Ontogeny of Ultraviolet-Sensitive Cones in the Retina of Rainbow Trout

*(Oncorhynchus mykiss)*

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ABSTRACT

In order to facilitate emerging models of retinal development, we developed electroretinogram and in situ hybridization protocols to examine the ontogeny of photoreceptors in the retina of a land-locked salmonid, the rainbow trout (*Oncorhynchus mykiss*). We cloned cDNA fragments corresponding to the rod opsin and each of the four cone opsin gene families, which we utilized to produce riboprobes. We established the specificity of the in situ hybridization protocol by examining subcellular signal localization and through double-labeling experiments. We confirm the assumption that the accessory corner cones in the square mosaic are the ultraviolet wavelength-sensitive (UVS) cone photoreceptor (i.e., they express an SWS1 opsin) and observed UVS cones throughout the retina of small trout. Larger fish have a decrease in sensitivity to short wavelength light stimuli and the distribution of UVS cones in the mature retina is limited to the dorsal-temporal quadrant. These larger fish also possess differentiated UVS cones in the peripheral germinal zone (PGZ), including within areas peripheral to mature retina lacking UVS cones. These data are consistent with the loss of putative UVS cones from the PGZ of a migratory salmonid of another genus, and thus the disappearance of UVS cones appears to be general to the Family Salmonidae, regardless of life history strategy. The generation, differentiation, and subsequent loss of UVS cones in the smolt PGZ is a dramatic example of the supposition that the mechanisms of PGZ development recapitulate the retinal embryogenesis of that species. J. Comp. Neurol. 461:294–306, 2003. © 2003 Wiley-Liss, Inc.

Indexing terms: teleost; in situ hybridization; proliferative growth zone; retinal development; electroretinogram

The vertebrate retina is a part of the central nervous system (CNS) that has served as an effective example of CNS function, development, and evolution. The teleost retina has various properties, including a layered structure, a regular mosaic of photoreceptors, and continuous growth throughout life (Lyall, 1957a,b), which have been particularly valuable in this regard. For example, the study of the visual system in goldfish and carp has revealed much about the mechanisms underlying color vision (Kamermans and Spekreijse, 1999), whereas the zebrafish retina has become a popular model to study CNS development (Bilotta and Saszik, 2001; Li, 2001). Furthermore, the diversity of habitats and life histories that teleosts have exploited allow an examination of the evolution of CNS function and development.

Salmonids, including salmon and trout, comprise a large group of closely related species with a variety of

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h Habitats, feeding strategies, and life history strategies. The variety of habitats individuals experience, often including lake, stream, and marine environments, create different challenges for the growing salmonid visual system. Changes in visual function associated with changing habitat have been reported in several teleost species (e.g., Alexander et al., 1994; Hawryshyn et al., 1989; Helvik et al., 2001; Shand et al., 1999, 2002), representing an opportunity to observe substantial neural development within an established CNS structure. Thus, a comparative approach could be valuable to understanding control of developmental events and how these control points have evolved to optimize visual systems to various habitats. Of particular interest in this regard are species such as Oncorhynchus mykiss, which include populations that remain landlocked (rainbow trout) and others that migrate to marine environments (steelhead salmon) (Parkyn and Hawryshyn, 2000).

Salmonids have various other features that facilitate a study of retinal development, such as light-induced degeneration of rod outer segments (Allen and Hallows, 1997; Allen et al., 2001) and disappearance of accessory corner cones (ACC) from their square cone mosaic. Because the loss of this cone type coincides with a decrease in visual sensitivity to ultraviolet (UV) light1 the ACC have been putatively identified as UV wavelength-sensitive (UVS) cones (Kunz, 1987). This is consistent with the morphology of salmonid UVS cones detected using microspectrophotometry (Hawryshyn et al., 2001; Hawryshyn and Harosi, 1994) and with the position of UVS cones in the mosaics of goldfish, zebrafish, and killifish as identified by in situ hybridization (Hisatomi et al., 1996, 1997; Raymond et al., 1993). Evidence has been provided through electron microscopy that the disappearance of UVS cones in Atlantic salmon is mediated by apoptosis (Kunz et al., 1994). This has been suggested as a useful model of retinal apoptosis (Kunz et al., 1994). Interestingly, UVS cones reappear into the retina of at least some salmonids (Beaudet et al., 1997; Novales Flamarique, 2000), which represents the only known example of cone regeneration during natural development.

An important feature of teleost retinas is their continued growth throughout the life of the fish. The peripheral germinal zone (PGZ) allows the eye of many nonamniotic vertebrates to grow by generating new cells at the rim of the retina and forms a developmental timeline that seems to recapitulate retinal genesis or retinal repair (Harris and Perron, 1998; Olson et al., 1999; Perron et al., 1998). Presumably reflecting rapid retinal growth, the PGZ is substantial in rainbow trout and this timeline (i.e., spatiotemporal coordination) is expanded in comparison to other popular teleost models (Olson et al., 1999). For example, the PGZ of young rainbow trout has been used to examine the development of ganglion cell electrical properties (Olson et al., 2000). The PGZ of older Atlantic salmon has been utilized as a timeline to study the generation of rod photoreceptors in the context of UVS cone apoptosis (Kunz et al., 1994). The study of proliferative events led to renewed interest in a population of retinal stem cells in the inner nuclear layer of the mature retina. This population of cells had been identified in larval fish (Hagedorn and Fernald, 1992; Johns, 1982). Subsequent to Julian et al.'s (1998) description, these proliferative events have been demonstrated in the retina of other adult teleosts (Cid et al., 2002; Otteson et al., 2001; Vithalani and Hyde, 2000). This feature appears to exist in other salmonids (Ahlbert, 1976).

