernacchii* (Boulenger) and *T. hansoni* (Boulenger), the shallow benthic dragonfish (Bathydraconidae) *Gymnodraco acuticeps* (Boulenger), and the deep water (600 m) *T. loennbergii* (Regan), were caught with baited traps, and the icefish (Channichthyidae) *Pagetopsis macropterus* (Boulenger) by diving at around 40 m. The pelagic nototheniid *Pleuragramma antarcticum* (Boulenger) was caught with otter trawl in the Ross Sea, and the mackerel icefish *Champsocephalus gunnari* (Lönnberg) by otter trawl from the Antarctic Peninsular water. Finally, the cool temperate nototheniid *Notothenia angustata* (Hutton) was obtained from the shallow rocky shore at the entrance of Otago Harbor (45.5°S, 170°E) of South Island, New Zealand. After capture, all Antarctic notothenioid species were kept in flow-through seawater aquarium tanks under dim fluorescent lighting for 1–3 months.

Liver and spleen were flash frozen in liquid nitrogen for subsequent DNA extraction. Whole eyes were dissected from the fish, the lens was removed, and the eye cup was flash frozen in liquid nitrogen for subsequent RNA isolation. For microspectrophotometry, fish were dark adapted for several hours in aquarium tanks. Eye capsules were then dissected in dim red light at 4°C, and lightly fixed for 15–30 s in 2% glutaraldehyde in notothenioid fish PBS (0.1 mol l^{-1} phosphate buffer, 0.1 mol l^{-1} NaCl, pH 7.4), and stored in light-tight bottles with fresh notothenioid PBS containing penicillin (100 U ml^{-1}), streptomycin (0.1 mg ml^{-1}), and amphotericin B (0.25 μg ml^{-1}) at 4°C. For retinal histology, either the lens and vitreous humour was removed from the eye prior to fixation or fixative was injected into the vitreous humour of the intact eye with a 30-gauge needle. In both cases, the eye capsules were then fully immersed in 4% (w/v) paraformaldehyde in notothenioid PBS.

Microspectrophotometry

Absorption spectra of individual photoreceptors were determined using a computer-controlled modified Liebman dual beam microspectrophotometer. By means of an infrared converter, the measuring beam (normally about 2 μm^2 cross section) was aligned to pass transversely through a given photoreceptor outer segment, while the reference beam passed through a clear space adjacent to the photoreceptor. Spectra were scanned from 750 nm to 350 nm in 2 nm steps and back from 351 nm to 749 nm. To estimate the peak absorbance (λ_{max}) of each outer segment, a standardised computer program was employed. A detailed description of the experimental procedures and methods of analysis have been published previously (Bowmaker et al., 1991a).

Genomic DNA extraction

High molecular mass genomic DNA was prepared from frozen liver or spleen. Tissue was ground with a pre-chilled pestle and mortar, and the pulverized tissue was digested (1:10 w/v) in lysis buffer (10 mmol l^{-1} Tris-HCl, pH 8.0, 100 mmol l^{-1} NaCl, 250 mmol l^{-1} EDTA, 0.5% SDS and 100 μg ml^{-1} proteinase K) at 60°C. The digest was then extracted twice with an equal volume of Tris-HCl-buffer-saturated phenol (pH 8.0), and then once each with phenol–chloroform and chloroform. The genomic DNA in the supernatant was dialyzed against 0.5× TE (5 mmol l^{-1} Tris-HCl, 0.5 mmol l^{-1} EDTA, pH 8.0), treated with RNaseA (50 μg ml^{-1}), re-dialyzed and stored at 4°C until use.

RNA isolation

Frozen eye cups were ground to a fine powder in a pre-chilled pestle and mortar, and total RNA was extracted with the Ultraspec RNA isolation reagent (Biotecx, Houston, TX, USA), which is based on the single-step acidic phenol extraction of Chomczynski and Sacchi (1987).

Southern blot and hybridisation

Genomic DNA was digested with the restriction enzyme *Eco*RI, separated by gel electrophoresis, transferred to a nitrocellulose membrane by capillary action in 20× SSC and fixed by baking at 80°C for 3 h. The blot was incubated with radiolabelled probes for the four vertebrate cone opsins, SWS1, SWS2 (short-wave sensitive 1 and 2), Rh2 (middle-wave sensitive) and LWS (long-wave sensitive). The SWS1 probe was cloned from *D. mawsoni* retinal cDNA and encompassed the entire coding region. The SWS2, Rh2 and LWS clones were obtained from retinal cDNA isolated from black bream, *Acanthopagrus butcheri* (J.A.C. and D.M.H., unpublished data). In all cases, the probes were cloned into the pGEM-T-Easy plasmid (Promega, Southampton, UK). The opsin inserts were excised by restriction enzyme digestion and radioactive

probes were synthesised using the Ready-To-Go™ DNA Labelling Beads (α-dCTP) (Amersham Biosciences, Little Chalfont, Bucks, UK), following the manufacturer's instructions. The membranes were prehybridised for 3 h at 50–65°C (depending on probe specificity) in a buffer containing 0.05 mol l^{-1} PO_4, 4× SSC, 5× Denhardt's reagent, 5 mg ml^{-1} denatured, fragmented salmon sperm DNA, 0.3% (w/v) SDS and 0.15% sodium pyrophosphate (NaPPi). The membrane was then hybridised against the SWS1, SWS2, Rh2 or LWS opsin probes overnight at 50–60°C. The blot was washed twice in 6× SSC, 0.5% (w/v) SDS and twice in 3× SSC, 0.5% SDS at 50–65°C before exposure to X-ray film.

Northern blot and hybridisation

Approximately 6 μg of total retinal RNA of each species were electrophoresed on a 1.2% agarose/2.2 mol l^{-1} formaldehyde gel, vacuum blotted onto Hybond N (Amersham) nylon membrane, and UV-crosslinked (Stratalinker, Stratagene, La Jolla, CA, USA). The membrane was prehybridised at 55°C in QuickHyb solution (Stratagene), and hybridised at 55°C to opsin probes generated from random-primed, ^{32}P-labelled sea bream opsin cDNA (SWS2, Rh2 and LWS) and *P. borchgrevinki* opsin (SWS1) cDNA. Hybridised blots were washed exhaustively in 2× SSC/0.1% SDS, followed by 0.1× SSC/0.5% SDS up to 50°C, and autoradiographed on X-ray film.

PCR cloning and sequencing of SWS1, SWS2 and Rh2 opsins

Standard polymerase chain reaction (PCR) conditions were used with either 30 ng cDNA or 100 ng of genomic DNA in a 50 μl reaction volume. A cDNA fragment of about 800 bp of SWS1 opsin was first obtained from *P. borchgrevinki* retinal RNA by reverse transcription-PCR amplification. The first strand cDNA was synthesised using Superscript II reverse transcriptase (Invitrogen), and used for the PCR amplification of a cDNA fragment with degenerate primers VSWS1F and VSWS1R (Table 1) designed from an alignment of vertebrate SWS1 opsins. Sequencing of the RT-PCR product and BLASTP of translated sequence verified that the fragment was from an SWS1 mRNA. SWS1-specific primers were designed based on this partial sequence to generate overlapping 5′ and 3′ fragments inclusive of UTRs by 5′RACE (rapid amplification of cDNA ends) and 3′RACE. For 5′RACE, the first strand cDNA was dC-tailed with terminal deoxynucleotide transferase and amplified using the SWS1-specific reverse primer paired with the 5′ RACE adaptor primer (5′RACE kit, Invitrogen). The 3′RACE product was amplified with the SWS1-specific forward primer paired with lock-docking oligo(dT) ($NV(T)_{20}$). Primers to the 5′UTR and 3′UTR (P.borchSWS1F and P.borchSWS1R) were used to amplify full-length SWS1 cDNA from *P. borchgrevinki*, *D. mawsoni* and *T. loennbergii* first strand cDNA.

