INCIDENCE AND REGULATION OF OPSIN EXPRESSION
IN THE RETINA OF SALMONID FISHES

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Christiana L.Y. Cheng
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APPROVAL

Name: Christiana Ling-Yu Cheng

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Examining Committee:

Chair: Dr. M. Moore, Professor

Dr. I. Novales Flamarique, Assistant Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. W. Davidson, Professor
Department of Molecular Biology and Biochemistry, S.F.U.

Dr. N. Haunerland, Professor
Department of Biological Sciences, S.F.U.

Dr. G. Rintoul, Assistant Professor
Department of Biological Sciences, S.F.U.
Public Examiner

Dr. D. Stenkamp, Associate Professor
Department of Biological Sciences, University of Idaho
External Examiner

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Date Approved
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ABSTRACT

The retinas of many vertebrates have cone photoreceptors that express multiple visual pigments. In many of these animals, including humans, the original cones to appear in the retina (which express opsins maximally sensitive to UV or blue light) may change opsin types giving rise to new cone phenotypes. In my dissertation, I used various cellular, molecular and biophysical techniques to determine the spatial and temporal progression of opsin appearance, the distribution of UV and blue cones, and the role of thyroid hormone (TH) in regulating opsin expression during retinal development of salmonid fishes.

The sequence of cone opsin appearance in salmonid fishes is SWS1 (UV), followed by LWS (red), RH2 (green) and SWS2 (blue) opsin. All opsins are first expressed in a small patch of cells in the centrotemporal retina, and expression then proceeds towards the dorsal retina. Single cones express a UV opsin at hatching (λmax of the visual pigment ~ 365 nm), and these cones later transform into blue cones by opsin switch (λmax of the blue visual pigment ~ 434 nm). Cones undergoing the UV to blue opsin switch exhibit a spectral absorbance profile characterized by blue absorbance at the base and UV absorbance at the tip of the outer segment. Double cones contain green (λmax ~ 510 nm) or red (λmax ~ 565 nm) visual pigments (one per member) and, like the rods (λmax ~ 508 nm), do not exhibit opsin changeover.

Treatment of juvenile fish with TH induced a premature switch from UV to blue opsin expression. This appears to be regulated at the transcriptional level by TH-mediated repression of the UV opsin promoter. Cessation of induced hyperthyroidism reverted the opsin switch, suggesting that additional factors are required to maintain it, as occurs during natural development.

This thesis provides direct evidence for an opsin switch in the retina of a vertebrate and demonstrates that this switch is mediated by TH. These are fundamental cellular mechanisms that establish the chromatic organization of the retina as evidenced by ongoing findings in other vertebrates including mammals.

Keywords: ultraviolet opsin; blue opsin; visual pigment; opsin switch; cone photoreceptor; cone mosaic; thyroid hormone; retinal development; salmon

Subject Terms: visual pigments; rhodopsin; photoreceptors; colour vision; eye; thyroid hormone
To My Parents and My Brother
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## GLOSSARY

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATU</td>
<td>Accumulated temperature units</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHSE</td>
<td>Chinook salmon embryonic cell</td>
</tr>
<tr>
<td>coBL</td>
<td>Coho-derived blue opsin riboprobe</td>
</tr>
<tr>
<td>coUV</td>
<td>Coho-derived UV opsin riboprobe</td>
</tr>
<tr>
<td>d/s</td>
<td>Ratio of double to single cones</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMSP</td>
<td>Dichroic microspectrophotometry</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FL</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>HBW</td>
<td>Half bandwidth</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>Maximum absorbance</td>
</tr>
<tr>
<td>LWS opsin</td>
<td>Long wavelength-sensitive (red light-sensitive) opsin</td>
</tr>
<tr>
<td>M opsin</td>
<td>Middle wavelength-sensitive (green light-sensitive) opsin</td>
</tr>
<tr>
<td>mRNA</td>
<td>Message ribonucleic acid</td>
</tr>
<tr>
<td>MSP</td>
<td>Microspectrophotometry</td>
</tr>
<tr>
<td>NaB$_4$O$_7$</td>
<td>Sodium tetraborate decahydrate</td>
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</tbody>
</table>
NaOH  Sodium hydroxide
NBT/BCIP  5-bromo-4-chloro-3-indolyl phosphate with 4-nitroblue tetrazolium chloride
OCT medium  Optimal cutting temperature medium
PCR  Polymerase chain reaction
RH1  Rhodopsin (dim light-sensitive)
RH2  Rhodopsin-like (green light-sensitive) opsin
RMS  Root mean square
RNA  Ribonucleic acid
RORβ  Retinoid-related orphan receptor, beta isoform
rpe  Retinal pigmented epithelium
rtBL  Rainbow trout-derived blue opsin riboprobe
rtUV  Rainbow trout-derived UV opsin riboprobe
RXRγ  Retinoid X receptor, gamma isoform
S opsin  Short wavelength-sensitive (UV/blue-light sensitive) opsin
SWS1  Short wavelength-sensitive (UV light-sensitive) opsin
SWS2  Short wavelength-sensitive (blue light-sensitive) opsin
T₃  Thyroid hormone
T₄  Thyroxine
TH  Thyroid hormone
TRE  Thyroid hormone response element
TUNEL  Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
UV  Ultraviolet
LIST OF ORIGINAL PUBLICATIONS

This dissertation is based largely on the following research articles, reproduced by permission of the journals below.

Chapter 2


In this study I was responsible for the synthesis of the W and blue opsin riboprobes, the histology, in situ hybridization, and the analysis of data. I had a limited role in writing and editing the manuscripts.

Chapter 3


My contribution to this study involved production of the red, green and rod opsin riboprobes, performing RNA isolation, RT-PCR, in situ hybridization, and analyzing the data. I had a role in writing and editing the manuscript.

Chapter 4


I was responsible for the overall research experiment. I had a role in writing and editing the manuscript.
Chapter 5


In this study, I carried out the histology and in situ hybridization experiments and I had a role in writing and editing the manuscript.

Chapter 6


I was responsible for the overall design of the cell culture work; in addition to contributing to the histology and in situ hybridization. I was solely responsible for the plasmid construction and cell transfection. I wrote the paper under the guidance of my supervisor.
CHAPTER 1
GENERAL INTRODUCTION
AND LITERATURE REVIEW

1.1 Retinal Development

Retinogenesis entails the developmental transition from proliferating
neuroepithelial cells to the highly specialized and multilaminar array of distinct types of retinal neurons. Considerable progress has been made in the discovery of intracellular regulatory mechanisms and extrinsic signaling molecules controlling cell fate specification, differentiation, and complex patterning of the retina (for reviews, see Stenkamp, 2007; Adler and Raymond, 2007). Despite this progress, the mechanisms directing photoreceptor competence and maturation have just started to unravel.

Photoreceptors are highly specialized neurons in the retina that mediate phototransduction. The two types of photoreceptors, rods and cones, are derived from a common pool of retinal progenitor cells. Studies on photoreceptor development have identified some of the key factors regulating this process. NEUROD1 and CRX play a role in restricting multipotent progenitors to commit to a photoreceptor cell fate (Yan and Wang, 2004; Ochocinska and Hitchcock, 2007) while NRL and NR2E3 influence the committed precursors towards a rod lineage. In the absence of these factors, the photoreceptor precursors are thought to become a proto-cone by default (Mears et al., 2001; Peng et al., 2005). The transcription factors TRβ2, RXRγ and RORβ determine the cone subtype identity (Ng et al., 2001; Roberts et al., 2005, 2006; Srinivas et al., 2006; Applebury et al., 2007).
1.2 Photoreceptors and Opsin Expression

Rods and cones differentiate from a common pool of progenitor cells resulting in two types of photoreceptors that are different in morphology and express a unique set of genes which contribute to their distinct function. Rods are tubular in shape, are orders of magnitude more sensitive to light than cones, and have a predominant visual pigment (rhodopsin) with maximum sensitivity in the green part of the spectrum ($\lambda_{\text{max}} \approx 500 \text{ nm}$) (Hárosi, 1994; Novales Flamarique, 2005). In contrast, cones show two major morphological types (single and double cone) and their predominant visual pigment can be most sensitive to either ultraviolet (UV, $\lambda_{\text{max}} \approx 365 \text{ nm}$), violet ($\lambda_{\text{max}} \approx 400 \text{ nm}$), blue ($\lambda_{\text{max}} \approx 430 \text{ nm}$), green ($\lambda_{\text{max}} \approx 520 \text{ nm}$), or red ($\lambda_{\text{max}} \approx 565 \text{ nm}$) light (Walls, 1963; Hárosi, 1994; Yokoyama, 2000). Single cones have circular cross section while double cones consist of two apposed cells sharing a double membrane partition and having elliptical cross-section (Walls, 1963; Engström, 1963). The visual pigments in cones and rods are composed of an opsin protein covalently bound to a chromophore (the aldehyde of either vitamin A$_1$ or vitamin A$_2$). It is the combination of two or more cone types having visual pigments with different $\lambda_{\text{max}}$ that allows for the discrimination of wavelength and that dictates the spectral (colour) sensitivity of an animal.

1.2.1 Cone mosaics in lower vertebrates

In lower vertebrates and, to a lesser degree, in primates, cone photoreceptors form repeating geometric formations termed mosaics (Lyall, 1957; Engström, 1963; Ahlbert, 1969). The disposition and chromatic organization of these mosaics is crucial to all aspects of vision and their study can reveal important insights into general mechanisms of cell pattern formation and function (Cook and Chalupa, 2000). Two major types of
mosaics are common: the square mosaic, in which cross sections of double cones at the nuclear level (and, sometimes, at the ellipsoid level) form the sides of a square (Engström, 1963; Ahlbert, 1976; Marc and Sperling, 1976; Novales Flamarique, 2001), and the row mosaic, in which double cones align to form rows that alternate with rows of single cones (Branchek and Bremiller, 1984; Raymond et al., 1993). The square mosaic unit is composed of a single “centre” cone in the middle of the square, with single “corner” cones sometimes present at the corners, facing the partitions of neighbouring double cones (Lyall, 1957; Engström, 1963; Ahlbert 1969, 1976). These two mosaics often coexist as observed in the retina of green sunfish (Cameron and Easter, 1993), goldfish (Wan and Stenkamp, 2000) and salmonid fishes (Novales Flamarique, 2001). In addition to the square and row mosaic, hexagonal mosaics have also been observed in flatfishes, as in the retina of larval flounder (Hoke et al., 2006). These mosaics transform to a square mosaic with double cones and single cones during metamorphosis (Hoke et al., 2006).

1.2.2 Cellular mechanisms underlying cone mosaic formation

The cellular and molecular mechanisms that generate cone mosaic patterns in the vertebrate retina are unknown but two hypotheses have been put forth. The first hypothesis postulates that cone mosaics may arise from a cascade of cell interactions whereby a given spectral cone type would influence the fate of neighbouring uncommitted cells to differentiate into the next spectral type (Raymond and Barthel, 2004). Such sequential development of photoreceptors occurs in the fruit-fly, Drosophila melanogaster, where a set of “founder cells” initiate a position-dependent differentiation cascade that results in the mosaic of rhabdoms characteristic of the compound eye of this
animal (Cook and Desplan, 2001). For a similar mechanism to operate in lower vertebrates, opsin expression would need to follow a precise developmental sequence, and each opsin class would need to be expressed in a particular cone type occupying a precise position in the unit mosaic. This is the case in cyprinid fishes (goldfish and zebrafish) and in chick, where the order of opsin appearance is: RH1 (rhodopsin), followed by LWS (red), RH2 (green), SWS1 (UV/violet) and SWS2 (blue) opsin (Raymond et al., 1995; Stenkamp et al., 1996; Stenkamp et al., 1997; Takechi and Kawamura, 2005; Bruhn and Cepko, 1996; Adler et al., 2001). A recent study using goldfish has demonstrated that rhodopsin does not play a role in differentiation of cones, as judged by opsin expression (Wan and Stenkamp, 2000). A role for the first cone opsin in establishing the chromatic organization of the mosaic through a hypothetical opsin cascade (Raymond and Barthel, 2004) has not been ruled out.

The second hypothesis proposes that photoreceptor mosaic patterning is achieved by differential adhesion between cell subtypes (Galli-Resta, 2001; Mochizuki, 2002; Tohya et al., 2003). This hypothesis is based on the assumption that pattern formation involves cells with a ‘pre-fix’ cone type and that each cone type possesses unique surface molecules having an affinity for non-self molecules. Computer simulations have demonstrated that these constraints can generate the square and row mosaic patterns (Tohya et al., 1999, 2003). A recent study has shown that cell surface signalling interaction mediated by Notch and Delta transmembrane proteins is required for cone patterning in zebrafish (Bernardos et al., 2005). Additional surface molecules that would mediate the adhesion mechanism have yet to be identified.
1.2.3 Cone mosaics in higher vertebrates

With the exception of the blue cone mosaic in primates, mammals do not exhibit discernable mosaics (Bumsted et al., 1997). Nonetheless, these retinas may show separation of cone spectral phenotypes along a gradient as occurs in the retinas of mouse and human. In mouse, blue cones are primarily found in the ventral retina while green cones are located in the dorsal retina (Szél and Röhlich, 1992; Szél et al., 1992; Applebury et al., 2000). In the human fovea, blue cones are absent while green and red cones densities are highest in this area of the retina (Curcio et al., 1991; Bumsted and Hendrickson, 1999). These compartmentalizations of cone types may serve dedicated ‘ecological’ functions, though comparison of retinal patterns from various rodents have failed to give a clear ‘raison d’être’ (Röhlich, 1994; Szél et al., 1994, 2000; Applebury et al., 2000). The cellular mechanisms that establish gradients of cone types in mammals have yet to be deciphered. However, recent studies suggest that thyroid hormone and a photoreceptor-specific thyroid hormone receptor, TRβ2, may participate in establishing the mouse cone gradient (Ng et al., 2001; Roberts et al., 2006; Applebury et al., 2007).

1.2.4 Photoreceptor plasticity

The spatial arrangement of photoreceptor types is dynamic throughout development. Several studies have indicated that such chromatic reorganization of photoreceptors cannot be accounted for by cell death and regeneration alone, suggesting that photoreceptors must express different opsins sequentially (Szél et al., 1994). In the mouse for instance, the embryonic retina has cones that express only blue opsin while the pup retina has green opsin expressing cones in the dorsal retina (Applebury et al., 2000). During the process of forming this graded retina, cones appear to express both opsins.
(Röhlich et al., 1994; Lyubarsky et al., 1999; Applebury et al., 2000). A sub-population of these dual opsin cones may be responsible for generating the green cone population. Likewise, in humans, dual opsin expressing cones appear in the circumferential growth zone as the embryo proceeds from a retina with only blue cones to a retina with blue, green, and red cones (Xiao and Hendrickson, 2000; Cornish et al., 2004).

In lower vertebrates, such hypothetical opsin switches within a cone are sometimes accompanied by restructuring of the cone mosaic. This is the case in the winter flounder where the larva has single cones arranged in an hexagonal mosaic, all expressing green opsin (Mader and Cameron, 2006). Following metamorphosis, cones are arranged in a square mosaic and all photoreceptors, including the rods, express new opsin. Other fishes also seem to undergo photoreceptor opsin switches including further species of flatfish (the black beam, Shand et al., 2002; the Atlantic halibut, Helvik et al., 2001), eels (Archer et al., 1995; Hope et al., 1998), and cichlids (Spady et al., 2006). Even in fishes in which cones may not switch expression between opsin gene families, like the zebrafish, opsin switching within a gene family may still occur (Takechi and Kawamura, 2005). Indeed, zebrafish express several types of green and red opsin genes with variable spectral distributions throughout development. In the adult retina, expression of red and green opsin is patterned topographically, with longer wavelength subtypes in the ventral and peripheral regions and shorter wavelength subtypes in the central and dorsal area (Takechi and Kawamura, 2005). In cichlids, changes in opsin expression appear to correlate with changes in the photic environment (Carleton and Kocher et al., 2001; Parry et al., 2005), presumably to optimize some crucial aspect of visual function such as spectral sensitivity. It is likely that this correlation may also hold
for other fishes (e.g. the flatfishes), though specific ecological tasks optimized by these opsin switches have yet to be identified.

1.3 Nuclear Hormone Receptor Signalling

The nuclear hormone receptor superfamily comprises molecular receptors that respond to the presence of a hormone, and includes the retinoic acid receptors, thyroid hormone receptors, and steroid hormone receptors (Nagy and Schwabe, 2004). Nuclear hormone receptors regulate various developmental processes by modulating the expression of target genes at the transcriptional level. In the absence of ligand, or hormone, nuclear hormone receptors often associate with co-repressors leading to transcriptional repression. When ligand becomes available, the binding of ligand induces a conformational change of the receptor leading to the dissociation of co-repressors and recruitment of co-activators. This new interaction results in gene transcription (Nagy and Schwabe, 2004; McKenna and O’Malley, 2002).

Nuclear receptors bind to specific DNA sequences in the promoter regions of their target genes known as response elements. For example, the response element for thyroid hormone receptor, T3 response element (TRE), is composed of two “AGGTCA” half sites separated by four nucleotides (for a review, see Yen, 2001).

