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Chromatic organization of cone photoreceptors in the retina of rainbow trout: single cones irreversibly switch from UV (SWS1) to blue (SWS2) light sensitive opsin during natural development

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Summary

The retinas of salmonid fishes have single and double cones arranged in square to row formations termed mosaics. The square mosaic unit is formed by four double cones that make the sides of the square with a single (centre) cone in the middle, and a single (corner) cone at each corner of the square when present. Previous research using coho salmon-derived riboprobes on four species of anadromous Pacific salmon has shown that all single cones express a SWS1 (UV sensitive) visual pigment protein (opsin) at hatching, and that these cones switch to a SWS2 (blue light sensitive) opsin during the juvenile period. Whether this opsin switch applies to non-anadromous species, like the rainbow trout, is under debate as species-specific riboprobes have not been used to study opsin expression during development of a trout. As well, a postulated recovery of SWS1 opsin expression in the retina of adult rainbow trout, perhaps *via* a reverse process to that occurring in the juvenile, has not been investigated. Here, we used *in situ* hybridization with species-specific riboprobes and microspectrophotometry on rainbow trout retina to show that: (1) single cones in the juvenile switch opsin expression from SWS1 to SWS2, (2) this switch is not reversed in the adult, i.e. all single cones in the main retina continue to express SWS2 opsin, and (3) opsin switches do not occur in double cones: each member expresses one opsin, maximally sensitive to green (RH2) or red (LWS) light. The opsin switch in the single cones of salmonid fishes may be a general process of chromatic organization that occurs during retinal development of most vertebrates.

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Key words: UV cone, blue cone, double cone, opsin, cone mosaic, retinal development, *in situ* hybridization, microspectrophotometry, thyroid hormone, fish.

Introduction

Photoreceptor development involves a series of differentiation events that include the expression of visual pigment protein (opsin) in a specialized region of the cell termed the outer segment (Ebrey and Koutalos, 2001). There are five opsin families (Yokoyama, 2000): four of them (SWS1, SWS2, RH2 and M/LWS) are generally expressed in cone photoreceptors and one family (RH1) is expressed in rods (Ebrey and Koutalos, 2001). In lower vertebrates, the various opsins are confined to different cone morphological types (single and double cones, the latter type absent in mammals) and these form square and row mosaics across the retina (see Novales Flamarique, 2001). Opsins that are maximally sensitive to UV–violet (SWS1) and blue (SWS2) light are commonly found in single cones whereas RH2 and M/LWS opsins, which are maximally sensitive to green and red light, respectively, occur in double cones (e.g. Hárosi, 1994; Ebrey and Koutalos, 2001). Though recent work has greatly advanced our understanding of the role that some receptors and their ligands play in regulating opsin expression (Prabhudesai et al., 2005; Roberts et al., 2005; Roberts et al., 2006; Srinivas et al., 2006; Mader and Cameron, 2006; Applebury et al., 2007), the molecular mechanisms that establish the chromatic organization of cone mosaics are not understood, and the plasticity in opsin expression within varying mosaics has only recently begun to be investigated (Shand et al., 2002; Parry et al., 2005; Hoke et al., 2006; Cheng et al., 2006).

The retinas of salmonid fishes, like those of most other teleosts (Lyall, 1957a; Engström, 1963), have cone mosaics that form a square or row, or take some intermediate formation (Lyall, 1957b; Ahlbert, 1976; Beaudet et al., 1997). The square mosaic unit consists of four double cones, whose elliptical cross-sections form the sides of the square, and one single cone, with circular cross-section, located at the centre of the square and, when present, at each corner (Lyall, 1957b; Bowmaker and Kunz, 1987; Beaudet et al., 1997). The double cones are made up of two apposing members sharing a common double membrane partition. In a perfect square mosaic, corner cones face the partitions of neighbouring double cones while centre cones are at the site of interception of these partitions, were they

to be imaginarily extended. The row mosaic consists of double cones whose partitions are not as perpendicular to each other as in the square mosaic, and spacing between single cones is often not as regular as in the square mosaic either (Beaudet et al., 1997; Novales Flamarique, 2001; Novales Flamarique, 2002). In many areas of the salmonid retina, these two mosaics coexist and, at least in the retina of the juvenile (~10 g in weight), the same cones can align in a row at the double cone ellipsoid level and in a square at the nuclear level (Novales Flamarique, 2001).

Salmonid fishes hatch as yolk sac alevins and proceed to absorb their yolk sacs over a period of weeks to months, depending on water temperature (Groot and Margolis, 1991; Novales Flamarique, 2005). Around the time of full yolk sac absorption, the fish rise from the stream gravel and adopt an active life style, as an alevin, feeding on various forms of zooplankton. As the alevin grows, it becomes a parr (fish >5 cm), which is a stage characterized by pronounced vertical bands along the body. This is followed by a physiological transformation termed smoltification that readies the fish (now termed a smolt) for life in sea water (Hoar, 1988). The smolt will enter the ocean and return to its home stream as an adult several years later to reproduce and begin the life cycle anew. Though all salmonids share a similar life cycle, different species will vary in their life history strategies (Groot and Margolis, 1991), with some (anadromous) species migrating to the ocean and others staying in fresh water (migrating to lakes). The rainbow trout is a landlocked salmonid belonging to the latter (non-anadromous) group.

Previous studies on several anadromous salmon species have shown that the yolk sac alevins hatch with all single cones expressing an opsin maximally sensitive to UV light (SWS1 opsin, λ_{max} ~365 nm) and double cones that express opsins maximally sensitive to green light (RH2 opsin, λ_{max} ~510 nm) and red light (LWS opsin, λ_{max} ~565 nm), one per double cone member (Cheng and Novales Flamarique, 2004; Novales Flamarique, 2005; Cheng et al., 2006; Cheng et al., 2007). Shortly prior to the time of full yolk absorption, the alevins start switching single cone opsin expression from SWS1 to one most sensitive to blue light (SWS2 opsin, λ_{max} ~434 nm) (Cheng et al., 2006). This opsin switch, from UV (SWS1) to blue (SWS2), starts in the ventral retina and proceeds toward the dorsal retina such that the young smolt has UV opsin-expressing cones only in the upper retina (Cheng et al., 2006; Cheng et al., 2007). The switch is followed by a loss of corner cones, primarily from the ventral retina, though the extent of the loss varies between salmonid species (Cheng et al., 2006; Bowmaker and Kunz, 1987). In adult Pacific salmon, the remaining single cones in the main (non-peripheral) retina express only blue sensitive opsin (Cheng and Novales Flamarique, 2007). The topography of spectral cone types in post-juvenile non-anadromous salmonid species, such as the rainbow trout, is unknown. In addition, the chromatic organization of single cones at younger stages has been the subject of controversy as species-specific riboprobes have not been used to analyse the retina of rainbow trout during embryonic development and at early juvenile stages, when the opsin switch occurs in other salmonid species (Cheng et al., 2006; Cheng et al., 2007).

Previous studies have also hypothesized that the corner cones lost during the juvenile period are regenerated and express UV opsin when the rainbow trout matures (Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006). These authors postulate a role for thyroid hormone in triggering the loss and reappearance of corner cones, as circulating levels of this hormone are known to increase at different stages during the life of salmonids, including the embryonic period (Greenblatt et al., 1989), at smoltification (Hoar, 1988) and during sexual maturation (Sower and Schreck, 1982). It has been shown, however, that the young rainbow trout smolt has similar corner cone topography to the adult, implying that there is no regeneration of corner cones following smoltification (see Martens, 2000; Novales Flamarique, 2001). Whether the remaining corner cones express UV opsin after smoltification or whether the production of this opsin is upregulated in the adult retina is unknown. Answers to these questions are crucial to understand the role, if any, of thyroid hormone in modulating the chromatic organization of the cone mosaic in salmonid fishes.

In this study we performed parallel *in situ* hybridization experiments on the retinas of alevin to adult rainbow trout using coho-derived riboprobes used in previous studies (Cheng et al., 2006; Cheng et al., 2007) and similar probes to those derived from rainbow trout by Allison et al. (Allison et al., 2003). We also measured visual pigment absorbance from isolated photoreceptors to identify whether the opsins expressed in the various cone types were consistent with the opsin mRNAs identified by *in situ* hybridization using the riboprobes. Thus, in addition to revealing the chromatic organization of the rainbow trout retina throughout the life of the animal, we also assessed whether the riboprobes and related methodology used by different laboratories gave the same results or not. The latter is very important to resolve, as discrepancies in results have been attributed by Allison et al. (Allison et al., 2006) to the use of riboprobes of different origin (coho *vs* rainbow trout), nucleotide length, and related methodology (e.g. incubation time of sections in proteinase K treatment). The suggestion of a difference in results due to riboprobe (species) origin has been put forward (Allison et al., 2006) despite a >97% sequence identity between our coho-derived UV and blue riboprobes and the corresponding mRNA sequences for the UV and blue opsins in rainbow trout (Cheng et al., 2006).

Materials and methods

Animals

Wild stock rainbow trout (*Oncorhynchus mykiss*) were obtained from the Fraser Valley Trout hatchery (Abbotsford, British Columbia, Canada) at stages ranging from the alevin to the adult. Fish size statistics (weight ± s.d., total length ± s.d., N=8) were as follows: alevin (0.27±0.046 g, 3.6±0.17 cm), large alevin/parr (3.7±1.2 g, 7.2±0.75 cm), smolt (54±13 g, 18±1.3 cm) and adult (42±3.4 cm). Fish were maintained in 7°C water and were exposed to the natural daylight cycle at the hatchery, where all the tissue sampling took place. Fish were sampled during the months of March–June. Four of each alevin and smolt fish were transported live from the hatchery to the aquatic facility at Simon Fraser University where they were kept for 7 days while microspectrophotometry experiments were conducted. Holding and experimental procedures at the hatchery and at Simon Fraser University were in accordance with the

guidelines set by the Canadian Council for Animal Care, and all experimental protocols were further approved by the Animal Care Committee of Simon Fraser University.