Degenerate PCR was also used to amplify the Rh2 opsins from four species, *D. mawsoni*, *P. borchgrevinki*, *N. angustata* and *G. acuticeps*. Degenerate primers Green1F and Green596R were used to amplify an approximately 600 bp product from *D. mawsoni* cDNA, which showed homology to teleost Rh2 opsins. The 3′ cDNA sequence was obtained by 3′ RACE using the FirstChoice™ RLM-RACE kit (Ambion, Austin, TX,

Table 1. *List of PCR primers used in the amplification of opsin sequences*

Primer	Primer sequence 5′-3′
VSWS1F	TGGGCCTTYTACCTNCARRCNGCCTTYATGGG
VSWS1R	GCTCTTRGAGAARAASGCVGGGATGGTGAC
5′ RACE adaptor primer	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
P.borch SWS1F	TTATACAGTCGGAGGTCACGATGGGGAAG
P.borch SWS1R	CATCCTTTAATCAGTCGTCATTTTGTCCCATCG
D.mawsUVF	GCGC**GAATTC**CACCATGGGGAAGGACTTCCAC
D.mawsUVR	CGGC**GTCGAC**GCAGACACTGAGGACACCTC
D.mawsGF	GCGC**GAATTC**CACCATGGAGACCAATGGCACAG
D.mawsGR	CGGC**GTCGAC**GCAGACACAGAGGACACTTCTG
Blue400F	YYGTGGTCTCTKGCTGT
Blue818R	CCAGCATACCAAGAAGCC
T.loen3′I	GCAGAGTCTGCCTCCACC
MZbluestop	CTAWGCAGGYSCCYACTTTRG
T.loenF	CGGATGGAGCAGGTAC
T.loenR	GGTGGAGGCAGACTCTGC
Green1F	ATGAAYGGCTGARGGMAA
Green596R	CATATGATTCATTGTTGTAGCC
Green3′O	TCCAGATACATTCCTGAGGG
Green3′I	GCTGGAATTGGTGTTGGGA
GreenWkO	CTAGAAGCTTGAACATGATGGG
GreenWkI	TAAGGACTTCTAACAATCCC
UNI33	$(T)_{15}$GTTTGTTGT$(G)_7$TT
UNI17	TTTTTGTTTGTTGTGGG

Underlined bases identify *Eco*RI and *Sal*I restriction enzyme sites for cloning into the expression vector pMT.

USA) and the *D. mawsoni*-specific primers Green3′O and Green3′I. To extend the 5′ sequence and to obtain upstream sequence information, a genomic walking method with genomic DNA was used as described by Dominguez and Lopez-Larrea (1994) with the initial PCR performed with the universal primer UNI33 and the *D. mawsoni*-specific GreenWkO primer. The inner nested PCR was performed with the universal primer UNI17 and the gene-specific GreenWkI primer. The complete Rh2 sequences for *P. borchgrevinki*, *N. angustata* and *G. acuticeps* were amplified with the primers D.mawsGF and D.mawsGR.

The SWS2 opsin DNA was PCR amplified from *T. loennbergii* genomic DNA with the degenerate primers blue400F and blue818R using a low annealing temperature of 48–54°C. This fragment encompassed exons 3 to 5. To extend the 3′ sequence of the gene, a PCR with the primers T.loen3′I and MZbluestop was performed on *T. loennbergii* genomic DNA.

All PCR-amplified products were analysed by gel electrophoresis and extracted using Wizard™ columns (Promega). The eluted DNA was cloned into the pGEM-T-Easy™ vector (Promega) and sequenced on an ABI 3100 using the BigDye® Terminator v3.1 Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA) V.2 or V.3 and vector-specific primers.

Expression of recombinant opsins

The entire coding sequences for *D. mawsoni* SWS1 and Rh2 opsins were amplified from retinal cDNA with *Pfu* DNA polymerase, using primer pairs D.mawsUVF/D.mawsUVR and D.mawsGF/D.mawsGR containing *Eco*RI and *Sal*I restriction sites (shown underlined in Table 1). The resulting products were then cloned *via* these restriction sites into the expression vector pMT4, which contains the sequence for the 1D4 epitope from bovine rod opsin downstream of and in-frame with the *Sal*I site (Franke et al., 1988).

The pMT vector containing either the SWS1 or Rh2 coding sequence was transfected into HEK-293T cells with Genejuice (Invitrogen) according to the manufacturer's instructions. Thirty 90 mm plates were used per transfection and the cells were harvested 48 h post-transfection, and washed with 1× PBS. The visual pigments were regenerated in 1× PBS with 40 μmol l^{-1} 11-*cis* retinal in the dark. The pigments were then incubated with 1% (w/v) dodecyl-maltoside and 20 mg ml^{-1} phenyl methyl sulphonyl fluoride (PMSF) and isolated by passage over a CNBr-activated Sepharose binding column coupled to an anti-1D4 monoclonal antibody (Molday and MacKenzie, 1983).

Absorption spectra were recorded in the dark using a dual-path spectrophotometer (Spectronic Unicam, Cambridge, UK). Pigments were either bleached by exposure to light for 15 min or acid denatured by incubation with 10.8 μl 1 N HCl for 10 min. The λ_{max} value for each pigment was determined by subtracting the bleached or acid denatured spectrum from the dark spectrum to produce a difference spectrum. This was then fitted to a standard Govardovskii rhodopsin A1 template (Govardovskii et al., 2000) and the λ_{max} determined.

In situ *hybridisation*

Enucleated eyes were fixed in 4% (w/v) paraformaldehyde in 1× PBS overnight and then washed briefly in fresh 1× PBS, and stored in 1× PBS until use. Whole mounts were prepared by removing the retinal pigment epithelium (RPE) and placing 1 cm wide strips, photoreceptor layer side up, on glass slides. Coverslips were placed on top and weighted down so as to keep the sample flat. The slides were left in 25% (w/v) sucrose in 1× PBS overnight, and then stored at –80°C (with coverslips removed). The retinal tissue to be sectioned was left with RPE attached and cut into 0.5 cm squares, placed in 25% (w/v) sucrose overnight and then embedded in OCT medium. Sections 2 μm thick were cut on a cryostat.

Digoxigenin (DIG)-labelled antisense and sense RNA probes were synthesised from the *D. mawsoni* SWS1 and Rh2 opsin clones and from the sea bream SWS2 opsin clone in pGEM-T-Easy using a DIG RNA labelling kit (Roche Diagnostics Ltd., Lewes, UK). The level of probe cross-hybridisation, as assessed by dot blot analysis, was found to be very low (data not shown). For hybridisation, retinal whole mounts and sections were rinsed in 1× PBS and left to dry for 2 h. Whole mounts were washed in PBS with 0.1% (w/v) Tween (PBST), treated with proteinase K (10 mg ml^{-1}) for 1–5 min, fixed with 4% (w/v) paraformaldehyde in 1× PBS for 20 min and then washed in PBST before being incubated in hybridisation buffer for 1 h at 60°C. Retinal sections were treated as described above but without proteinase K treatment. DIG-labelled RNA probe was then added at an approximate concentration of 0.5 μg ml^{-1} and left to hybridise overnight at 60°C. After multiple washes in SSC and PBST, hybridisation was detected with anti-DIG alkaline phosphatase Fab fragment and labelled for visualisation with a solution of Nitrobluetetrazoleum (NBT) (18.75 mg ml^{-1}) and BCIP (9.4 mg ml^{-1}). Slides were viewed under a light microscope and images taken with a Nikon digital camera.

Histology

Enucleated eyes were transferred into 2% (w/v) paraformaldehyde, 2% (w/v) glutaraldehyde in 1× PBS overnight. The large size of the *D. mawsoni* eyes (a diameter of approximately 5 cm) made manipulation of the whole retina very difficult, so fragments were dissected from the eyecup, with the RPE intact, and dehydrated before storage in historesin for 24–120 h at 4°C. The fragments were then embedded in Technovit embedding hardener (Heraeus, Hanau, Germany) and flat mounted on to slides. The eyes of *T. hansoni* are considerably smaller (1.5 cm diameter) so the whole retina with RPE attached was dissected and flat mounted. 0.5–2.5 μm thick sections were cut with a glass blade and dried on to slides. The cells were stained with 1% (w/v) Toluidine Blue before being coverslipped. Images were captured using a Nikon digital camera attached to a light microscope.