To date, the identification of cone types in the salmonid retina has relied on spectrophotometric analysis and histological sectioning and the latter method is most reliable when a square mosaic is revealed by tangential sectioning. However, a square mosaic does not occur throughout salmonid retina and a variety of methods (e.g., cell fate mapping and TUNEL detection) we employ are typically completed using radial sections that allow the visualization of cone nuclei and the various retinal layers. Furthermore, we are interested in comparing the ontogeny of photoreceptors among the variety of salmonids and this could be more practical with a protocol that allows easy assessment of cone distributions.

In order to address these issues and facilitate these important models of retinal development and repair, we have undertaken the development of tools to examine salmonid photoreceptor distributions. Here we report on the utility of electroretinograms, as well as the development of in situ hybridization protocols allowing us to label rods and each of the cone photoreceptors in rainbow trout. We confirm that accessory corner cones are the UVS cones and find that small (parr) rainbow trout possess UVS cones throughout their retinas. Larger rainbow trout have a UVS distribution limited to the dorsal temporal quadrant of the mature retina and this is coordinated with a decrease in visual sensitivity to UV stimuli. Furthermore, we find that in areas adjacent to mature retina without UVS cones, UVS cones are generated, differentiated, and disappear within the peripheral germinal zone.

MATERIALS AND METHODS

Tissue used for in situ hybridization was obtained from rainbow trout (Oncorhynchus mykiss) from Fraser Valley Trout Hatchery (Abbotsford, British Columbia, Canada). The fish were maintained in 350-L flow-through tanks at the University of Victoria, with a mean water temperature of 15 ± 1°C. A 12L:12D photoperiod was provided by standard fluorescent lights. Fish were maintained in these conditions for a minimum of 2 months prior to sampling.

To sample the retinas, fish were deeply anesthetized in 300 mg/l MS-222 (tricaine methanesulphonate, Crescent Research Chemicals, Phoenix, AZ, USA) until euthanized. Retinas were dissected during the last half of the light cycle in an attempt to maximize the amount of cone opsin mRNA present in the photoreceptors. Cone opsin mRNA has been shown to be highest before dark onset in various vertebrates (Pierce, 1999; Pierce et al., 1993; von Schantz et al., 1999), and at least the circadian rhythm of rod opsin mRNA content (highest immediately before light onset) has been shown to match this pattern in teleosts (Koren-Hagedorn and Fernald, 1992). Retinas used to generate some samples of cDNA, and partial clones of long wavelength-sensitive (LWS) and short wavelength-sensitive (SWS) opsin cDNAs (see below), were isolated from rainbow trout obtained from Lune Fish Farm (Oster Island, Norway).

1UV light is defined here as radiation with wavelength of 300–400 nm.

2In teleost fishes UVS cones express opsin proteins from the SWS1 gene family. Short wavelength-sensitive (SWS) cones (also referred to as “blue-sensitive cones”) express opsins from the SWS2 gene family. See Hunt et al. (2001) for further discussion of nomenclature.
Care of the fish and all procedures were in accordance with and approved by the University of Victoria Animal Care Committee under the auspices of the Canadian Council for Animal Care.

Cloning of partial opsin cDNAs and riboprobe production

Partial clones of SWS and LWS opsins were obtained by amplifying trout mRNA using degenerate primers designed to conserved regions of retinal opsins, and thus can amplify each of the cone and rod opsins (Forward 5’-AAGAACYTCGMCCTACCTCTTTA; Reverse 5’-GTCTCAGAACTGTACATGCT-3’). Second round, nested primers, consisted of the following: UVS opsin partial clone forward primer 5’-GACAGGCCATCTACTAACATTCT-3’, reverse primer 5’-GCCGTAACAGCAGTGAGGAC-3’; rod opsin partial clone forward primer 5’-CCAGITTCT-CTACCTCTATGTGCT-3’; reverse primer 5’-GGGCGTCTTTCCCTCTCAGA-3’. Second round, nested primers, consisted of the following: UVS opsin partial clone forward primer 5’-GACAGGCCATCTACTAACATTCT-3’, reverse primer 5’-GCCGTAACAGCAGTGAGGAC-3’; MWS opsin partial clone forward primer 5’-ACAGGACCAGG- GATTTAGGAGG-3’, reverse primer CACCGAGCCGC- CCATAACCATT-3’. Rod opsins were obtained by amplifying trout mRNA using standard guanidine isothiocyanate protocols (Chomczynski and Sacchi, 1987). PCR conditions for each clone were as follows: first round consisted of a 42°C RT reaction followed by 94°C for 5 minutes, and 30 cycles of 45 seconds 94°C; 45 seconds 55°C; 1 minute 72°C. The second round of PCR used 1 µl of the first round reaction as template under the following conditions: 94°C for 5 minutes, and 30 cycles of 45 seconds 94°C; 45 seconds 60°C; 1 minute 72°C, and finishing with a 5 minute hold at 72°C. First round primers consisted of the following: UVS opsin partial clone forward primer 5’-GGGCGTCTTGTAGATTTGTCGACC-3’, reverse primer 5’-TAGTCTTTGTTTCTGCGTGT-3’; MWS opsin partial clone forward primer 5’-AATGGCAGCTGAAGGAAAGAT-3’, reverse primer 5’-GCGAAGGACGCGGAGGATGC-3’; rod opsin partial clone forward primer 5’-CCAGITTCT-CTACCTCTATGTGCT-3’; reverse primer 5’-GGGCGTCTTTCCCTCTCAGA-3’.