Preparation of opsin riboprobes

Rainbow trout partial cDNAs for the various opsins were generated by RT-PCR amplification of juvenile total RNA isolated from homogenized retina of parr fish (weight ~7 g). For the UV (SWS1) and blue (SWS2) opsins, primers were designed to match the sequences of opsin probes described by Allison et al. (Allison et al., 2003). A few bases at the 5′ and 3′ end of each probe sequence were omitted to allow for optimal PCR conditions; these omissions were less than 1.2% and 3.6% of the total UV and blue riboprobe sequences, respectively, used by Allison et al. (Allison et al., 2003) and were functionally insignificant for hybridization purposes. The primers were as follows: rtUV forward 5′-AACCGCTGAACTACATCCT-3′, rtUV reverse 5′-TAACACAGAATGAAGGAG-CA-3′; rtBL forward 5′-GATCCCATCTCAAC-TACATT-3′, rtBL reverse 5′-ATGAGAGGGT-TGTAGACTGT-3′. Our UV probe (rtUV) corresponds to bases 237–822 of the published *Oncorhynchus mykiss* SWS1 opsin mRNA (GenBank accession no. AF425074) while our blue probe (rtBL) corresponds to bases 403–1111 of the published *Oncorhynchus mykiss* SWS2 opsin mRNA (GenBank accession no. AF425075). We also generated riboprobes against the green (RH2) and red (LWS) opsins; the primers for these were: rtGR forward 5′-AAAATAGGCAAAAGGTTC-AC-3′, rtGR reverse 5′-TAGACGGCAAGACA-ATAGTA-3′ (GenBank accession no. AF425076; our probe corresponds to bases 1–192 of this sequence); and rtRE forward 5′-AGCAAGAC-AAGACAACAGAA-3′, rtRE reverse 5′-TGAG-AGGATGACCACTATGA-3′ (GenBank accession no. AF425073; our probe corresponds to bases 33–273 of this sequence). The cDNAs were cloned into pCRII-TOPO vectors (Invitrogen, La Jolla, CA, USA) and sequenced by AmpliTaq Dye terminator cycle sequencing (UBC Sequencing laboratory). The identity of each sequence was confirmed by comparing it with the GenBank nucleotide sequence database (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/). To generate a given cRNA probe, a PCR fragment containing the partial cDNA clone of interest and an RNA promoter amplified from the pCRII-TOPO vector were used to generate sense and antisense riboprobes by *in vitro* transcription. Riboprobes were labelled with either digoxigenin (DIG) or fluorescein (Roche Diagnostics, Laval, Canada).

A similar procedure was used to generate the coho-derived UV (coUV) and blue (coBL) riboprobes. The primers for these were as follows: coUV forward 5′-GGGCTTTGTGTTCTTTGC-TG-3′, coUV reverse 5′-GGTACTCCTCGTTGTTTGTG-3′ (GenBank accession no. AY214148; our probe corresponds to bases 111–574 of this sequence); coBL forward 5′-AAA-CCTTGGTAGTGGGGATT-3′, coBL reverse 5′-CATAGA-AGATAGCACTGCCC-3′ (GenBank accession no. AF425075; our probe corresponds to bases 119–312 of this sequence).

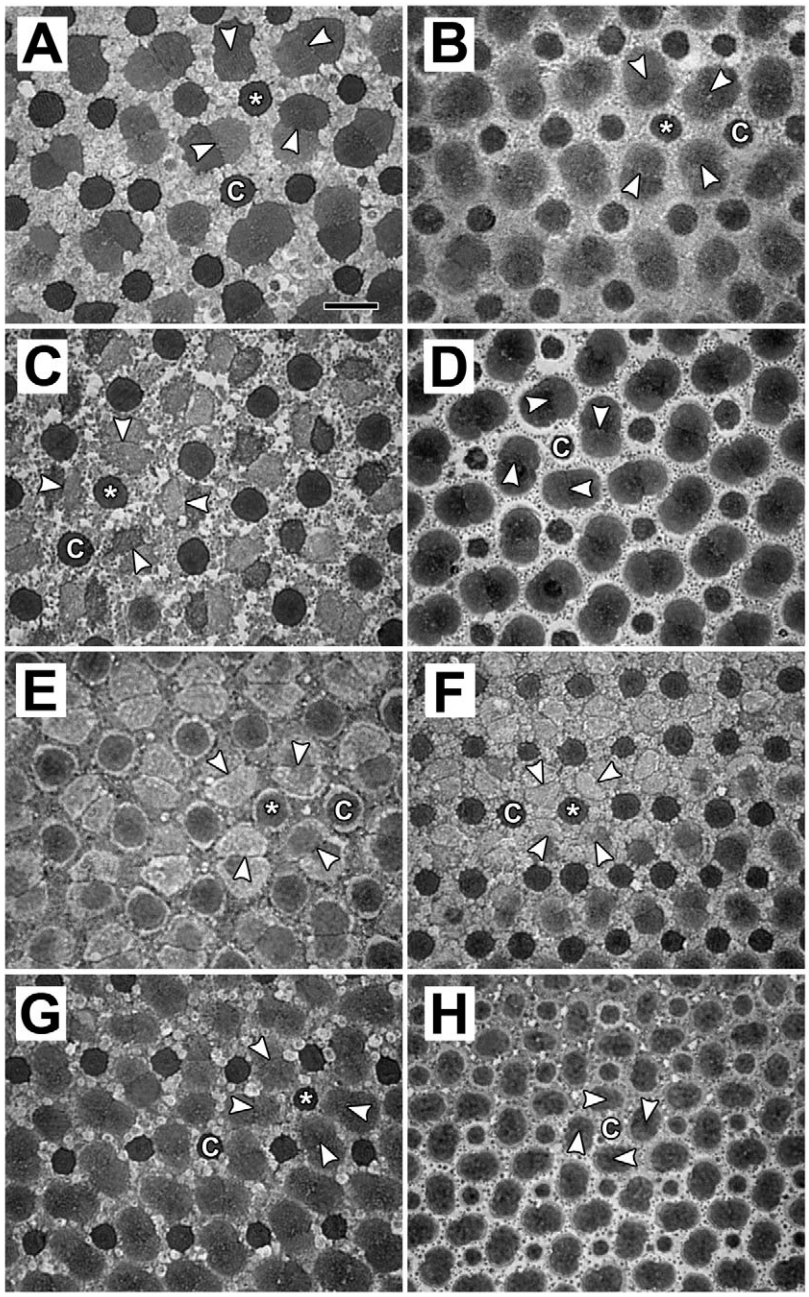

Fig. 1. Cone mosaic formations in the light-adapted retina of adult rainbow trout. (A) Square mosaic with corner cones from the centro-dorsal retina, (B) row mosaic from the centro-temporal retina, and (C,D) square mosaic with and without corner cones from the centro-ventral and centro-nasal parts of the retina, respectively. An asterisk indicates a corner cone, a white arrowhead points to the partitioning membrane of a double cone, and 'c' refers to a centre cone. (E,F) Square to row mosaics from the proximal (E) and distal (F) dorsal retina. (G,H) Square mosaics with few to no corner cones from the proximal (G) and distal (H) ventral retina. Cones are smaller and more closely packed towards the distal (peripheral) retina. Magnification bar (in A) is 25 μm and relates to all panels.

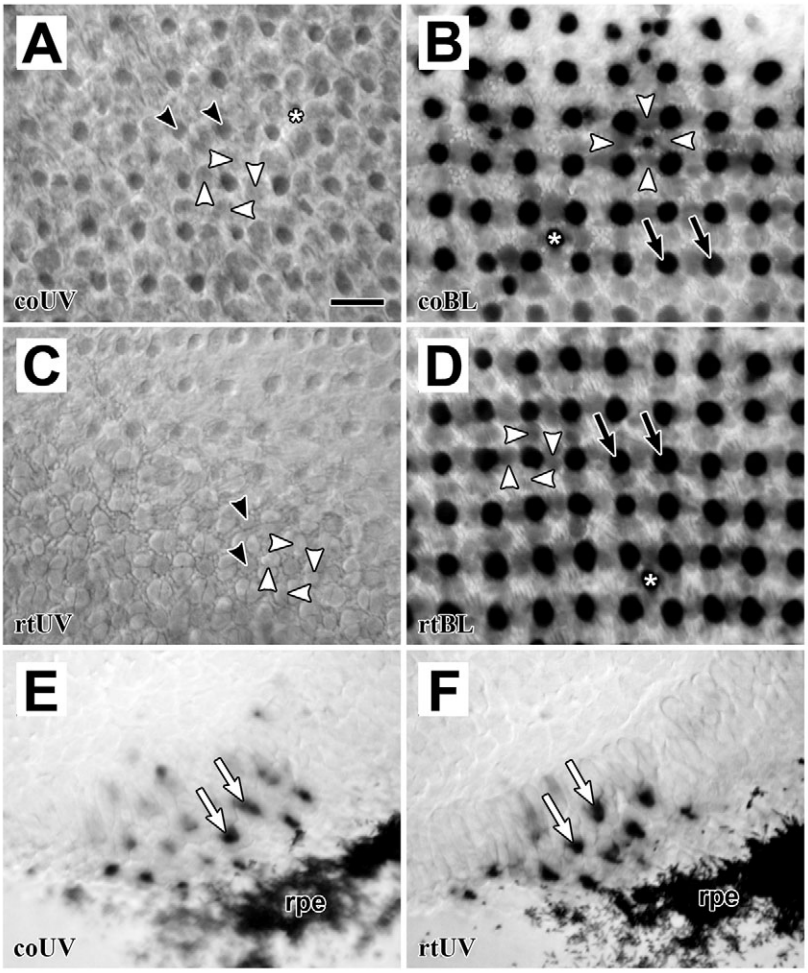

Fig. 2. Micrographs of sections from the smolt retina following *in situ* hybridization with the UV or blue riboprobes derived from coho (coUV, coBL) or rainbow trout (rtUV, rtBL). (A–D) Serial sections from the mid-ventral retina show that the UV riboprobes (coUV in A and rtUV in C) do not label any cones while the blue riboprobes (coBL in B and rtBL in D) label all single cones, regardless of position in the square mosaic. (E,F) Serial sections from the dorso-nasal periphery show multiple cells labelled by the UV riboprobes (coUV in E and rtUV in F). Black arrowheads point to unlabelled single cones, black arrows point to single cones labelled with the blue riboprobes, and white arrows point to single cones labelled with the UV riboprobes; rpe, retinal pigment epithelium. The riboprobe used in each section is specified at the bottom of each panel. Other symbols and nomenclature as in Fig. 1. Magnification bar (in A) is 25 μm and relates to all panels.

