ABSTRACT Lampreys are one of the two surviving groups of jawless vertebrates, whose ancestors arose more than 540 million years ago. Some species, such as *Geotria australis*, are anadromous, commencing life as ammocoetes in rivers, migrating downstream to the sea, and migrating back into rivers to spawn. Five photoreceptor types and five retinal cone opsin genes (*LWS*, *SWS1*, *SWS2*, *RhA*, and *RhB*) have previously been identified in *G. australis*. This implies that the ancestral vertebrates possessed photopic or cone-based vision with the potential for pentachromacy. Changes in the morphology of photoreceptors and their spectral sensitivity are encountered during differing aquatic phases of the lamprey lifecycle. To understand the molecular basis for these changes, we characterized the visual pigments and measured the relative levels of opsin expression over two lifecycle phases that are accompanied by contrasting ambient light environments. By expressing recombinant opsins *in vitro*, we show that *SWS1*, *SWS2*, *RhA*, and *RhB* visual pigments possess *λ*<sub>max</sub> values of 359, 439, 497, and 492 nm respectively. For the *LWS* visual pigment, we predict a *λ*<sub>max</sub> value of 560 nm based on key spectral tuning sites in other vertebrate *LWS* opsins. Quantitative reverse transcriptase-polymerase chain reaction reveals that the retinal opsin genes of *G. australis* are differentially regulated such that the visual system switches from a broad sensitivity across a wide spectral range to a much narrower sensitivity centered around 490–500 nm on transition from marine to riverine conditions. These quantitative changes in visual pigment expression throughout the lifecycle may directly result from changes in the lighting conditions of the surrounding milieu.—Davies, W. L., Cowing, J. A., Carvalho, L. S., Potter, I. C., Trezise, A. E. O., Hunt, D. M., Collin, S. P. Functional characterization, tuning and regulation of visual pigment gene expression in an anadromous lamprey. *FASEB J.* 21, 2713–2724 (2007)

Key Words: color vision · lifecycle · recombinant protein
Over the lifecycle of *G. australis*, the downstream migrants are diurnal, traveling toward the sea during the day and burrowing at night. At sea, these lampreys frequent the brightly lit surface waters where they are predated on by albatross. On returning to the stream system, where the water is more turbid, *G. australis* becomes photophobic, burrowing during the day and moving upstream at night (11). Morphological (12) and spectral analyses reveal changes in the appearance and sensitivity of the photoreceptors during the protracted lifecycle.

The postmetamorphic visual system of *G. australis* is well-developed, and five morphologically distinct photoreceptor types have been characterized (13). The peak spectral sensitivity of the visual pigments of three receptor types in downstream and upstream migrants, respectively, have been previously determined by microspectrophotometry (MSP) as 610 and 616 nm (long wavelength sensitive cone; C1), 515 and 515 nm (medium wavelength sensitive cone; C2), and 506 and 500 nm (medium wavelength sensitive rod-like cone; C3), all characterized with a chromophore based on vitamin-A₂ (porphyropsin; ref. 14). The final two photoreceptor types (C4 and C5) could only be distinguished at the ultrastructural level in the retina of downstream migrants (13) and, due to their small size and relatively low density, their spectral characterization has yet to be determined.

The morphology of the photoreceptors of the downstream and upstream phases in the lifecycle of *G. australis* differ. In the downstream phase, all photoreceptors are large (7–12 μm in diameter) and densely packed, with three receptor types possessing dense aggregations of yellow short wavelength-absorbing pigment within their myoid (14). In the upstream phase, all photoreceptors are large (7–12 μm in diameter) and although the same complement of photoreceptors exist, the yellow myoidal pigment is absent in the C2 photoreceptor and is replaced by a (pigment-free) ellipsosome. Large photoreceptors and the presence of an ellipsosome are features typically associated with increasing sensitivity. Therefore, there appears to be a high degree of retinal plasticity underlying the transition of this species from its downstream phase (reliant on high densities of small photoreceptors sampling a photopic environment) to its upstream phase (reliant on low densities of large photoreceptors sampling a scotopic environment; refs. 13, 14).

Molecular genetic studies reveal that *G. australis* expresses five cone visual pigment (opsin) genes: a long wavelength sensitive opsin (LWS), two short wavelength sensitive opsins (SWS1 and SWS2), and two middle wavelength sensitive opsins (RhA and RhB; ref. 15). Phylogenetic analysis has demonstrated that the LWS, SWS1, SWS2, and Rh visual pigment genes are orthologous to the major classes of opsin genes present in the gnathostome lineage of vertebrates and arose by several linear gene duplications before the separation of the jawless and jawed vertebrate lineages (15).