There are often multiple isoforms of a nuclear receptor, for example thyroid hormone receptor has four isoforms (TRα1, TRα2, TRβ1, TRβ2). Each isoform has a specific expression pattern which contributes to the tissue-specific and stage-dependent effect. In addition, each isoform can act as a monomer, homodimer or heterodimer with other members of the nuclear hormone receptor family (Forrest et al., 2002).
1.4 A Role for Thyroid Hormone in Opsin Regulation

Thyroid hormone is a crucial molecule for vertebrate development and its role has been the focus of recent studies in rodent, chick, amphibian and fish (for reviews, see Forrest et al., 2002; Harpavat and Cepko, 2003; and Bernal, 2007). Thyroid hormone can induce a range of actions in distinct tissues or cell-types and it can have different effects throughout development. The effects of thyroid hormone are controlled by the availability of receptors and the availability of ligand, which in turn depends on the distribution of the enzymes that control its production: the deiodinase enzymes.

Changes in the levels of thyroid hormone receptors have been associated with the timing of cone neurogenesis in teleosts (Jones et al., 2002), chick (Sjöberg et al., 1992), mice (Applebury et al., 2007) and humans (Lee et al., 2006), suggesting a conserved role of such receptors during photoreceptor development. Mice lacking a photoreceptor-specific isoform of thyroid hormone receptor (the TRβ2 receptor) have a selective loss of green cones and a concomitant increase in blue cones (Ng et al., 2001). Transgenic mice carrying targeted mutations in TRβ2 have shown that binding to both the DNA response element and the ligand is required to regulate opsin expression (Applebury et al., 2007). Consistent with these observations, injection of T3 into wild type mice or TRβ2-deficient mice showed that T3 decreases blue opsin and increases green opsin expression and that the changes are mediated by TRβ2 (Roberts et al., 2005). Similarly, in salmon, thyroid hormone also affects opsin expression in that treatment with this compound leads to a downregulation in UV opsin mRNA and an upregulation in blue opsin mRNA as assessed by quantitative RT-PCR (Veldhoen et al., 2006). However, the effects of thyroid
hormone on the chromatic organization of photoreceptors and on cone differentiation in salmonid fishes have not been explored.

1.5 Salmonid Fishes as Model Organisms to Study Photoreceptor Development

Salmonid fishes vary substantially in their life history strategies (Groot and Margolis, 1991) though the basic life cycle is similar to all. All species hatch as yolk-sac alevins and, following a variable stay in freshwater, proceed to the ocean to spend most of their adult life (non-anadromous morphs stay in freshwater, usually in a lake, Groot and Margolis, 1991). Around the time of full yolk sac absorption, the fish rises from the stream gravel and adopts 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 process of adaptation from freshwater to saltwater called smoltification, and it involves several hormones including growth hormone, prolactin, and thyroid hormone (Hoar, 1988; Clarke et al., 1996). Following several years in saltwater (anywhere between 2 and 7 on average, Groot and Margolis, 1991), the salmon becomes sexually mature and returns to freshwater to reproduce and begin the life cycle anew. For anadromous (ocean-migrating) salmonids, juvenile residence time in freshwater after hatching is usually 1-3 months for pink (Oncorhynchus gorbuscha) and chum (Oncorhynchus keta) salmon, 4-6 months for chinook salmon (Oncorhynchus tschawytscha), 8-16 months for coho salmon (Oncorhynchus kisutch), and 1-4 years for Atlantic salmon (Salmo salar) and the trouts. Non-anadromous (landlocked) salmonids, such as rainbow trout (Oncorhynchus mykiss), remain in freshwater (migrate to lakes) throughout development. The variability in life
history strategies within this family of fishes makes the group particularly attractive to study genetic and environmental factors that guide the evolution of visual system function (Ahlbert, 1969, 1976; Bowmaker and Kunz, 1987; Schmitt and Kunz, 1989; Beaudet et al., 1997).

In addition to their interesting and diverse life strategies, these animals possess single and double cones distributed in regular mosaic across the retina. Furthermore, opsin expression is associated with specific cone types such that short wavelength opsins (UV, blue) occur in the single cones while red and green opsins occur in the double cones (Hárosi, 1994). This correlation and the fact that photoreceptor development occurs to a large extent outside the egg (as a yolk sac embryo) makes these animals particularly amenable to exogenous treatment and genetic manipulation. These advantages, which are not found in other models (like the mouse), have been further enhanced in recent years by the development of the salmon genome project. This resource will be essential to future investigations into the genetic basis of photoreceptor development in salmonids.

1.6 Objectives of Dissertation

I undertook this research with the following objectives:

- to establish the chromatic organization of the salmonid retina during development and throughout ontogeny,

- to assess a suspected role of thyroid hormone in regulating opsin expression

The first goal entailed comparing the sequence of opsin induction and the chromatic organization of cone photoreceptors at the various life stages to develop a
unifying pattern of opsin expression in these fishes. The second goal involved investigations using thyroid hormone to assess any potential role of this compound in controlling opsin expression.

To achieve these goals, I used a combination of biophysical and molecular techniques (microspectrophotometry, RNA isolation, cloning, reverse transcriptase – polymerase chain reaction, in situ hybridization), histology, and cell culture methods. Together, results from this combination of techniques led to a comprehensive understanding of opsin expression in the retina of salmonid fishes and the role that thyroid hormone plays in controlling this expression.
CHAPTER 2
PHOTORECEPTOR LAYER OF SALMONID FISHES: TRANSFORMATION AND LOSS OF SINGLE CONES IN JUVENILE FISH

Christiana L. Cheng, Íñigo Novales Flamarique, Ferenc I. Hárosi, Jutta Rickers-Haunerland, and Norbert H. Haunerland

Department of Biological Sciences
Simon Fraser University
8888 University Drive
Burnaby, British Columbia
V5A 1S6, Canada

2.1 Introduction

Several studies have shown that the number of single cones (in particular corner cones) varies between retinal locations in salmonid fishes (Ahlbert, 1976; Novales Flamarique, 2001, 2002), and that some of these cones disappear as the juvenile fish grows (Lyall, 1957; Bowmaker and Kunz, 1987; Kunz, 1987; Beaudet et al., 1993; Raymond et al., 1993; Kunz et al., 1994; Novales Flamarique, 2000, 2002). Early studies that combined histological techniques, microspectrophotometry, and electrophysiological recordings from the visual system of salmonid fishes suggested that the corner cones contained an ultraviolet (UV) visual pigment, the centre cones a blue visual pigment, and that the paired cones were double cones containing green and red visual pigment, respectively (Bowmaker and Kunz, 1987; Beaudet et al., 1993; Hawryshyn and Hárosi, 1994; Novales Flamarique, 2000). It was generally accepted that the decrease in UV sensitivity in young salmonid fishes was due to the loss of corner cones by programmed cell death, or apoptosis (Kunz et al., 1994; Allison et al., 2003). However, the resolution of techniques used in previous studies was insufficient to establish such conclusions.

This study combined histological techniques and in situ labeling with riboprobes to investigate cone mosaic structure and the spectral phenotype in the single cones of four species of Pacific salmon, in the Atlantic salmon, and in the steelhead and rainbow trout. These investigations were accompanied by microspectrophotometric (MSP) observations of visual pigment absorbance from isolated photoreceptors (Hárosi, 1987; Novales Flamarique and Hárosi, 2000) to further assess opsin expression during the early juvenile period. From the labeling and MSP results, we show that single cones can switch their spectral phenotype from UV to blue by regulating the production of the appropriate
opsins as the fish grow older. Combining histological and labeling results, retinal maps were constructed that reveal the extent of single cone transformation and loss of corner cones, and the approximate timing of both events. The results also show whether the transformation targets specific cones (e.g. only the centre cones in the mosaic) or not. These observations are important to guide future investigations into molecular mechanisms that may play a role in the transformation and corner cone disappearance events.

2.2 Materials and Methods

2.2.1 Animals

Wild stock coho (*Oncorhynchus kisutch*), chum (*O. keta*), chinook (*O. tschawytscha*), and pink (*O. gorbuscha*) salmon were obtained from the Capilano River, Chilliwack River, Big Qualicum River and Inchcreek hatcheries at developmental stages ranging from the hatched fish (alevin) to the larger juvenile (smolt). Wild stock rainbow trout and steelhead trout (*Oncorhynchus mykiss*) at similar stages were obtained from the Fraser Valley Trout hatchery, and aquaculture-stock Atlantic salmon (*Salmo salar*) was obtained from a hatchery owned by Omega Group Corporation Ltd. All collection sites are located in Vancouver Island and in the Lower Mainland of British Columbia, Canada. Fish statistics at the different developmental stages investigated are presented in Table 1. Fish were reared in open troughs with flowing river water (temperature range: 4-6 °C during the study period) and were subjected to the natural daylight cycle. All holding and experimental procedures were approved by the Animal Care Committee of Simon Fraser University, which follows the guidelines set by the Canadian Council for Animal Care.
2.2.2 Preparation of the UV and blue riboprobes

Coho ultraviolet-sensitive opsin (CohoUV) cDNA was generated by RT-PCR amplification from juvenile coho total RNA isolated from homogenized retina of smolt fish (weight ~ 8 g) (RNAqueous-Midi; Ambion). CohoUV cDNA was synthesized (Ready-To-Go RT-PCR beads, Amersham Biosciences) using primers that were designed from a published UV opsin sequence for coho salmon (accession # AY214148, our probe corresponds to bases 111-574 of this sequence) (UV forward primer = 5' - GGG CTT TGT GTT CTT TGC TG -3'; UV reverse primer = 5'- GGT ACT CCT CGT TGT TG T -3'). Reverse transcription with UV reverse primer was carried out at 42 °C for 15 min. Cycling parameters for the subsequent PCR were: 95 °C x 5 min, 32 cycles of 95 °C x 30 sec, 56 °C x 30 sec, and 72 °C x 1 min, and 1 cycle of 72 °C x 10 min. CohoUV cDNA was gel purified and cloned into pCRII-TOPO cloning vector (Invitrogen) and sequenced by AmpliTaq Dye terminator cycle sequencing (UBC Sequencing Laboratory). The identity of the sequence was confirmed by comparing it to nucleotide sequence databases using the BLASTn program (Basic local alignment search tool, Altschul et al., 1997). To fabricate the cRNA probe, a PCR fragment containing the CohoUV insert and an RNA promoter amplified from the pCRII-TOPO vector was used to generate digoxigenin (DIG)-labeled RNA sense and antisense riboprobes by in vitro transcription (DIG RNA labeling kit, Roche Diagnostics).

Partial coho blue-sensitive opsin (Cohobl) cDNA was generated similarly to the CohoUV cDNA, except that the primers were designed from a published blue opsin sequence for rainbow trout, Oncorhynchus mykiss (accession # AF425075, our probe corresponds to bases 119-312 of this sequence) (blue forward primer = 5' - AAA CCT...
TGG TAG TGG GGA TT -3'; blue reverse primer = 5' -CAT AGA AGA TAG CAC TGC CC -3').

2.2.3 Tissue preparation of histology

Light-adapted fish were sacrificed by quick spinal bisection and decerebration. The eyeballs were then removed, the iris and lens discarded, and the remaining eyecup immersed in either primary fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer, pH = 7.4) for histological analysis (Beaudet et al., 1997; Novales Flamarique, 2001), or in cryo-fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH = 7.4) for cryoembedding and in situ hybridization (Forsell et al., 2001). After 24 hr 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 8-21 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). Care was taken to cut a ~0.5-1 mm thick strip around the entire periphery of the retina; in fishes, this area comprises the growth zone and is of interest because it recapitulates events during retinal development (Johns, 1982). 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 (Beaudet et al., 1997; Novales Flamarique, 2001). 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 dH₂O and 1% Methylene blue in 1% NaB₄O₇ solution) to reveal the cone mosaic. Using a grid
system on the computer monitor, the density of double cones and single cones (centre cones + corner cones) in a 32700 \(\mu\text{m}^2\) area was then counted and the ratio of double to single cones (d/s) computed for each sector of the retina (d refers to the two member double cone pair). A d/s ratio closer to 2 indicates a total loss of corner cones while a ratio closer to 1 indicates full corner cone presence (see Beaudet et al., 1997).

The contours of pieces mapped back to the original retina were used to trace maps of double cone to single cone (d/s) ratio, or of UV cone percentage within the single cone population (from the in situ labelling results, see next section). Five such retinal maps were superimposed on a grid and averaged visually to give the final maps shown in the Results section. Retinas used to obtain an average map were of similar size, thus minimizing contour differences due to age.

2.2.4 Tissue preparation for in situ hybridization

Retinal pieces for in situ hybridization analysis were cryo-protected in sucrose solution (30% sucrose, 0.1 M phosphate buffer in O.C.T medium) overnight at 4 °C and cryo-embedded in 100% O.C.T. medium (Cedar Lane Laboratories) (Forsell et al., 2001). These blocks were cut tangentially or radially, in 5-7 \(\mu\text{m}\) steps, to reveal the cone mosaic or the photoreceptor layer, respectively. The resulting cryosections were used for in situ hybridization with the UV and/or blue riboprobe(s) as per published studies (Raymond et al., 1993; Stenkamp et al., 1996; Forsell et al., 2001). Briefly, the procedure involved rehydrating the sections, permeabilizing them in 10 \(\mu\text{g/ml}\) proteinase K (Sigma) for 7 min, followed by exposure to 0.1 M triethanolamine containing 0.25% acetic anhydride, dehydration, and hybridization overnight at 56 °C with 1-5 \(\mu\text{g}\) cRNA probe in hybridization solution containing 50% formamide, dextran sulfate, and goat serum.
Sections were then treated with RNase A (Sigma) and incubated with anti-DIG Fab fragments conjugated to alkaline phosphatase (1:3000; Roche Diagnostics) overnight at room temperature. The DIG-labelled probes were visualized using 5-bromo-4-chloro-3-indolyl phosphate with 4-nitroblue tetrazolium chloride (NBT/BCIP; Roche Diagnostics). Sense probes were used as negative controls and did not hybridize in any of the retinas. Digital images of sections were acquired with an E-600 Nikon microscope equipped with a DXM-100 digital camera and DIC optics. Because of the minute size of some of the retina pieces sectioned and the unavoidable slant in some of the embedded tissue, we sometimes obtained sections in which only a few rows of cones were visible. In such cases, density counts were obtained from multiple consecutive sections by matching images on the computer. This way, an analogous area to that analyzed by histology could be obtained.

We performed two types of cryosection collections. In the first type, serial sections were deposited on the same slide for in situ hybridization with the UV riboprobe. The labelled cones were counted for each retinal sector examined and the results from five similar size retinas pooled together to give the UV percentage maps shown in the Results section. In the second type of collection, serial sections were alternatively deposited on two slides. One of the slides was used for in situ hybridization with the UV riboprobe, while the other slide was used for in situ hybridization with the blue riboprobe. These slides were processed together allowing for comparison of hybridization results with the two riboprobes. Unless otherwise stated, results for each species and developmental stage are presented as a comparison of consecutive sections, one treated with the UV riboprobe (and positioned on the left part of the figure panel), and the other
treated with the blue riboprobe (and positioned immediately to the right of the corresponding UV riboprobe-treated section).

2.2.5 Microspectrophotometry (MSP) of photoreceptors

Individual fish were dark adapted overnight. Following this adaptation period, the animal was killed under dim red light conditions, one eye enucleated and the retina removed under infrared illumination while keeping its natural orientation. The retina was then divided into sectors from which small pieces were cut, 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 aided by 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, 2002). The DMSP was 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 x 0.6 μm. The wavelength scale was calibrated with a spectral lamp to an accuracy of ± 1 nm. In general, MSP-derived absorbance values are contaminated by random noise, which increases in the UV range due to a drop-off of available photons in the measuring beam. The root mean square (RMS) error of absorbance is about 30% of the peak-to-peak excursions of absorbance (noise). Repeated
scans averaged together reduces the RMS error by the square root of \( n \), where \( n \) is the number of independent scans. Under conditions of normal measurements, we rarely see evidence of photoproduct accumulation in cones, probably because they are short-lived and their chromophores diffuse out of the light path. For a given photoreceptor, measurements were obtained at multiple locations along the outer segment with increasing distance from the base or tip. Absolute absorbance spectra were computed in 2 nm increments from the obtained transmittances (each spectrum consisted of an average of eight scans). The solid spectra (fits) were derived from experimental data by Fourier filtering (Hárosi, 1987). High quality spectra from similar photoreceptor types (as determined by the bandwidth at half maximum, HBW, and overall goodness of fit, Hárosi, 1987, 1994) were averaged together to give the absorbance curves presented (e.g. Fig. 2.6, 2.18). In the case of single cones expressing two opsins with similar absorbance profile along the outer segment, spectra were pooled together regardless of the initial scan site and direction (base to tip or tip to base) since this had no effect on the absorbance profile.