Additional details can be found in previous studies (Cheng et al., 2006; Cheng et al., 2007).

Tissue preparation for histology

Light-adapted fish were killed by quick spinal bisection and decerebration. The eyeballs were removed, the iris and lens discarded, and the remaining eyecup immersed in either primary fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.06 mol l^{-1} phosphate buffer, pH 7.4) for histological analysis, or in cryo-fixative (4% paraformaldehyde in 0.06 mol l^{-1} phosphate buffer, pH 7.4) for cryoembedding and *in situ* hybridization (Cheng et al., 2006). After 24 h fixation at 4°C, the retinas were extracted from the eyecups, flattened by making small peripheral incisions, and their contours traced by projecting the image onto a screen using an overhead projector. Each retina was then cut into 4–28 pieces (depending on size) whose locations were mapped back onto the original retina by matching the composite projected image onto the original (Beaudet et al., 1997; Novales Flamarique, 2001; Cheng et al., 2006). Retina pieces for histological analysis were then incubated in secondary fixative (1% osmium tetroxide), washed briefly in distilled water, dehydrated through a series of solutions of increasing ethanol concentration, infiltrated with mixtures of propylene oxide and EPON resin and embedded in 100% EPON blocks (Novales Flamarique, 2001; Cheng et al., 2006). Retinal blocks were cut tangentially in 1 μm steps and the sections stained with Richardson's solution (1:1 mixture of 1% Azure II in dH_2O and 1% Methylene blue in 1% NaB_4O_7 solution) to reveal the cone mosaic. Digital images of sections were acquired with an E-600 Nikon microscope equipped with a DXM-100 digital camera and differential interference contrast (DIC) optics.

We used a grid system on the computer monitor to count the density of double cones and single cones (centre cones plus corner cones) over an area of retinal section that varied between 5760 and 11 520 μm^2 for the alevin and was 23 040 μm^2 for the other stages. The ratio of double to single cones (d/s) was then computed for each sector of the retina (d refers to the two-member double cone pair). The contours of pieces mapped back to the original retina were used to trace maps showing some combination of cone density and double cone to single cone (d/s) ratio. For a given stage, retinas (N=4) used to obtain an average map were of similar size, minimizing contour differences due to age.

Tissue preparation for in situ hybridization

Retinal pieces for *in situ* hybridization analysis were rinsed 3× 30 min in 0.06 mol l^{-1} phosphate buffer and cryo-protected in sucrose solution [30% sucrose, 0.06 mol l^{-1} phosphate buffer, PBS, in O.C.T. medium (Cedar Lane Laboratories, Hornby, Ontario, Canada)] overnight at 4°C. The pieces were then cryo-embedded in 100% O.C.T. medium. These blocks were cut tangentially or radially, in 7–10 μm steps, to reveal the cone mosaic or the photoreceptor layer, respectively. Sections were collected and deposited serially on a number of slides corresponding to the number of riboprobes being tested in parallel. For example, comparison of labelling by the UV and blue riboprobes from rainbow trout and coho salmon was carried out on four separate slides, each treated with a different riboprobe (rtUV, rtBL, coUV or coBL). This method of serial deposition of sections permitted comparison of labelling between riboprobes for the same retinal area (Cheng et al., 2006). In addition, we also carried out double labelling

experiments with two riboprobes at a time to further verify the results obtained by parallel processing of independent riboprobe/slide sets.

The methods for *in situ* hybridization on cryosections were modified as needed from previous studies (Forsell et al., 2001; Cheng et al., 2006). Briefly, the procedure involved rehydrating the sections, permeabilizing them in 10 μg ml^{-1} proteinase K (Sigma, St Louis, MO, USA) for 5, 10 or 13 min [as per the protocol in Allison et al. (Allison et al., 2003), for rainbow trout riboprobes], followed by exposure to 0.1 mol l^{-1} triethanolamine containing 0.25% acetic anhydride, dehydration, and hybridization overnight at 50°C with 1 μg riboprobe in hybridization solution containing 50% formamide and dextran sulphate. Sections were then washed in 50% formamide in 2× SSC at 60°C, treated with 20 μg ml^{-1} RNase A (Sigma) and incubated with appropriate Fab fragments conjugated to alkaline phosphatase (1:3000; Roche Diagnostics) for 2 h at room temperature. The riboprobes were visualized using NBT/BCIP or FastRed (Roche Diagnostics). Sense probes were used as negative controls and did not hybridize in any of the retinas.

Double labelling of cryosections was carried out using the above methodology with the following modifications. The hybridization step included two different riboprobes, one labelled with DIG, the other with fluorescein. The DIG-labelled probes were visualized using NBC-BCIP. The colour reaction was stopped by washing the sections in glycine-HCl (0.1 mol l^{-1}, pH 2.2). To visualize the fluorescein-labelled riboprobes, the sections were then incubated with anti-fluorescein Fab fragments conjugated to alkaline phosphatase (1:3000) for 2 h and stained with FastRed. The same microscopy set-up used to obtain images from EPON-embedded sections was employed to photograph cryosections. These were used to obtain mean percentages of single cones that expressed UV opsin mRNA.

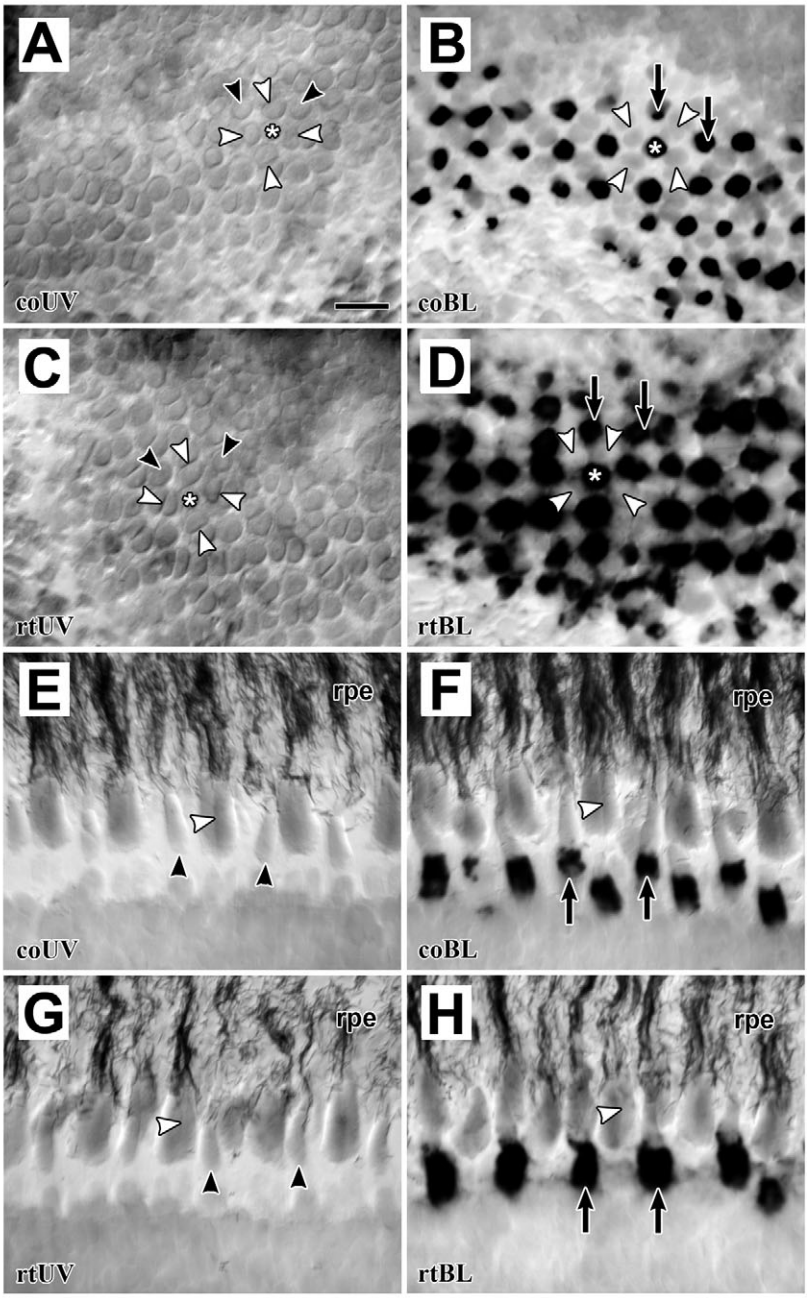

Fig. 3. Micrographs of sections from the adult retina following *in situ* hybridization with the two sets of UV and blue riboprobes. (A–D) Serial sections from the upper dorsal retina show that the UV riboprobes (coUV in A and rtUV in C) do not label any cones while the blue riboprobes (coBL in B and rtBL in D) label all single cones, regardless of position in the square mosaic. (E–H) Radial serial sections from the same area as in A–D show lack of labelling by the UV riboprobes (coUV in E and rtUV in G) and labelling of all single cones by the blue riboprobes (coBL in F and rtBL in H). Note that, in the light-adapted retina, the double cone ellipsoids (see white arrowheads pointing to the partitions) are located closer to the retinal pigment epithelium with respect to the single cones. Other symbols and nomenclature as in Fig. 2. Magnification bar (in A) is 25 μm and relates to all panels.