Riboprobe was generated using standard protocols (Barthel and Raymond, 2000). Briefly, the plasmids were linearized with an appropriate restriction enzyme and riboprobes were generated using T7 RNA polymerase (Gibco-BRL) or SP6 RNA polymerase (Amersham-Pharmacia, Arlington Heights, IL) as appropriate. Reaction mixtures for these enzymatic reactions contained either digoxigenin (DIG) or fluorescein (FL)-labeled nucleotide triphosphates (Roche Biochemicals, Nutley, NJ). Successful probe production was assessed by gel electrophoresis with ethidium bromide staining and compared to a standard riboprobe included on the gel. In some cases riboprobes were also analyzed by dot blots of a dilution series of riboprobes, with standards, which were performed as per the manufacturer’s protocols.

Whole-mount in situ hybridization

Whole-mount in situ hybridization procedures were modified from established protocols (Henrique et al., 1995). Fish were maintained in dark for approximately 1 hour before dissection and eyes were enucleated under deep red light. Neural retina, with a portion of the optic nerve intact, was separated from other ocular tissues. These retinae were fixed in 4% formaldehyde buffered in phosphate-buffered saline (PBS), pH 7.4, overnight at 4°C. Retinae were washed several times in PBS and dehydrated through a graded series into methanol and stored at −20°C. Retinae were rehydrated through a graded series into PBS containing 0.1% Tween-20 (PTW) and dissected into pieces of appropriate size. For labeling with MWS or LWS opsin riboprobe, the tissue was treated at room temperature for 30 minutes with 10 µg/ml proteinase K (Sigma, St. Louis, MO). For labeling with SWS or UVS opsin riboprobe, retinae were treated with 200 µg/ml proteinase K for 45 minutes. The tissue was then rinsed in PTW and secondarily fixed in 4% formaldehyde and 0.1% glutaraldehyde in PBS and prehybridized at 60°C in hybridization mix (Henrique et al., 1995). Excess DIG-labeled riboprobe (approximately 1 µg/ml) was hybridized to tissue overnight at 60°C in hybridization mix. Posthybridization washes, at 60°C, consisted of two 30-minute washes in hybridization mix and a 10-minute wash in 1:1 hybridization mix : maleic acid buffer (MAB) (100 mM maleic acid, 150 mM NaCl, pH 7.5) with 0.1% Tween-20 (MABT). DIG-
labeled probe was detected with monoclonal sheep anti-DIG conjugated to alkaline phosphatase (anti-DIG-AP) and visualized with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt; Gibco-BRL), plus nitroblue tetrazolium (Gibco-BRL), with levamisole (Sigma). Alternatively, color development utilized FastRed (Roche Biochemicals) in 0.1 M Tris-HCl, pH 8.2. Results were visualized and documented using a 16-bit color MicroImager II digital camera (QImaging, Burnaby, BC) mounted on a Zeiss Axioskop 2 with Atto Arc 2 HBO 100 Mercury Arc Lamp. Differential interference contrast (DIC), brightfield, or multiple fluorescent images were merged and equalized using Northern Eclipse 5.0 (Empix Imaging) and Adobe PhotoShop 4.0 (Adobe Systems, Mountainview, CA).

**In situ hybridization on sectioned material**

Methods for in situ hybridization on sectioned material were modified from established protocols (Barthel and Raymond, 2000; Helvik et al., 2001a). Light-adapted eyes, with lenses removed, were fixed in 4% paraformaldehyde in PBS overnight at 4°C and rinsed with PBS. Retinas, including retinal pigmented epithelium, were dissected away from other ocular tissues and cut into quadrants. Quadrants were kept overnight in 25% Tissue-Tek OCT compound with 25% sucrose in PBS. Quadrants were frozen in this solution to acquire either radial (along the long-axis of the photoreceptors) or tangential (orthogonal to the latter, through the photoreceptor mosaic) sections. A Microm HM5000 (Zeiss) or CM1850 (Leica) cryostat was used to cut the latter, through the photoreceptor mosaic) sections.

Sections were stored in PBS overnight at 4°C. Posthybridization washes were performed at 60°C in 50% formamide in 2X SSC. Probes were detected with anti-DIG-AP or monoclonal sheep anti-fluorescein conjugated to alkaline phosphatase (anti-FL-AP; Roche Biochemicals), as appropriate, and visualized with BCIP/NBT or FastRed.

Double-labeling on sectioned material was accomplished using the above methods on sectioned material with the following modifications. The hybridization step included two different antisense riboprobes, one of which was DIG-labeled, while the other was FL-labeled. After visualization of one riboprobe, the sections were incubated in glycine-HCl, pH 2.2, washed several times in PBS, and fixed in 4% paraformaldehyde to deactivate the alkaline phosphatase conjugated to the antibody. The tissue was thoroughly washed in MAB and the antibody to the second label applied (e.g., anti-FL-AP was applied if anti-DIG-AP was applied during the first round of immunohistochemistry). The second antibody was visualized with the other alkaline phosphatase substrate (e.g., FastRed if BCIP/NBT was used for the first round of color development).