2.3 Results

2.3.1 Photoreceptor distributions in the light- and dark-adapted retina of salmonid fishes

Tangential sections from the light-adapted retina of the coho alevin showed that the cones were organized in a square mosaic composed of double cones, whose elliptical cross sections formed the sides of the square, and single cones located at the center and at the corners of the unit square (Fig. 2.1a). In general, corner cones, also known as accessory corner cones, faced the membrane partitions of neighbouring double cones.
Centre cones were usually bigger than corner cones and were located at the hypothetical intersection of neighbouring double cone partitions. This square mosaic was characteristic of all the species studied, though Atlantic salmon alevins often had corner cones missing from the ventral retina (see also Novales Flamarique, 2002). The same retina examined by radial sections showed that the double cones were displaced towards the retinal pigment epithelium with respect to the single cones, such that the outer segments of the latter spanned the upper ellipsoid region of the double cones (Fig. 2.1b). Rod outer segments were located further into the retinal pigment epithelium with respect to the outer segments of double cones (Fig. 2.1b). In the dark-adapted retina, the double cone mosaic was not discernable (Fig. 2.1c). This was due to retinomotor movements that resulted in rods migrating towards the outer nuclear layer and cones stretching towards the retinal pigment epithelium (Fig 2.1d). The result was a collection of cone inner segment ellipsoids scattered among the rod outer segments (Fig. 2.1c,d).

By the time salmonids had reached the smolt stage, the retina was made up of three distinct morphological regions. In the dorsal retina, the cone mosaic at the level of the double cone ellipsoids was primarily square and comprised single center and corner cones (Fig. 2.2a). The central retina, however, consisted mainly of intercalating rows of single and double cone ellipsoids with both center and corner cones present (as determined by double to single cone ratios (dc/sc) < 1.5, Fig. 2.2b, Table 2.2). The ventral retina was characterized by a square mosaic that lacked most corner cones (Fig. 2.2c). Cones in the ventral retina were markedly smaller than those in the central and dorsal retina.
2.3.2 Specificity of riboprobes

The UV and blue riboprobes were 464 and 194 base pairs long, respectively, and showed 47% identity between them (Fig. 2.3). Further analyses using ClustalW (Chenna et al., 2003) revealed a 55% sequence identity between the UV riboprobe and the complete mRNA sequence of coho blue opsin (accession # AY214149, penalty gap: 200, Fig. 2.4) and a 35% sequence identity between the blue riboprobe and the complete mRNA sequence of coho UV opsin (AY214148, penalty gap: 200, Fig. 2.5). The distant similarity between the two riboprobes and between each riboprobe and the non-corresponding opsin sequence (i.e. UV riboprobe-blue opsin or blue riboprobe-UV opsin) suggest a lack of cross-hybridization between non-corresponding riboprobe-opsin pairs, as demonstrated by the combination of in situ hybridization and MSP results (see below). A further BLASTN search revealed the following results: (1) over 97% sequence identity between the UV riboprobe and the complete mRNA sequence of UV opsin from chum (accession # AY214143.1), chinook (accession # AY214138.1), pink (accession # AY214153.1), Atlantic salmon (accession # AY036959.1), rainbow trout (accession # AF425074.1), and sockeye salmon, *Oncorhynchus nerka* (accession # AY214158.1), (2) 85-88% sequence identity between the UV riboprobe and the complete mRNA sequence of cichlid, halibut, zebrafish and carp UV opsin, and (3) over 98% sequence identity between the blue riboprobe and the complete mRNA sequence of blue opsin from chum (accession # AY214144.1), chinook (accession # AY214139.1), rainbow trout (accession # AF425075.1), and sockeye salmon (accession # AY214159.1). These similarities between the UV and blue riboprobes with the corresponding opsin sequences in the Salmonidae family of fishes strongly suggest cross-hybridization of the riboprobes with the corresponding opsin mRNAs across species, as the Results show.
To demonstrate that our UV and blue riboprobes were specific for the corresponding opsins, we performed a series of experiments where adjacent pieces of retina were examined by MSP and by *in situ* hybridization. Results from both techniques corroborated each other and confirmed our conclusions from bioinformatic analyses. In the coho alevin, all single cones labeled with the UV riboprobe and none labeled with the blue riboprobe (Fig. 2.6a-d). In agreement with these findings, all the single cones (n=11) measured by MSP in the adjacent piece of retina contained a UV visual pigment with maximum absorbance ($\lambda_{\text{max}}$) $\sim$ 368 nm (Fig. 2.6i). In the smolt, the distal dorso-temporal retina showed a predominance of UV-labeled single cones (Fig. 2.6e,f). MSP recordings from six cones in this region of the retina of one fish resulted in four containing UV visual pigment ($\lambda_{\text{max}}$ $\sim$ 364 nm) and two housing a blue visual pigment ($\lambda_{\text{max}}$ $\sim$ 433 nm). In the lower half of the ventral retina of the smolt, all the single cones labeled with the blue riboprobe and none labeled with the UV riboprobe (Fig. 2.6g,h). Accordingly, all the single cones measured by MSP in the adjacent piece of retina (n=10) contained a blue visual pigment with $\lambda_{\text{max}}$ $\sim$ 434 nm (Fig. 2.6j). These results demonstrate that our riboprobes are specific for the corresponding opsin mRNAs. The riboprobes also cross-hybridized with the corresponding opsin mRNAs in other salmonid fishes. The following is a summary of results for each species.

### 2.3.3 Coho salmon

The coho salmon alevin had a square mosaic throughout the retina in which single cones (whether center or corner cones) labeled *exclusively* with the UV riboprobe (Fig. 2.7a-d). A one-way ANOVA performed on sectors 1 and 6 (see Fig. 2.8a) showed that the average number of single cones measured histologically was statistically the same as
the number of single cones labeled with the UV riboprobe in each sector (sector 1: $F_{1,9} = 0.99, p = 0.35$; sector 2: $F_{1,9} = 0.01, p = 0.93$; $n = 5$ per location) after differences in tissue shrinkage were accounted for (on average, the shrinkage difference between plastic sections and cryosections was 10%). The labeling was restricted to the inner segment (Fig. 2.7a,c), consistent with the location of the endoplasmic reticulum in rainbow trout photoreceptors (Schmitt and Kunz, 1987) and with the localization of similar riboprobe labeling in the cones of other fishes (Raymond et al., 1993; Stenkamp et al., 1996, 1997; Forsell et al., 2001). When the fish was about 2-3 g, single cones in the lower ventral retina of the coho alevin (now having absorbed its yolk sac), started labeling with the blue riboprobe (Fig. 2.7e,f, 8). Histological sections from this area of the retina showed corner cones beginning to disappear from the mosaic (results not shown). As the fish continued to grow, fewer single cones labeled with the UV riboprobe and more labeled with the blue probe, such that after 1 year (weight $> 8$ g), UV labeling of single cones was restricted to the distal dorso-temporal retina (Fig. 2.8). The majority of the retina labeled with the blue but not with the UV riboprobe (Fig. 2.7g-j), though much of the dorsal retina still had corner cones (Fig. 2.8, 2.7h). The few cones that retained UV visual pigment in larger (smolt) fish were primarily located in the upper retina and could be either corner or center cones (Fig. 2.7k,l).

The highest densities of double cones were found in the ventral and dorsal periphery of the alevin, and in the ventro-temporal periphery of the smolt (Fig. 2.8a,b, Table 2.2). Higher cone densities are commonly associated with retinal areas of enhanced visual acuity (Engström, 1963; Ahlbert, 1976; Beaudet et al., 1997).
2.3.4 Chum salmon

Young chum salmon alevins (0.3-0.6 g in weight) showed the same cone mosaic and the same labeling of single cones with the UV riboprobe as did the coho salmon (Fig. 2.9a,b). A one-way ANOVA performed on sectors 1 and 6 revealed that the number of single cones labeled with the UV riboprobe was statistically the same as the total number measured by histology after shrinkage was accounted for (sector 1: $F_{1.9}=0.27$, $p=0.62$; sector 2: $F=0.45$, $p=0.52$; $n=5$ per location). When the fish reached ~0.7 g in weight, single cones in the lower retina started transforming phenotype from UV to blue in an event that proceeded across the retina towards the dorsal half. This transformation event was followed closely by a progressive loss of corner cones (Fig. 2.10). With the exception of the peripheral growth zone, this transformation led to a smolt retina where UV cones were extremely rare (Fig. 2.10d), but where most single cones (regardless of position in the mosaic) labeled with the blue but not with the UV riboprobe (Fig. 2.9e-f). As in the coho smolt, the dorso-temporal retina of the chum smolt had corner cones (Fig. 2.10b), the vast majority of which (>95%) contained blue visual pigment (Fig. 2.10d). The highest double cone densities were found peripherally, namely in the ventral and ventro-temporal areas of the retina (Fig. 2.10a,b, Table 2.2).

2.3.5 Chinook salmon

The young chinook alevin had a full square mosaic where all single cones labeled with the UV riboprobe (Fig. 2.11a,c) but none labeled with the blue riboprobe (Fig. 2.11b,d). Fish that were ~1.7 g in weight had corner cones missing from most of the ventral retina (Fig. 2.12a) and blue labeling was extensive throughout this part of the retina (Fig. 2.12c). As the fish grew towards the smolt stage, the majority of single cones
failed to label with the UV riboprobe (Fig. 2.11e) but labeled, instead, with the blue riboprobe (Fig. 2.11f). Some remaining corner cones could be found that labeled with the UV riboprobe (Fig. 2.11g) but these were scarce; the majority of cones, whether center or corner in position, labeled with the blue riboprobe (Fig. 2.11h, 2.12). The smolt retina had varying densities of corner cones throughout the dorso-temporal retina (Fig. 2.12b, Table 2.2), with a reduced area of UV labeling dorsal to the optic nerve head (Fig. 2.12d). The highest double cone densities occurred in the ventral retina (Fig. 2.12a,b).

2.3.6 Pink salmon

All single cones in the recently-hatched pink salmon (~ 0.4 g in weight) label with the UV but not with the blue riboprobe, and that these cones undergo a transformation to the blue phenotype as the alevin grows (Fig. 2.13). In this study, we provide histological counts of morphological and spectral cone types (Fig. 2.14) to compare with the other species examined. Absorbance measurements for all photoreceptor types are presented, as well as absorbance profiles indicating that some single cones undergo UV opsin down-regulation without any apparent changeover to the blue opsin (see Fig. 2.18, 2.20).

At the time of collection, the pink salmon examined were being released from the hatchery onto the Big Qualicum river and would have been in the ocean (coastal waters) within 30 min of release. Fish transported to the SFU Aquatic facility showed the beginning of transformation in the ventral retina around 0.8 g in weight. By the time fish were ~ 1.5 g in weight, a gradient of increasing UV cone density from ventral to dorsal retina was apparent (Fig. 2.14b). This transformation event was associated with a loss of single cones closely trailing the transformation front (Fig. 2.14a). As in the other salmon
species, the highest double cone densities occurred in the ventral and ventro-temporal parts of the retina (Fig. 2.14a).

2.3.7 Atlantic salmon

The retina of young Atlantic salmon alevins (~ 0.4 g in weight) had corner cones that labeled with the UV riboprobe (Fig. 2.15a) and center cones that labeled with the blue riboprobe (Fig. 2.15b). The UV label was most extensive in the dorsal retina (Fig. 2.15c). By the time the fish had reached ~ 1 g in weight, the majority of corner cones, whether in the dorsal or in the ventral retina, had disappeared (Fig. 2.16a). Only the distal dorsal retina had corner cones at this young age, and UV labeling was associated with corner cones in this region of the retina (Fig. 2.15d, 2.16c). In agreement with these results, most of the retina in these and larger fish had single (center) cones that failed to label with the UV riboprobe (Fig. 2.15e, 2.16) but labeled, instead, with the blue riboprobe (Fig. 2.15f). This early segregation of UV and blue labeling to corner and center cone types, and the accelerated loss of single cones contrasts with results from Pacific salmon species. By the time the fish had attained the parr stage (~ 4 g in weight), corner cones were rare across the retina (Fig. 2.16b), and only the peripheral growth zone had UV cones (Fig. 2.16d). The highest densities of double cones occurred in the ventral and temporal peripheral areas (Fig. 2.16a,b).

2.3.8 Rainbow trout and steelhead trout

Results from these genetically-identical morphs were similar. Because retinal maps of cone densities at all post-hatching stages (Novales Flamarique, 2001), and UV visual pigment distributions at the parr-to-smolt stages (Allison et al., 2003) are available
for rainbow trout, our analysis was restricted to the identification of single cone phenotypes in the alevin-to-parr stages. In the alevin (weight up to ~2 g), all single cones labeled with the UV riboprobe (Fig. 2.17a,c), while blue labeling was non-existent (Fig. 2.17b) or extremely rare (Fig. 2.17d). As the fish grew (weight > 3 g), progressively more single cones in the ventral retina failed to label with the UV riboprobe but started to label with the blue riboprobe, and these could be either center or corner cones (Fig. 2.17e,f). Larger fish showed a ventral retina that was devoid of UV cones, but all single cones (including remaining corner cones) labeled with the blue riboprobe (Fig. 2.17g,h). By the time the fish were ~9 g in weight, UV labeling was restricted to the corner cones in the distal dorso-temporal retina. The fact that UV label following the transformation was restricted to some of the remaining corner cones is somewhat similar to the situation in the Atlantic salmon. However, the extent of corner cone loss in the Atlantic salmon is much more pronounced than in the rainbow trout, and it occurs, like the potential transformation, at a much younger age.

2.3.9 Absorbance of visual pigments

The juvenile salmonid fishes examined had a visual system based on five visual pigments (UV, blue, green, red, and rhodopsin, Fig. 2.18, Table 2.3). In the *Oncorhynchus* species, all single cones in small juvenile fish (approximate weight < 0.7 g) had a UV visual pigment with \( \lambda_{\text{max}} \) in the range 361-370 nm. By contrast, in larger juveniles (>8 g in weight) the majority of single cones contained a blue visual pigment with \( \lambda_{\text{max}} \) spanning the range 432-436 nm. In the Atlantic salmon, the smallest fish examined (weight ~0.24 g, total length ~3 cm) had already UV and blue single cones. In all species, the double cones were green/red pairs; the green pigment \( \lambda_{\text{max}} \) was in the
range 494-518 nm, while that of the red was in the range 563-578 nm. The range of bandwidths at half maximum (HBW) for the green and red visual pigments were 4101-4351 cm⁻¹ and 3645-4149 cm⁻¹, respectively. All five species of salmon had rod photoreceptors containing a rhodopsin with \( \lambda_{\text{max}} \) in the range 505-515 nm and with HBW in between 4134-4296 cm⁻¹ (Table 2.3). Together, these results indicate retinas based primarily on vitamin A₁ (Hárosi, 1994).

2.3.10 Absorbance profiles of single cones undergoing UV opsin down-regulation

The changes in single cone absorbance between the recently-hatched alevin and the larger smolt paralleled the riboprobe labeling results and indicated that the majority of single cones were changing opsins from UV to blue (also supported by double label in situ co-localization of UV and blue riboprobes to single cones; Fig. 2.13). To obtain more concrete evidence for this transformation, we originally carried out in situ hybridization labeling observations of single cones from chum salmon as the fish grew by 0.2 g increments and compared the results with visual pigment absorbance measurements from cones in analogous regions of the retina (Fig. 2.19). The chum salmon was chosen for this initial study because it progresses from the (fresh water) alevin to the (salt water-ready) smolt very quickly (within 1-3 months; Groot and Margolis, 1991). Very young chum salmon (0.4-0.7 g in weight) showed ubiquitous labeling of single cones with the UV riboprobe (Fig. 2.19b, 2.9a). However, when the fish reached ~0.7 g in weight, single cones in the lower retina started transforming phenotype from UV to blue. These transition cones showed either a decrease in UV visual pigment absorbance from outer segment tip to base (Fig. 2.19a), or absorbance of blue and UV visual pigment at the tip with single blue absorbance at the base (Fig. 2.19c). By the time the fish reached 2 g in
weight, the majority of single cones in the ventral retina were blue-type (Fig. 2.19e). Cones undergoing the transformation showed less intense labeling (Fig. 2.19d) to no labeling (Fig. 2.19f) with the UV riboprobe in accordance with the lower UV absorbances measured by MSP.

Additional measurements on the retinas of the other salmon species revealed single cones with absorbance characteristics analogous to those measured in chum salmon (Fig. 2.20). In coho and pink salmon, cones were found with primarily UV visual pigment at the tip of the outer segment and blue visual pigment at the base (Fig. 2.13, Fig. 2.20a). Other cones had a progressive decline in UV absorbance from tip to base without any sign of blue opsin absorbance at the base (Fig. 2.20b,c). In Atlantic salmon, cones were found with increased blue absorbance from tip to base (Fig. 2.20d). In chinook salmon, we found single cones that showed blue absorbance at the base and a mixture of UV and blue absorbance at the tip. Average traces from one such cone are presented in Fig. 2.21. Upon exposure of the tip to 440 nm light for 2 minutes, the blue peak disappeared leaving the UV peak. Further bleaching for 2 minutes with 365 nm light reduced the UV peak significantly and created a new maximum around 465 nm (which is indicative of a photoproduct as reported in previous studies, Hárosi and Hashimoto, 1983; Loew et al., 1996). Most of the transition cones that did not show any trace of blue absorbance were smaller than those that showed some dual mixture of visual pigments; these cones may therefore be the corner cones since, in salmonid fishes, they are usually smaller than the center cones (Bowmaker and Kunz, 1987; Beaudet et al., 1997; Novales Flamarique, 2005).
Absorbance measurements from double cones and rods in fish undergoing the single cone transformation did not reveal the presence of multiple visual pigments within a photoreceptor (Fig. 2.22). For any of these photoreceptors, the absorbance curves obtained at different levels of the outer segment were similar. The only trend was a slight decrease in absorbance from base to tip.