Microspectrophotometry of photoreceptors

Individual fish were dark adapted for 3 hours. Following this adaptation period, the animal was killed by quick spinal bisection and decerebration, one eye enucleated, and the retina removed under infrared illumination. The retina was divided into pieces; a few of these were teased apart with tweezers and mounted in a drop of Ringer's solution between two No. 1.5 glass microscope coverslips. After sealing around the edges to prevent evaporation, the preparation was mounted on the sliding/gliding stage of the microscope in the dichroic microspectrophotometer (DMSP) where photoreceptors could be viewed under infrared illumination using a closed-circuit television system. The DMSP is a computer-controlled, wavelength-scanning, single-beam photometer that simultaneously records average and polarized transmitted light fluxes through microscopic samples (Hárosi, 1987; Novales Flamarique and Hárosi, 2000; Novales Flamarique and Hárosi, 2002). The DMSP was

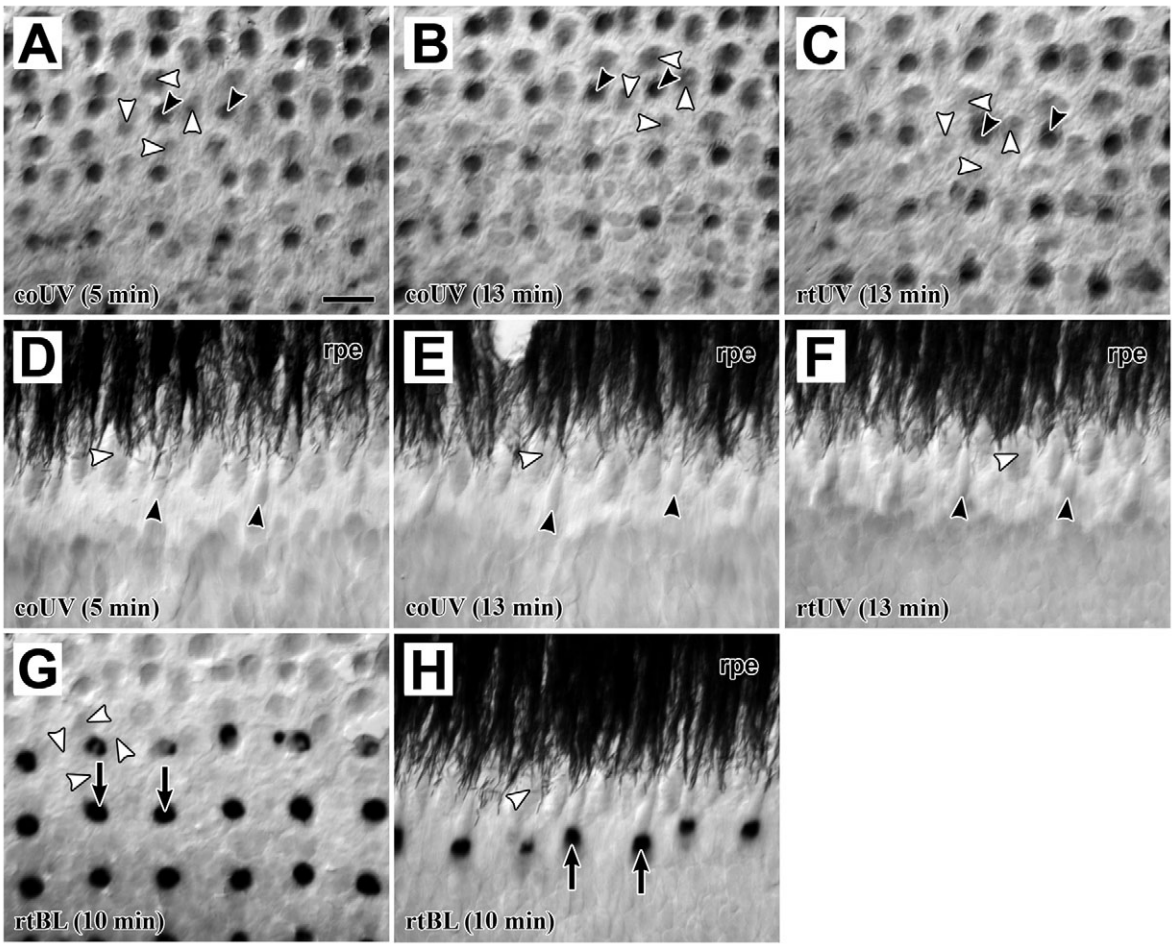

Fig. 4. Micrographs of sections from the adult retina following *in situ* hybridization with the UV and blue riboprobes after variable proteinase K incubation times. (A–F) Serial sections from the mid-ventro-temporal retina show that the coho UV riboprobe (coUV) does not label cones after 5 min (A,D) or 13 min (B,E) of proteinase K exposure, and this is the same result as obtained using the rainbow trout UV riboprobe (rtUV) after 13 min of proteinase K exposure (C,F). (G,H) Serial tangential (G) and radial (H) sections corresponding to those in A–F show that all single cones are labelled by the blue riboprobe (rtBL) after 10 min of proteinase K treatment. The riboprobes used and the time of proteinase K exposure (in parentheses) are shown at the bottom of each panel. Note that proteinase K exposure times used by Allison et al. (Allison et al., 2003) for the rainbow trout UV and blue riboprobes were 13 min and 10 min, respectively. Other symbols and nomenclature as in Fig. 2. Magnification bar (in A) is 25 μm and relates to all panels.

equipped with ultrafluar (Zeiss) objectives: 32/0.4 for the condenser and 100/1.20 for the objective. With the aid of reference measurements recorded through cell-free areas, individual photoreceptor outer segments were illuminated sideways with a measuring beam of rectangular cross-section of *ca* 2×0.6 μm. Absolute absorbance spectra were computed in 2 nm increments from the obtained transmittances (each spectrum consisted of an average of 8 scans). The solid spectra (fits) were derived from experimental data by Fourier filtering (Hárosi, 1987).

Results

Cone types and mosaics in the retina of rainbow trout

In the retina of adult rainbow trout, the centro-dorsal and centro-temporal regions showed square (Fig. 1A) and row (Fig. 1B) mosaics complete with corner cones (i.e. a d/s ratio of ~1). This was also the case in the centro-ventral retina, near the optic nerve head (Fig. 1C), but the square mosaic lacked corner cones in other retinal locations such as the centro-nasal region (Fig. 1D). Row mosaics with high densities of single cones were found in the dorsal retina (Fig. 1E,F) whereas square mosaics lacking corner cones were primarily located in the ventral retina (Fig. 1G,H). The highest cone densities were always found in the retinal periphery. The same mosaics and cone density trends were found in younger fish, though the smallest fish analysed (alevins having just absorbed their yolk sacs) had overwhelmingly square mosaics complete with corner cones. These mosaic observations are similar to those reported for other salmonid species (e.g. Ahlbert, 1976; Novales Flamarique and Hawryshyn, 1996; Novales Flamarique, 2000; Novales Flamarique, 2002; Cheng et al., 2006; Cheng and Novales Flamarique, 2007) and for rainbow trout obtained from a domesticated aquaculture strain (Beaudet et al., 1997).

Labelling by riboprobes

Treatment of serial sections with the coho-derived riboprobes against UV opsin (SWS1) and blue opsin (SWS2) mRNAs

resulted in the same labelling patterns as those obtained with analogous riboprobes derived from rainbow trout (Figs 2–4). In the ventral retina of smolt rainbow trout, the coho-derived UV opsin riboprobe (coUV) failed to label any of the cones, including the single cones (Fig. 2A). The same result was obtained with the UV riboprobe derived from rainbow trout (rtUV, Fig. 2C). In contrast, both the coho-derived blue opsin riboprobe (coBL, Fig. 2B) and that obtained from rainbow trout (rtBL, Fig. 2D) labelled all single cones, including a minority of corner cones. In peripheral regions, where UV opsin expression has been reported in adult salmon (Cheng and Novales Flamarique, 2007), both UV opsin riboprobes (coUV, Fig. 2E; and rtUV, Fig. 2F) labelled single cells.

We obtained similar results when treating serial sections from adult rainbow trout with both sets of riboprobes. Though the dorsal retina showed complete mosaics with corner cones (d/s ~1), both UV riboprobes failed to label any cone type, as seen in tangential (Fig. 3A,C) and radial (Fig. 3E,G) sections. In contrast, both blue opsin riboprobes labelled all single cones, whether centre or corner cones, as observed in tangential (Fig. 3B,D) and radial (Fig. 3F,H) sections. In these experiments (Figs 2, 3), labelling by rtBL was generally more pronounced than that by coBL, probably because treatment with proteinase K in the *in situ* protocol was longer for rtBL (10 min) (Allison et al., 2003) than for coBL (5 min) (Cheng et al., 2006). As well, the difference in length between riboprobes may have contributed to these results.

Further experiments that varied the duration of proteinase K treatment in the *in situ* hybridization protocol showed that the same labelling pattern was obtained with analogous riboprobes. Serial sections of ventral retina in adult rainbow trout showed that coUV did not label any cone regardless of whether proteinase K incubation time was 5 min (Fig. 4A,D) or 13 min (Fig. 4B,E), and this was the same result as obtained with rtUV (Fig. 4C,F). All single cones labelled, however, with the blue riboprobe (e.g. rtBL, Fig. 4G,H). In general, longer proteinase K treatment enhanced both specific and background labelling without altering the pattern of cones labelled.