In some cases, sections were counterstained with 200 μg/ml Hoechst 33258 (Sigma) in PBS. This stains nucleic acids and weakly stains actin filaments, allowing visualization of the nuclei and general cell structure using a standard DAPI filter set. Slides were mounted in 1:1 PBS:glycerol, with 6.25 μg/ml n-propylgallate for the sections developed with FastRed.

**Immunohistochemistry**

SWS cones were labeled using our rabbit polyclonal antibody raised against goldfish SWS opsin, previously shown to label rainbow trout single cones (Veldhoen et al., 1999). Sectioned material was produced as above. The tissue was blocked in 1:50 horse serum in PTW. The anti-SWS opsin serum was applied 1:10 in PTW with 1:50 horse serum and detected with 1:100 fluorescein-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). For double-labeling experiments with in situ hybridization, the anti-SWS opsin primary antibody was applied after color development of the in situ hybridization.

**Electroretinograms**

All experiments were conducted at least 1 hour after light onset and completed 1 hour before dark onset. Fish were anesthetized by immersion in 100 mg/ml MS-222 and immobilized with an injection of Flaxedil (0.01 mg/g body weight). Fish were placed in a foam cradle, within a Faraday cage, and the gill epithelia irrigated with oxygenated water. Fish were further anesthetized with an injection of Maranil (metomidate hydrochloride, Wildlife Laboratories, Fort Collins, CO, USA, 0.003 mg/g body weight). A chlorided silver electrode (0.01 in, A-M Systems, Everett, WA, USA) was placed on the surface of the iris of the left eye. A reference electrode was placed in the right nare and the ground was placed on the body musculature.

Stimuli were presented in a manner similar to previously reported protocols (Beaudet et al., 1993; Deutschlander et al., 2001; Novales Flamarique and Hawryshyn, 1996; Parkyn and Hawryshyn 2000). Briefly, a constant bright yellow background light, provided by a tungsten-halogen bulb filtered through 500 nm long pass and neutral density filters, was used to isolate the UVS and SWS cone mechanisms. Electroretinogram (ERG) responses were recorded in response to increasing intensities of monochromatic stimuli. Stimuli were generated by a 300 W xenon-arc lamp (Oriel, Stratford, CT, USA) and controlled by a holographic-grating monochromator (ISA, Louisville, KY, USA) and quartz neutral density wedge (Melles-Griot). Stimuli were presented as 500 μsec flashes controlled by a shutter (Vincent Associates, Rochester, NY, USA) with 20-second interstimulus interval. Background and stimulating light were mixed in a trifurcated light pipe and projected onto the right eye through a quartz plate diffusing element. Custom-designed software was used for optical system calibration, stimulus control, data acquisition, and on-line analysis.

To determine sensitivity we measured the b-wave amplitude (Fig. 1A). Responses were amplified, filtered (3-300 Hz, Grass Instruments, Quincy, MA, P-5 Preamplifier), displayed on an oscilloscope, and acquired by a data acquisition board (16-bit A/D, National Instruments, Baltimore, MD). Response versus intensity functions were fit with a Naka-Rushton equation (Naka and Rushton, 1966a,b) using a nonlinear least-squares fit. This function was then used to interpolate the threshold of light intensity required to generate a criterion response (Fig. 1B). The criterion response was determined for each fish by choosing a value above the baseline recording of responses.
to low-light intensities, within the linear portion of the response versus intensity curves. Sensitivity was the inverse of threshold intensity within various wavelengths. Spectral sensitivity was normalized to 420 nm, averaged, and standard error was calculated. As described previously (Coughlin and Hawryshyn, 1994a; Deutschlander et al., 2001; Parkyn and Hawryshyn, 2000), the spectral sensitivity functions were fit using a linear-additive model of the cone mechanisms. The bright yellow background allows the assumption that responses to UV stimuli do not contain contributions from the β-band of MWS and LWS cone mechanisms. The best-fit weighting (i.e., contributions) of the UVS \( K_{UV} \) and SWS \( K_{B} \) cone mechanisms was then determined using a nonlinear least-squares fit to the spectral sensitivity data. Normalizing the data to the 420 nm response allows a comparison of the UVS cone mechanism’s sensitivity relative to that of the SWS cone mechanism.

RESULTS

Sequencing data revealed partial cDNAs with high sequence identity to each of the teleost opsin gene families as determined by BLAST search (NCBI). The five sequences utilized to produce riboprobes against UVS, SWS, MWS, LWS, and rod opsins had lengths of 596, 734, 803, 563, and 758 basepairs and are reported in GenBank (gene family/accession numbers SWS1/AF425074, SWS2/AF425075, RH2/AF425076, LWS/AF425073, and RH1/F425072, respectively). Each of these sequences is predicted to produce an amino acid sequence that would span several transmembrane domains. Full-length sequences for these cDNAs have been acquired from \( O. mykiss \) (data not shown; reported in GenBank under the above accession numbers) and other salmonid species (Data not shown) that further confirm their assignment to the five opsin gene families through comparisons of sequence identity.