2.4 Discussion

2.4.1 Phenotypic transformation and corner cone loss in the retina of salmonid fishes

Salmonid fishes of the genus *Oncorhynchus* (whether salmon or trout) hatch with a full complement of UV cones. Following a transformation period during which most single cones undergo UV opsin down-regulation, the majority of single cones retain a single blue opsin. The transformation event appears to be closely related to the loss of corner cones since both events overlap in retinal surface area and both progress from ventral to dorsal retina. In the Atlantic salmon, there is segregation of UV and blue visual pigment into corner and center cones at a very young age (see also Forsell et al., 2001). Some cones, however, have absorbance profiles that indicate the co-expression of UV and blue opsins. This observation, and the early loss of corner cones in the young alevin (see Novales Flamarique, 2002), suggests that the transformation, if it occurs, may be taking place during embryonic development. Alternatively, the transformation may not occur at all in Atlantic salmon, as may be the case in other animals that express multiple opsins within a cone type (e.g. the tiger salamander, Makino and Dodd, 1996). Whatever the differences in the timing and extent of both events between salmonid species, the
general trend is one of eradication of corner cones from the ventral retina and restricted UV cone presence in the dorsal retina.

Among the *Oncorhynchus* species, the loss of single cones was fastest for pink and chum salmon, two species that migrate to coastal waters within 1-3 months of hatching (~0.3-0.6 g in weight, Groot and Margolis, 1991). Yolk-sac absorption in these species is very fast and the fish are silver coloured (a sign of smoltification) when entering coastal waters at this very young age (Hoar, 1988). The transformation in these species started at ~0.6-0.8 g in weight. In chinook and coho salmon, the transformation started later (weight ≥ 1 g), especially in the coho salmon. The slowest onset of the transformation occurred in the rainbow trout, where corner cone loss from the ventral retina was apparent at >3 g. Thus, it appears that the transformation and loss of corner cones starts later for salmonids with longer freshwater residence times. At present, we cannot explain the accelerated onset of corner cone loss (and potential transformation) in the Atlantic salmon, since fresh water residence time for wild salmon of this species is at least 1 year. Atlantic salmon commonly used in research, however, is of farm origin (as in this study). It is well known that the neuroendocrine system of farmed fish is altered by selective breeding practices and artificial rearing conditions to promote fast growth. Since hormones (e.g. thyroid hormone) are likely involved in the signaling pathway that commands the onset of transformation and corner cone loss, and since the levels of this hormone vary with rearing conditions, it may not be surprising that farmed Atlantic salmon may exhibit an accelerated onset of both events. In the Pacific salmon species studied, the onset of these events appears to occur after the time of full yolk-sac
absorption, an event that is preceded by a spike in blood thyroid hormone (Greenblatt et al., 1989).

Several studies have shown that UV sensitivity diminishes in juvenile salmonid fishes as they grow (e.g. Hawryshyn et al., 1989; Beaudet et al., 1993; Novales Flamarique, 2000). Until recently, this loss of UV sensitivity had been associated with a loss of corner cones (Beaudet et al., 1993; Novales Flamarique, 2000; Allison et al., 2003), or alternatively, for the rainbow trout, with apoptosis of corner cones in the peripheral retina coupled to retinal growth (Novales Flamarique, 2001). Our results show that the UV-blue transformation is the primary event leading to diminished UV sensitivity. In some studies, the loss of UV sensitivity has been associated with a progressive shift in the peak of the behaviourally-measured spectral sensitivity curve under short wavelength isolation conditions from 360 nm to 390 nm to 430 nm (Hawryshyn et al., 1989; Browman and Hawryshyn, 1992). The animals used in these studies were parr (~ 8 g) to large smolts (> 30 g), and originated from different populations, including non-wild stocks. According to our observations, the majority of these animals would have been at some intermediate stage in the UV-blue transformation. Thus, a shift in the behaviourally-measured spectral sensitivity curve would be expected from the transitional cone stages described in this study. Previous studies of UV sensitivity in rainbow trout suffer from inconsistent topographical delivery of the light stimulus on the retina (see Novales Flamarique, 2001), hence it is impossible to ascertain from these studies when the loss of UV sensitivity begins exactly, and to compare with the timing of onset of the UV-blue transformation.
Our finding of a UV-to-blue transformation in the single cones of rainbow trout has not been reported by previous investigators (Allison et al., 2003). There may be two reasons for this. First, fish examined in previous studies were large (> 7 g) compared to the young fish (1-3 g) that show the beginning of the single cone transformation. Second, our UV riboprobe, although 100% similar to that used by Allison et al. (2003), is shorter by ~ 250 bases. It may be that our UV riboprobe binds to other, yet unidentified, UV opsin transcripts.

2.4.2 Evidence for regulation of UV opsin production leading to change in opsin type or potential apoptosis

The observed decrease in UV visual pigment absorbance from outer segment tip towards the base in single cones during the time of transformation (Fig. 2.20, 2.21) is contrary to normal expectations. If a conical outer segment were uniformly packed with visual pigment, as might be assumed on the basis of measurements from various photoreceptor types (Hárosi, 1975), the peak absorbance measured from the side would increase from the tip towards the base (as observed for the remaining photoreceptor types, Fig. 2.22). This is because the pathlength equals the diameter at the place of measurement and, all other parameters being equal, absorbance is proportional to pathlength. Thus, absorbance should increase towards the base. The fact that we observed the opposite implies that the UV pigment was being depleted at the base. As normal renewal of visual pigment occurs by insertion of new opsin into the lipid bilayers at the base of an outer segment, which then slowly make their way towards the tip (Besharse, 1986), the pattern of distribution reflects the history of opsin production. The observed pattern is consistent with the UV opsin gene being down-regulated.
Cone outer segments differ from those of rods in that the lipid bilayers maintain continuity within the plasma membrane (as opposed to the disks in rods). Newly incorporated visual pigment may therefore diffuse throughout the outer segment. Several studies have suggested, however, that redistribution of visual pigment in the outer segment occurs slowly and that the edge region of the cone lipid bilayer presents a significant barrier to axial redistribution (see Besharse, 1986). Our results support these hypotheses in that we were able to measure gradients of visual pigment co-expression within the outer segment. This indicates that other processes besides diffusion must control axial distribution of visual pigment.

The two types of absorbance gradients observed in the outer segments of single cones during the time of transformation suggest different cell fates. In one case, UV opsin is progressively displaced by blue opsin, thereby transforming the cone from UV to blue phenotype. In the other case, the UV opsin is not replaced by blue opsin; instead, the outer segment is being depleted of opsin altogether. The latter observation suggests that the cone is undergoing apoptosis (programmed cell death), which encompasses the termination of gene activity. The situation may be somewhat similar to the decreased opsin production in rhodopsin knock-out mice leading to apoptosis of rod photoreceptors (Humphries et al., 1997; Lem et al., 1999). Our in situ hybridization and histological studies show that the transformation event is followed very closely by (or is concurrent to) the loss of corner cones (see Kunz et al. 1994 for histological evidence indicating that this corner cone loss is due to apoptosis). It is therefore likely that cones in which UV opsin is undergoing down-regulation without a concomitant increase in blue opsin
production are indeed undergoing apoptosis. Their overall smaller size further suggests that these single cones are the corner cones of the square mosaic.

2.4.3 Double cones and rods do not undergo opsin changeover in juvenile salmonids

All measurements of visual pigment absorbance from double cones and rods revealed the same opsin within a given photoreceptor type (Fig. 2.22, Table 2.3). Small variations in $\lambda_{\text{max}}$ of the visual pigments could be attributed to alterations in chromophore content (vitamin A$_1$/A$_2$ ratio; Bridges, 1972; Hárosi, 1994). Such changes are known to occur in salmonid retinas due primarily to variations in water temperature affecting the endocrine system (Tsin and Beatty, 1977; Alexander et al., 1994; Novales Flamarique, 2005).

2.4.4 UV opsin precedes blue opsin in salmonid retinal development

The delayed occurrence of blue opsin in the retina of salmonid fishes is not consistent with the cascade of opsin expression reported for goldfish (Stenkamp et al., 1996, 1997) and with similar early findings from zebrafish (e.g. Raymond et al., 1995). In these species, the order of cone opsin appearance was reported to be: red, green, blue, and UV. There are now several indications, however, that this sequence is not correct for zebrafish [Takechi and Kawamura (2005) have shown that blue opsin is expressed last in zebrafish], and that it is not universal. In cichlids, for instance, MSP measurements of single cones from juvenile fish have revealed only UV visual pigment (Carleton et al., 2000), suggesting that cichlids also express UV opsin prior to blue opsin. In flatfishes, where development involves a pronounced metamorphosis (including the appearance of paired cones and the re-organization of the cone mosaic, Evans and Fernald, 1993), larval
forms have predominantly green sensitive cones, while red cones account for less than 10% of the cone population (Evans et al., 1993; Helvik et al., 2001; Forsell et al., 2001). This indicates that it is the green opsin and not the red that appears first in flatfishes. In rodents and in primates, including humans, the order of cone opsin appearance is: UV (or blue, for species lacking a UV opsin gene), green (for rodents), and green/red at about the same time for humans (Szél et al., 1993; Szél et al., 1994; Bumsted et al., 1997; Cornish et al., 2004). The early appearance of the UV cone opsin in salmonid fishes is consistent with this sequence.

2.4.5 Co-expression of visual pigments and opsin changeover in other vertebrates

Microspectrophotometric and DNA sequencing studies on the rod photoreceptors of the European eel, *Anguilla anguilla*, have indicated a changeover in rhodopsin from one maximally sensitive to 501 nm to one peaking at 482 nm (Wood et al., 1982; Wood and Partridge, 1993; Archer et al., 1995). Similar changes in rhodopsin have also been suggested for several deep water fishes (Partridge et al., 1992). Other studies that have compared cone opsin gene sequences or visual pigment absorbance from a variety of juvenile fishes also suggest differential expression of visual pigments within a cone type (Shand et al., 1988; Shand, 1993; Carleton and Kocher, 2001; Britt et al., 2001; Shand et al., 2002; Loew et al., 2002). It is therefore likely that changes in cone opsin type similar to the one described here for salmonid fishes are widespread among this group of vertebrates. In addition, the co-expression of opsins that occurs in humans (Xiao and Hendrickson, 2000; Cornish et al., 2004), in rodents (Röhlich et al., 1994; Applebury et al., 2000; Lukáts et al., 2002; Parry and Bowmaker, 2002), in the salamander (Makino and Dodd, 1996) and, perhaps, in the gecko (Loew et al., 1996), suggests that such cone
transformations are common in mammals, amphibians and reptiles. As in rodents
(Röhlich et al., 1994; Applebury et al., 2000; Lukáts et al., 2002; Parry and Bowmaker,
2002), UV expression appears to be the main pathway for all single cones in young
salmonid fishes. In the mouse and the guinea pig (Applebury et al., 2000; Parry and
Bowmaker, 2002), co-expression of UV/green opsins persists in the adult retina resulting
in a spatial gradient whereby the upper retina has relatively more green visual pigment
and the lower retina contains relatively more UV visual pigment, with a transitional zone
of higher co-expression of both pigments near the central retina. In salmonids, co-
expression of UV/blue opsins does not persist in the adult retina but the transformation
results in a centro-dorsal spatial gradient of increasing UV pigment bearing cells.

2.4.6 Determinants of corner cone loss and functional role(s) associated with the
UV cone

The loss of corner cones in salmonid fishes has traditionally been associated with
the process of smoltification (Hawryshyn et al., 1989; Beaudet et al., 1993; Deutschlander
et al., 2001; Allison et al., 2003). Treatment with thyroid hormone is believed to induce
the loss of corner (UV) cones and related UV sensitivity in rainbow trout (Browman and
Hawryshyn, 1992; Deutschlander et al., 2001). However, all these studies used fish that
were undergoing the UV-blue opsin changeover (weight > 7 g), a confounding factor that
compounds problems due to inconsistencies in stimulus delivery and insufficient
resolution of the histology (see Novales Flamarique, 2001). The present results dispute
the association between corner cone loss and the smoltification process. Pink and chum
salmon enter saltwater without parr marks (an indication of smoltification; Hoar, 1988)
but with a full complement of corner cones. By contrast, Atlantic salmon lose the
majority of corner cones and UV opsin at the alevin stage, at a size ~ 30 times smaller than that at which smoltification occurs in this species (Clarke et al., 1996). Clearly, smoltification is not associated with the loss of corner cones in these three fishes. In the rainbow trout, the loss of corner cones begins at the advanced alevin stage (see Novales Flamarique, 2001), at a time when the fish has not started the smoltification process either.

The timing of the transformation and loss of corner cones also questions the importance of UV cones in the behaviour/ecology of salmonid fishes. Ultraviolet sensitivity mediated by UV cones is thought to improve contrast enhancement of zooplankton prey (Loew et al., 1993; Browman et al., 1994), and to aid in the detection of the polarization of light for prey contrast enhancement (Novales Flamarique and Browman, 2001) and for orientation (Hawryshyn et al., 1990). The present observations do not support these functions. For instance, the Atlantic salmon loses its UV cones at a very early age, when the fish has barely absorbed its yolk sac and started to feed on zooplankton. Similarly, the transformation occurs in the early alevin stage of pink, chum, and chinook salmon, at a time when the fish are dependent on zooplankton for their sole food source. Young salmon feed by attacking prey from below, thereby forming an image in the ventral retina. If UV cones were important for detecting prey, then one would expect the transformation and apoptosis events to proceed from the dorsal to the ventral retina, leaving UV cones in the ventral retina where the image is formed and where cone density (and hence visual acuity) is high. The opposite is in fact the case. Zooplankton images taken under different spectral backgrounds show that contrast in the blue wavelengths rivals, or is better than, that in the UV (Novales Flamarique and
Hence, the changeover to a blue opsin and pruning of corner cones may be ecologically and energetically favourable. The latest behavioural studies also dispute the role of UV sensitivity in the feeding performance of juvenile rainbow trout (Rocco et al., 2002).

The involvement of UV cones in polarization sensitivity of salmonid fishes is similarly questionable. Besides the illumination and recording problems that plagued previous studies claiming polarization sensitivity/orientation in salmonid fishes (see Horváth and Varjú, 2004; Novales Flamarique, 2001), the restricted distribution of UV cones to the dorsal retina of juvenile salmonids at the time of migration to the open ocean would make the UV component of the celestial polarization signal hard to detect. And yet, it is this celestial polarization signal mediated by UV cones that has been argued to account for salmonid orientation (Hawryshyn et al., 1990; Parkyn et al., 2003). The present results add to a mounting body of evidence that fails to support a role for UV cones in polarization sensitivity of salmonid fishes. The sensory capability itself has not been conclusively established in these animals (see Horváth and Varjú, 2004).

In contrast to the traditional roles postulated for UV cones in past studies, our results suggest a novel function for UV cones in shaping the chromatic organization of the retina. This hypothesis is supported by the following observations: (1) UV opsin is one of the first opsins expressed during salmonid retinal development (unpublished results), (2) UV opsin is confined to single cones, which form a regular mosaic pattern throughout the retina, and (3) down-regulation of UV opsin begins when the animal starts an active life style (suggesting that this opsin is no longer needed after development).
Future investigations that block UV opsin expression in the retina of the embryo should reveal any role for the UV cone during development.