The darker appearance of some non-labelled cones in some of the sections was due to enhanced contrast of the distal ellipsoid under the polarization optics used. It is to be noted that both non-labelled double and single cones exhibited some darkness but at different 'levels' of the (oblique) cross-section (e.g. Fig. 4A,B), in accordance with the radial positioning of these two morphological cone types in the light-adapted retina. Nonetheless, such artifacts were clearly distinguishable from riboprobe labelling based on any of the following label attributes: colour, texture (appearance) and positioning.

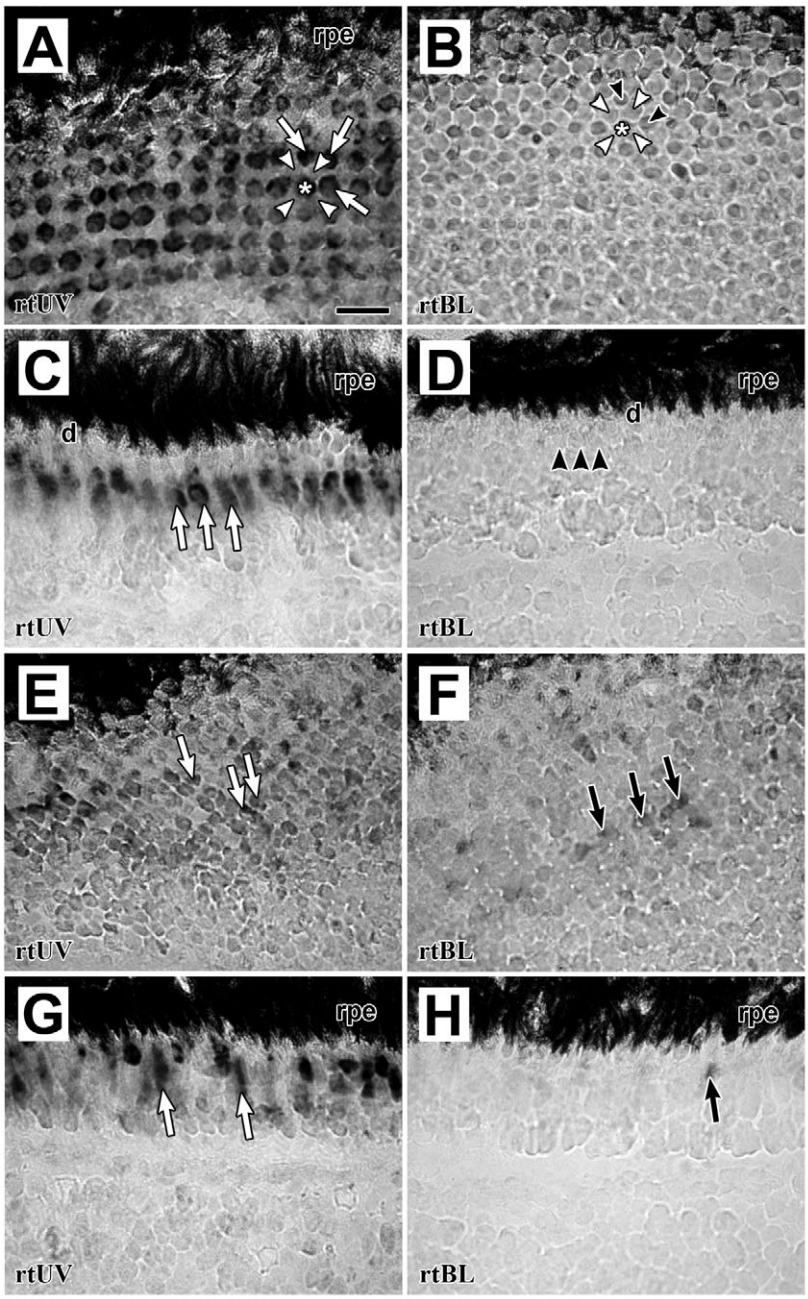

Fig. 5. Micrographs of sections from the retina of the alevin following *in situ* hybridization with the rainbow trout UV and blue riboprobes. (A–D) Serial sections from the dorsal retina show a full square mosaic in which all single cones are labelled by the UV riboprobe (A,C) and none are labelled with the blue riboprobe (B,D). (E–H) Serial sections from the lower ventral retina show that most single cones are labelled by the UV riboprobe (E,G) but some are also labelled with the blue riboprobe (F,H). d, double cone; other symbols and nomenclature as in Fig. 2. Magnification bar (in A) is 25 μm and relates to all panels.

Distribution of cone spectral types during the ontogeny of rainbow trout

The young alevin, having recently absorbed its yolk sac, had cone photoreceptors arranged in a square mosaic (Fig. 5). All single cones in the dorsal retina were labelled exclusively with the UV riboprobe (rtUV, Fig. 5A,C) while no cone was labelled with the blue riboprobe (rtBL, Fig. 5B,D).

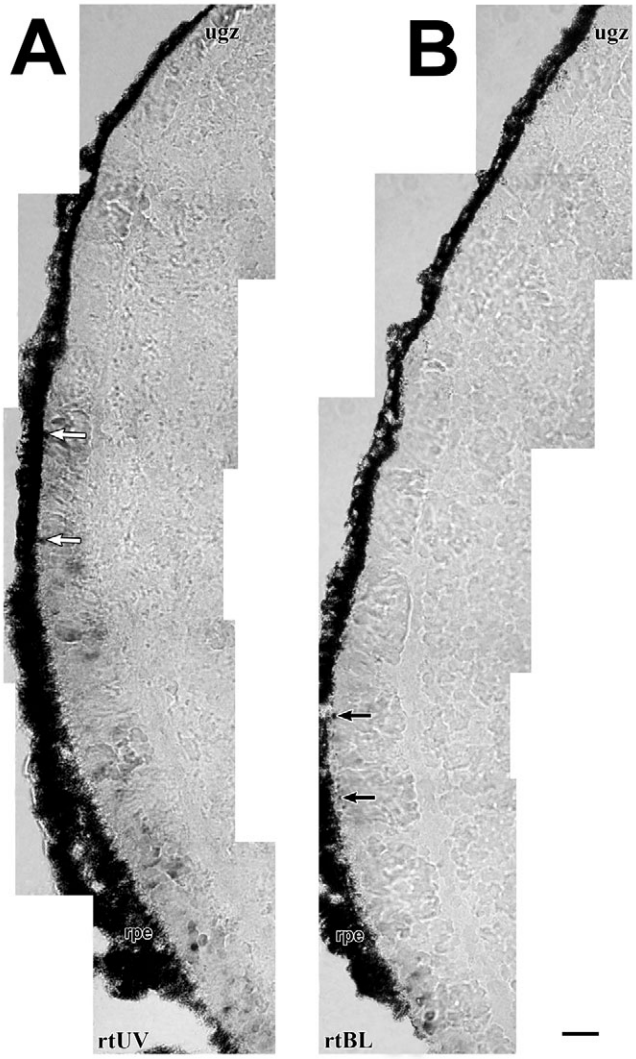

Fig. 6. Composites of micrographs from the distal ventral retina of the alevin showing the progression of retinal development from the peripheral undifferentiated growth zone (ugz), at the top of each figure, toward the (main) central retina, at the bottom of each figure. Labelling by the UV riboprobe (rtUV, A) appears closer to the ugz than labelling by the blue riboprobe (rtBL, B). Symbols and nomenclature as in Fig. 5. Magnification bar (at the bottom right of the figure) is 25 μm.

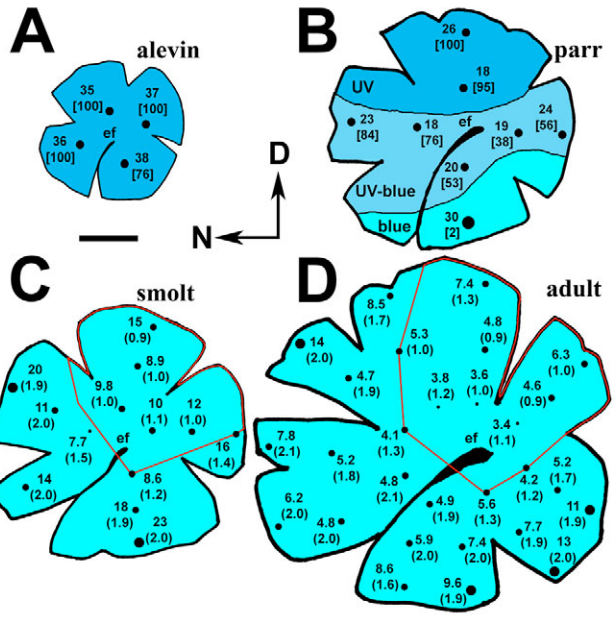

Fig. 7. Retinal maps of cone distributions in the retina of rainbow trout from the alevin to the adult (*N*=4 per map). (A,B) Mean cone density and percentage of single cones that express UV opsin mRNA (in brackets) in the retina of the alevin (A) and parr (B). All locations had square mosaics complete with corner cones such that d/s was ~1. The overwhelming majority of single cones in the alevin retina expressed UV opsin. Shown for the parr retina (B) are the approximate regions with cones expressing only blue opsin mRNA (blue), a combination of UV and blue opsin mRNA (mostly as corner cones expressing UV opsin mRNA and centre cones expressing blue opsin mRNA, but with some cones co-expressing the two transcripts; UV-blue), and cones expressing only UV opsin mRNA (UV). (C,D) Mean cone density and associated d/s ratio (in parentheses) in the retina of the smolt (C) and the adult (D). All single cones in the main (non-peripheral) retina expressed blue opsin mRNA. For each retina, the area within the red perimeter had high corner cone densities (d/s ratio <1.4). Cone densities are expressed in thousands per square millimetre. A larger circle indicates that the mean cone density for that location was at least 1 s.d. above the mean from all locations pooled together. A smaller circle indicates the opposite. In salmonid fishes, the embryonic fissure (ef) runs from the ventral to the central retina (approximate location of the optic nerve head), pointing toward the temporal retina. D, dorsal, N, nasal. Magnification bar is 0.11 cm in A, 0.12 cm in B, 0.33 cm in C and 0.39 cm in D.