The previous reports of changes in the level of a short wavelength-absorbing pigment and the spectral sensitivities of three of the five photoreceptor types during the lifecycle of this anadromous lamprey appear to correlate with concurrent changes in the light environment as it moves from the clear surface layers of the ocean to the turbid water of rivers. The aim of this study was therefore to 1) characterize the peak spectral sensitivities of the full complement of visual pigments during the downstream and upstream phases of the lifecycle of *Geotria australis* by employing *in vitro* expression of recombinant opsins and regeneration with 11-cis-retinal, 2) relate the spectral sensitivities of the visual pigments encoded by the five opsin genes to the key residues shown to be important for spectral tuning, and 3) quantify the relative levels of opsin gene expression in both the downstream and upstream phases of the anadromous lifecycle using quantitative polymerase chain reaction (qPCR) methods.

**MATERIALS AND METHODS**

**RNA source and preparation**

A duplicate cohort of three independent downstream (75–110 mm in total length) and upstream (560–640 mm in total length) migrants of the southern hemisphere lamprey *Geotria australis* were obtained from streams and rivers in southwestern Australia using an electric fish shocker. In our experiments, the retina of the downstream phase is considered to be a preadaptation for the marine phase (14) based on ultrastructural studies that reveal a salt-acclimated retina is identical to the retina of a downstream migrant and the fact that the duration of the downstream phase can be small (~6 wk). All animals were housed in freshwater aquaria that mimicked their natural environment as far as possible (constant temperature of 17°C under a 12 h light/dark cycle). After euthanasia by an overdose of methane tricaine sulfonate salt (MS 222; 1 in 2000 dilution), the right and left eyes were removed and a small horizontal incision made through the cornea before placement in RNA Later solution (Ambion, GeneWorks, Hindmarsh, Australia) to minimize RNA degradation. All tissue samples were initially stored at 4°C overnight to maximize the diffusion of the RNA preservation solution throughout the whole tissue and then stored at −80°C until required for further analysis. All procedures were performed in accordance with the ethical guidelines of the National Health and Medical Research Council of Australia and the Animal Ethics Committee of The University of Queensland (AEC No: PHY/PH/074/03/NHMRC/ARC). Fresh tissue was homogenized in a solution containing guanidinium thiocyanate, and total RNA was isolated using the Nucleospin RNA II purification kit according to the manufacturer’s instructions (Macherey-Nagal, Integrated Sciences, Chatswood, Australia).

**Generation of recombinant lamprey opsin constructs**

The full-length coding region of the lamprey (downstream migrant) LWS, SWS1, SWS2, RhA, and RhB opsin sequences were isolated by PCR and reconstructed as described previously (15). Forward and reverse primers (Table 1) were used to amplify the cDNA sequences as a single fragment with the addition of EcoRI and SalI restriction sites at the 5’- and 3’-ends of the coding region, respectively, for
insertion into a mammalian expression vector. The forward primer for each opsin contained a translation start codon (AUG) present within a Kozak consensus sequence to ensure efficient translation of the recombinant visual pigment. The reverse primers were designed to replace the stop codon of the opsin protein with a Sall restriction site contained within a sequence that would encode a C-terminal bovine rod opsin 1D4 epitope (ETSQVAAP) that would be recognized by the anti-Rh1D4 monoclonal antibody. Subsequent to PCR and restriction enzyme digestion, the EcoRI-Sall fragment was subcloned into the vector pMT4, as described previously (16).

Expression and purification of opsin proteins

Human embryonic kidney (HEK-293T) cells were transiently transfected with 7 μg per plate of opsin-pMT4 recombinant expression vector by GeneJuice (Merck, Chemicals Ltd., Nottingham, 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 μM cis-retinal in the dark. The membrane-bound pigments were solubilized and purified by immobilon-affinity chromatography using the anti-Rh1D4 antibody coupled to a CNBr-activated Sepharose column as described previously (17), eluted, and stored on ice.

Spectrophotometric analysis of recombinant visual pigments

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 either bleached by exposure to fluorescent light for 10 min (SWS2, RhA, and RHb visual pigments) or acid-denatured with 26 mM hydrochloric acid (SWS1 visual pigment). Spectrophotometric recordings were repeated three times per sample, and the bleached or acid-denatured spectra were subtracted from the dark absorbance spectra to produce difference spectra for the calculation of the peak absorbance ($\lambda_{max}$) value for each expressed lamprey visual pigment. The resultant visual spectra were overlaid with visual pigment templates (18), and best-fit spectral curves were obtained using the Solver add-in function in Microsoft Excel to vary the $\lambda_{max}$. As absorbance spectra are distorted by the underlying absorbance and scatter of the protein, difference spectra were used as the more accurate estimation of the $\lambda_{max}$ values.