Table 2. *List of notothenioid fish species*

Family	Common name	Species	Dwelling and depth range*
Nototheniidae	Cod icefishes	*Dissostichus mawsoni*	Pelagic; 88–1600 m; usually near-bottom range
		Notothenia angustata	Demersal; 0–100 m
		Pagothenia borchgrevinki	Cryopelagic; 0–30 m
		Pleuragramma antarcticum	Pelagic; 0–700 m
		Trematomus loennbergii	Bathydemersal; 65–832 m; mostly ⩾300 m
		Trematomus hansoni	Demersal; 5–550 m
		Trematomus bernacchii	Demersal; ⩽200 m
Channichthyidae	Crocodile icefishes	*Pagetopsis macropterus*	Demersal; 5–655 m; mostly 5–40 m
		Champsocephalus gunnari	Pelagic; 0–700 m
Bathydraconidae	Antarctic dragonfish	*Gymnodraco acuticeps*	Demersal; 0–550 m; mostly ⩽50 m

*Data from Gon and Heemestra (1990).

Phylogenetic analysis

Neighbor-joining (Saitou and Nei, 1987) was used to construct phylogenetic trees from opsin nucleotide coding 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).

Results

The notothenioids studied represent three families, the Nototheniidae or cod icefishes (seven species), the Channichthyidae or crocodile icefishes (two species), and the Bathydraconidae or Antarctic dragon fish (one species). Most of these species can be found in shallow water (0–100 m), although for many, their maximal depth range can extend to at least 500 m (Table 2). One species, the mid-water *Dissostichus mawsoni*, is deeper dwelling, with a depth range extending to 1600 m. Seven of the species studied, namely *Pagothenia borchgrevinki*, *Trematomus hansoni*, *T. bernacchii*, *T. loennbergii*, *Gymnodraco acuticeps*, *Pagetopsis macropterus* and *Champsocephalus gunnari*, are near-shore species where ice cover is generally continuous throughout the year. The remaining two species, *D. mawsoni* and *Pleuragramma antarcticum*, are migratory and schooling respectively, so will spend some time in open water in the summer, although they are also frequently found under sea ice. These Antarctic notothenioids therefore spend a significant proportion, if not all, of the year under ice cover of various thicknesses. Notothenioids do not show a diurnal vertical migration. They generally sit on the sea bottom as they lack a swim bladder and are negatively buoyant. Regular large excursions in the water column at freezing seawater temperatures would therefore be energetically too costly. A 24 h light:dark cycle is not present in the high-latitude Antarctic. Larval forms of these species are also recovered from under the ice.

Table 3. *Peak absorbance of visual pigments in rods and cones from microspectrophotometry*

	SWS, single cone	MWS, single cone	MWS, double cone	Rods
Pagothenia borchgrevinki				
Mean absorbance	No pigment	488.7±0.8	489.2±0.8	498.5±1.2
Density/number		0.030/12	0.037/18	0.034/8
Trematomus loennbergii				
Mean absorbance	426.4±1.3	490.1±6.5	489.8±1.0	500.0±0.5
Density/number	0.020/8	0.011/1	0.023/8	0.026/12
Trematomus hansoni				
Mean absorbance	417.6±7.3	490.1±0.9	489.5±1.1	501.0±0.7
Density/number	0.005/4	0.025/11	0.026/7	0.017/10
Trematomus bernacchii				
Mean absorbance	416.1±2.8	489.6±0.8	489.1±1.1	502.3±0.6
Density/number	0.009/9	0.028/6	0.031/12	0.022/9
Dissostichus mawsoni				
Mean absorbance	414.3±2.9	490.4±1.3	490.8±0.6	503.7±1.7
Density/number	0.012/6	0.016/8	0.030/15	0.014/4

Peak absorbance (λ_{max}) in nm (± S.D.) of the mean absorbance spectra including the mean maximum transverse absorbance and the number of cells that passed selection criteria.

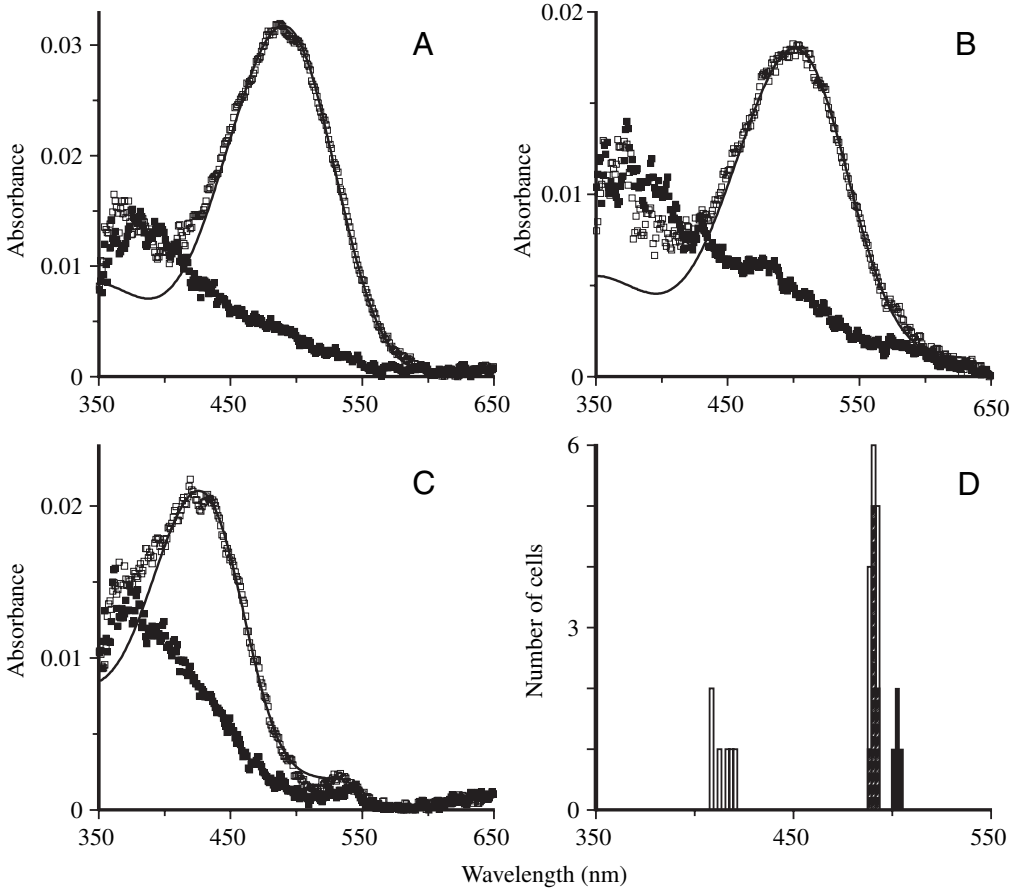

Fig. 1. Absorbance spectra of rods and cones. (A) Middle-wave sensitive double cones from *T. bernacchii*. (B) Rods from *T. hansoni*. (C) Short-wave sensitive single cones from *T. loennbergii*. Open symbols, before bleaching; filled symbols, after white light bleaching. Solid lines are visual pigment templates (Govardovskii et al., 2000) with λ_{max} at 490 (A), 501 (B) and 426 nm (C). (D) Distribution histogram of rods and cones from *D. mawsoni*. Filled bars, rods; hatched bars, MWS single cones; open bars MWS double cones and SWS single cones; bin size 2 nm.

Microspectrophotometry

Microspectrophotometry (MSP) was carried out on the photoreceptor outer segments from the retinae of five species, *T. loennbergii*, *T. hansoni*, *T. bernacchii*, *D. mawsoni* and *P. borchgrevinki*. All five species possessed a single class of rod with a λ_{max} around 500 nm (Table 3), in addition to morphologically identical double cones and both small and large single cones. Both members of the double cones and a number of single cones contained a middle-wave sensitive (MWS) pigment with λ_{max} close to 490 nm. In *T. hansoni*, *T. bernacchii* and *D. mawsoni*, the remaining single cones contained an SWS pigment with a λ_{max} at about 415 nm, but in *T. loennbergii*, the pigment was long-wave shifted to about 426 nm (Table 3). In *P. borchgrevinki*, a population of single cones was identified that appeared not to contain a photosensitive pigment; it is assumed that these were SWS cones in which the pigment had become denatured as a result of the fixation of the tissue. Examples of mean absorbance spectra of the rod and cone pigments and the distribution of the λ_{max} of individual cells are shown in Fig. 1.