In the lower half of the ventral retina, UV riboprobe labelling was not as intense as in the rest of the retina (Fig. 5E,G) and several cells were labelled faintly with the blue riboprobe (rtBL, Fig. 5F,H). Further analyses of the peripheral ventral retina showed that UV opsin mRNA expression appeared first (i.e. labelling was close to the undifferentiated growth zone, Fig. 6A) and was followed in time by blue opsin expression (labelling further away from the undifferentiated growth zone, Fig. 6B). Cone densities were highest in the ventral retina (Fig. 7A). These results are consistent with published reports on various salmonid species using coho-derived riboprobes (Cheng et al., 2006; Cheng et al., 2007).

The retina of larger alevins (also termed parr) (e.g. Veldhoen et al., 2006; Allison et al., 2006) showed a pattern of stratified labelling from the ventral to the dorsal retina (Fig. 7B, Fig. 8).

Double labelling experiments with the rainbow trout riboprobes demonstrated co-expression of UV and blue mRNA in single cones undergoing the transformation from UV to blue spectral phenotype (see also Cheng and Novales Flamarique, 2004; Cheng et al., 2006). These cones exhibited a colour (purple) that was intermediate between that of cones expressing UV opsin mRNA (red) and that of cones expressing blue opsin mRNA (blue; Fig. 8A,B). Co-expressing cones were most common in the centro-dorsal retina (Fig. 8A,B) at the advancing front of the single cone transformation (Fig. 7B). In the centro-ventral to mid-ventral retina, the cone mosaic was square with centre cones expressing blue opsin mRNA and corner cones expressing UV opsin mRNA (Fig. 8C). Single cones expressing blue opsin mRNA (Fig. 8D) or in the process of switching

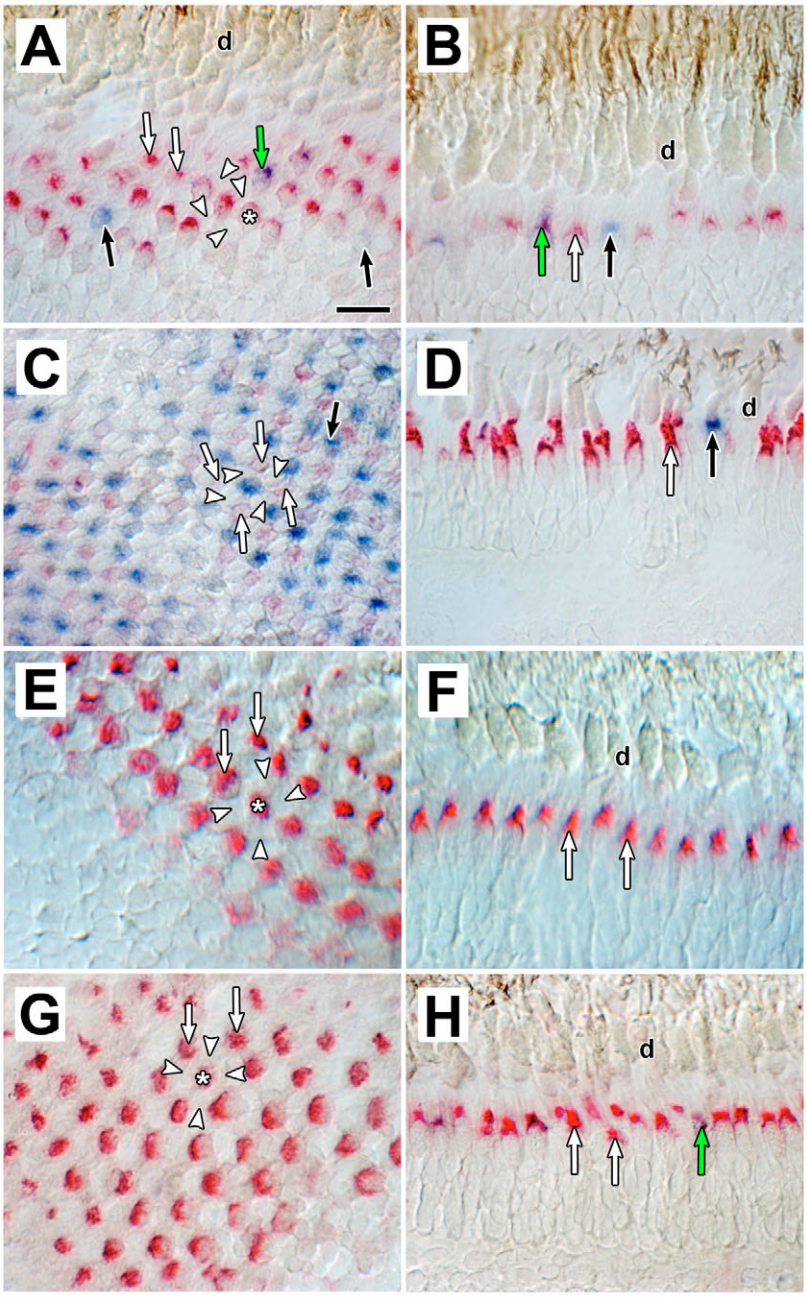

Fig. 8. Micrographs of sections from the large alevin/parr retina following double label *in situ* hybridization with the rainbow trout UV and blue riboprobes. (A,B). Tangential (A) and radial (B) sections from the centro-temporal retina showing single cones expressing UV opsin mRNA (red colour), blue opsin mRNA (blue colour), and both UV and blue opsin mRNAs (purple colour, green arrow). (C) Tangential section from the centro-ventral retina showing corner cones expressing UV opsin mRNA and centre cones expressing blue opsin mRNA. (D) Radial section from the nasal periphery showing single cones expressing UV or blue opsin mRNA. (E–G) Tangential (E) and radial (F) sections from the mid-dorsal retina show that all single cones express UV opsin mRNA exclusively, as do single cones from the distal dorsal retina (G). (H) Radial section from the distal temporal retina showing that the majority of cones express UV opsin mRNA, though some show early signs of co-expression (green arrow). Other symbols and nomenclature as in Fig. 5. Magnification bar (in A) is 25 μm and relates to all panels.

opsins (Fig. 8H) were present in the nasal and temporal periphery, though most cones in these peripheral areas were labelled solely with the UV riboprobe. Cones in the mid- (Fig. 8E,F) and distal (Fig. 8G) dorsal retina were labelled exclusively with the UV riboprobe. These distinct labelling patterns revealed a progression in UV opsin down-regulation from the ventral to the dorsal retina (Fig. 7B).

In the smolt, corner cones continued to be present in a large area of the dorso-temporal retina (Fig. 7C). None of the cones were labelled with the UV opsin riboprobe (rtUV, Fig. 9A) and all were labelled with the blue opsin riboprobe (rtBL, Fig. 9B). Sections from the mid-dorsal (Fig. 9C,D) and temporal (Fig. 9E) retina showed that the single cones were labelled exclusively with the blue riboprobe (rtBL). On one occasion, however, a faint UV label was detected in two seemingly disappearing cones near the embryonic fissure (Fig. 10B). Double cones were green/red pairs that alternated their labelling pattern around the square mosaic unit (Fig. 10A). This double cone labelling pattern was the same in the adult retina (Fig. 10C,D) and in the retina at other stages (results not shown).

As was the case in the smolt, the adult had single corner cones over a large area of dorso-temporal retina (Fig. 7D). None of these single cones were labelled with the UV opsin riboprobe (rtUV, Fig. 11A) but all were labelled with the blue opsin riboprobe (rtBL, Fig. 11B). This labelling pattern was consistent throughout the entire (non-peripheral) retina including areas without corner cones (Fig. 11C–F). As in the smolt, cone densities were highest in the ventro-temporal and dorso-nasal regions of the retina (Fig. 7C,D).

Photoreceptor visual pigments

Our visual pigment absorbance measurements from individual photoreceptors (Fig. 12) corroborated the *in situ* hybridization results. All the single cones measured (N=56) in the young alevin (Fig. 12A) had a UV visual pigment with mean (±s.d.) maximum absorbance (λ_{max}) of 373±6 nm, and double cones (N=25) with mean λ_{max} (±s.d.) of 494±7 nm (green member) and 564±6 nm (red member). In contrast, all the single cones (N=48) measured from the retina of smolt fish contained a blue visual pigment with mean λ_{max} (±s.d.) of 437±5 nm (Fig. 12A), and double cones (N=35) with mean λ_{max} (±s.d.) of 523±6 nm (green member) and 578±10 nm (red member; Fig. 12B). Compared with the visual pigments in the alevin, for the same cone types, those in the smolt had higher wavelengths of maximum absorption. The rod opsin visual pigment was similar at the two stages, with mean λ_{max} (±s.d.) of 510±5 nm (Fig. 12B). These means indicate retinas based on a mixture of vitamin A_1 and A_2 chromophores (Hárosi, 1994).

Discussion

Opsin switch in the single cones of rainbow trout

Our results demonstrate that single cones in the rainbow trout retina switch opsins from SWS1 (maximally sensitive to UV light) to SWS2 (maximally sensitive to blue light) in a transformation event that begins in the ventral retina and proceeds toward the dorsal retina. This event starts before full yolk sac absorption (Cheng et al., 2007) and continues throughout the juvenile period such that the adult (sexually mature) rainbow trout lacks UV expression throughout the main retina. These results are similar to those obtained by analysis of retinas from multiple Pacific salmonid species with the coho-derived riboprobes used in this study (Cheng and Novales Flamarique, 2004; Cheng et al., 2006; Cheng et al., 2007).