Phylogenetic analysis of vertebrate Rh opsin genes

Sequence alignments of Rh1 and Rh2 protein sequences across a variety of vertebrate classes (from teleosts to mammals) were produced and subjected to phylogenetic analysis using the MEGA Version 3.1 computer package (19). With the use of codon-matched alignment, a neighbor-joining tree (20), bootstrapped with 1000 replicates, was generated using a Jones-Taylor-Thornton (JTT) substitution matrix (21).

qPCR analysis of opsin expression

First-strand cDNA was prepared from total RNA (1 μg) extracted from the retinae of downstream and upstream migrants of the southern hemisphere lamprey G. australis. Reverse transcription was performed using random 9-mer oligonucleotides (750 ng) and Superscript II reverse transcriptase (Invitrogen, Mount Waverly, Australia) at 42°C for 2 h. Removal of the initial RNA strand from the cDNA sample was achieved by digestion with RNase H (New England Biolabs, Australia) at 37°C for 15 min and purification through a silica-membrane column, using the UltraClean PCR Clean-up Kit, according to the manufacturer’s instructions (Mo Bio Laboratories, Geneworks, Australia). Individual visual pigment transcripts were quantified using gene-specific primers designed specifically to amplify LWS, SWS1, SWS2, RhA, and RhB opsin transcripts (Table 2). In addition, forward and reverse primers were designed to amplify transcripts transcribed from the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to use as an internal control to correct for sample to sample variation.

Initially, a codon-matched nucleotide alignment of each opsin expressed in the retina of G. australis was prepared. Regions of divergence were determined, and 30-mer oligonucleotides were designed manually with nonconserved nucleotides present at the 3′-end of all forward and reverse primers to facilitate amplification of specific transcripts. Each primer was subjected to in silico analysis using the blastn algorithm (http://www.ncbi.nlm.nih.gov/blast) and a PCR-simulation program (Amplify Version 3.0, http://engels.genetics.wisc.edu/amplify/) to ensure specific hybridization to individual lamprey opsin transcripts with minimal primer-dimer formation. All primer combinations traversed at least one exon-exon boundary, resulting in amplicons <150 bp with an average GC content of 68%. Triplicate qPCRs were performed on three independent cDNA templates (40 ng), using 1× Dynamo PCR SYBR-Master Mix (Finzymes, GeneWorks, Hindmarsh, Australia) and forward and reverse primers (500 nM), for both experimental opsin and endogenous GAPDH.

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Table 1. Oligonucleotide sequences used to generate the recombinant G. australis opsin constructs for expression analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>PE-LWS-F</td>
<td>5'-GCCGAGATTCGACCATGTCGCGAGGACGAGAGTT-3'</td>
</tr>
<tr>
<td>PE-LWS-R</td>
<td>5'-CCCGGTCGACGCCCGCGGTAGCAGGAGATTT-3'</td>
</tr>
<tr>
<td>PE-SWS1-F</td>
<td>5'-GCCGAGATTCGACCATGTCGCGGAGAAGAGTT-3'</td>
</tr>
<tr>
<td>PE-SWS1-R</td>
<td>5'-CCCGGTCGACGCCCGCGGTAGCAGGACGAGTT-3'</td>
</tr>
<tr>
<td>PE-SWS2-F</td>
<td>5'-GCCGAGATTCGACCATGTCGCGGAGAAGAGTT-3'</td>
</tr>
<tr>
<td>PE-SWS2-R</td>
<td>5'-CCCGGTCGACGCCCGCGGTAGCAGGACGAGTT-3'</td>
</tr>
<tr>
<td>PE-RHA-F</td>
<td>5'-GCCGAGATTCGACCATGTCGCGGAGAAGAGTT-3'</td>
</tr>
<tr>
<td>PE-RHA-R</td>
<td>5'-CCCGGTCGACGCCCGCGGTAGCAGGACGAGTT-3'</td>
</tr>
<tr>
<td>PE-RHB-F</td>
<td>5'-GCCGAGATTCGACCATGTCGCGGAGAAGAGTT-3'</td>
</tr>
<tr>
<td>PE-RHB-R</td>
<td>5'-CCCGGTCGACGCCCGCGGTAGCAGGACGAGTT-3'</td>
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Eco RI site (GAATTC) in the forward (F) primes and Sal I site (GTGAC) in the reverse (R) primes are underlined.
reactions. A MJ Research Opticon II qPCR Detector was used to detect SYBR-green reporter dye fluorescence and data were analyzed offline. A typical protocol took 2 h to complete and included an initial denaturation step at 94°C for 10 min, followed by 40 cycles of a 94°C for 10 s (denaturation), 58°C for 30 s (annealing), 72°C for 30 s (extension), and a final extension step at 81°C for 10 s to negate any spurious primer-dimer amplification before a plate read. To confirm primer specificity, melting and standard curves were generated for each amplicon using a 10-fold serial dilution (1 pg to 10 ng) of input template. The level of fluorescence, as an indication of amplicon accumulation with an increasing PCR cycle number, was measured in real time and the threshold cycle (CT) calculated. The CT value is defined as the cycle number where the level of fluorescence is above a background threshold. The baseline range and threshold level were manually adjusted to where the fluorescence increases exponentially and the CT is linearly proportional to the logarithm of the input template concentration. Quantification of opsin gene expression, relative to GAPDH, was performed using the comparative threshold cycle method as described previously (22, 23). Statistical comparisons were made using Student’s t tests (unpaired, two-tailed) and a P value of <0.05 was taken to indicate a change in transcript level was of statistical significance.