Southern and northern hybridisation

In order to determine which cone opsin genes are present in the notothenioid fish genome, Southern analysis of genomic DNA from *D. mawsoni* was carried out using cone opsin gene probes. As shown in Fig. 2, the SWS1, SWS2 and Rh2 probes identified single bands in *Eco*RI digests, confirming the presence of the SWS1, SWS2 and Rh2 genes. In contrast, the LWS probe did not generate a signal, indicating that this gene is not present in the genome of this species.

Northern analysis of retinal RNA from six species (Fig. 3) demonstrated the expression of SWS1, SWS2 and Rh2 opsins. In confirmation of the Southern analysis, the LWS probe did not hybridise in any of the six species examined, whereas there was a signal for the SWS2 (faint for *P. borchgrevinki* and *P. antarcticum*) and Rh2 probes in all six species. Surprisingly,

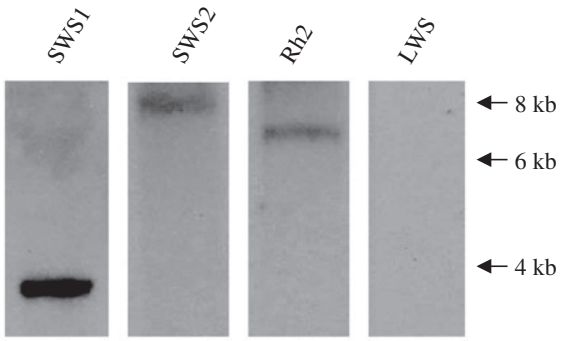

Fig. 2. Southern hybridisation of *D. mawsoni* genomic DNA digested with *Eco*RI. The blot was probed with SWS2, Rh2 and LWS opsin probes amplified from sea bream retinal cDNA and with an SWS1 opsin probe amplified from *D. mawsoni* cDNA.

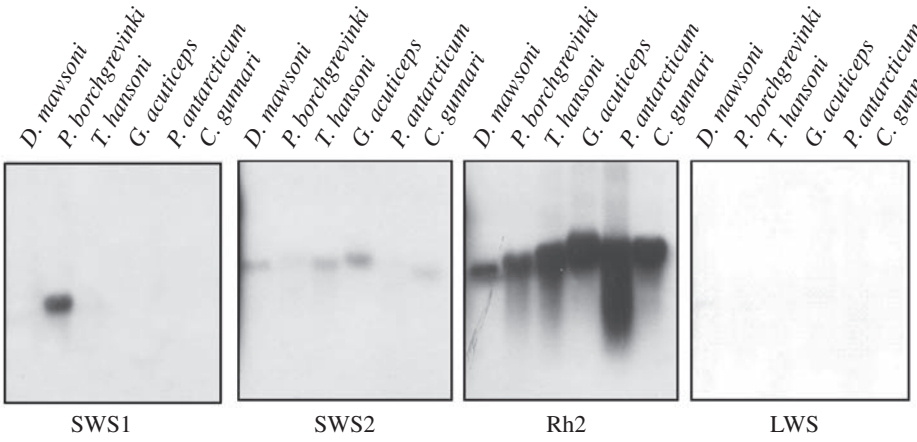

Fig. 3. Northern blots of retinal RNA from six species of notothenioid fish. The blots were hybridised with opsin probes amplified and cloned either from sea bream opsin cDNA (SWS2, Rh2 and LWS) or from *P. borchgrevinki* (SWS1) cDNA.

only RNA from the *P. borchgrevinki* retina hybridised with the SWS1 probe. SWS1 opsins are clearly expressed in the other species since SWS1 coding sequences were amplified from retinal cDNA. The restriction of signal only to the RNA from the *P. borchgrevinki* retina may reflect a quantitative difference, with rather more SWS1 photoreceptors in the cryopelagic species, *P. borchgrevinki*, that is found at the underside of sea ice, than in the other species that live at deeper depths.

Amplification and sequencing of opsin genes

SWS1 opsins

The SWS1 coding sequence was amplified from retinal cDNA using a combination of RT-PCR and 5′ and 3′ RACE. Primer details are in Table 1. Full coding sequences were obtained for five species, *D. mawsoni*, *N. angustata*, *P. borchgrevinki*, *T. loennbergii* and *P. macropterus* (Fig. 4A). These opsins showed an average of 90.7% amino acid identity, with no consistent differences between the two notothenioid families that these species represent.

The λ_{max} of the SWS1 class of visual pigments varies in different vertebrate species between the UV (generally around 360 nm) and violet (>390 nm) regions of the spectrum. Since UVS cones were not identified by MSP, the λ_{max} of the pigment in *D. mawsoni* was determined as a recombinant opsin expressed in HEK 293T cells and regenerated *in vitro* with 11-*cis*-retinal. As shown in Fig. 4B, a Govardovski visual pigment