2.5 Tables

Table 2.1: Range of total lengths and weights for the salmonid species examined

Higher sampling was carried out in the 0.4-3 g range, as this is when the single cone transformation commenced (see text). (n = 15 per stage)

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Total length (cm)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coho salmon</td>
<td>alevin-to-parr</td>
<td>1.2-7.3</td>
<td>0.17-3.5</td>
</tr>
<tr>
<td></td>
<td>parr-to-smolt</td>
<td>7.1-14.2</td>
<td>3.6-19.8</td>
</tr>
<tr>
<td>Chum salmon</td>
<td>alevin-to-parr</td>
<td>4.1-5.8</td>
<td>0.34-1.1</td>
</tr>
<tr>
<td></td>
<td>parr-to-smolt</td>
<td>6.0-15.4</td>
<td>1.2-19.2</td>
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<td>Chinook salmon</td>
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<td>3.2-7.6</td>
<td>0.26-2.8</td>
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<td></td>
<td>smolt</td>
<td>13.8-28.4</td>
<td>14.0-33.5</td>
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<td>Pink salmon</td>
<td>alevin-to-parr</td>
<td>3.1-6.6</td>
<td>0.19-1.6</td>
</tr>
<tr>
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<tr>
<td></td>
<td>parr-to-smolt</td>
<td>6.0-12.6</td>
<td>6.5-20.3</td>
</tr>
<tr>
<td>Rainbow/ Steelhead trout</td>
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<td>3.2-9.6</td>
<td>0.21-10.4</td>
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Table 2.2: Average ratio of double to single cone (d/s) and density

Average ratio of double to single cone (d/s) and density for each retinal location examined per species and developmental stage (see Fig. 5,8,10,11,13; n = 5 per location). Density is that of double cones (per mm²); d/s is the ratio of double to single cones. Abbreviations are as follows: a/p, alevin-to-parr (weight range: 0.4-0.7 g for coho, chum, and Atlantic salmon, and 1.3-1.9 g for pink and chinook salmon); p/s, parr-to-smolt (weight range: 5-7 g); s, smolt (> 1 year old, weight range: 9-13 g for coho, 12-15 g for chum, and 16-20 g for chinook); Atlantic, Atlantic salmon.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Location</th>
<th>D/S (± SD)</th>
<th>Density (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coho</td>
<td>a/p</td>
<td>1</td>
<td>1.03 (0.050)</td>
<td>11825 (201)</td>
</tr>
<tr>
<td>Coho</td>
<td>a/p</td>
<td>2</td>
<td>1.19 (0.37)</td>
<td>10886 (2122)</td>
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<tr>
<td>Coho</td>
<td>a/p</td>
<td>3</td>
<td>1.06 (0.16)</td>
<td>7197 (737)</td>
</tr>
<tr>
<td>Coho</td>
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Table 2.3: Statistics of the visual pigments in the five species of salmon studied.

Error measures are standard deviations of the means.

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2.6 Figures and Figure Legends

Figure 2.1: Micrographs of sections from light-adapted and dark-adapted retina of alevin coho salmon (fish weight: 0.52 g, total length: 4.1 cm). (a) The light-adapted retina shows a square mosaic in tangential sections. The square mosaic consists of double cones (d) that make the sides of the unit square, and single cones located at the centre (black arrowhead) and at the corners of the square (asterix). At the ellipsoid level, the cross sections of double cones are elliptical while those of single cones are circular. A common partition separates the two members of a double cone (white arrowhead). (b) Radial section through the light-adapted retina shows a stratified photoreceptor layer whereby cones lie vitreal to rod outer segments (r), and the latter are embedded within the retinal pigment epithelium (rpe). (c) In contrast, the dark-adapted retina does not exhibit a recognizable cone mosaic: rod outer segments are found scattered amid the cone inner segments (which stain lighter). (d) Radial section through the dark-adapted retina showing the mixture of cones and rods resulting from photoreceptor migration. The rpe becomes detached from the photoreceptor layer in the dark-adapted retina and commonly falls off during retinal removal from the eyecup. Magnification bar (in a) = 27 μm holds for all panels.
Figure 2.2: Cone mosaic formations in the retina of the coho smolt

Cone mosaic formations in the light-adapted retina of the coho smolt (fish weight: 13.5 g, total length: 11.7 cm). (a, b) Tangential section from the dorso-temporal retina (a) and from the central retina (b) showing a mosaic complete with corner cones. (c) Tangential section from the ventral retina showing a square mosaic without corner cones (white arrows). In salmonid fishes, cones are largest in the central retina, smaller in the upper half of the dorsal retina and smallest in the lower half of the ventral retina. Magnification bar (in a) = 27 μm holds for all panels. Nomenclature and symbols as in Fig. 2.1.
Figure 2.3: Sequence alignment of salmon UV and the blue opsin riboprobes

Analysis using ClustalW revealed a 47% identity between the UV and blue opsin riboprobes. An asterix (*) indicates the nucleotides shared by both sequences.
Figure 2.4: Sequence alignment of the UV opsin riboprobe and the coho blue opsin mRNA (GenBank accession #AY214149)
Figure 2.5: Sequence alignment of the blue opsin riboprobe and the coho UV opsin mRNA (GenBank accession #AY214148)
Figure 2.6: Micrographs of sections from dark-adapted coho retinas and absorbance spectra of single cones

(a-d) Tangential (a,b) and radial (c,d) sections show that, in the central retina of the alevin (weight: 0.35 g, total length: 3.4 cm), all single cones are labeled by the UV riboprobe (white arrows, a,c) while none label with the blue riboprobe (dark arrowheads, b,d). In accordance with these results, all single cones recorded by MSP possessed a UV visual pigment with $\lambda_{\text{max}} \approx 368$ nm ($n = 7$). (e-h) Tangential sections from the retina of the smolt (weight: 12.1 g, total length: 12.5 cm) show that the distal dorso-temporal retina has a mixture of UV (white arrows, e) and blue (black arrows, f) cones with a predominance of UV cones. Accordingly, MSP recordings from six single cones in this region of the retina resulted in four having UV visual pigment. In the ventral retina, however, no label with the UV riboprobe was observed (black arrowheads, g) but all single cones labeled with the blue riboprobe (black arrows, h). MSP recordings from this part of the retina resulted in single cones containing blue visual pigment ($\lambda_{\text{max}} \approx 434$ nm) exclusively ($n = 7$). Magnification bar (in a) = 16 µm, holds for (a-f), and = 27 µm for (g,h).
Micrographs of sections from light-adapted retina of the coho salmon (weight: 0.31 g, total length: 3.6 cm) (a-f) and smolt (weight: 12 g, total length: 12.7 cm) (g-j). (a,b) Tangential sections from the central retina show a square mosaic in which all single cones label with the UV riboprobe (a) and none label with the blue riboprobe (b). (c,d) Radial sections show that the UV label is restricted to the myoid region of the single cones (c), while the blue riboprobe fails to produce any labeling (d). In the light-adapted retina, double cones (d) are displaced toward the retinal pigment epithelium (rpe) with respect to single cones (see also Fig. 2.1 b). (e,f) As the alevin grows in size (weight ~ 1-3 g), the ventral retina shows a mixture of UV (e) and blue (f) labeled cones. (g-j) Tangential (g,h) and radial (i,j) sections from the centro-ventral retina of the coho smolt show that single cones do not label with the UV riboprobe (g,i) but instead label with the blue riboprobe (h,j). At this stage, most of the corner cones are missing but those that remain (asterix) also label with the blue riboprobe in this region of the retina. (k,l) In the centro-dorsal retina, UV-labeled cones are rare; nonetheless, they can be corner (k) or centre (l) cones. As in Fig. 2.6, white arrowheads indicate double cone partitions; white arrows and black arrows are UV-labeled and blue-labeled single cones, respectively; and non-labeled single cones are indicated by black arrowheads. Magnification bar (in a) = 16 μm holds for (a-j), and = 27 μm for (k,l).
Retinal maps of cone distributions in the retina of coho salmon

Retinal maps of cone distributions in the retina of coho salmon at the alevin-to-parr stage (a,c) and at the smolt stage (b,d) (n=5 per map). Top maps (a,b) show the distribution of double to single cone (d/s) ratio in the retina of the alevin-to-parr and in the smolt, respectively. Bottom maps (c,d) are corresponding maps to (a,b) but illustrating the percentage of single cones that express UV opsin. See legends for d/s ratio and percentage categories. The average d/s ratio and double cone (d) density for each location (filled circle) shown on the maps is given in Table 2.2. Within a given retinal map, a larger circle indicates that the average double cone density for that location was at least 1 standard deviation 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. The approximate area of the alevin-to-parr retina (a,c) is indicated by the inner circle within the smolt retina in (d),(n=5 per map) D, dorsal, N, nasal. Magnification bar = 0.15-0.2 cm (a,c) and 0.5-0.6 cm (b,d).
Figure 2.9: Micrographs of sections from the retina of chum salmon

(a, b) The retina of the alevin (weight: 0.52 g, total length: 4.4 cm) shows a square mosaic where all single cones label with the UV riboprobe (a) and none label with the blue riboprobe (b). (c,d) In the smolt (weight: 14 g, total length: 17.8 cm), the majority of corner cones are missing and the remaining (centre) cones do not label with the UV riboprobe (c) but label with the blue riboprobe (d). (e,f) In radial sections of the smolt retina, the single cones, located vitreal to the double cones, fail to label with the UV riboprobe (e) but label with the blue riboprobe (f). Symbols and nomenclature as in Fig. 2.7. Magnification bar (in a) = 16 µm holds for all panels.
Figure 2.10: Retinal maps of cone distributions in the retina of chum salmon

Retinal maps of cone distributions in the retina of chum salmon at the alevin-to-parr stage (a,c) and at the smolt stage (b,d). Same presentation and nomenclature as in Fig. 2.8. Magnification bar = 0.15-0.2 cm (a,c) and 0.6-0.7 cm (b,d).
Figure 2.11: Micrographs of retinal sections from chinook salmon

(a-d) Tangential (a,b) and radial (c,d) sections from the retina of the alevin (weight: 0.27g, total length: 3.5 cm) show that all single cones label with the UV riboprobe (a,c) and none label with the blue riboprobe (b,d). (e,f) In contrast, the ventral retina of the late parr/young smolt (weight: 4 g, total length: 7.8 cm) shows a full square mosaic with corner cones, but none of the single cones label with the UV riboprobe (e), and all label with the blue riboprobe (f). (g,h) Tangential sections from the central retina in the young smolt show that some corner cones remained labelled with the UV riboprobe (g) while most label with the blue riboprobe (h). Symbols and nomenclature as in Fig. 2.7. Magnification bar (in a) = 16 μm, holds for all panels.
Figure 2.12: Retinal maps of cone distributions in the retina of chinook salmon

Retinal maps of cone distributions in the retina of chinook salmon at the late alevin-to-parr stage (a,c) and at the smolt stage (b,d). Same presentation and nomenclature as in Fig. 2.8. Magnification bar = 0.12-0.25 cm (a,c) and 0.7-0.8 cm (b,d).
Figure 2.13: Absorbance measurements and double in situ labeling of single cones in Pacific pink salmon with the UV- and blue riboprobes

(a) Cones show increased absorbance in the ultraviolet at the tip of the outer segment (right) and increased absorbance of blue light at the base, with intermediate absorbance in between (traces are composite averages from seven cones). (b) Tangential retinal section showing single cones labelled by in situ hybridization with probes specific for mRNAs encoding UV-opsin (shown in blue) or blue-opsin (in red); cones coexpressing both mRNAs appear purple (arrows). (c, d) Single cones are labelled with only the UV riboprobe before the switch in opsin expression (c), and with only the blue riboprobe afterwards (d). Magnification bar = 5.5 μm (b), 7.8 μm (c) and 13 μm (d). Source: Cheng, C.L., and Novales Flamarique, I., 2004. (Reproduced by permission).
Figure 2.14: Retinal maps of cone distributions in the retina of pink salmon

Retinal maps of cone distributions in the retina of pink salmon at the alevin-to-parr stage (a,b). Same presentation and nomenclature as in Fig. 2.8. Magnification bar = 0.14-0.26 cm.
(a,b) Tangential sections from the yolk-sac alevin (weight: 0.24 g, total length: 3.2 cm) show a complete square mosaic in which corner cones label with the UV riboprobe (a) and centre cones label with the blue riboprobe (b). (c) Radial section showing that the UV riboprobe label is restricted to single cones. (d) In the young fish (weight: 1.1 g, total length: 5.1 cm), the distal dorsal retina retains some corner cones and these are labelled by the UV riboprobe. (e,f) In the majority of the retina, however, corner cones at this young stage have disappeared and the remaining (center) cones do not label with the UV riboprobe (e) but label with the blue riboprobe (f). Symbols and nomenclature as in Fig. 2.7. Magnification bar (in a) = 16 µm, holds for all panels.
Retinal maps of cone distributions in the retina of Atlantic salmon at the alevin-to-parr stage (a,c) and at the parr-to-smolt stage (b,d). Same presentation and nomenclature as in Fig. 2.8. Magnification bar = 0.12-0.17 cm (a,c) and 0.3-0.34 cm (b,d).
Figure 2.17: Micrographs of retinal sections from the rainbow/steelhead trout

(a,b) Tangential sections from the central retina of the alevin (weight: 1.6 g, total length: 5.1 cm) show a complete mosaic where all single cones label with the UV riboprobe (a) and none label with the blue riboprobe (b). (c,d) As the fish grows (weight ~ 2-3 g), the distal ventral retina shows some loss of corner cones among the UV cone population (c) and the blue riboprobe begins to label some single cones (black arrowhead, d). (e,f) Further growth sees the loss of UV label extend to single cones in the centro-ventral retina (e, black arrowhead) with a concomitant increase in blue labeling (f). (g,h) At the advanced parr stage (weight: 7.8 g, total length: 8.1 cm), the centro-ventral retina shows a mosaic devoid of most corner cones, and the majority of the remaining single cones (whether at the centre or corner cone position) do not label with the UV riboprobe (g) but label, instead, with the blue riboprobe (h). Symbols and nomenclature as in Fig. 2.7. Magnification bar (in a) = 16 μm, holds for all panels.
Figure 2.18: Absorbance spectra of isolated photoreceptors in salmonid fishes

(a-c) Coho, (d-f) chum, (g-i) chinook, (j-l) pink, and (m-o) Atlantic salmon. Visual pigments are identified as follows: UV, ultraviolet; B, blue; G, green; R, red; rod, rhodopsin. Only spectra from cones containing a single visual pigment type were averaged (range of n = 6-21). The green and red visual pigments reside in the double cones (one visual pigment per double cone member). See Table 2.3 for visual pigment statistics.
Figure 2.19: Single cones undergoing UV opsin down-regulation

Visual pigment absorbance, and staining with the UV riboprobe, of single cones from alevin chum undergoing UV opsin down-regulation. These cones show reduced UV absorbance from outer segment tip to base (a, n=9; 0.02 O.D. units were added to successive traces, from base to tip for clarity), or UV-blue absorbance at the tip (arrow points to the shoulder in UV absorbance due to blue absorbance) with blue absorbance at the base (c, n=8; 0.01 O.D. units were added to the tip trace for clarity). Each trace is the average from several single cones (n) that exhibited a similar profile of absorbance along the outer segment. Transition cones also show less label (d) in comparison with single cones expressing only UV opsin (b). Following the transformation, single cones from the ventral retina have only blue visual pigment absorbance (e) and, thus, do not stain with the UV riboprobe (f, black arrowheads point to single cones). The average ± SD outer segment dimensions (in μm) of the cones whose average absorbance profile is shown in (a) were: 2.3 ± 0.16 (base), 2.0 ± 0.14 (tip), and 6.2 ± 0.16 (length); the corresponding dimensions for the cones in (c) were: 2.1 ± 0.17 (base), 1.8 ± 0.11 (tip), and 5.9 ± 0.21 (length). The cones whose absorbance is depicted in (e) had similar dimensions to those associated with (c). Abbreviations: a, accessory corner cone; c, centre cone. Other symbols and nomenclature as in Fig. 2.7. Magnification bar (in b) = 7 μm, holds for (d,f).
Figure 2.20: Visual pigment absorbance of single cones undergoing UV opsin down-regulation.

Each trace is the average from several single cones (n) that exhibited a similar profile of absorbance along the outer segment. (a) Average absorbance from cones in coho salmon showing UV-blue absorbance at the tip, with blue absorbance at the base of the outer segment (n=7; 0.02 O.D. units were added to the tip trace for clarity). The average ± SD outer segment dimensions (in μm) of these cones were: 2.4 ± 0.27 (base), 1.9 ± 0.18 (tip), and 6.1 ± 0.32 (length). (b) Average absorbance from cones in coho salmon showing reduced UV absorbance from tip to base (n=7; 0.02 O.D. units were added to successive traces for clarity). The average ± SD outer segment dimensions (in μm) of these cones were: 2.3 ± 0.16 (base), 1.9 ± 0.10 (tip), and 6.0 ± 0.19 (length). (c) Average absorbance from cones in pink salmon showing reduced UV absorbance from tip to base (n=8; 0.02 O.D. units were added to successive traces for clarity). The average ± SD outer segment dimensions (in μm) of these cones were: 2.1 ± 0.15 (base), 1.8 ± 0.18 (tip), and 6.0 ± 0.24 (length). (d) Average absorbance from cones in Atlantic salmon showing a progressive increase in blue absorbance from tip to base (n=6; 0.02 O.D. units were added to the tip trace for clarity). The average ± SD outer segment dimensions (in μm) of these cones were: 2.0 ± 0.14 (base), 1.6 ± 0.09 (tip), and 5.8 ± 0.25 (length). Arrows in (a) and (d) point to shoulders in UV absorbance due to blue absorbance.
Figure 2.21: Average absorbance of a small single cone in alevin chinook salmon expressing two opsins, UV and blue

Each trace is the result of 3 spectral scans; 0.02 O.D. units were added to each successive trace for clarity. The outer segment dimensions (in µm) of this cone were: 1.9 (base), 1.6 (tip), and 5.6 (length). Initial measurements from the base of the cone outer segment revealed a single peak at 430 nm, indicative of blue opsin. Absorbance measurements from the tip showed two peaks at 367 nm and 431 nm, indicative of UV and blue opsins. Following a 2 minute bleach at 440 nm of the tip, the resulting average absorbance had a single peak at 366 nm. An additional 2 minute bleach at 365 nm resulted in the disappearance of the UV peak, and the suggestion of a 465 nm peak, which may be due to a long-lived photointermediate.
Figure 2.22: Average visual pigment absorbance from the double cones and the rod of chinook salmon undergoing the transformation.