RESULTS

Regeneration and spectral sensitivity of visual pigments

To characterize spectrally each of the five visual pigments in Geotria australis, an in vitro recombinant spectrophotometric assay was used. Dark spectra for each reconstituted visual pigment was determined across a wide range of wavelengths (200 nm to 700 nm) and the pigment was then either bleached (SWS2, RhA, RhB, and LWS visual pigments) or acid denatured (SWS1 visual pigment). λ_{max} values were obtained by fitting a Govardovskii template (18) to the difference absorbance spectra for each regenerated visual pigment.

As shown in Fig. 1, expression of four out of the five lamprey visual pigments (SWS1, SWS2, RhA, and RhB) was successful. Unfortunately, despite several attempts with different clones, the LWS visual pigment consistently failed to produce a pigment. Expression of the SWS1 opsin yielded a λ_{max} value of 358 nm, confirming the ultraviolet sensitivity (UVS) of this pigment (Fig. 1A). Furthermore, this value is almost identical to the

<table>
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<th>Primer</th>
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<tr>
<td>RT-LWS-F</td>
<td>5’-GCCCATGATCTCTGCTACCTTGAAGTCCTG-3’</td>
</tr>
<tr>
<td>RT-LWS-R</td>
<td>5’-CCCGAAGATCTGGAAGATCATGAC-3’</td>
</tr>
<tr>
<td>RT-SWS1-F</td>
<td>5’-ACCTTCTCCTGCTTCGCGCATGCTG-3’</td>
</tr>
<tr>
<td>RT-SWS1-R</td>
<td>5’-AAAAACTCTTCTGCTTCGCGCATGCTG-3’</td>
</tr>
<tr>
<td>RT-SWS2-F</td>
<td>5’-GGGGTTCCTTCTACATGGCTGCGAT-3’</td>
</tr>
<tr>
<td>RT-SWS2-R</td>
<td>5’-TTAATTTCACGCTTGCTTCTGGCCTG-3’</td>
</tr>
<tr>
<td>RT-RHA-F</td>
<td>5’-GTCGTTCTCCTGCTTCGCGCATGCTG-3’</td>
</tr>
<tr>
<td>RT-RHA-R</td>
<td>5’-TTAATTTCACGCTTGCTTCTGGCCTG-3’</td>
</tr>
<tr>
<td>RT-RHB-F</td>
<td>5’-GTTCTTTCACGCTTGCTTCTGGCCTG-3’</td>
</tr>
<tr>
<td>RT-RHB-R</td>
<td>5’-TTAATTTCACGCTTGCTTCTGGCCTG-3’</td>
</tr>
<tr>
<td>RT-GAPDH-F</td>
<td>5’-AAATTACGCCACAGGTGTCTGGCTTCTGGCCTG-3’</td>
</tr>
<tr>
<td>RT-GAPDH-R</td>
<td>5’-AAAAATCGCGCCACAGGTGTCTGGCTTCTGGCCTG-3’</td>
</tr>
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Figure 1. Absorption spectra of regenerated G. australis SWS1 (A), SWS2 (B), RhA (C), and RhB (D) visual pigments. For each pigment, dark (closed circles) and bleached (SWS2, RhA, and RhB) or acid-denatured (SWS1) spectra (open circles) are shown, with fitted Govardovskii (18) templates (line) in insets to determine λ_{max} values.
predicted $\lambda_{\text{max}}$ of 360 nm for ancestral SWS1 pigments (24). The $\lambda_{\text{max}}$ of the regenerated SWS2 pigment was 439 nm (Fig. 1B), which resides within the blue-range (440–460 nm). Reconstitution of RhA and RhB opsins with 11-cis-retinal generated $\lambda_{\text{max}}$ values of 497 (Fig. 1C) and 492 nm (Fig. 1D), respectively, which is consistent with the middle wavelength sensitivity of Rh1 and Rh2 visual pigments.

Structural characterization of each visual pigment opsin

Amino acid sequences of the five opsin genes (LWS, AAR14680; SWS1, AAR14684; SWS2, AAR14681; RhA, AAR14682; and RhB, AAR14683) expressed in the retina of *G. australis* were analyzed for structurally important residues, especially given that the LWS opsin failed to regenerate in vitro. Each opsin sequence was subjected to protein secondary structure prediction analysis using the TMHMM Server Version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and seven transmembrane domains (TMDs), two extracellular domains (ECDs), three intracellular domains (ICDs), an extracellular N terminus, and an intracellular C terminus were predicted at locations consistent with those determined by crystallography (25) for the bovine rhodopsin polypeptide (NP_001014890; Fig. 2).