These partial cDNA clones were used to produce riboprobes that labeled cells of expected morphology. Whole-mount preparations and tangential sections reveal that the double cone partitioning membranes point towards the cones containing SWS opsin mRNA and UVS opsin mRNA is within single cones at the corners of the square mosaic (Fig. 2). The latter, the accessory corner cone, disappears coincident with the fish’s loss of sensitivity to UV light (Beaudet et al., 1993; Browman and Hawryshyn, 1992, 1994; Kunz, 1987; Novales Flamarique, 2000), and has been shown to contain a UVS pigment using microspectrophotometry (Hawryshyn et al., 2001; Hawryshyn and Harosi, 1994). Thus the assumption that the accessory corner cone is the UVS cone (Beaudet et al., 1997; Browman and Hawryshyn, 1992, 1994; Hawryshyn et al., submitted; Kunz et al., 1994; Martens, 2000; Novales Flamarique, 2000), which is central to the interpretation of data in these articles, generally appears to be true. This is consistent with other species examined to date (Hisatomi et al., 1996, 1997; Raymond et al., 1993), and appears to be a common feature of teleosts with square mosaics. Whole-mount in situ hybridizations revealed that MWS and LWS opsin mRNA was localized to double cones (data not shown). Using radial sections (Fig. 3), MWS or LWS opsin mRNA was localized to one member of double cones, while SWS or UVS opsin mRNA was localized to subpopulations of single cones. These identifications are consistent with cone morphologies of photoreceptors as identified by microspectrophotometry (Hawryshyn et al., 2001; Hawryshyn and Harosi, 1994) and appear in a variety of teleosts. Consistent with other species, including other salmonids, the UVS cone nuclei were vitreal to the outer limiting membrane (OLM) and found among the rod nuclei, while SWS cone nuclei were scleral to the OLM with other cone nuclei. Negative controls for each probe, consisting of the sense strand of riboprobe, or no probe, revealed no such labeling.

Because the UVS and SWS cones are similar in morphology and the specificity of our labeling protocol is key to

![Fig. 1. Electroretinogram responses to increasing intensities of light. A: Responses to increasing intensities of stimulus are presented towards the bottom of the figure. Stimulus intensity, in log photons · cm⁻² · s⁻¹, is presented near the end of each trace. The b-wave amplitude (in μV) as measured from the peak of the a-wave (first negative peak) to the peak of the b-wave (first positive peak), is plotted versus stimulus intensity (B) to determine the threshold stimulus required to elicit a criterion response. In this example a stimulus intensity of 13.84 log photons · cm⁻² · s⁻¹ is required to elicit the criterion of 60 μV.](image-url)
interpreting results, we sought to further characterize these labels. In radial sections it was clear that the UVS opsin mRNA was localized to single cones (Fig. 3A). Double-labeling of the SWS opsin mRNA and protein using in situ hybridization and our anti-SWS opsin antisemum, respectively, showed that the signals colocalized to the same photoreceptors (Fig. 3B). The mRNA was perinuclear, polarized, and consistent with the position of the endoplasmic reticulum in goldfish (Raymond, 1985) and rainbow trout (Schmitt and Kunz, 1989) cones. The antisemum has previously been shown to label SWS cones in rainbow trout (Veldhoen et al., 1999) and labeled protein sclerad to the SWS opsin mRNA signal. Presumably because the in situ hybridization protocol includes protease digestions, heat, and organic solvents, the antibody labeling of SWS cones was not consistent across the retina in this double-label protocol. Where this double-labeling was achieved, however, the colocalization to cones containing SWS opsin mRNA was apparent. We also undertook double-label in situ hybridization to show that the UVS and SWS opsin mRNAs localize to separate populations of single cones (Fig. 3C). These cones have appropriate morphology and occur with an appropriate periodicity. We obtained the same results regardless of the order of signal development or the type of molecule (DIG or FL) used to label the riboprobe. Using a similar approach, we focused on the specificity of the MWS and LWS opsin mRNA labels. We noted that the MWS opsin mRNA is localized to the shorter “accessory” member of the double cone (Fig. 3D). We also utilized double-labeling with MWS and LWS opsins and found the signals localized to separate members of the double cone pair (Fig. 3E). Rod opsin mRNA was present in a band corresponding to rod nuclei in the outer nuclear layer, with more punctuate labeling near the outer limiting membrane (Fig. 3F).

The loss of UVS cones during ontogeny (see below) was consistent with a decreased sensitivity to UV light as revealed by ERGs on the same individuals examined by in situ hybridization (Fig. 4). This difference is apparent upon qualitative observation of the data and is also revealed by quantifying the contributions of the UVS cone mechanism in parr (K_UV = 0.556) vs. smolt (K_UV = 0.325) fish. This decrease in sensitivity to UV light is expected based on psychophysical data (Browman and Hawryshyn, 1994; Hawryshyn et al., 1999; Hawryshyn et al., 1999) and recordings from the optic nerve (Beaudet et al.; 1993, Deutschlander et al., 2001; Parkyn and Hawryshyn, 2000). The high sensitivity to short wavelength light relative to longer wavelengths (Fig. 4A), under the adapting conditions utilized, demonstrates that the responses to short wavelength light are not due to β-band absorption of other cone photoreceptors.
We confirmed that an independent UVS cone was measurable with ERGs by adding UV light into the background adapting light. As expected, this caused a decrease in sensitivity to UV light relative to that of other wavelengths within individual fish that had sensitivity to UV light (data not shown).