Traces are the average of 3-5 photoreceptors; 0.02 O.D. units were added to each successive trace for clarity. Absorbance profile from base to tip of: (a) the green member of the double cone, (b) the red member of the double cone, (c) a small rod, and (d) a large rod. The outer segment dimensions (in µm) of the green member of the double cone were: 2.6 (base), 2.2 (tip), and 7.1 (length), and those of the red member were: 2.7 (base), 2.2 (tip), and 7 (length). The outer segment dimensions (in µm) of the large rod were: 3.5 (base), 2.5 (tip), and 36 (length), and those of the small rod were: 2.1 (base), 1.6 (tip), and 19 (length). Regardless of measurement location along the outer segment, all these photoreceptor types showed a single absorbance trace whose $\lambda_{max}$ was statistically similar between measurements within a photoreceptor type. In general, the absorbance decreased in magnitude from base to tip.
CHAPTER 3
THE ULTRAVIOLET OPSIN IS THE FIRST OPSIN EXPRESSED DURING RETINAL DEVELOPMENT OF SALMONID FISHES

Christiana L. Cheng, Kathlyn J. Gan, and Iñigo Novales Flamarique

Department of Biological Sciences
Simon Fraser University
8888 University Drive
Burnaby, British Columbia
V5A 1S6, Canada

3.1 Introduction

Our studies detailed in Chapter 2 have shown that the cones of salmonid fishes are organized in a square mosaic similar to that in goldfish (Stenkamp et al., 1997) and in most other fishes (Engström, 1963). In contrast to goldfish, zebrafish, and chick, the single cones of Pacific salmonid fishes undergo a switch in opsin from UV to blue (Chapter 2, Novales Flamarique, 2005). This switch to a longer wavelength absorbing opsin is analogous to that postulated for mammals (some rodents and human) where green and red cones are believed to arise from blue cones (Lukáts et al., 2005; Xiao and Hendrickson, 2000; Cornish et al., 2004). The common property of dual opsin cones shared by salmonid fishes and some mammals (Lukáts et al., 2005; Parry and Bowmaker, 2002; Xiao and Hendrickson, 2000; Applebury et al., 2000; Lukáts et al., 2002; Röhlich et al., 1994) raises the possibility that the order of opsin expression during development may be the same between these groups. If such were the case, the UV opsin of fish and violet/blue opsin of mammals (both in the SWS1 opsin gene family) (Yokoyama, 2000) may play important roles in the chromatic organization of the retina in these animals.

This study combined reverse transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization to determine the spatial and temporal progression of opsin appearance and the localization of opsins to specific morphological photoreceptor types during retinal development of salmonid fishes (genus *Oncorhynchus* and *Salmo*).

3.2 Materials and Methods

3.2.1 Animals

Wild stock chinook salmon (*Oncorhynchus tshawytscha*) were obtained from the Capilano River hatchery at various stages of embryonic development starting at the eyed
egg. Two sets of 10-20 embryos were collected at each sampling date for opsin transcript detection by reverse transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization.

Salmonid development, like that of other fishes, is primarily a function of water temperature. Developmental stage is therefore routinely characterized by the Accumulated Temperature Units (ATUs), which is the sum of the rearing water temperature, sampled daily, since fertilization. Results of this research are therefore expressed in relation to ATUs. The chinook salmon used were reared at 12 °C. At this temperature, the eye spot on the egg appeared at ~290 ATUs, hatching started at 432 ATUs (all 2 million eggs at the hatchery hatched within a period of 3 days: 432-456 ATUs), and the embryos had completely absorbed their yolk sac (corresponding, in nature, to the swim-up stage from the gravel as an alevin) by ~810 ATUs.

Wild stock coho (O. kisutch) and chum (O. keta) salmon were obtained from the Capilano and the Big Qualicum River hatcheries, respectively. These salmon were raised in river water which varied in temperature between 3.5 and 8.5 °C. Wild stock rainbow trout (O. mykiss) were obtained from the Fraser Valley Trout hatchery, and aquaculture stock Atlantic salmon (Salmo salar) were supplied by a hatchery owned by Omega Salmon Group Ltd. The rearing temperature at these two facilities was 7 °C. With the exception of coho salmon, the sampling regime for the other species was less rigorous than that for chinook salmon. Results from all species were compared to establish general trends in opsin development in this family of fishes.

Care and use of experimental animals was approved by the Animal Care Committee of Simon Fraser University, which follows the guidelines set by the Canadian
3.2.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

Embryos were decapitated and the heads immersed in RNA-later solution (Ambion) and stored at 4 °C. Several hours later, the eyes were separated from the heads and the lenses removed. The resulting eyecups were immersed in new RNA-later solution and stored at 4 °C. Opsin cDNAs were generated by RT-PCR amplification of DNAse-treated total RNA isolated from homogenized eyecups (RNAqueous-4PCR, Ambion). Reverse transcription was carried out at 42 °C for 15 min. Cycling parameters for the subsequent PCR were: 95 °C x 5 min, 32 cycles of 95 °C x 30 sec, 56 °C x 30 sec, and 72 °C x 1 min, and 1 cycle of 72 °C x 10 min. Primers used for RT-PCR were designed from published opsin sequences to span across introns to distinguish amplicons from DNA contamination. Reaction products were analyzed by electrophoresis on 1% agarose gels in 1 x Tris Borate EDTA (TBE) containing 0.5 µg/ml ethidium bromide, and photographed. The primers used were: UV opsin forward 5'- GGG CTT TGT GTT CTT TGC TG-3', reverse 5'- GGT ACT CCT CGT TGT TTG TG-3'; blue opsin forward 5'-ATG AAC ACA ATG AGG TCG AA-3', reverse 5'-TTA ACC AGC AGA AGA CAC TT; red opsin forward 5'- TGA GAG GAT GAC CAC TAT GA-3'; green opsin forward 5'- ATG CAG AAC GGC ACA GAA GG-3', reverse 5'-TTA TGC AGG GCC CGC AGA AG-3'; rod opsin forward 5'-CCC TTT CCA TCT CTC TTT CT-3', reverse 5'-CCA TGA GTG AGT ACG CCG CC-3'; and β-actin forward 5'-CCC ATG GAG CAC GGT ATC ATC AC-3', reverse 5'-GCG TGG GGC AGA GCG TAA CCT TC-3'.
3.2.3 Preparation of opsin riboprobes

Opsin partial cDNAs were generated by RT-PCR from total RNA isolated from eyecups as described in Chapter 2. The primers used were: UV opsin partial cDNA forward 5’- GGG CTT TGT GTT CTT TGC TG-3’, reverse 5’- GGT ACT CCT CGT TGT TTG TG-3’ (accession # AY214148, our probe corresponds to bases 111-574 of this sequence); blue opsin partial cDNA forward 5’- AAA CCT TGG TAG TGG GTA TAC TTT TCA ATG TCC TAC TGA TTA AAC GGA AAG ACA AGA CAA CAG AA-3’, reverse 5’- CAT AGA AGA TAG CAC TGC CC-3’ (accession # AF425075, our probe corresponds to bases 119-312 of this sequence); red opsin partial cDNA forward 5’- AGC AAG ACA AGA CAA CAG AA-3’, reverse 5’- TGA GAG GAT GAC CAC TAT GA-3’ (accession # AF425073, our probe corresponds to bases 33-273 of this sequence); green opsin partial cDNA forward 5’- AAA ATA GGC AAA AGG TTC AC-3’, reverse 5’- TAG ACG GCA AGA CAA TAG TA-3’ (accession # AF425076, our probe corresponds to bases 1-192 of this sequence); rod opsin partial cDNA forward 5’- CCC CCA TCT CTC ACT-3’, reverse 5’- CCA TGA GTG AGT ACG CCG CC-3’ (accession # AF425072, our probe corresponds to bases 8-184 of this sequence). The cloning of cDNAs and the fabrication of the cRNA probes were the same as described in Chapter 2.

3.2.4 In situ hybridization and quantification of opsin expression

Embryos were decapitated and the heads fixed overnight at 4 °C in a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS). The tissue was then rinsed in PBS (3 x 30 min), cryo-protected in sucrose solution (30% sucrose, 0.1 M PBS in O.C.T medium) overnight at 4 °C, and cryo-embedded in 100% O.C.T. medium (Cedar Lane Laboratories). The resulting blocks were cut in 5-7 μm steps to reveal: (1)
transverse sections of the head (coronal cuts of the brain) such that the nasal retina appeared first and the temporal retina last, or (2) longitudinal sections of the head (sagittal sections of the brain) such that the central retina appeared first and the cornea last. The cryosections were used for \textit{in situ} hybridization with the various opsin riboprobes as in Chapter 2.

Serial sections were collected cyclically on poly-L-lysine coated slides for \textit{in situ} hybridization treatments with each of the four cone opsin probes and the rod probe. These slides, along with positive and negative controls (made up of slides containing retinal sections from older fish), were processed simultaneously permitting comparison of results between probes. To elucidate the order of opsin appearance, absence or presence of label was noted for each probe and counts of labelled cells were conducted at early stages of opsin expression over a similar stretch of radially-oriented retina (20 µm length of photoreceptor layer). This stretch was chosen by careful examination of all sections and selecting consecutive sections that showed the highest numbers of labelled cells for each probe. This was possible because the site of initiation and expansion dynamics were similar for all early appearing opsins (see Results). Based on the dimensions of the eye at each stage analyzed, the number of sections required to cut it entirely, the extent of labelling on sections, and the presence of ocular landmarks (the embryonic fissure and the lens), we were able to deduce the approximate pattern of opsin expression during development.

3.2.5 \textbf{Immunocytochemistry}

Embryos were collected for immunocytochemical analysis using the mouse zpr-1 monoclonal antibody (Zebrafish Monoclonal Facility, University of Oregon, Eugene).
These samples were embedded in O.C.T. medium and sectioned as described previously. Following 3 washes for 5 min with 50 mM PBS, the cryosections were blocked with PBST (2% normal goat serum in PBS/0.3% Triton X-100) for 1 hr. These were then incubated with the zpr-1 antibody (1:100) in PBST for 2 hr at 4°C, washed in PBST, and incubated in goat anti-mouse IgG conjugated to FITC antibody (1:500) in PBST for 1 hr. After several washes with PBST, the slides were mounted and photographed with the E-600 Nikon microscope, equipped for fluorescence imaging.

3.2.6 Photoreceptor ultrastructure

The stages at which opsin mRNA was first detected by RT-PCR and in situ hybridization were analyzed for photoreceptor ultrastructure using a Hitachi H7600 Transmission Electron Microscope (TEM). Whole heads were fixed overnight at 4°C in primary fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.06 M PBS), followed by post-fixation in secondary fixative (1% osmium tetroxide in 0.06 M PBS) for 1 hr at 4°C. After a brief wash, the tissue was dehydrated through a series of solutions of increasing ethanol concentration, infiltrated with mixtures of propylene oxide and EPON resin and embedded in 100% EPON resin (Novales Flamarique and Hawryshyn, 1996). Blocks were cut with a Leica UC6 ultramicrotome to obtain 70 nm thick sections that were collected on 200 μm size grids. The grids were stained with 2% uranyl acetate followed by lead citrate, and the sections photographed using the TEM.
3.3 Results

3.3.1 Opsin expression by RT-PCR

In chinook salmon, expression of UV opsin mRNA by RT-PCR was first detected at 288 ATUs (2 of 8 amplifications). This partial transcript was 600 bases long and was likely the un-spliced version of the 464 base-long transcript first detected at 317 ATUs (Figs. 3.1, 3.2). From 317 ATUs onwards, all amplifications resulted in UV opsin product, with increased intensity of the band as a function of total ATUs (Fig. 3.1). At 326 ATUs, expression of green opsin transcript was clearly visible in half of the samples (4 of 8 amplifications). At 368 ATUs the green opsin band was more prominent than at 374 or 432 ATUs, even when the loaded sample volume was 3 times more for the latter two dates. Red opsin transcript first appeared at 336 ATUs (1 out of 8 amplifications) but only consistently at 368 ATUs (Fig. 3.1). Blue opsin transcript was the last to be expressed, around 432 ATUs (Fig. 3.1). Rhodopsin transcript was present at 317 ATUs, concomitant with the appearance of UV opsin transcript (Fig. 3.1). These results suggest the following sequence of opsin appearance: rod, UV, green, red and blue.

3.3.2 Expression of cone opsins by in situ hybridization

The sequence of cone opsin expression revealed by RT-PCR in chinook salmon was different from that revealed by in situ hybridization of opsin riboprobes on cryofixed retinas. In this case, expression of UV and red opsin mRNAs was first detected at 350 ATUs. Out of 20 embryos processed in the range 350-362 ATUs, 2 had retinas that labelled exclusively with the UV riboprobe while 7 labelled with both the UV and red riboprobes. A one way ANOVA performed on average cell counts over a 20 μm stretch of photoreceptor layer revealed that the number of UV cones (average ± SD: 1.2 ± 1.8)
was not statistically different from that of red cones (0.8 ± 1.3) (F1,39 = 0.689, p = 0.412).
In subsequent days, however, red cones became more numerous than UV cones; for instance, at 432 ATUs (Fig. 3.3), the average number of UV cones (9.8 ± 3.3) over a 20 μm stretch of photoreceptor layer was significantly lower than the number of red cones (13 ± 3.2) (F1,19 = 4.76, p = 0.043). At higher ATUs (> 1 week post-hatching), UV and red cones showed similar labelling intensity (Fig. 3.3). Labelling with the green riboprobe was first detected at 456 ATUs, with multiple cells clearly labelled at 468 ATUs (Fig. 3.3). Both the red and green riboprobes labelled one member of the double cone, forming a square mosaic pattern similar to that established by the UV cones (Figs. 3.3M-O). Blue opsin mRNA was first detected at 690 ATUs (Fig. 3.8A) and the label was restricted to single cones that occupied the centre position (Figs. 3.8B,C) in the square mosaic formation characteristic of cone photoreceptors in the salmonid retina (Novales Flamarique and Hawryshyn, 1996; Chapter 2). These labeling patterns are consistent with microspectrophotometric observations of visual pigment absorbance from the various morphological types of photoreceptors in the retina of salmonid fishes (Hawryshyn and Hárosi, 1994; Chapter 2), attesting to the specificity of the probes. The order of cone opsin appearance obtained by in situ hybridization was therefore: UV, red, green and blue.

3.3.3 Spatio-temporal dynamics of cone opsin expression

The first opsin mRNAs to appear (UV and red) were found within a small patch of cells located in the centro-temporal retina (Figs. 3.4A-C). That these cells were committed cone photoreceptors was verified by immunocytochemical labelling with the zpr-1 antibody (Figs. 3.4D,E). In salmonid fishes, we have found that this antibody labels
cone photoreceptors (Figs. 3.4F,G), as is the case in zebrafish and goldfish (though, in these species, it primarily labels double cones) (Wan and Stenkamp, 2000). At the time of first opsin mRNA detection by in situ hybridization, the cones had not developed outer segments yet, though the inner segments were clearly visible (Fig. 3.4H), which was not the case in retinas at the time of first opsin mRNA detection by RT-PCR (Fig. 3.4I).

With higher ATUs, expression proceeded towards the temporal and dorsal retina with little expansion towards the ventral retina. This pattern of expansion was visible in both transverse and longitudinal sections, as illustrated by UV and red riboprobe labelling (Figs. 3.5-3.7). At 468 ATUs, UV label increased from the nasal (Fig. 3.5A) to the temporal retina (Fig. 3.5I). In accordance with this pattern, longitudinal sections revealed strong labelling in the central and temporal retina with diminished to no labelling in the ventral and nasal retina (Fig. 3.6). The same pattern was observed for red opsin mRNA expression (Fig. 3.7). By 690 ATUs, UV and red opsin mRNAs were detectable in all parts of the retina except in the peripheral area ventral to the embryonic fissure. At this stage, green opsin mRNA was distributed over a large area of centro-temporal retina following similar expansion dynamics to that of the UV and red opsins (results not shown).

Blue labelling was first observed in the centro-temporal retina (Fig. 3.8, the same area where the mRNAs of other opsins first appeared). By 768 ATUs, blue label was present across the lower half of the ventral retina, nasal to the embryonic fissure (Fig. 3.8D). This labelling was opposite to that of the UV label (Fig. 3.8E), which remained present in the upper ¾ of the retina. The spatial progression of blue opsin mRNA expression was therefore different from that of the other cone opsins.
3.3.4 Rhodopsin expression

Rhodopsin mRNA expression was first detected in the centro-temporal retina (Figs. 3.9A,B) and it progressed temporally and dorsally (Figs. 3.9C-E), as per the majority of cone opsins. Localization of rhodopin mRNA was present in the budding inner segments at early stages of expression (Figs. 3.9A,B), and then became concentrated to small cell bodies located more vitreal and in between cone inner segments (Figs. 3.9F), in accordance with rod inner segment positioning in the light-adapted retina of salmonid fishes (Novales Flamarique and Hawryshyn, 1996; Chapter 2).