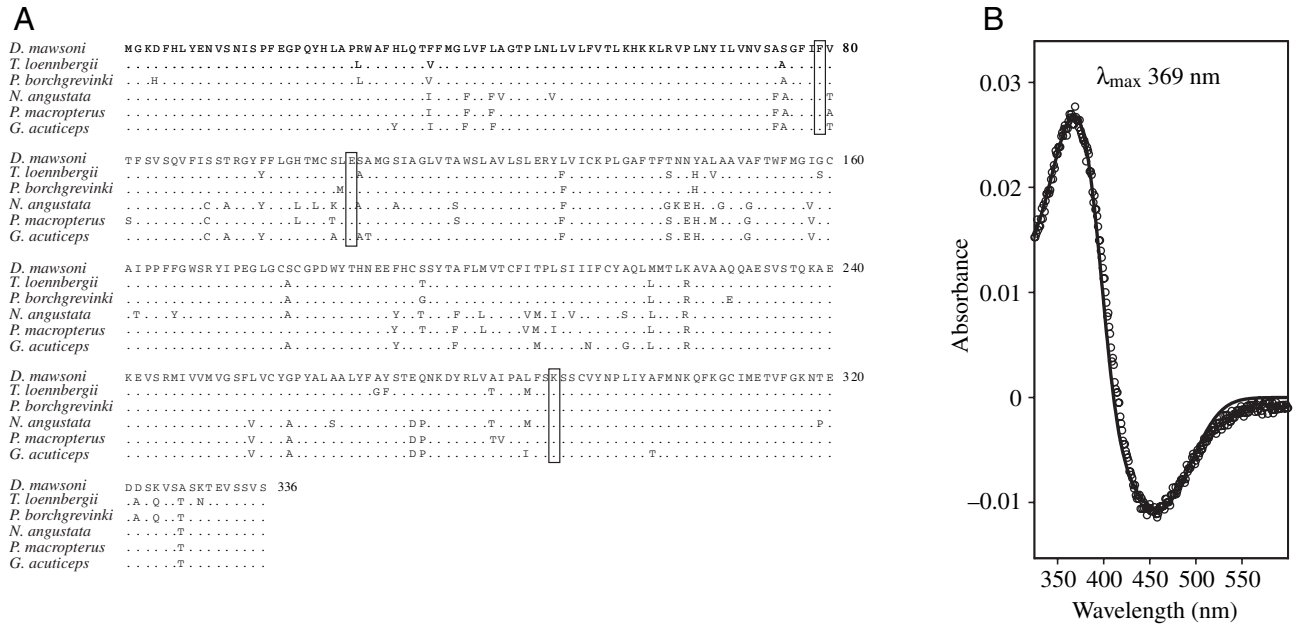

Fig. 4. SWS1 opsins. (A) Deduced amino acid sequences from six species of notothenioid fish (GenBank accession numbers AY927651-6). The Phe residue at the equivalent site to 86 in the bovine opsin sequence that is critically important for tuning into the UV is boxed, together with the counterion Glu at the equivalent site to 113 and the Lys at the equivalent site to 296 that binds the chromophore. (B) *In vitro* absorbance spectrum for the pigment from *D. mawsoni*. Recombinant SWS1 opsin was produced in transfected HEK 293T cells and the pigment regenerated with 11-*cis*-retinal. The difference spectrum shown with a fitted Govardovski visual pigment template, was obtained by subtracting the acid-denatured spectrum from the dark spectrum.

```
T. loennbergii  ----------------------------------------------------------INALTVACTIQNKKLRSHLNYIL 80
Goldfish        MKQVPEFHEDFYIPIPLDINNLSAYSPFLVPQDHLGNQGIFMAMSVFMFFIFIGGAS..I..IL....F..........

T. loennbergii  VNLAVSNLLVSCVGSLTAFLSFANKYFILGPLACNIEGFIATLGGMVSLWSLAVVALERWLVICKPLGHFIFKPDHAIAC 160
Goldfish        ...SIA..F.AIF..PLS.Y..F.R...F.AT..K....L.......G........F...........N.T..TP....G

T. loennbergii  CALTWVFALIASVPPLPIFGWSRYIPEGLQCSCGPDWYTTNNKYNNESYVMFLFGFCFAVPFATIVFCYSQLLITLK-AV 240
Goldfish        .I.P.IS..A..L.--.L.....................................C.......G......G.......L.A

T. loennbergii  KAQAESASTQKAEREVTRMVVIMVFGFLVCWLPYASFALWDVNNRGQTFDLRLASVPSVFSKSSAIYNPVIYVLLNKQFR 320
Goldfish        ....D...........K...V..L......A.....S..I.SH..EE....M..I..CL..A.TV........M.....

T. loennbergii  TCMMKILGMGGGDDDESSSTTSVTEVSKVAPA-                                              353
Goldfish        S....MVCGKNIEE..A..SSQ..Q..S...EK
```

Fig. 5. Deduced amino acid sequences for the partial sequence of SWS2 opsin from *T. loennbergii* (GenBank accession number AY771356), aligned with goldfish SWS2 opsin. Boxed residues identify known spectral tuning sites.

template (Govardovskii et al., 2000) fitted to the difference spectrum gave a λ_{max} of 369 nm, confirming the presence of a UVS SWS1 pigment in this species.

Previous work (Cowing et al., 2002; Fasick et al., 2002; Parry et al., 2004) has shown that the amino acid present at site 86 is critically important for the production of UV- or violet-sensitivity, with phenylalanine (Phe) present in all teleost, amphibian, reptilian and mammalian UVS pigments sequenced