Comparison of the opsin amino acid sequences of *G.
australis and bovine rod opsin demonstrated that the critical residues involved in the maintenance of the tertiary structure of the opsin molecule are present (Fig. 2). With the use of the conventional numbering system of the bovine rod opsin polypeptide sequence (Fig. 3A; gray circles), these key sites include 1) three conserved cysteine (C) residues at positions 110 (TMD3), 185 (ECD2) and 187 (ECD2) that are involved in disulphide bond formation (26), except for a threonine (T) residue at position 185 in the G. australis LWS opsin, which is also conserved throughout the rest of the vertebrate LWS opsin class; 2) a conserved glutamate (E) at position 113 (TMD3) that provides the negative counterion to the proton of the Schiff’s base (27); 3) a conserved glutamate (E) at position 134 (TM3) that provides a negative charge to stabilize the inactive opsin molecule (28); 4) a conserved lysine (K) at position 296 (TM7) that is covalently linked to the chromophore via a Schiff’s base (29); 5) conservation of one or both of the cysteine (C) residues at putative palmitoylation positions 322 and 323 (both at the carboxy-terminus; ref. 30) in all lamprey opsins (SWS1, SWS2, RhA, and RhB), except for the LWS opsin; 6) the presence of a number of serine (S) and threonine (T) residues in the carboxy-terminus, which are potential targets for phosphorylation by rhodopsin kinases in the deactivation of metarhodopsin II (31, 32, 33); and 7) the conserved glycosylation sites at positions 2 and 15 of the amino terminus in the MWS opsins (RhA and RhB) identified in the retina of G. australis (32). Overall, each opsin identified in the retina of G. australis, including the LWS opsin, possesses all of the critical amino acids required to produce a functional visual pigment; the failure of the LWS opsin to produce a pigment in vitro cannot therefore be due to the absence of these structurally important residues.

Putative amino acids involved in spectral tuning of visual pigments

Functionally, a total of 22 tuning sites have been shown to influence the spectral sensitivity of vertebrate visual pigments (24, 34–37), most of which are in close proximity to the photosensitive chromophore (Fig. 3A; black circles). For LWS visual pigments, five tuning sites (164, 181, 261, 269, 292) have been identified, with the amino acids present at these sites in the ancestral LWS opsin proposed to be SHYTA respectively and giving a \( \lambda_{\text{max}} \) value of 560 nm based on a vitamin-A\(_1\) chromophore (38). The LWS opsin expressed in the retina of G. australis possesses an identical complement of residues (SHYTA) at these critical positions and would be expected to yield a pigment with a \( \lambda_{\text{max}} \) value of 560 nm if based on a vitamin-A\(_1\) chromophore (Fig. 3B).

The predicted ancestral tuning sites and spectral sensitivities (based on a vitamin-A\(_1\) chromophore) for SWS1 and SWS2 visual pigments are F46/F49/T52/F86/S90/T93/A114/S118 (FIFTFTAS) with a \( \lambda_{\text{max}} \) of 360 nm (39) and F46/I49/V52/T93/A164/L207/A269 (FIVTALA) with a \( \lambda_{\text{max}} \) of 439 nm (36), respectively. The sequence of the putative tuning sites present in the G. australis SWS1 visual pigment is FITFSTAS, which only differs from the proposed ancestral vertebrate sequence at position 49 (I instead of F). Therefore, the predicted \( \lambda_{\text{max}} \) is consistent with the value of 358 nm.