3.3.5 Other salmonid species

Figure 3.10 shows a summary of RT-PCR and in situ hybridization results for all the salmonid species studied. Regardless of the rate of development, several observations appear common. First, there is a delay of at least 1 day between detection of an opsin transcript by RT-PCR and corresponding detection by in situ hybridization. Second, the UV and red opsin mRNAs are always among the first transcripts to be detected by in situ hybridization. Third, detection of green opsin mRNA by in situ hybridization is much delayed compared to detection by RT-PCR; by the latter technique, green opsin mRNA is detected before or around the time of UV and red opsin mRNA expression. Fourth, except for the Atlantic salmon, blue opsin mRNA is detected last and, by in situ hybridization, it appears after hatching and closer to full yolk sac absorption (swim up, alevin, stage) or later. In contrast, by in situ hybridization, mRNAs for UV and red opsins appear up to 2 weeks before hatching, and those for rhodopsin and green opsin appear just prior to or around the time of hatching. We have previously shown that, unlike Pacific salmonids, the young Atlantic salmon shows blue cones shortly after
hatching (Chapter 2), in agreement with the early expression of this opsin reported here for this species.

3.4 Discussion

Both the RT-PCR and in situ hybridization results show that the UV and red opsins are expressed almost simultaneously during salmonid retinal development. Our detailed observations of chinook salmon embryos and results from rainbow trout and Atlantic salmon (see Fig. 3.10) further indicate that UV opsin expression precedes that of red opsin. The salmonid UV opsin belongs to the family of opsins that also encompasses the violet/blue opsins of mammals (the SWS1 opsin gene family) (Yokoyama, 2000). Red opsins from both groups of vertebrates also belong to the same (M/LWS) opsin gene family (Yokoyama, 2000). Hence, the sequence of cone opsin expression from SWS1 to M/LWS is the same for salmonids and mammals. Also as in mammals (Bumsted et al., 2004; Chen et al., 2005), rhodopsin expression in salmonid fishes appears to be delayed with respect to that of the first cone opsin (Fig. 3.10, in situ hybridization results). This occurs despite the presence of precocial rods that form a square mosaic around the single centre cones (Novales Flamarique and Hawryshyn, 1996). In cyprinid fishes (Raymond and Barthel, 2004; Stenkamp et al., 1996), precocial rods express the first opsin (rhodopsin) and form rows that lead the opsin expression front.

Another point in common between salmonid fishes and mammals is the presence of dual opsin expressing cones during retinal development. In salmonid fishes, the centre cones, which like the rest of the single cones express UV opsin at hatching (Fig. 3.3M) (Novales Flamarique, 2005; Chapter 2), progressively start changing over to blue opsin closer to the time of yolk sac absorption (Fig. 3.8; the salmonid blue opsin belongs to the
SWS2 opsin gene family) (Yokoyama, 2000). This switch originates in the same area where other opsin transcripts are first detected, and eventually reaches the dorsal retina. In mammals (rodents, primates), the first cones to appear express a SWS1 (violet/blue) opsin (Bumsted et al., 1997; Szél et al., 1994; Xiao and Hendrickson, 2000). Later on, dual opsin expressing cones are found, and it is believed that a sub-population of these gives rise to the longer wavelength (red and green opsin) cones by opsin switch (Lukáts et al., 2005; Szél et al., 1994; Xiao and Hendrickson, 2000; Röhlich et al., 1994). Co-expression of photoreceptor opsins has not been reported in goldfish (Stenkamp et al., 1996), zebrafish (Prabhudesai et al., 2005), or chick (Adler et al., 2001).

The origin of opsin expression and its spatial progression in the salmonid retina are different from those described for goldfish (Stenkamp et al., 1996) and zebrafish (Raymond et al., 1995; Raymond and Barthel, 2004). The origin of expression in the centro-temporal retina of salmonid fishes differs from the centro-ventral position described for goldfish (Stenkamp et al., 1996). With the exception of the blue opsin, expression of the other opsins in salmonid fishes then progresses towards the temporal and dorsal retina, in similar fan-shape dynamics as described for cyprinids (Raymond and Barthel, 2004). Expression of blue opsin in salmonids seems to follow a wave of single cone transformation (from UV-to-blue opsin expression) that starts in the centro-ventro-temporal retina and progresses in opposite direction to the other opsins. Differences in patterns of expression between opsins have also been observed in the chick retina (Bruhn and Cepko, 1996). In this animal, green and red opsins are first detected slightly nasal to the optic disc while violet and blue opsins are expressed in cell patches at this site and in the temporal retina. Expression then proceeds peripherally with a bias towards the
temporal retina. Rhodopsin, in the chick, is first detected surrounding the embryonic fissure in the centro-ventral retina and expression is then confined to the ventral retina (Bruhn and Cepko, 1996). The reasons for these differences in opsin dynamics within and between species are unknown.

Analogous discrepancies in opsin sequence and time of appearance to those reported in this study derived from RT-PCR vs. in situ hybridization have also been found in the chick (Adler et al., 2001). It has been suggested that inhibitory pathways, perhaps acting through the retinal pigment epithelium, maintain opsin expression at very low levels in the retina at early stages of development (Adler et al., 2001). Such opsin transcripts could be amplified by RT-PCR, but their importance to early photoreceptor differentiation is unknown. In salmonid fishes, there are no morphological signs of photoreceptor differentiation at the time when opsin messages are first detected by RT-PCR. The purpose for the up-regulation of green opsin message, observed at 368 ATUs (Fig. 3.2), is also puzzling. It is possible that such up-regulation is due to the transient expression of different opsin isoforms, as occurs during retinal development of the zebrafish (Takechi and Kawamura, 2005). The reason for such isoforms, which have very close absorbance profiles, is also unknown.
3.5 Figures and Figure Legends

Figure 3.1: Opsin and actin (control) transcripts detected by RT-PCR during retinal development of chinook salmon

Bands shown are representative of at least half of the amplifications for any given date. The gene families to which the various salmonid opsins belong to are noted on the right side of the figure.
Figure 3.2: Un-spliced version of the UV opsin transcript

The UV opsin RT-PCR product is aligned with the UV opsin gene, which was obtained by screening the Atlantic salmon genomic DNA library (see Chapter 6). The un-spliced version of the transcript, which has an intron intact, would be 590 bases in length. The differences in nucleotide sequence could be ascribed to expression of species-specific subtypes.
Figure 3.3: Micrographs of cryosections from the retina of chinook salmon labeled by in situ hybridization with one of the four cone opsin riboprobes (UV, red, green, or blue)

(A-D) At 374 ATUs, only UV and red opsin mRNAs are detected (black arrows point to labelled cells in all panels). (E-H) At 432 ATUs, the number of cells labelled with the red riboprobe is on average slightly higher than that labelled by the UV riboprobe (see text for details). (I-L) At 468 ATUs, a small number of green labelled cells are visible. (M-P) Oblique sections from embryos at 672 ATUs show that the UV riboprobe labels the single cones, while the green and red riboprobes label opposite members of the double cone pair (a white arrowhead points to the membrane partition that separates the two members of a double cone). At this stage of development there is still no labelling with the blue riboprobe. Abbreviations: rpe, retinal pigment epithelium. Magnification bar (in A) = 11 μm holds for all panels.
Figure 3.4: Micrographs of cryosections and EPON embedded sections from the retina of chinook salmon

(A) UV opsin expression starts around 374 ATUs in the centro-ventro-temporal retina (black rectangle). (B) Labelled UV cones (black arrows) within the black rectangle depicted in (A). (C) Labelled red cone (black arrow) within an analogous area to that in (A), photographed from the following (serial) section. (D-G) Labelling by fluorescence with the zpr-1 antibody in 374 ATU embryos (D,E) showing restricted label to the differentiating photoreceptor layer, and similar labelling in 10 g chinook smolts showing label of cone photoreceptors (F,G); d, double cone; s, single cone. Black and white arrows point to the same structures under bright field and fluorescence imaging, respectively. (H,I) Radial sections showing photoreceptor ultrastructure in embryos at 374 ATUs (H) and 317 ATUs (I). Arrows in (H) point to budding inner segments; n, nucleus. Magnification bar in (A) = 160 μm (A), 16 μm (B-G), 2.1 μm (H) and 2 μm (I). Retinal directions: C, central, and D, dorsal.
Figure 3.5: Transverse head cryosections from 468 ATU chinook salmon showing UV opsin labeling

(A,B) Nasal, (C,D) centro-nasal, (E,F) central, (G,H) centro-temporal, and (I,J) temporal regions of the retina. For each set of panels, the one on the right shows the area encompassed by the rectangle depicted in the adjacent left panel. White arrows point to the limits of labelling, black arrows point to individual labeled cells. Nomenclature as in Fig 3.3; l, lens; retinal directions in (A) apply to (A, C, E, G, I). Magnification bar (in A) = 160 μm, holds for panels (A, C, E, G, I), and is 16 μm for panels (B, D, F, H, J).
Figure 3.6: Longitudinal head cryosections from 468 ATU chinook salmon showing UV opsin labeling

(A,D) Central, (B,E) centro-temporal, and (C,F) temporal regions of the retina. The retinal area within the rectangle depicted in each of the upper panels is shown on the corresponding lower panel. Retinal directions: D, dorsal, N, nasal (in A) hold for (A-C); ef, embryonic fissure. Other nomenclature as in Fig 3.4. Magnification bar (in A) = 160 μm, holds for panels (A,B,C), and is 16 μm for panels (D,E,F).
Figure 3.7: Transverse and longitudinal head cryosections from 468 ATU chinook salmon showing red opsin labeling

(A,B) Nasal, (C,D) temporal, and (E,F) centro-temporal and centro-dorsal regions of the retina. For each set of panels, the one on the right shows the area encompassed by the rectangle depicted in the adjacent left panel. Nomenclature as in Figs 3.4 and 3.5; retinal directions in (A) apply to (C). Magnification bar (in A) = 160 μm, holds for panels (A,C,E), and is 16 μm for panels (B,D,F).
Figure 3.8: Chinook salmon at various ATUs showing blue and UV opsin labeling

(A,B) Radial sections showing increased labelling of single cones in the centro-ventro-temporal retina with incremental ATUs. (C) Tangential section showing that the blue label is restricted to the centre cones of the square mosaic. (D,E) Serial longitudinal sections showing labelling by the blue riboprobe in the ventral retina (D) and corresponding label by the UV riboprobe in the dorsal retina (E). ATUs are shown on the lower left corner of each panel. Nomenclature as in Figs 3.2, 3.4 and 3.5; retinal directions in (D) apply to (E). Magnification bar (in A) = 16 μm, holds for panels (A,B,C), and is 160 μm for panels (D,E).
Figure 3.9: Transverse and longitudinal head cryosections from chinook salmon showing rhodopsin labeling

(A,B) Initial site of label in the centro-temporal retina at 432 ATUs. (C,D) Enhanced labelling in the centro-temporal retina 3 days after first rhodopsin mRNA detection. For each set of panels in (A-D), the one on the right shows the area encompassed by the rectangle depicted in the adjacent left panel. (E) Longitudinal section showing that early rhodopsin label stretches along the centro-temporal retina. (F) Labelling of discrete cell inner segments located in between and vitreal to the double cone ellipsoids.

Nomenclature as in Figs 3.2, 3.4, 3.5 and 3.7; retinal directions in (A) apply to (C). Magnification bar (in A) = 160 μm, holds for (A,C,E), and is 16 μm for (B,D,F).
Figure 3.10: Onset of opsin expression

Approximate onset of expression, shown by arrows, for the various opsin mRNAs for each species of salmonid fish investigated. U, R, G, B, and r correspond to UV, red, green, blue and rod opsin mRNAs, respectively. Also shown are the ranges of ATUs at which main developmental stages [eyed egg, hatching into a yolk sac embryo (*), and full yolk sac absorption and swim-up as an alevin (**)] occurred for each species. Chum embryos at 411 and 438 ATUs (available previous samples to those reported) did not reveal any opsin mRNA expression by RT-PCR and in situ hybridization, respectively. Similarly, no opsin mRNA expression was observed by in situ hybridization in 425 ATU Atlantic salmon embryos. RT-PCR analysis was not carried out for Atlantic salmon.
CHAPTER 4
PHOTORECEPTOR DISTRIBUTION IN THE RETINA OF ADULT PACIFIC SALMON: CORNER CONES EXPRESS BLUE OPSIN

Christiana L. Cheng and Iñigo Novales Flamarique

Department of Biological Sciences
Simon Fraser University
8888 University Drive
Burnaby, British Columbia
V5A 1S6, Canada

4.1 Introduction

For many years it was believed that the loss of corner cones and associated UV sensitivity in salmonid fishes took place at smoltification, that this loss was complete, and that it was mediated by the thyroid hormone precursor, T4 (Hawryshyn et al., 1989; Browman and Hawryshyn, 1992). Since thyroid hormone has also been associated with sexual maturation of salmon (Sower and Schreck, 1982; Youngson and Webb, 1993), it was hypothesized that the corner cones present in the retina of adult fish were regenerated and expressed UV opsin (Beaudet et al., 1997). It is now known that corner cones are not completely lost from the juvenile retina and that this loss and the decrease in UV opsin is variable and starts before smoltification, at the young alevin stage (Chapter 2; see also diminished UV opsin transcript levels in the ventral vs. the dorsal retina of ~ 5 g rainbow trout, termed parr, Veldhoen et al., 2006). Whether the corner cones present in the adult retina of salmonid fishes express UV opsin and whether they are regenerated is unknown.

In this study, we investigated these unknowns using histological methods that involved cell density counts and in situ labeling with cRNA probes against the various opsin mRNAs in salmon retina. We also amplified opsin transcripts using reverse transcriptase polymerase chain reaction (RT-PCR) to compare with the in situ labeling results. If corner cones expressed UV opsin in the adult retina, then these cells would be labeled with our UV riboprobe. Alternatively, we expected corner cones to express blue opsin, as this is the only other opsin that has been found in the single cones of salmonid fishes (Novales Flamarique, 2005; Chapters 2 and 3).
4.2 Materials and Methods

4.2.1 Animals

Wild stock coho (*Oncorhynchus kisutch*), chum (*O. keta*), chinook (*O. tschawytscha*), and pink (*O. gorbuscha*) salmon spawners (i.e. adult, sexually mature, fish) were obtained from the Chilliwack and Capilano river systems (British Columbia, Canada). Eyes were collected from fish killed by blow to the head; for each eye, the iris, lens and some ocular fluid were removed on site and the resulting eye cup immersed in fixative. Left eyes from three individuals per species (plus three additional eyes from precocial chinook jacks) were immersed in primary fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.06 M phosphate buffer, pH = 7.4) for histological analysis while the corresponding right eyes were immersed in cryofixative (4% paraformaldehyde in 0.06 M phosphate buffer, pH = 7.4) for cryoembedding and *in situ* hybridization. Two coho were transported back live in oxygenated tanks to the laboratory for analyses using RT-PCR. The average fork length ± SD for the various fish groups were: 70 ± 2.4 cm (coho), 81 ± 2.6 cm (chum), 92 ± 3.2 cm (chinook), 60 ± 2.3 cm (chinook jack), and 72 ± 3.1 cm (pink). The chinook jack is a sexually mature chinook that returns to reproduce ~1-3 years before chinook spawners of the same cohort do. Because of the smaller size of the chinook jack retina compared to the “normal” spawner, we decided to group these retinas on their own. All animal experimentation procedures were approved by the Animal Care Committee of Simon Fraser University, which follows the guidelines set by the Canadian Council for Animal Care.
4.2.2 **Histology**

Fixed 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 14-22 pieces whose locations were mapped back onto the original retina by matching the composite projected image onto the original (see Beaudet et al., 1997; Novales Flamarique, 2001). The procedure for fixing and embedding retina pieces was the same as described in Chapter 2, as was the method for quantifying double and single cones.