A

```
D. mawsoni      METNGTEGNNFYIPMSNRTGIVRSPYEYQQYYLADPIMFKLLAFYMLFLIFTGTPINALTLLVTIQNKKLQTPLNLILVN  80
P. borchgrevinki ..............................................V.................................
N. angustata    .....A.....................N...........V.....V.............................F....
G. acuticeps    ............V..............N...M.......IQ.....................................

D. mawsoni      LAVAGLIMCAFGFTITFVSSINGYFILGPTFCAIEGFMATLGGQVSLWSLVVLAVERYIVVCKPMGSFKFSNTHAGIGVG 160
P. borchgrevinki ......V..........I...S...........M.....................................
N. angustata    ..................I..M.....................E.A.................S..............
G. acuticeps    .S..............I..............M...L.....................................

D. mawsoni      ITWVMASTCAGPPLLGWSRYIPEGMQCSCGPDFYTLAPGFNNESYVMYMFTVHFFTPVFLIFFSYGSLVLTVKAAAAQQQ 240
P. borchgrevinki ...L..IA..A...F.....L...L.......Y..............L.V......G.....................
N. angustata    ......FA......F.....L...........Y...........F....S.I.....................
G. acuticeps    ......FA......F.....L...L.......Y..............L.C......I.....................

D. mawsoni      ESESTQKAEREVTRMCILMVFGFLLAWVPYASFAGWIFLNKGAAFTALTAAIPAFFAKSSALYNPVIYVLLNKQFRNCML 320
P. borchgrevinki ................................A...M.....................................
N. angustata    ................L.......V.....................S.............................
G. acuticeps    ................L.......V.....................................................

D. mawsoni      STIGMGRMVEDETSVSASKTEVSSVS                                                  346
P. borchgrevinki ..V...G...................
N. angustata    ......G...................
G. acuticeps    .S....G.....S.............
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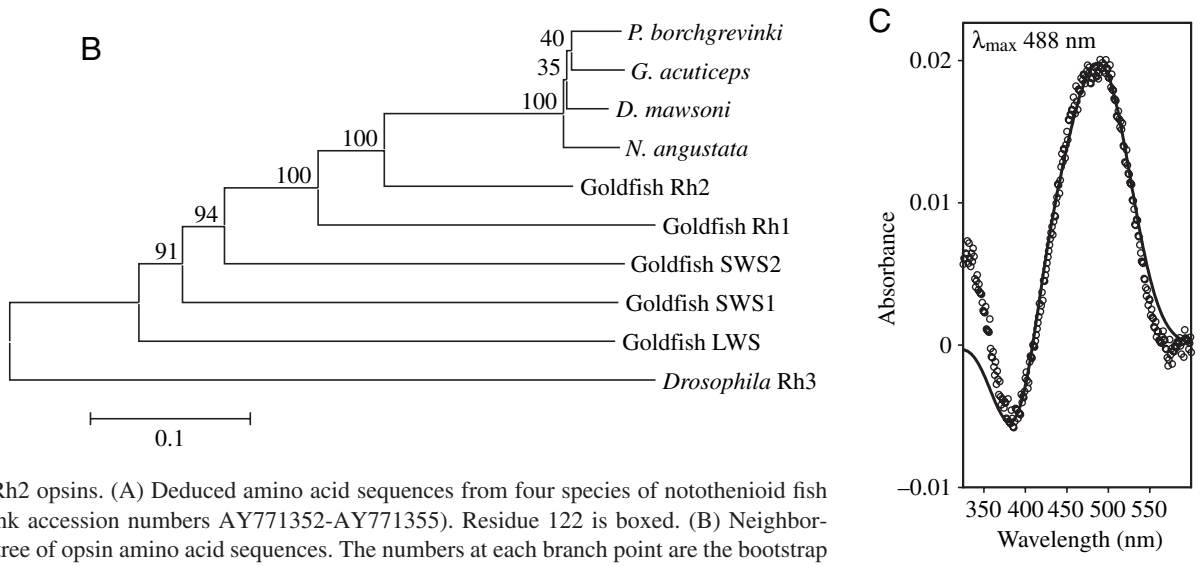

Fig. 6. Rh2 opsins. (A) Deduced amino acid sequences from four species of notothenioid fish (GenBank accession numbers AY771352-AY771355). Residue 122 is boxed. (B) Neighbor-joining tree of opsin amino acid sequences. The numbers at each branch point are the bootstrap values derived from 1000 bootstraps. The scale bar is calibrated as 0.1 substitutions per site, with the length of each branch proportional to divergence. *Drosophila* Rh3 (GenBank accession number NM_079687) was used as an outgroup. Goldfish opsin GenBank accession numbers: Rh1 L11863, Rh2 L11866, SWS1 D85863, SWS2 11864, LWS L11867 (C) *In vitro* absorbance spectrum for the pigment from *D. mawsoni*. The difference spectrum shown with a fitted Govardovski visual pigment template, was obtained by subtracting the bleached spectrum from the dark spectrum.

to date (Hunt et al., 2004). Since Phe86 is present in all six of the notothenioid SWS1 pigments, and the opsin in *D. mawsoni* has been shown to encode a UVS pigment by *in vitro* expression, it is likely that all six species possess a UVS SWS1 pigment.

SWS2 opsins

The SWS2 opsin gene sequence was amplified from *T. loennbergii* genomic DNA using degenerate PCR primers Blue400F and Blue818R (Table 1). This generated a 1 kb fragment that included exons 3–5. A 500 bp fragment at the 5′ end was then amplified by a genomic walk (Dominguez and Lopez-Larrea, 1994) with gene-specific primers (Table 1). This extended the sequence into the first exon to within 200 bp of the start codon. However, further sequence could not be obtained at the 5′ end as the universal first round walking primer consistently bound to the same site within the first exon. Expression was validated by RT-PCR in *P. borchgrevinki* and *G. acuticeps*, but no product could be amplified from *D. mawsoni* or *N. angustata* mRNA (data not shown).

The deduced amino acid sequence is shown in Fig. 5, aligned with the goldfish SWS2 sequence. This sequence was then used to design a primer pair, T.loenF and T.loenR (Table 1), that successfully amplified a short 250 bp fragment of the SWS2 coding sequence (data not shown) from retinal cDNA of two species, *P. borchgrevinki* and *G. acuticeps*, thereby confirming expression of the SWS2 gene in the notothenioid fish retina.

Rh2 opsins

The coding sequence for the Rh2 opsin gene was initially amplified from *D. mawsoni* retinal cDNA using degenerate primers Green1F and Green596R (Table 1). From this 600 bp fragment, 3′ RACE and genomic walking primers (Dominguez and Lopez-Larrea, 1994) were designed to complete the coding region. Primers designed to the 5′ and 3′ ends of the coding region were then used to amplify full coding sequences from *P. borchgrevinki*, *G. acuticeps* and *N. angustata* cDNA. The translated sequences are shown aligned in Fig. 6A. Phylogenetic analysis (Fig. 6B) shows that the notothenioid sequences form a clade with goldfish Rh2 with a bootstrap value of 100. The sequences are approximately 93% identical to each other at the amino acid level and show an 84% identity with cichlid (*Metriaclima zebra*, GenBank acc. no. AF247122), and a 74% identity with cyprinid (goldfish, GenBank acc. no. L11866) Rh2 opsins. Their identity as Rh2 cone pigments was further confirmed by *in vitro* expression of the *D. mawsoni* opsin in HEK 293T cells followed by regeneration of the pigment with 11-*cis*-retinal. The difference spectrum for the pigment (Fig. 6C) fits a Govardovskii template at 488 nm, which closely matches the value of 490 nm obtained from single and double cone photoreceptors by MSP, and differs from the rod pigment which has a λ_{max} of around 500 nm as determined by MSP (Table 3).

Although the amino acid sequences of the Rh1 and Rh2 opsins are similar, Rh2 pigments show higher rates of regeneration and meta II decay than Rh1 pigments, and this difference has been shown, for chicken pigments, to be largely dependent on the residue present at site 122 (Imai et al., 1997). Interestingly, the notothenioid Rh2 pigments differ at this site, with three species following chicken Rh2, with Gln, but one, *N. angustata*, following chicken Rh1, with Glu (Fig. 6A).

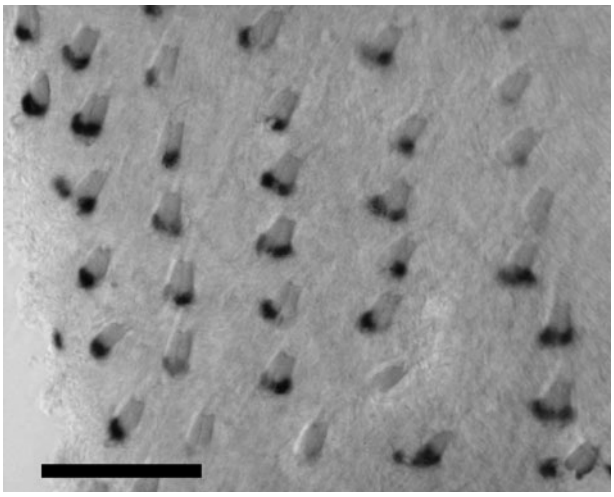

Fig. 7. *In situ* hybridisation of flat mounts of *D. mawsoni* retina probed with anti-sense Rh2 DIG-labelled cRNA. Note double cones with clear outer segments and labelled inner segments. Scale bar, 100 μm.

In situ *hybridisation*

All *in situ* hybridisation experiments were carried out on retinal tissue from *D. mawsoni*. Positive labelling was found in flat whole mounts of peripheral retina probed with antisense (Fig. 7) but not with sense Rh2 DIG-labelled cRNA (data not shown). Labelling was localised to the inner segments of double cone receptors. The pattern of labelling of these double cones is, however, variable, with either both partners, only one partner, or neither partner labelled. This result contrasts with the MSP data where no differences in λ_{max} between the two partners of a pair were found. Although it is possible that a population of double cones with different pigments in the partners was missed by MSP, labelling is uneven between partners, as evidenced by the variation in the labelling of cells shown in Fig. 7, so it is probable that the Rh2 mRNA transcript in some cells was below the level of detection by *in situ* hybridisation.

En face cryosections probed with the antisense Rh2 showed hybridisation in both peripheral and central retina (Fig. 8) whereas neither the SWS1 nor SWS2 probes showed positive labelling (data not shown).

Transverse sections from peripheral and central retina showed Rh2-positive labelling in both central and peripheral sections, whereas SWS2-positive labelling was confined to the peripheral retina (Fig. 9). No SWS1-positive labelling was found in any sections.