In situ hybridization revealed that UVS cones were present throughout the retina of parr trout ranging from 3–7 g. This is consistent with results using tangential sections of retina from Atlantic salmon, brown trout (Kunz, 1987; Kunz et al., 1994), and sockeye salmon (Novales Flamarique, 2000). The distribution of UVS cones in the mature retina of larger (>30 g) trout was limited to the dorsal retina (Fig. 5). This is consistent with recent topographical mapping by tangential sectioning of trout retina (Hawryshyn et al., submitted; Martens, 2000). The reduced distributions of UVS cones in large fish are consistent with reduced sensitivity to UV light in these same individual fish as determined by ERGs. In areas where UVS opsins were not detected, adjacent sections were shown to contain SWS opsins (Fig. 5), so that degradation of target mRNA was deemed unlikely. In addition to the examples detailed herein, we observed the same qualitative distribution of UVS cones in the retinae of six other individual trout of this size.

We quantified the in situ hybridization labeling of UVS cones and expressed it as a ratio to the number of SWS cones detected on adjacent sections (Fig. 6). We are mostly interested in the qualitative result of presence versus absence of UVS cones and UVS opsin mRNA. In our experience, quantification of cone photoreceptor densities is more accurate using plastic sections (e.g. Beaudet et al., 1997; Hawryshyn et al., submitted; Martens, 2000) or immunohistochemistry (Allison, Dann, and Hawryshyn, unpubl. results). Thus, Figure 6 is presented to highlight the robust nature of our protocol, including the use of an effective positive control for areas where UVS cones were not detected. Within the context of these qualifiers, we note that the nasal border of the UVS cone distribution seems to gradually decline in density, and thus could represent an area where UVS cones are being removed from the retina.

UVS cones were detected in the PGZ of smolts, in areas peripheral to mature retina that lack UVS cones (Fig 5, 6). The UVS cones were detected robustly in the PGZ but were absent from adjacent mature retina in the same section (Fig. 5D,E). Acting as a positive control, adjacent sections showed SWS cones throughout the retina (Fig. 5, 6), indicating that the state of the tissue did not play a role in the lack of UVS cone labeling in mature retina. Several UVS cones in the smolt PGZ had outer segments (Fig. 3A), implying full differentiation. Expression of opsin mRNA can also be considered an indication of differentiation. This is consistent with the presence of ACC in the PGZ of smolt retina in sockeye (Novales Flamarique, 2000), Atlantic salmon, and brown trout (Kunz, 1987; Kunz et al., 1994). This implies that the UVS cones either transdifferentiate (i.e., transmute) into some other cell type (e.g., rods), become dormant, or die via programmed cell death. Adjacent to the differentiated UVS cones, more centrally, we consistently detected degenerating UVS cones. These cells, in the appropriate position and containing UVS opsin mRNA, lacked clear cone morphology. This is consistent with EM evidence from Atlantic salmon, which suggests that differentiated ACC disappear via PCD (Kunz et al., 1994).

**DISCUSSION**

Salmonids are an attractive model for studying retinal development. Subsequent to embryogenesis, substantial developmental changes occur within the salmonid retina. This further retinal development is characterized by a loss of UVS cones and visual sensitivity to UV light (Deutschlander et al., 2001; Hawryshyn et al., 1989; Kunz, 1987; Novales Flamarique, 2000), and shifting visual pigments (i.e., chromophore A1/A2 ratio) (Alexander et al., 1998), allowing the study of how visual systems change to accommodate changing photic environments. Differences exist in life history strategies (e.g., the timing of migrations) between species or between populations within the same species. These differences can be interpreted in a framework of a well-established phylogeny to examine both visual ecology and how retinal development has evolved (Parkyn and Hawryshyn, 2000). Importantly, the UVS cones reappear at sexual maturity, when the fish return to their natal streams (Beaudet et al., 1997; Novales Flamarique, 2000). This represents the only known regeneration of cone photoreceptors during the natural life history of a vertebrate. Thyroid hormone treatment can act to induce events of interest (e.g., UVS cone disappearance and regeneration) (Alexander et al., 1998; Browman and Hawryshyn, 1992, 1994), providing the important advantage of a controlled onset time (Reme et al., 1998). The investigation of neural development in salmonids can utilize tools of transgenesis (Devlin et al., 2001), and genome sequencing is under way in a variety of salmonid species (reviewed in Milchert et al., 2002; Phillips and Rab, 2001). We sought to facilitate these models by developing tools to readily examine visual sensitivity to...
Figure 3
UV light via ERGs and to label each of the rod and cone photoreceptor types via in situ hybridization.

To date, the examination of sensitivity to UV light in salmonids has been limited to psychophysical techniques or recordings from higher-order retinal neurons, optic tectum, or the torus semicircularis (Beaudet et al., 1993; Coughlin and Hawryshyn, 1994b; Deutschlander et al., 2001; Hawryshyn et al., 1989). ERGs have long been used to assess visual function in salmonids (Ali and Kobayashi, 1968; Allen and Munz, 1983; Millodot, 1967), but the study of sensitivity to UV light is particularly difficult, having been achieved in only a few teleosts (Chen and Stark, 1994; Hughes et al., 1998). ERGs are simpler and more reliable than other protocols, and do not require surgery. The latter point facilitates developmental studies because individual fish can be recorded from multiple times allowing one to examine within-individual differences between developmental stages or before/after treatment.