Figure 3. Spectral tuning of opsin molecules expressed in the retina of G. australis, modeled on bovine rod opsin. A) A schematic representation of the three-dimensional structure of a visual pigment, showing the opsin seven transmembrane domains (TMD I–VII), 3 extracellular domains (ECD I – III), 3 intracellular domains (ICD I–III), amino- and carboxy-termini (N or C), and the retinal chromophore. Modified from Baldwin (51). Amino acids are represented by white circles, except when highlighted gray or black to show residues critical for maintaining protein structural integrity or spectral sensitivity, respectively. Sites for glycosylation (residues 2 and 15), disulphide bonding (residues 110 and 187), attachment of the chromophore (residue 296), and palmitoylation (residues 322 and 323) are also indicated. B) Tables showing the positions and sequence of tuning sites implicated in the spectral tuning of ancestral LWS, SWS1, SWS2, Rh1 and Rh2 visual pigments and the equivalent residues present in G. australis visual pigments. *Denotes tuning sites that underlie a red-shift of 44 nm in the spectral sensitivity of the SWS2 visual pigment of the Japanese common newt (Cynops pyrrhogaster) compared to the bullfrog (Rana catesbeiana) (35).
obtained for the regenerated lamprey SWS1 visual pigment and can be accounted for therefore by the residues present at these tuning sites (Fig. 3B). The most important site for UV-sensitivity (UVS) is however 86 (40) with F resulting in a UVS pigment in all nonavian UVS pigments (37). The sole amino acid difference at position 49 is unlikely therefore to modulate the spectral sensitivity to any significant extent. Unlike SWS1, the tuning sites determined for the G. australis SWS2 visual pigment (FLFLALA) vary to a greater extent from the proposed ancestral sequence, at positions 49 (L instead of I), 52 (F instead of V) and 93 (L instead of T; Fig. 3B). Despite this, the predicted $\lambda_{\text{max}}$ value for the ancestral pigment and actual $\lambda_{\text{max}}$ value for the lamprey lie within the blue-range and are identical at 439 nm, thus contrasting with a previous study that showed that amino acid changes at these three positions generate a small spectral shift (36). In addition to the residues at these sites, changes at seven further sites have together been shown to cause a red-shift of 44 nm in the newt SWS2 visual pigment ($\lambda_{\text{max}}$ of 474 nm) compared to that of the bullfrog ($\lambda_{\text{max}}$ of 430 nm; ref. 35). The residues present at these sites in the G. australis SWS2 sequence (S91, A94, M122, S127, C211, F261, S292) are identical to those in the bullfrog SWS2 opsin, implying that they represent the ancestral state.

To date, no ancestral sequence has been proposed for the vertebrate Rh1/Rh2 class of visual pigments. Seven tuning sites have, however, been shown to modulate the spectral sensitivities of Rh1 and Rh2 visual pigments (D83/E122/M207/H211/W265/A292/A295; DEMHWAA; ref. 39). Sequence alignments of Rh1 and Rh2 protein sequences across a variety of vertebrate classes (from teleosts to mammals) were produced and subjected to phylogenetic analysis. The resulting neighbor-joining tree (20) suggested that the residues present at key tuning sites in the ancestral Rh1 and Rh2 opsins are DEMHWAA and DEMHWAAS, respectively (Fig. 4). Comparison of the amino acids at these positions in G. australis RhA and RhB protein sequences shows that RhA (NEMHWAA) possesses a similar complement of residues to the proposed ancestral sequence for Rh1 with the exception of a single amino acid difference at position 83 (N instead of D; Fig. 3B). The sequence for RhB (DELHWAS) is similar to the ancestral sequence proposed for Rh2 but with a single amino acid difference at position 207 (L instead of M) (Fig. 3B). Spectral analysis of isolated rod-like photoreceptors from the retina of an upstream migrant of the northern hemisphere lamprey Petromyzon marinus (the lamprey) identified a porphyropsin (vitamin-A2) with a $\lambda_{\text{max}}$ value of 525 (41). When analyzed in the context of a vitamin-A3 chromophore using the method of Whitmore and Bowmaker (42), this visual pigment would be predicted to give a spectral sensitivity peak absorbance of 496 nm, which is almost identical to the $\lambda_{\text{max}}$ value of 497 nm for the regenerated G. australis RhA visual pigment. Similarly, a $\lambda_{\text{max}}$ value of ~500 nm (based on a vitamin-A1 chromophore) has been found for a regenerated “rhodopsin” of another northern hemisphere lamprey, Lampetra fluviatilis (the river lamprey; ref. 43). Unlike most Rh1 pigments, which possess D83 and exhibit $\lambda_{\text{max}}$ values close to 500 nm (39), the RhA pigment identified in G. australis, as well as Petromyzon marinus (the sea lamprey; U67123) and Lethenteron japonicum (the Artic lamprey; M63632) all contain N83. In bovine Rh1 visual pigments, a D83N mutation causes a decrease in the $\lambda_{\text{max}}$ value by 6 nm (44), which may explain the small short wavelength shift in the peak spectral absorbance of regenerated lamprey RhA visual pigments and the presence of D or N at site 83 in some deep-sea fish has been implicated in the spectral tuning of rod visual pigments (45).

Comparison between the spectral tuning sites present in G. australis RhA (NEMHWAA) and RhB (DELHWAS) identifies three candidate amino acid differences (N83D, M207L, A295S) for this spectral shift. In bovine Rh1 visual pigments, D83N and A295S mutations cause a decrease in the $\lambda_{\text{max}}$ value by 6 nm (44) and 5 nm (46), respectively. In contrast, a L207M mutation in the Rh2 visual pigment of the coelacanth causes a 6 nm increase in the $\lambda_{\text{max}}$ value (47). If additive therefore, these three amino acid differences would be expected to cause a net short wavelength shift of 5 nm. When applied to the RhA and RhB visual pigments of G. australis, the $\lambda_{\text{max}}$ value of the RhB pigment is indeed short wavelength shifted with respect to the RhA pigment by the expected 5 nm, thus the spectral difference between RhA and RhB visual pigments may be entirely explained by the A295S difference.