Histology

In order to determine the spatial organisation of cone photoreceptors in species adapted to different depths, flat whole mounts of retinae from two species, *D. mawsoni*, which inhabits depths down to 1600 m, and *T. hansoni*, which generally lives in shallow waters and is found down to 550·m (Table 2), were examined. Note, however, that both species had been maintained after capture in identical conditions of a flow-through tank under continuous dim light, so any differences in morphology do not just reflect differences in dark adaptation of the individuals under study. As shown in Fig. 10, the retina of *D. mawsoni* is organised with the septum between double cone partners always orientated in the same direction. This is typical of a row mosaic. There are some gaps in the mosaic caused by missing single cones, although in general, the mosaic is retained across all regions of the retina. In contrast, the retina of *T. hansoni* is organised into a square mosaic in which adjacent double cones lie at right angles to each other. Central cones are present but corner cones are lacking. The overall packing of cone photoreceptors is lower in the *D. mawsoni* retina, thereby allowing for a higher rod photoreceptor density.

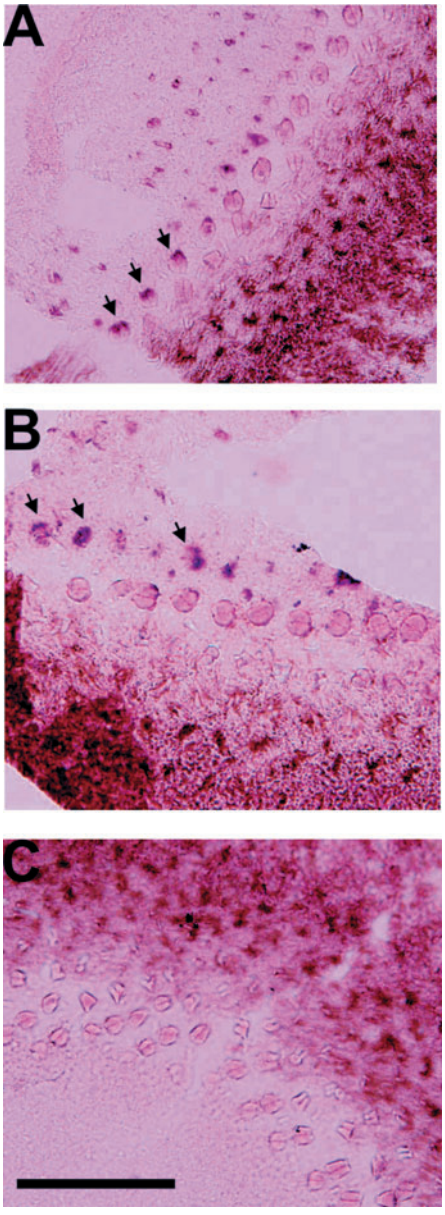

Fig. 8. *In situ* hybridisation of *en face* sections of *D. mawsoni* retina. (A) Central retina probed with anti-sense Rh2 DIG-labelled cRNA. (B) Peripheral retina probed with anti-sense Rh2 DIG-labelled cRNA. (C) Control probed with sense Rh2 DIG-labelled cRNA. Labelled cells are indicated with arrows. Scale bar, 100 μm.

Discussion

The ancestral visual pigment complement of jawed vertebrates almost certainly comprised a single rod pigment class and four cone pigment classes with λ_{max} values in the UV, blue, green and yellow regions of the spectrum. The combination of direct sampling of visual pigment spectra *in situ* by MSP, Southern analysis of opsin genes, and northern analysis of opsin gene transcripts in the retinae of notothenioid fish is consistent in demonstrating the retention of the rod and three of the cone classes, a UV-sensitive SWS1 opsin, a blue-sensitive SWS2 opsin, and a green-sensitive Rh2 opsin. The LWS opsin would appear to have been discarded and the lack of any signal from the Southern analysis indicates that this happened sufficiently long ago for the defunct sequence to have diverged such that it no longer hybridises with the LWS gene probe.

As down-welling light passes through a body of water, it becomes progressively attenuated with a maximum penetration in the clearest oceanic water to around 1000 m. This attenuation is greater, however, at longer and shorter wavelengths, such that after a few hundred metres, down-welling light is reduced to a narrow band of radiation between 470 and 480 nm in the blue-green region of the spectrum (Jerlov, 1976). In Antarctic waters, light penetration is further reduced by snow and thick sea-ice cover with a peak transmission around 500 nm (Pankhurst and Montgomery, 1989; Perovich et al., 1998). These reduced light levels under the ice will therefore limit photopic vision to the upper 150 m and scotopic vision to a maximum depth of around 300–400 m (Morita et al., 1997; Pankhurst and Montgomery, 1989) in summer months. In the winter, these limitations will be substantially greater.

The longwave spectral attenuation of light as it passes through ice and water is the most probable evolutionary basis for the loss of the LWS pigment and red-sensitive photoreceptors in notothenioid fish. In fact, the loss of long-wave sensitivity is not unusual in fish, particularly in species from low-light environments such as the deep ocean (Douglas et al., 2003; Levine and MacNichol, 1979; Loew and Lythgoe, 1978; Yokoyama and Tada, 2000; Yokoyama et al., 1999) and deep lakes. The species flock of cottoid fish in Lake Baikal is an example of the latter situation; amongst these species, only the very surface dwelling have retained red cones (Bowmaker

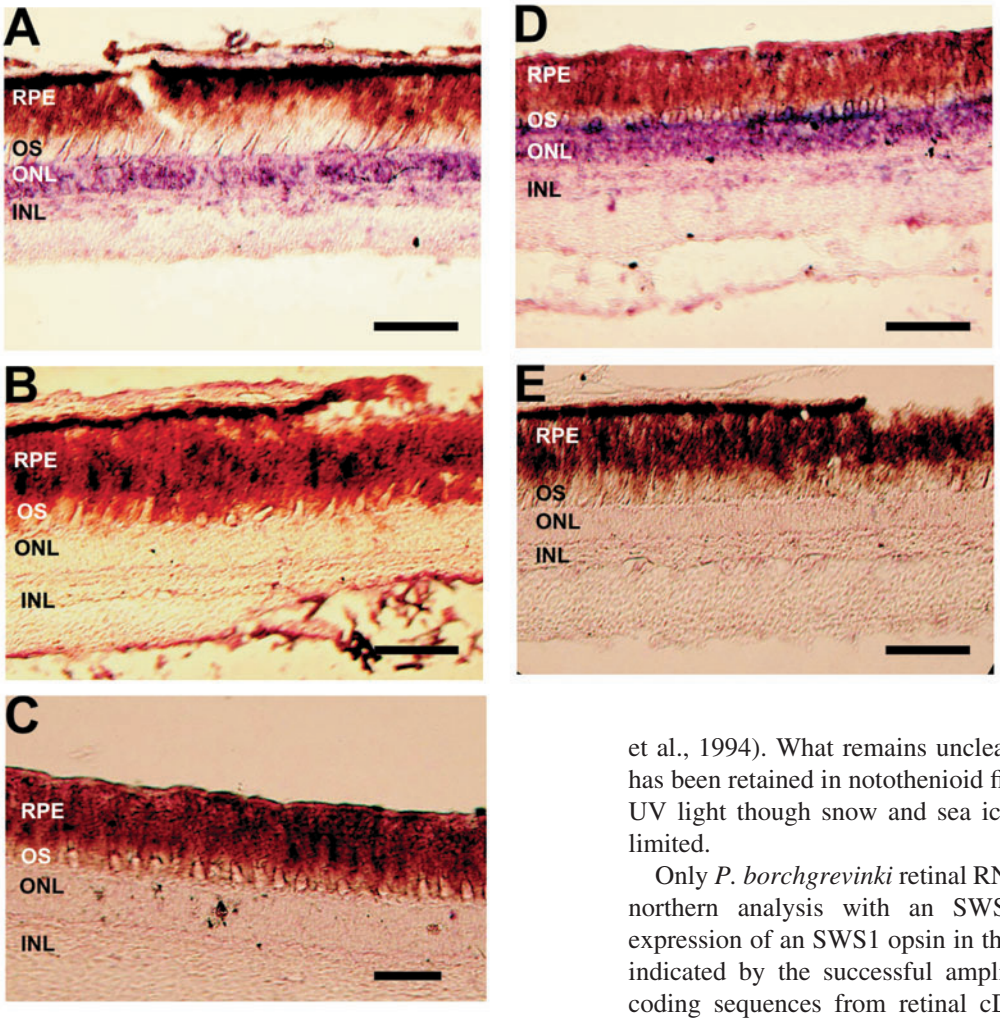

Fig. 9. *In situ* hybridisation of transverse cryosections of *D. mawsoni* retina. (A) Peripheral retina with SWS2 anti-sense probe. (B) Central retina with SWS2 anti-sense probe. (C) Control peripheral retina with SWS2 sense probe. (D) Peripheral retina with Rh2 anti-sense probe. (E) Control peripheral retina with Rh2 sense probe. Positive regions are stained blue. INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments; RPE, retinal pigmented epithelium. Scale bar, 100 μm.

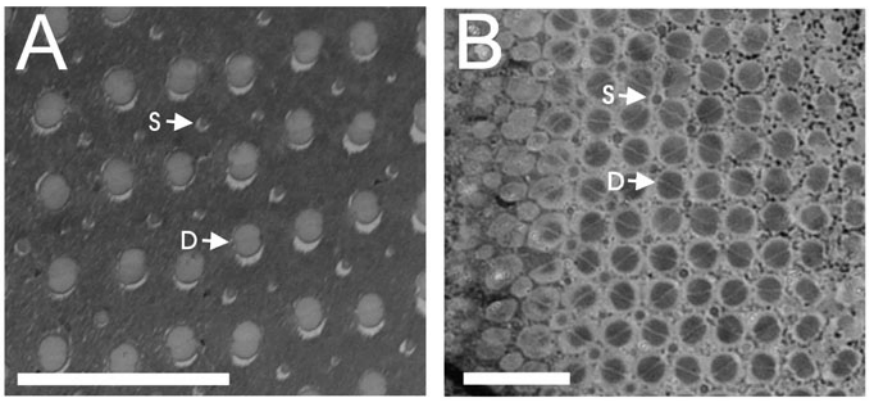

Fig. 10. Flat whole-mounted retinae from (A) *D. mawsoni* and (B) *T. hansoni*. S, single cones; D, double cones. Scale bar, 100 μm.

et al., 1994). What remains unclear is why the UVS pigment has been retained in notothenioid fish where the penetration of UV light though snow and sea ice would also be extremely limited.

Only *P. borchgrevinki* retinal RNA gave a positive signal by northern analysis with an SWS1 opsin probe, although expression of an SWS1 opsin in the retinae of other species is indicated by the successful amplification of the full SWS1 coding sequences from retinal cDNAs. Presumably, only a small number of SWS1 receptors are present in these latter species such that the SWS1 mRNA levels are below detection level by northern analysis. No UV cones were identified by MSP, although this may be a sampling problem arising from the small number of UV cones that may be present in the retina. UV sensitivity of the SWS1 pigment, if present, is a consistent finding in teleost fish and the UV sensitivity of the pigment in *D. mawsoni* was confirmed directly by *in vitro* expression and regeneration. The key amino acid for this UV sensitivity is Phe at site 86 (Hunt et al., 2004); all five notothenioid fish studied possessed this residue so it is probable that the other four species also possess a UV pigment. Many fish possess UV sensitivity while immature to aid plankton foraging, but it is then lost upon maturity (Bowmaker and Kunz, 1987; Loew et al., 1993). Notothenioid fish may also follow this pattern, with a low number of UV cones retained into the adult, although the positive signal by northern analysis seen with *P. borchgrevinki* retinal RNA may be associated with a higher frequency of UV cones in this cryopelagic species that lives

in the platelet ice layer on the underside of surface sea ice; the higher levels of UV light just below the ice that this species will encounter may have led to the retention of more UV cones than in other species.

SWS2 opsin expression in retinal RNA was confirmed by northern analysis in six species, by PCR in two species, *P. borchgrevinki* and *G. acuticeps*, and by the amplification, cloning and sequencing of 800 bp of the SWS2 gene from the genomic DNA of a third species, *T. loennbergii.* Surprisingly, although northern analysis gave a positive, albeit faint, signal with *P. borchgrevinki* retinal RNA, no blue cones were identified by MSP. This may again be due to sampling limitation although a population of single cones was identified by MSP that appear to have outer segments devoid of pigment. It may be that these cones originally contained the blue-sensitive SWS2 pigment but that fixation had denatured the pigment.