The topography of UV, blue and dual opsin-expressing cones leading to the smolt retina suggests that the single cone transformation progresses as a wave that starts in the ventro-temporal retina. This is consistent with observations of blue opsin mRNA expression first in the ventro-temporal retina of salmonid embryos, and its subsequent progression toward the ventral and, later, toward the dorsal retina (Cheng et al., 2007). The molecular determinants that control the opsin switch driving this progression are presently unknown, though thyroid hormone is a likely candidate. In the developing mouse, for instance, this ligand establishes a ventro-dorsal retinal gradient that, together with the presence of $TR\beta_2$ receptor, induces a dominant expression of green opsin (RH2) in dorsal cones (Ng et al., 2001; Roberts et al., 2006; Applebury et al., 2007). The resulting ventro-dorsal gradient of decreasing SWS1 opsin expression in the mouse is somewhat opposite to that in the juvenile rainbow trout, and is probably mediated by similar diffusible factors.

Chromatic organization of single cones in the rainbow trout retina

In accordance with previous findings from other salmonid species (Novales Flamarique, 2005; Cheng et al., 2006; Cheng et al., 2007), this study shows that the vast majority of single cones express UV opsin mRNA in the rainbow trout alevin that has recently absorbed its yolk sac. This organization is modified via the UV-to-blue opsin switch resulting in retinal regions with varying proportions of UV to blue opsin-expressing cones. There is therefore no general association between single cone position in the mosaic and spectral phenotype, as determined by opsin mRNA expression. The opsin switch affects primarily centre cones in the centro-ventral retina of the young fish, leading to a temporary mosaic that displays centre cones expressing blue opsin mRNA and corner cones expressing UV opsin mRNA (Fig. 8). It is this transient mosaic, originating from a specific region of retina, that is the cause of a misconceived generalization stating that corner cones are UV cones and centre cones are blue cones (Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006). Following the single cone transformation, the retina of the smolt shows that the remaining single cones (whether centre or corner in position) express only blue opsin mRNA. Interestingly, most corner cones have disappeared from the ventral retina of the smolt, whereas they remain in the dorso-temporal retina at this and later stages (see also Novales Flamarique, 2001). These findings suggest that the switch in opsin expression is uncoupled from corner cone disappearance.

Consistency of riboprobe labelling reconciles literature findings

The labelling results obtained with our coho-derived riboprobes and those obtained from rainbow trout [similar probes to those used by Allison et al. (Allison et al., 2003)] were equivalent. In particular, both sets of riboprobes showed an opsin switch in the single cones of rainbow trout juveniles, and an overall lack of association between single cone position in the mosaic and opsin content. These results stand in contrast with those presented in several previous studies (e.g. Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006) that

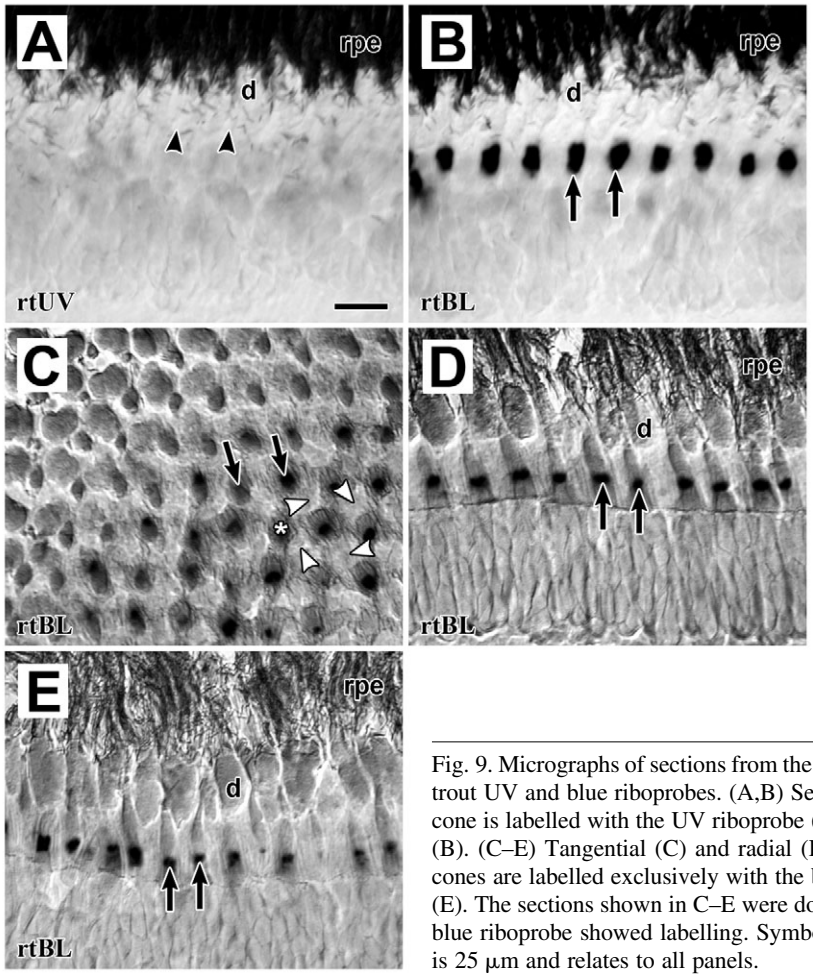

Fig. 9. Micrographs of sections from the smolt retina following *in situ* hybridization with the rainbow trout UV and blue riboprobes. (A,B) Serial radial sections from the distal nasal retina show that no cone is labelled with the UV riboprobe (A) and all single cones are labelled with the blue riboprobe (B). (C–E) Tangential (C) and radial (D) sections from the mid-dorsal retina show that all single cones are labelled exclusively with the blue riboprobe, as are single cones from the temporal retina (E). The sections shown in C–E were double labelled with the UV and blue riboprobes, but only the blue riboprobe showed labelling. Symbols and nomenclature as in Fig. 5. Magnification bar (in A) is 25 μm and relates to all panels.

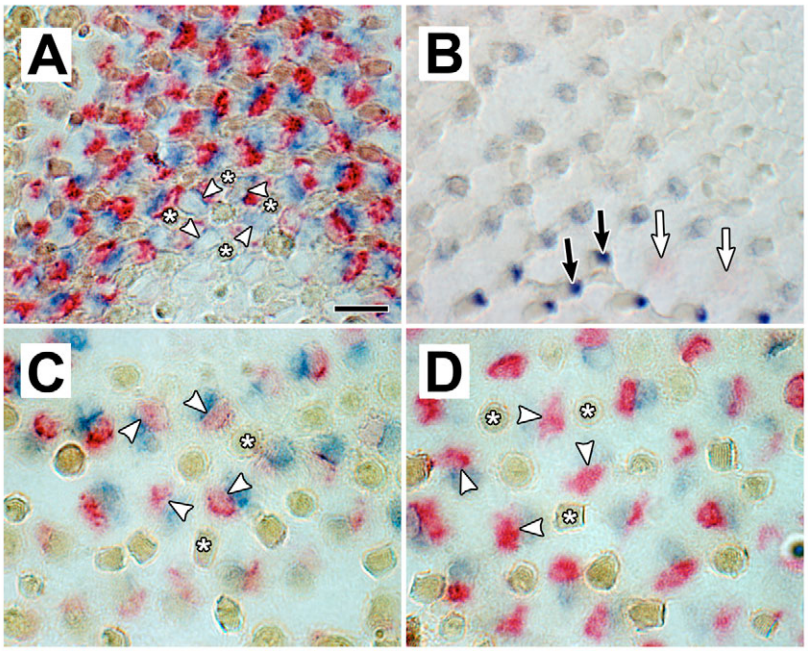

Fig. 10. Micrographs of sections from smolt and adult retinas following double label *in situ* hybridization with the green (rtGR) and red (rtRE) riboprobes or with the UV (rtUV) and blue (rtBL) riboprobes. (A) Tangential section from the centro-temporal retina of the smolt showing that each member of a double cone expresses a mRNA encoding green opsin (blue colour) or red opsin (red colour), and that these alternate around the unit mosaic. (B) Tangential section from the centro-dorsal retina of the smolt, adjacent to the embryonic fissure, shows two faint UV riboprobe labels (though no associated cone morphology is clearly discernable) among the single cone population. (C,D) Tangential sections from the centro-dorsal (C) and centro-temporal (D) retina of adult rainbow trout showing the same labelling pattern of double cones as in the smolt retina. Symbols and nomenclature as in Fig. 5. Magnification bar (in A) is 25 μm and relates to all panels.

deny the single cone transformation in rainbow trout and that assign a specific opsin to a specific cone position in the mosaic (according to these studies, corner cones express only UV opsin while centre cones express only blue opsin).