Differential expression of opsins throughout the lamprey life-cycle

The adult stage of the lifecycle of G. australis consists of two migratory phases: 1) a downstream migration from a spectrally-poor freshwater river environment to a spectrally-rich marine phase (8) and 2) an upstream migration involving a return to the original spectrally-poor freshwater setting from the marine environment (11). To investigate changes in opsin gene expression during these two migratory stages, retinal tissue was collected from downstream and upstream migrants. Complimentary DNA was prepared from total RNA extracted from the retinai of three different animals and subjected to qPCR to determine the relative mRNA expression levels compared to an internal GAPDH control.

To obtain meaningful and accurate data when using qPCR, it is essential to show that primers designed to each transcript of interest are specific and do not cross-hybridize with closely related transcripts in the cDNA pool under a chosen set of experimental conditions. Under qPCR conditions, forward and reverse primers designed for a particular lamprey opsin were tested for specificity against plasmids containing full-length clones of LWS, SWS1, SWS2, RhA, and RhB opsins. In all cases, a 150 bp amplicon was observed for
PCR reactions containing a discreet primer set and a corresponding cloned opsin cDNA (Fig. 5A). As the actual expression level for each opsin is unknown, it is vital to establish that the primer sets remain specific over a large range of input template concentrations. By using a 10-fold serial dilution of template, standard curves were generated demonstrating that each primer set was specific over a range of 1 pg to 10 ng of input template (Fig. 5B) with amplification efficiencies close to 100% (Fig. 5C), thus allowing direct comparison between the relative expression levels.

Overall, all five opsins are expressed in the retinae of both downstream and upstream migrants of *G. australis*. The different opsin mRNA levels however showed substantial differences over the two lifecycle stages studied (Fig. 6). In the retina of downstream migrants, about a third of the total opsin mRNA present was from the *LWS* gene, SWS1 transcripts accounted for slightly less than a third, SWS2 and RhB mRNA levels were 15% each, with RhA expression accounting for less than 10%. For upstream migrants, the total transcript level for these five opsins was reduced by 4-fold when compared to the levels in downstream migrants. This decrease arises from a 9-, 6-, 4-, and 2-fold diminished
expression of LWS ($P<0.05$), SWS1 ($P<0.05$), SWS2 ($P<0.01$), and RhB transcripts ($P<0.1$), respectively (Fig. 6A), with the expression level of RhA in upstream migrants remaining unchanged. Thus, RhA was the predominantly expressed visual pigment mRNA in the upstream migrant retina, accounting for $\sim30\%$ of all opsin transcripts detected. Of the remaining, 70%, 25%, and 20% are accounted for by RhB and SWS1 opsin, respectively, and 15% by LWS and SWS2 opsins (Fig. 6B).

In summary, the predominantly expressed retinal opsins of downstream migrants are sensitive to short (SWS1) and long (LWS) wavelengths, encompassing a wide range from UV to the red end of the visual spectrum. In contrast, opsin gene expression underlying spectral sensitivity switches in the retina of upstream migrants to become more middle wavelength or green light sensitive, with RhA and RhB the predominantly expressed retinal visual pigments (Fig. 6C).

**DISCUSSION**

Five morphological photoreceptor types (C1–C5) were previously identified in the retina of G. australis (14), only three of which (C1–C3) have been characterized by MSP. Due to their small size, low density, and topographical heterogeneity, the spectral characterization of C4 and C5 by MSP has thus far been unsuccessful (13). G. australis expresses five opsin genes (15), with each deduced amino acid sequence adhering closely to the secondary conformation of other G-coupled proteins and containing all of the critical residues required for correct assembly and functional expression. Thus, we sought to characterize each visual pigment by expressing them in vitro. Four opsins in G. australis were successfully expressed in vitro and regenerated with 11-cis-retinal to give $\lambda_{max}$ values of 358 (SWS1), 439 (SWS2), 497 (RhA), and 492 nm (RhB), all of which are similar to the spectral sensitivities calculated for other vertebrate opsins based on amino acid differences present in those species (24, 36), with the key residue F86 underlying the UV sensitivity of the SWS1 visual pigment in G. australis (40). In contrast, the LWS visual pigment consistently failed to regenerate in vitro, despite possessing all the key residues shown to be important in producing a functional pigment. Since no other LWS opsin transcript has been found in the retina of G. australis and, as discussed below, the spectral peak of the native pigment determined by MSP is consistent with the predicted peak based on the residues at the key tuning sites, it would seem unlikely that the LWS pigment is encoded by a different gene. Thus, the reasons for the failure of the lamprey LWS opsin to produce a pigment in vitro remain unclear.