All five species of notothenioid fish examined by MSP gave a λ_{max} for the MWS cones of around 490 nm, a common value for Rh2 pigments in teleost fish, and this value was also obtained for the *D. mawsoni* Rh2 pigment by *in vitro* expression and regeneration with 11-*cis* retinal. This also agrees exactly with the photopic spectral sensitivity as determined by electrophysiology in *P. borchgrevinki* (Morita et al., 1997) and in *T. bernacchii* (Pankhurst and Montgomery, 1989). Although the λ_{max} values for the pigment in the different notothenioid species are essentially identical, they do differ at site 122, which has been implicated in determining the rate of pigment regeneration and meta II decay in chicken rod and Rh2 cone pigments (Imai et al., 1997). In chicken rod, charged Glu122 is present with uncharged Gln in chicken Rh2. The pigment in *N. angustata* departs from this rule however, with Glu present rather than Gln, although it is difficult to see why the Rh2 pigment in *N. angustata* should be more rod-like than the pigments in the other three species. Moreover, a comparison of other fish rod and Rh2 cone opsin sequences shows that the residue present at site 122 is not tightly conserved, with Glu present in the Rh2 pigments of goldfish and *Metriaclima zebra*, and either Glu, Gln or Val present in the rod pigments of different species of deep-sea fish (Hunt et al., 2001). In view of this, it would seem unlikely that this site plays the same key role in the regulation of the rate of regeneration in fish as it does in chicken pigments. However, it would appear to have an impact on spectral tuning, with the substitution of Gln by Glu causing a 14–15 nm shortwave shift in zebrafish Rh2 pigments (Chinen et al., 2005).

MSP identified both partners of all the double cones as green sensitive, and single cones as either blue or green sensitive. *In situ* hybridisation with an Rh2 probe also demonstrated that double cones express the Rh2 pigment, both in peripheral and central retina. Although SWS single cones were routinely identified by MSP, the SWS2 probe showed positive staining only in transverse sections of peripheral retina, indicating either that blue cones are only present in the peripheral retina or that the expression level in the central retina is below detection by *in situ* hybridisation. No signal for UV cones was obtained by *in situ* hybridisation with the SWS1 probe, and no UV cones were identified by MSP, although PCR experiments did confirm that SWS1 mRNA is present in the retina of *D. mawsoni*.

This absence of identifiable UV cones raises a major question regarding the organisation of the different spectral cone classes within the retinal cone mosaic. In a number of species of *Trematomus,* there is a typical square cone mosaic with both central and corner single cones (Meyer-Rochow and Klyne, 1982; Miyazaki et al., 2002; Miyazaki et al., 2001). Miyazaki et al. have assumed that because the corner cones in many teleosts are UV sensitive, this will also be the case in the notothenioids. Our data, both molecular and MSP, indicate that this cannot be the case, at least not throughout the retina. During MSP experiments, we often encountered preparations where two small single cones were clearly aligned on either side of a double cone in the position of corner cones. These single cones were, however, green sensitive and spectrally identical to the double cones. We infer from this that at least in some regions of the retina, the mosaic squares are composed of double cones and corner single cones that are all green sensitive, surrounding a blue-sensitive central single cone.

The organisation of the cone receptor mosaics is different in the retinae of two related nototheniid species, *D. mawsoni* and *T. hansoni*. The deeper dwelling *D. mawsoni* (mainly around 1600 m, but sometimes up at 300–500 m to feed) has double and single cones positioned in a row mosaic. All double cones in a single row lie in the same orientation, with single cones forming rows in between the doubles. Meyer-Rochow and Klyne (1982) described this as a hexagonal arrangement of double cones surrounding a pair of single cones, but this would appear to be a consequence of the slightly disordered row array (Fig. 10). Cone row arrays are commonly found in fish that have a reduced demand on photopic vision, e.g. in deeper dwelling species where vision is largely dependent on the scotopic system, and in species with a non-predatory lifestyle. The lack of a retinomotor response in the *D. mawsoni* retina (Meyer-Rochow and Klyne, 1982) is also consistent with a light environment that lacks significant fluctuations in intensity as found at depth in the ocean. The second species examined, *T. hansoni,* is demersal but lives in shallower water (down to 550 m). The retina of this species is organised into a typical square mosaic in which the double cones form the sides of a square, each orientated 90° to its closest neighbours. In contrast to the previous study by Miyazaki et al. (2001), there appears to be only a single morphologically distinct class of central single cones, with corner cones absent, which may explain the failure to identify UV cones by MSP. Two distinct retinal arrangements are therefore present in two related species within the same family. However, the visual pigments of the deeper dwelling species do not differ from the more shallow living fish: indeed, the λ_{max} values for rod, Rh2, and SWS2 pigments match almost exactly across all notothenioid species studied.

In general there is a trend in deep-water fish for the maximum sensitivities of the visual pigments of both rods and

cones to be displaced to shorter wavelengths with increasing depth of habitat, and for the cone population to be reduced and eventually lost. This is most evident in deep-sea fish that have pure rod retinae with λ_{max} around 470–480 nm (Bowmaker, 1995; Douglas and Partridge, 1997; Partridge et al., 1989), tuned to the maximum transmission of oceanic water and/or to the maximum emission of bioluminescence, and in the cottoid species flock of Lake Baikal (Bowmaker et al., 1994; Hunt et al., 1996). Why is similar spectral tuning not apparent in the different species of notothenioid fish and how is optimal sensitivity achieved further down the water column? Light penetrating through bare Arctic sea ice has a peak transmittance at around 500 nm and substantially reduced intensity, and the effect of a snow covering will be to reduce intensity further but not to alter the spectral characteristics (Perovich et al., 1998). Transmission on through the clear waters of the Antarctic (algal bloom is limited to the austral summer) would be expected to lead to a further attenuation of intensity and shift in the wavelength of maximal penetration towards 480 nm. Under these restricted photic conditions, the notothenioid rods with λ_{max} at about 500 nm and the dominant double cones with λ_{max} at 490 nm would appear to be tuned to the available down-welling light (Morita et al., 1997). Also, most bioluminescence peaks in the same spectral region (Nicol, 1969; Herring, 1983). Increased sensitivity is achieved in the deeper-dwelling species such as *D. mawsoni* by an increase in the proportion of rod photoreceptors and an increase in the length of the outer segments (Meyer-Rochow and Klyne, 1982). This is a common mechanism in deep-sea fish for photon capture in a dim light environment. Furthermore, the more shallow water species organise the cone receptors in a square mosaic to ensure optimal packing of cones in the retina, since these fish presumably rely more on photopic vision. Behavioural studies on both shallow and deep living species may help to identify how the different retinal arrangements affect chromatic and overall light sensitivity.

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