Previous examinations of retinal development in salmonids have relied on identification of cone types by their morphology. The examination of UVS cone ontogeny has relied on the tentative assignment of the accessory corner cone, in square mosaic, as being maximally sensitive to UV light. This histological method has limited interpretive power within other retinal mosaic types, which often occur in areas of interest adjacent to the PCZ (Beaudet et al., 1997; Martens, 2000). This methodology is also limited to tangential sections; however, radial sections are often required to visualize nuclear labeling in cell fate determination protocols (Julian et al., 1998). Thus, we developed a label for the UVS cones and confirm that, consistent with other fish species (Hisatomi et al., 1996, 1997; Raymond et al., 1993), the accessory corner cone expresses UVS opsin mRNA.

We utilized these tools to examine the changing distribution of UVS cones as the rainbow trout goes through metamorphosis, often referred to as smoltification.3 We found that UVS cones were present throughout the retina of young (parr) rainbow trout. This result is similar to migratory sockeye salmon (O. nerka) (Novales Flamarique, 2000), as well as salmonids from the genus Salmo (e.g., Atlantic salmon) (Kunz, 1987; Kunz et al., 1994). We found that in larger, postmetamorphic rainbow trout the distribution of UVS cones in the mature retina is limited to the dorsal temporal retina. In the areas where UVS cones were not detected, robustly labeled SWS cones were detected on adjacent retinal sections. Coordinated with this decrease in UVS cone distribution, we show that the sensitivity to UV light is reduced in postmetamorphic fish. Thus the decrease in UV sensitivity is coordinated with the UVS cone distribution as revealed by in situ hybridization. The results are also consistent with a recent histological examination of accessory corner cones in large rainbow trout (Hawryshyn et al., submitted; Martens, 2000). The results are also supported by optic nerve recordings of rainbow trout undergoing thyroid hormone treatment (mimicking metamorphosis) and steelhead smolts where the remaining sensitivity to UV light stimuli is localized to the dorsal retina (Deutschlander et al., 2001). It appears that the loss of UVS cones in rainbow trout, during natural development, is not as substantial as that of sockeye salmon, where UVS cones may disappear from the entire retina (Novales Flamarique, 2000). More data is needed before one can assess if this represents a difference in experimental method, species, developmen-

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3Smoltification is a term often reserved for ocean-going salmonids, which alter their physiology to osmoregulate during migration to marine environments. The developmental changes that occur in rainbow trout may not meet some definitions of smoltification; however, they do mimic many of the same physiological changes. Importantly, visual system changes at this time of development appear to be similar to those of ocean-going populations.
tal timeline, and/or a difference between anadromous (ocean migrating) vs. nonanadromous (landlocked) populations. Regardless, our results are not consistent with the suggestion that salmonid fishes lose the UVS cones solely because they experience a reduced number of UV photons in their environment during later stages of their life history: more UV light reaches the ventral retina than the dorsal retina at this time, yet it is in the dorsal retina where we have demonstrated that UVS cones remain.

Fig. 5. Distribution of ultraviolet wavelength-sensitive (UVS) cones, as revealed by in situ hybridization on radially sectioned retina. UVS cones were present throughout the retina of small (parr) trout and the dorsal temporal retina of smolts (A, dark precipitate; F, fluorescent signal). In smolts, UVS cones have disappeared from the ventral and nasal portions of the retina (B). In areas of retina where UVS cones were not detected, short wavelength-sensitive (SWS) opsin mRNA was detected on immediately adjacent sections (C,E), serving as a positive control for the labeling protocol. Differentiated UVS cones were present in the peripheral germinal zone (D) of ventral and temporal smolt retina (represented by fluorescent signal and with the periphery towards top left of panel), immediately adjacent to areas of mature retina that lack UVS cones. Compared to parr that had UVS cones throughout their retina, smolts had a decreased distribution of UVS cones (G,H,I). This is coordinated with a decrease in sensitivity to UV light (Fig. 4). These results were similar for all smolt retinas examined and a detailed map was created for retina from three individuals (G,H,I) where the lines indicate the detection of UVS opsin mRNA. The orientation of these lines is an artifact of tissue orientation during sectioning. The retina was traced using an overhead projector during dissection into pie-shaped pieces, allowing the location of sections to be documented. These pie-shaped pieces were each carefully mounted, sectioned radially, processed, and examined for the presence of UVS and short wavelength-sensitive opsin mRNA. D, dorsal; N, nasal. Scale bars = 50 μm in A,D,E,F; 100 μm in B,C; 2 mm in G; 5 mm in H,I.
We have established that thyroid hormone, which peaks during smoltification, plays a key role in salmonid visual development (Browman and Hawryshyn, 1994; Deutschlander et al., 2001; Plate et al., submitted). Thyroid hormone appears to play similar roles in *Xenopus* metamorphosis (Mann and Holt, 2001; Marsh-Armstrong et al., 1999). We have found that the retina of rainbow trout and sockeye have substantial levels of deiodinase enzymes (Plate et al., 2002). These deiodinases provide the ability to regulate the amount of activated thyroid hormone that reaches the retina, independent of serum hormone levels. The deiodinase expression in *Xenopus* retina peaks at metamorphosis, controls cell proliferation and differentiation, and is asymmetrical along the dorsoventral axis (Marsh-Armstrong et al., 1999). We are continuing to examine retinal deiodinase expression, particularly within the context of the presently described dorsoventral asymmetry of UVS cone disappearance during natural development. We also noted that retinal deiodinase activity levels differ between rainbow trout and sockeye, such that deactivation of thyroid hormone is higher in rainbow trout (Plate et al., 2002). These differences coincide with the apparent species differences in postmetamorphic UVS cone distributions.