There are several omissions and errors in these publications (Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006) to cast serious doubts on the findings presented. For instance, these authors used rainbow trout that were undergoing the UV-to-blue opsin switch (fish length >6 cm, weight >5 g), and thus must have analysed retinas that comprised a variety of mosaic configurations (Figs 7, 8). Yet, in their work, these authors present tangential micrographs from the ventral retina exclusively [Fig. 2B,C in Allison et al. (Allison et al., 2003)]. Our results show that this is the only part of the retina where the centre cones express blue opsin [especially following the loss of corner cones; Fig. 2B in Allison et al. (Allison et al., 2003)] and the corner cones express UV opsin [in the centro- to mid-ventral retina; Fig. 2C of Allison et al. (Allison et al., 2003)]. Curiously, the authors do not state where their sections originate but, instead, proclaim the labelling pattern as representative of the entire retina. In a later manuscript (Allison et al., 2006), a micrograph (their Fig. 3B) is presented from the dorsal retina of a parr fish in which single cones are labelled with a UV opsin antibody. Careful analysis of this figure (see Fig. S1 in supplementary material)

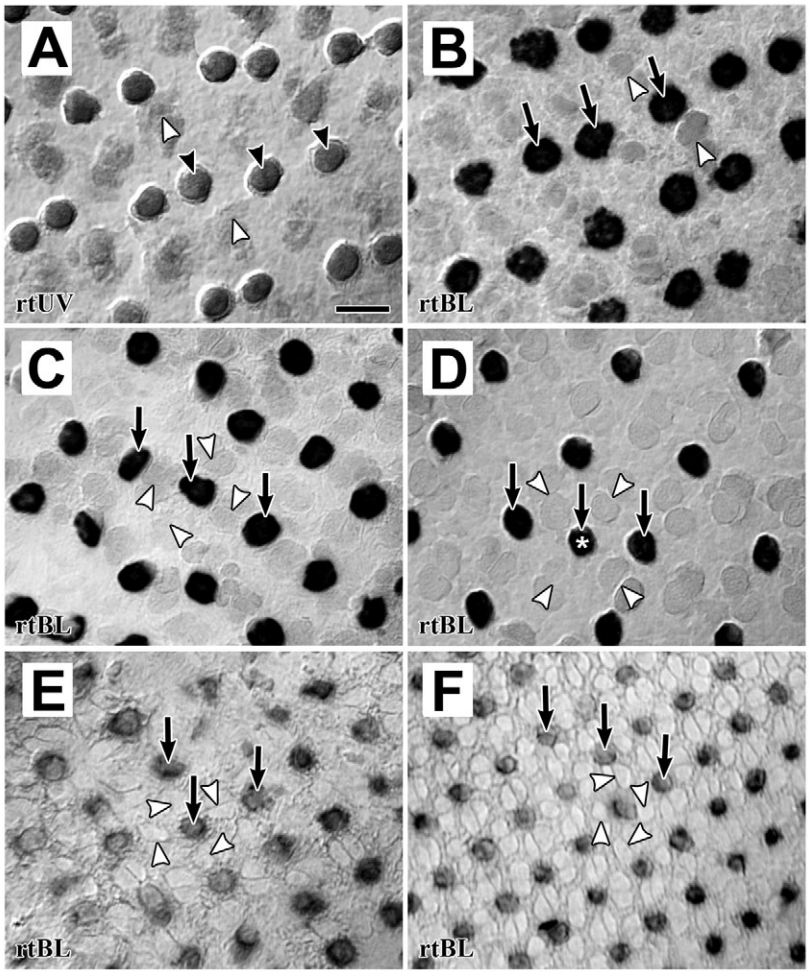

Fig. 11. Micrographs of sections from the retina of the adult following *in situ* hybridization with the rainbow trout UV and blue riboprobes. (A,B) Serial tangential sections from the dorsal retina show that no cone is labelled by the UV riboprobe (A) but all single cones are labelled with the blue riboprobe (B). (C–F) Tangential sections, double labelled with the UV and blue riboprobes, show exclusive labelling of all single cones by the blue riboprobe in the centro-temporal (C) and centro-ventral (D) retina, and in the temporal (E) and ventral (F) periphery. Symbols and nomenclature as in Fig. 5. Magnification bar (in A) is 25 μm and relates to all panels.

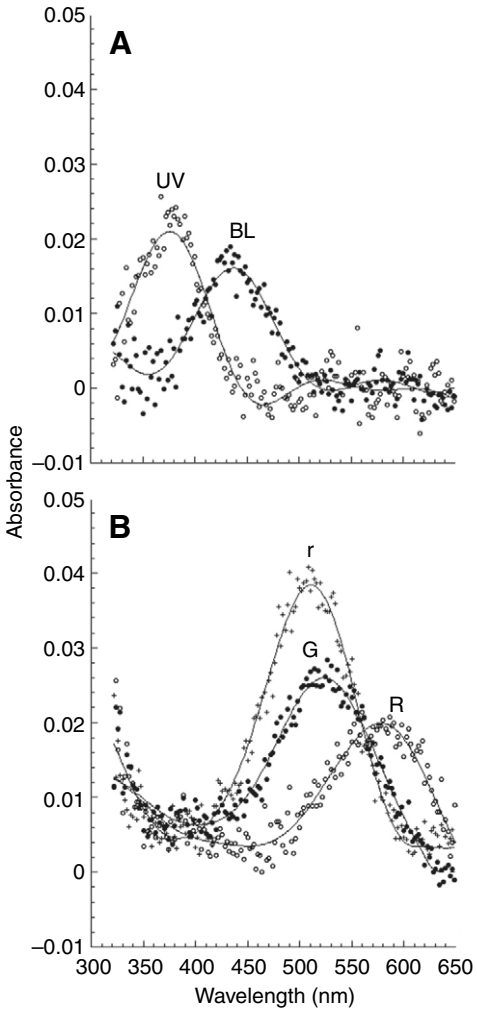

Fig. 12. Absolute absorbance spectra of isolated photoreceptors in rainbow trout. (A) Single cones in the young alevin contain a visual pigment maximally sensitive to ultraviolet light (UV) while single cones in the smolt retina contain a visual pigment maximally sensitive to blue light (BL). (B) Green (G) and red (R) visual pigments present in the double cones of the smolt (one pigment per member), and rod opsin pigment (r) contained in the rods.

shows both centre and corner cones labelled, in accordance with our *in situ* hybridization results, and in direct contradiction with what the authors state in the manuscript.

A potential source of discrepancy between our results and those from previous studies (Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006) may be the rearing conditions (e.g. water temperature, light regime) in which the fish were maintained. In our opinion, however, this is unlikely to be the case for multiple reasons. First, many studies have established that water temperature and photoperiod affect chromophore usage (vitamin A1 *vs* A2) in the retinas of fishes (e.g. Bridges, 1972), but a change in opsin type or mosaic structure resulting from such variations in rearing environment has never been documented in salmonid fishes (see Novales Flamarique, 2005). Second, we have examined the retinas of juvenile rainbow trout originating from multiple hatcheries (including those sampled by Hawryshyn and colleagues) and found that the mosaic structure and developmental pattern of opsin expression is common to all. Even adult fish of different origin show the same cone distributions (Beaudet et al., 1997) (present study). Third, the fish used in previous studies (Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006) were not approaching sexual maturation, a time when temperature and photoperiod may influence the animal's changing endocrinology (Sower and Schreck, 1982; Groot and Margolis, 1991) and potentially lead to alterations to the visual system.

Several studies from the same group of researchers further contend that thyroid hormone induces the disappearance of corner cones in rainbow trout (e.g. Browman and Hawryshyn, 1992; Deutschlander et al., 2001; Hawryshyn et al., 2003; Allison et al., 2006). This body of work suffers from two major drawbacks: (1) the use of fish that were undergoing the UV-to-blue cone transformation, the timing and extent of which varies between individuals, thereby introducing confounding variables into the analysis, and (2) inconsistencies in stimulus delivery and/or insufficient resolution of the histology (see Beaudet et al., 1997; Novales Flamarique, 2001), which makes comparison of results between individuals extremely difficult or impossible.

Recent work in rodents (Ng et al., 2001; Roberts et al., 2006; Applebury et al., 2007) and winter flounder (Mader and Cameron, 2006) has shown that thyroid hormone alters the type of opsin expressed by differentiating photoreceptors, but induction of hyperthyroid or hypothyroid states has no consequence on differentiated photoreceptors. This agrees with the higher levels of thyroid hormone receptor expression (particularly $TR\beta_2$) in the developing retina (Roberts et al., 2006; Applebury et al., 2007). In flatfishes (e.g. the winter flounder) (Hoke et al., 2006), metamorphosis involves a complete re-arrangement of the cone mosaic and expression of novel opsins in various cone types. Since thyroid hormone levels are elevated during this time of transformation (Inui and Miwa, 1985), it is likely that the primary role of this hormone in the retina is to regulate opsin expression, as in the mouse. It is only in the rainbow trout that thyroid hormone has been claimed to induce changes to the structure of the cone mosaic by triggering corner cone apoptosis (Allison et al., 2006). Other studies on rainbow trout (Julian et al., 1998) and brown trout (Candal et al., 2005) have failed to find any apoptotic cones in the retina after development, i.e. past the yolk sac alevin stage. These contradictory results suggest that Allison et al. (Allison et al., 2006) may have labelled with BrdU photoreceptor nuclei other than those of single cones, possibly those of rods, which can vary in radial positioning depending on the light adaptation state of the retina.

The above discrepancies, together with the labelling inconsistencies revealed by the present study, indicate that previous work on 'ultraviolet cones' in the rainbow trout retina (Hawryshyn et al., 2003; Allison et al., 2003; Allison et al., 2006) must be re-evaluated. In particular, experiments that examine the role of thyroid hormone in the salmonid retina should be carried out in young alevin fish, when the transformation has barely started and the majority of cones express UV opsin. Given the actions of nuclear receptor ligands in the retinas of other vertebrates (Prabhudesai et al., 2005; Roberts et al., 2005; Roberts et al., 2006; Srinivas et al., 2006; Mader and Cameron, 2006; Applebury et al., 2007), we suspect that thyroid hormone will modulate UV and blue opsin expression [an indication of which was given by Veldhoen et al. (Veldhoen et al., 2006)] but

will not induce single cone apoptosis (see Allison et al., 2006). Such results would be consistent with the presence of corner cones in the dorsal retina of the post-juvenile, all of which express blue opsin (i.e. these cones have undergone the opsin switch but have not been removed from the retina). Our results suggest that modulation of opsin expression and corner cone disappearance are independent processes.

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