The native visual pigments of G. australis are porphyropsins, which utilize 11-cis-3,4-didehydroretinal (vitamin-A$_2$) as a chromophore (14). Applying the formula of Whitmore and Bowmaker (42), the predicted $\lambda_{max}$ values for the four G. australis pigments successfully expressed in vitro would be 371 nm (SWS1), 452 nm (SWS2), 526 nm (RhA), and 519 nm.
nm (RhB) based on a vitamin-A₂ chromophore. The ‘five-sites’ rule proposed by Yokoyama (39) for spectral tuning of LWS visual pigments indicates that the spectral sensitivity of the LWS opsin would be around 560 nm based on a vitamin-A₁ chromophore, which is consistent with the $\lambda_{\text{max}}$ of 560/616 for the C1 photoreceptor found in downstream/upstream migrants obtained in vivo by microspectrophotometry (MSP) for the vitamin-A₂ pigment (equivalent to a $\lambda_{\text{max}}$ value of 553/557 nm for a vitamin-A₁ pigment). Thus, it is probable that the C1 (610/616 nm), C2 (515/515 nm), and C3 (506/500 nm) cones previously identified by MSP in downstream/upstream migrants of G. australis (14) predominantly express the LWS, RhA, and RhB genes, respectively, leaving the SWS1 and SWS2 visual pigments and the C4 and C5 photoreceptor types (13) unassigned. The identification of five morphologically distinct photoreceptors and five opsin genes suggest that the expression of each visual pigment may be restricted to one particular photoreceptor. However, we are not able to remove the possibility of different opsin mRNA transcripts being coexpressed, thus RNA fluorescence in situ hybridization or immunohistochemistry using opsin-specific probes will be required to resolve which cell expresses which opsin.

By using quantitative methods, we have been able to show that the retinal visual pigments of G. australis are differentially expressed, with decreasing expression in the order of LWS>SWS1>SWS2>RhB>RhA in downstream migrants and RhA>RhB>SWS1>LWS>SWS2 in upstream migrants. Therefore, opsin expression is temporally regulated with a switch from predominantly short and long wavelength sensitive visual pigments in the downstream phase, which may be a preadaptation to the marine phase of the lamprey lifecycle (14), to largely middle wavelength sensitive opsins in its upstream migration phase in a freshwater environment. This level of plasticity may have arisen as a direct response to changes in the ambient light environment from clear oceanic water to turbid riverine water. During its marine phase, G. australis is exposed to almost 24 h of sunlight within the upper parts of the water column, where it is highly susceptible to avian attacks (10). A large spectral difference in sensitivity may enhance contrast and aid the achromatic detection of these avian predators against a brightly lit background (49), while possessing the potential for using chromatic cues to locate new hosts. A change in opsin expression on migration from the ocean to the river system may also be reflected in the switch from a diurnal to a nocturnal lifestyle, where upstream migrants burrow during the day and travel upstream at night. Although the spectral transmission of the two water bodies has not been measured, the loss of the short wavelength-absorbing filter in some photoreceptor types, the development of a presumably sensitivity-enhancing ellipsosome, and the appreciable increase in photoreceptor size between the two phases (2–3 to 7–12 μm in diameter; ref. 14), all suggest that the increase in middle wavelength sensitive visual pigments in the upstream phase reflects a major change in the photosensitivity of the animal. Whether the quantitative changes in opsin expression are due to visual pigments becoming more highly concentrated within the outer segment discs (50) or the number of discs increasing to provide an increased membrane surface area for the insertion of visual pigment is currently unknown. The

![Figure 6. Opsin mRNA expression in the retinas of downstream and upstream G. australis migrants. A) Relative expression levels of visual pigment transcripts (LWS, SWS1, SWS2, RhA and RhB) for downstream and upstream G. australis migrants measured relative to GAPDH mRNA levels. B) Relative proportion of each opsin level compared to total opsin expression in retina of downstream and upstream G. australis migrants. C) Percentage of total retinal transcripts encoding for an opsin sensitive to a particular wavelength of light for downstream (black) and upstream (white) G. australis migrants. In all cases, error bars = 1 sE. LWS, long wavelength sensitive; SWS, short wavelength sensitive; Rh, rod-like cone opsin (medium wavelength sensitive). Data are mean ± sE; n = 3. Statistical difference in opsin expression between downstream and upstream migrants are indicated by (#) when P < 0.01 or (*) when P < 0.05.](image-url)
presence of an identical number of photoreceptor types and visual pigment genes suggest that these ancient animals possess the potential for pentachromacy. Our study shows that all five opsin genes are expressed simultaneously, thus arguing for the presence of a functional pentachromatic visual system. The relative levels of expression, however, vary during two contrasting phases of the lamprey lifecycle; visual plasticity would therefore appear to be a key component in the development of the earliest vertebrate visual system.

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