We are currently working to differentiate between hypotheses regarding the fate of the disappearing UVS cone. The disappearing UVS cones have been suggested to transmute (transdifferentiate) into another cell type (Lyall, 1957b). Alternatively, they may become dormant, similar to the proposed fate of rainbow trout photoreceptors following damage by light (Allen and Hallows, 1997) or ouabain (Kurz-Isler and Wolburg, 1982). The balance of current evidence seems to support the contention that UVS cones are dying by programmed cell death, as suggested by EM evidence from an anadromous species of another salmonid genus (Kunz et al., 1994).

UVS cones in the smolt peripheral germinal zone

We report that the PGZ of smolt retina contains UVS cones. Our in situ hybridization labeling of these cells confirms the putative identity of these disappearing cells, as was suggested by histological methods in Atlantic salmon (Kunz et al., 1994). This is especially interesting in areas generating mature retina that will lack UVS cones. These short-lived UVS cones express opsin mRNA (Figs. 5, 6) and have fully elaborated outer segments (Fig. 3A), and these data support the contention that they are fully differentiated. The generation, differentiation, and subsequent loss of UVS cones in the smolt PGZ is a dramatic example of the supposition that the mechanisms of PGZ development recapitulate the retinal embryogenesis of that species (Harris and Perron, 1998; Olson et al., 1999; Perron et al., 1998).

We are curious if these short-lived UVS cones are generated in the smolt PGZ for some particular function. Photoreceptors are metabolically expensive to produce, and yet our data support a wide phylogenetic distribution of UVS cone generation and disappearance in the PGZ of the Family Salmonidae. A similar distribution of UVS cones in the PGZ has been detected by in situ hybridization in Atlantic halibut (*Hippoglossus hippoglossus*; J.V. Helvik, unpubl. data). Ancestral teleosts are believed to have possessed four cone types (Bowmaker, 1998) and many extant teleosts have only three cone types (Engstom, 1963). Thus, one can conclude that mechanisms of retinal development, other than apoptosis of a fourth cone type, have readily evolved to generate the three-cone mosaic observed in the salmonid smolt. This view is supported by several examples where the presence and absence of four cone types occurs between species of the same family (Engstom, 1963). Taken together, this argues

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Fig. 6. Density of ultraviolet wavelength-sensitive (UVS) cones in smolt retina from two individuals, represented as a ratio to the number of short wavelength-sensitive (SWS) cones detected in immediately adjacent radial sections. Location within the retina was determined as in Figure 4G–I. Numbers of UVS cones within a field of view were counted and expressed as a ratio to the number of short wavelength-sensitive cones in a similar field of view (determined by utilizing landmarks in the tissue) in an immediately adjacent section. Fields examined along a section were separated by approximately 400 μm. Scale bars = 5 mm. D, dorsal, N, nasal.

We have established that thyroid hormone, which peaks during smoltification, plays a key role in salmonid visual development (Browman and Hawryshyn, 1994; Deutschlander et al., 2001; Plate et al., 2002; Hawryshyn et al., submitted). Thyroid hormone appears to play similar roles in *Xenopus* metamorphosis (Mann and Holt, 2001; Marsh-Armstrong et al., 1999). We have found that the retina of
in favor of the UVS cone serving some function in the smolt PGZ; if this were not true, other mechanisms of retinal development leading to a mosaic with three cone types would be favorable.

While all available markers suggest that the UVS cones in the smolt PGZ are fully differentiated, it is unlikely that they serve any visual function due to their position behind the iris, which would considerably limit the amount of light reaching them. It was suggested previously that the disappearing UVS cones may make space for rods (Kunz et al., 1994). It is also possible that the UVS cone is generated to affect the differentiation of other adjacent cell types. A population of horizontal cells, H3, receive input from UVS cones, and other cone types in some teleosts (Kamermans and Spekreijse, 1995). These horizontal cells could require the formation of contacts with UVS cones for their appropriate differentiation, maybe of their late forming (at least during embryogenesis) gap junctions (Schmitt and Kunz, 1989) that contribute to receptive field properties. If it is true that the UVS cone is generated in the smolt PGZ solely to affect the differentiation of adjacent cells, then this has broader implications for interpreting the overproduction and subsequent pruning of neurons during neural development. The neurotrophic theory speculates that the overproduction of neurons (in some developing structures 50% of neurons are eliminated) allows the selection of neurons that have formed proper connections and are appropriately positioned (Raff et al., 1993). Thus, the hypothesized roles of neuronal death is to control cell number and eliminate neurons that have formed inappropriate connections (Clarke et al., 1998). However, it is apparent that neuronal death plays other important, as yet unknown, roles in nervous system morphogenesis (de la Rosa and de Pablo, 2000; Kuan et al., 2000). The current example supports the contention that some of the excess neurons function to direct the differentiation of surviving neurons. This hypothesis is similar to that of the role of generating vestigial organs during development. This role for dying neurons during development may be widespread, as it would typically be difficult to detect or describe. Indeed, our example of disappearing UVS cones in the smolt PGZ is a clear example, solely because the degenerating neuron is readily identifiable.

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LITERATURE CITED