4.2.3 **Preparation of opsin riboprobes and in situ hybridization**

Opsin riboprobes and the *in situ* procedure used were the same as detailed in Chapter 2. In the case of double labeling experiments, sections were incubated first with *anti-DIG Fab fragments conjugated to alkaline phosphatase (1:3000)* for 2 hours, and the DIG-labeled probes visualized using NBT-BCIP. The colour reaction was stopped by washing the sections in glycine-HCl (0.1 M, pH 2.2). To visualize the fluorescein-labeled probes, the sections were then incubated with *anti-fluorescein Fab fragments conjugated to alkaline phosphatase (1:3000)* for 2 hours, and stained with Fast red (Roche) for 2 hours. Sense probes were used as negative controls and did not hybridize in any of the retinas. Sections from retinas of younger fish were used as positive controls. The same microscopy set-up used to collect images from EPON embedded sections was used to photograph cryosections.
4.2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Two retinas from freshly killed coho salmon were extracted from the eyecups, immersed in RNA-later solution (RNAqueous-Midi, Ambion), and stored at 4 °C. The following day, each retina was cut into small pieces corresponding to sectors of the main retina used for histological analyses. These were immersed in new RNA-later solution and stored at 4 °C. Ultraviolet opsin partial cDNA was generated by RT-PCR amplification of DNase-treated total RNA isolated from each homogenized piece of retina, as described previously. Primers used for RT-PCR were designed from the UV opsin sequence to span across introns to distinguish amplicons from DNA contamination. These primers were: UV opsin forward 5'- GGG CTT TGT GTT CTT TGC TG-3', reverse 5'- GGT ACT CCT CGT TGT TTG TG-3', β-actin was used as a control, β-actin forward 5'-CCC ATG GAG CAC GGT ATC ATC AC-3', reverse 5'-GCG TGG GGC AGA GCG TAA CCT TC-3'. Reaction products were analyzed by electrophoresis on 1% agarose gels in 1 x Tris Boreate EDTA (TBE) containing 0.5 µg/ml ethidium bromide, and photographed. These experiments were conducted to identify any UV opsin transcripts in the main retina of adult coho salmon that may had been missed by in situ hybridization, since the latter technique is not as sensitive.

4.3 Results

4.3.1 Distribution of morphologically-defined cone photoreceptor types

The cone photoreceptors were arranged in mosaics that varied from square to row at the ellipsoid level (Fig. 4.1). The square mosaic consisted of double cones forming the sides of the unit square and single cones in the centre and, when present, at the corners (Fig. 4.1A). Corner cones faced the partitioning membranes of adjacent double cones.
whereas centre cones were located at the intersection of the partitions, if these were to be imaginary extended. The row mosaic had double cones and single cones arranged primarily in rows, though the partitions from adjacent double cones could still trace the incomplete outline of a square (Fig. 4.1B). Although the differences between row mosaic and square mosaic are often blurry at the ellipsoid level (e.g. both mosaics can be viewed as alternating rows of double cones and single cones), in general, the partitions of neighbouring double cones, if extended, would intersect at an angle closer to 90° in a square vs. a row mosaic, and the single cones tend to be equidistant in a square mosaic.

In the central and centro-temporal retina, there were both square and row mosaics complete with corner cones (Figs. 4.1A,B). More distal dorsal areas showed square to row mosaics with fewer to no corner cones (Figs. 4.1C,D), which was also the pattern observed in sections going from the centro-nasal retina (Fig. 4.1E) to the nasal periphery (Fig. 4.1F). The ventral retina had few to no corner cones, the mosaic was square (Fig. 4.1G), and cone density increased dramatically towards the periphery (Fig. 4.1H). These patterns are summarized in Figure 4.2 where the d/s ratio is shown to be lowest (range: 1.0-1.3) in the centro-dorso-temporal retina, and the highest cone densities are found in the ventro-temporal and dorso-nasal peripheries. All salmon species studied showed the same patterns, which were also consistent with those reported by Beaudet et al. (1997).

4.3.2 Identification of cone spectral classes

Our in situ hybridization experiments resulted in similar findings for all salmonid species. Regardless of whether they were centre or corner cones, single cones labeled with the blue opsin riboprobe (Figs. 4.3A,B) and none of these cones labeled with the UV riboprobe (Fig. 4.3C). Each member of a double cone labeled with either the green or red
riboprobe, such that each double cone was a green-red pair (Figs. 4.3D, E). In tangential sections progressing from the pigment epithelium towards the ganglion cell layer, labeling by the green riboprobe appeared first indicating that the red cone is displaced slightly vitreal with respect to its green partner (Fig. 4.3D). The expression of either of these two opsins alternated along the unit square mosaic so that each corner cone was flanked by two green and two red cones (Fig. 4.3E). These results depict the chromatic organization sketched in Figure 4.4. The only UV labeling ever detected occurred in the dorsal periphery (Fig. 4.3F). These cells stained heavily and were often found in areas where the retina was not fully differentiated (growth zones). By comparison, the central retina of the young alevin (used as a control) showed labeling of all single cones with the UV riboprobe (Fig. 4.3G), as has been reported previously (Chapter 2). Labeling with the rod riboprobe was observed throughout the retina. This labeling was restricted to the small inner segments of rod photoreceptors that interdigitated between cones in the light-adapted retina (e.g. Fig. 4.3A). In accordance with the lack of UV labeling observed in the main retina by in situ hybridization, we did not amplify any UV opsin transcripts by RT-PCR analysis of isolated retinas. That UV transcripts were not found by RT-PCR in peripheral regions may reflect variations in the amount of peripheral retina extracted between fish or differential expression between individuals.

In general we found that opsin content, as judged by intensity of riboprobe labeling, was much lower in adult fish than in juveniles (Chapter 2) suggesting an overall downregulation of opsins with age. For instance, in chinook salmon, the precocial jacks showed much stronger labeling than normal spawners (which are older by 1-3 years).
Similar conclusions were reached by comparing absorbance of visual pigments obtained by microspectrophotometry (results not shown).

4.4 Discussion

This study demonstrates that UV opsin is not expressed in the main (non-peripheral) retina of the sexually mature Pacific salmon species studied. The corner cones present in the dorso-temporal retina of all species (Fig. 4.2; see also Beaudet et al., 1997) express blue opsin, as do the centre cones. There is therefore no association between single cone position in the mosaic and spectral phenotype, in accordance with our observations on the retinas of juvenile salmonid fishes (Chapters 2 and 3). A previous study on sockeye salmon (Novales Flamarique, 2000) showed that large fish (total length ~ 30 cm) had UV sensitivity following chromatic isolation of the UV cone mechanism (i.e. the UV cone and associated neural circuitry from the retina to the brain). This result was likely due to the use of a diffuser in the stimulus path proximal to the eye, as this would have stimulated cones throughout the retina including the periphery and the embryonic fissure (the latter being another growth zone where UV expression may occur). Using the same diffuser, a UV cone mechanism was revealed in the young sockeye salmon smolt when most corner cones had disappeared (Novales Flamarique and Hawryshyn, 1996). The results from the present study and other recent studies (Novales Flamarique, 2005; Chapter 2) indicate that UV opsin expression is irreversibly downregulated throughout the life of the animal starting at the yolk sac alevin stage, when the switch to blue opsin commences in the ventral retina (Chapter 3). The general downregulation of UV opsin transcripts has also been confirmed in rainbow trout smolts by quantitative PCR (Veldhoen et al., 2006).
Although UV opsin is not upregulated in the adult retina, the possibility of corner cone regeneration still exists. Figure 4.2 shows the overlap in corner cone distributions between the smolt retina (topography re-drawn from Chapter 2) and the adult retina for the various species studied. This comparison suggests that some regeneration may occur in the chinook salmon, especially in the upper half of the retina. A difference in corner cone distributions with age has also been reported for the sockeye salmon, where large fish have corner cones over a greater area of dorsal retina than the young smolts (Novales Flamarique, 2000). Nonetheless, because these sockeye salmon were reared at a constant temperature for 4 years, and because temperature is known to affect the endocrinology and the visual system of salmonid fishes (e.g. Hoar, 1988; Novales Flamarique, 2005), the corner cone distributions in that study may not be representative of natural ontogeny.

Over the last two decades, a series of studies have tested the hypothesis that corner cones are regenerated in adult rainbow trout and that this event is similar to that induced by external exposure of smolt fish to thyroxin (T₄) (Browman and Hawryshyn, 1994; Deutschlander et al., 2001, Allison et al., 2006). We have shown that these studies should be re-evaluated in light of the present study and our recent findings (see Beaudet et al., 1997; Novales Flamarique, 2001; Chapter 2). For example, the previous studies used fish that were undergoing the UV-to-blue cone transformation, a phenomenon that also occurs in rainbow trout (Chapter 5, results obtained using riboprobes and protocols described in Allison et al., 2006). This introduces a confounding variable into the analyses, especially when combined with insufficient resolution of the histology and variation in retinal illumination during in vivo recordings (see Beaudet et al., 1997; Novales Flamarique, 2001). In addition, regeneration of corner cones in the rainbow
trout was reported to take place in the ventral retina (Hawryshyn et al., 2003), yet Martens (2000) concluded that “thyroxin treatment decreased the ventral UV (corner cone) density rather than the expected regeneration”. These contradictory conclusions raise concerns regarding these data sets. The overall distribution of corner cones in the smolt rainbow trout retina is similar to that in T4 treated fish of the same size (Martens, 2000) and to that of the adult animal (Beaudet et al., 1997). Such similarities suggest that corner cones are not regenerated in the rainbow trout retina during natural ontogeny or as a result of thyroxin treatment.
Figure 4.1: Cone mosaic formations in the light adapted retina of adult Pacific salmon

(A) A square mosaic with corner cones from the centro-dorsal retina. An asterisk (*) indicates a corner cone while a black arrow head indicates a centre cone; the partitioning membrane separating double cone members is indicated by a white arrow head. (B) Row mosaic with corner cones from the centro-temporal retina; t indicates a triple cone. (C-F) Row mosaics devoid of corner cones from the proximal (C,E) and distal (D,F) dorsal and nasal retina, respectively. (G,H) Square mosaics without corner cones from the proximal (G) and distal (H) ventral retina. Cones are smaller and more closely packed towards the peripheral retina. Magnification bar (in A) = 25 μm holds for all panels.
Figure 4.2: Retinal maps of cone distributions in the retina Pacific salmon

Shown for each location is the average cone density and the associated d/s ratio (in parenthesis). Cone densities are expressed in thousands per square millimeter. A larger circle indicates that the average cone density for that location was at least 1 standard deviation above the mean from all locations pooled together. A smaller circle indicates the opposite. In each map, the full line polygon delineates the minimum area in which high corner cone densities were found (d/s ratio <1.4). Also embedded within each map is the approximate size of the corresponding smolt retina (reproduced from Chapter 2) and its associated area of high corner cone density (dashed polygon). 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. (n=3 per map) D, dorsal, N, nasal. Magnification bar = 0.55 cm (coho), 0.80 cm (chum), 0.61 cm (chinook), 0.46 cm (chinook jack), and 0.54 cm (pink).
Figure 4.3: Micrographs of tangential cryosections from the light adapted retina of adult Pacific salmon

(A-C) Sections from the central retina show a full square mosaic in which all the single cones label with the blue riboprobe (dark blue stain, A,B) and none label with the UV riboprobe (C). The rod riboprobe labels rod inner segments (red stain, black arrows), which occupy the space between cones. (D,E) The green and red riboprobes label alternating double cone members in the square mosaic; in this case, the blue and red stains indicate labeling by the green and red opsin riboprobes, respectively. (F) Labelled cones (white arrows) expressing UV opsin mRNA in the peripheral dorsal retina. (G) All single cones in the central retina of the alevin label with the UV opsin riboprobe (blue stain); the rhodopsin riboprobe labels rod inner segments in the juvenile retina (red stain). Other symbols as in Fig 4.1. Magnification bar (in A) = 15 μm holds for (D-G), and = 25 μm for (B,C).
Figure 4.4: Chromatic organization of the cone mosaic in adult Pacific salmon

Visual pigments in the cones are identified as follows: B, blue; G, green; R, red. In the young alevin, most of the single cones have UV visual pigment. As the fish grows, combinations of UV and blue can be found within the single cone population.
CHAPTER 5
CHROMATIC ORGANIZATION OF CONE PHOTOSENSORS IN THE RETINA OF RAINBOW TROUT: SINGLE CONES IRREVERSIBLY SWITCH FROM UV (SWS1) TO BLUE (SWS2) LIGHT SENSITIVE OPSIN

Christina L. Cheng and Iñigo Novales Flamarique

Department of Biological Sciences
8888 University Drive
Burnaby, British Columbia
V5A 1S6, Canada

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5.1 Introduction

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 analyze the retina of rainbow trout during embryonic development and at early juvenile stages, when the opsin switch occurs in other salmonid species (see Chapters 2 and 3).

Previous studies 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; 2006). These authors postulated 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 now been shown, however, that the young rainbow trout smolt has similar corner cone topography as the adult, implying that there is no regeneration of corner cones following induced smoltification (see Martens 2000; Novales Flamarique, 2001). Non-anadromous salmonids go through a transformation that is somewhat similar to smoltification though salt water tolerance is not achieved. Whether the remaining corner cones express UV opsin after induced smoltification or whether the production of this opsin is upregulated in the adult retina is unknown. Answers to these unknowns 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, parallel *in situ* hybridization experiments were performed on the retina of alevin to adult rainbow trout using the coho-derived riboprobes used in previous studies and similar probes to those derived from rainbow trout by Allison et al. (2003). Visual pigment absorbance from isolated photoreceptors was also measured 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. (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). This suggestion has been put forward 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 (Chapter 2).

5.2 Materials and Methods

5.2.1 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 ± SD, total length ± SD, 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 (total length ± SD = 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. 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.

5.2.2 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. (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 used by Allison et al. (2003) and were functionally insignificant for hybridization purposes (Fig. 5.1, 5.2). The primers were as follows: rtUV forward 5'- AAC CGC TGA ACT ACA TCC T -3', rtUV reverse 5' - TAA CAC AGA ATG AAG GAG CA -3'; rtBL forward 5'- GAT CCC ATC TCA ACT ACA TT -3', rtBL reverse 5' - ATG AGA GGG TTG TAG ACT GT -3'. Our UV probe (rtUV) corresponds to bases 237 to 822 of the published Oncorhynchus mykiss SWS1 opsin mRNA (Genbank accession # AF425074) (Fig. 5.1) while our blue probe (rtBL) corresponds to bases 403 to 1111 of the published Oncorhynchus mykiss SWS2 opsin mRNA (Genbank accession # AF425075) (Fig. 5.2). We also generated riboprobes against the green (RH2) and red (LWS) opsins, the primers
for these were: rtGR forward 5'- AAA ATA GGC AAA AGG TTC AC -3', rtGR reverse 5'-TAG ACG GCA AGA CAA TAG TA -3' (GenBank accession #AF425076, our probe corresponds to bases 1-192 of this sequence), and rtRE forward 5'- AGC AAG ACA AGA CAA CAG AA -3', rtRE reverse 5'- TGA GAG GAT GAC CAC TAT GA -3' (GenBank accession #AF425073, our probe corresponds to bases 33 -273 of this sequence). The cDNAs were cloned into pCRII-TOPO vectors (Invitrogen) and sequenced by AmpliTaq Dye terminator cycle sequencing (UBC Sequencing laboratory). The identity of each sequence was confirmed by comparing it to 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 either labelled with digoxigenin (DIG) or fluorescein (FL) (Roche Diagnostics).

The procedure for generating coho-derived UV and blue opsin riboprobes was described in Chapter 2.

5.2.3 Tissue preparation for histology and in situ hybridization

The procedure for tissue preparation was the same as detailed in Chapter 2.

5.2.4 In situ hybridization

The methods for in situ hybridization on cryosections were modified as needed from previous studies (Forsell et al., 2001; Chapter 2). Briefly, the procedure involved rehydrating the sections, permeabilizing them in 10 μg/ ml proteinase K (Sigma) for 5, 10 or 13 min [as per the protocol in Allison et al. (2003), for rainbow trout riboprobes],

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followed by exposure to 0.1 M 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 sulfate. Sections were then washed in 50% formamide in 2 x SSC at 60 °C, treated with 20 μg/ml RNase A (Sigma) and incubated with appropriate Fab fragments conjugated to alkaline phosphatase (1:3000; Roche Diagnostics) for 2 hours 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-labeling of cryosections followed the above methodology with the following modifications. The hybridization step included two different riboprobes, one labelled with digoxigenin (DIG), the other with fluorescein (FL). The DIG-labelled probes were visualized using NBT-BCIP. The colour reaction was stopped by washing the sections in glycine-HCl (0.1 M, 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 hours and stained with FastRed (Roche Diagnostics). The same microscopy set-up to obtain images from EPON-embedded sections was used to photograph cryosections. These were used to obtain average percentages of single cones that expressed UV opsin mRNA.

5.2.5 Microspectrophotometry (MSP)

The procedure for MSP measurements was the same as detailed in Chapter 2.
5.3 Results

5.3.1 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. 5.3a) and row (Fig. 5.3b) mosaics complete with corner cones (i.e. a d/s ratio ~ 1). This was also the case in the centro-ventral retina, near the optic nerve head (Fig. 5.3c), but the square mosaic lacked corner cones in other retinal locations such as the centro-nasal region (Fig. 5.3d). Row mosaics with high densities of single cones were found in the dorsal retina (Fig. 5.3e,f) whereas square mosaics lacking corner cones were primarily located in the ventral retina (Fig. 5.3g,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 analyzed (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, 2002; Chapters 2 and 4) and for rainbow trout obtained from a domesticated aquaculture strain (Beaudet et al., 1997).

5.3.2 Labeling by riboprobes

Treatment of serial sections with the coho-derived riboprobes against UV opsin (SWS1) and blue opsin (SWS2) mRNAs resulted in the same labeling patterns as those obtained with analogous riboprobes derived from rainbow trout (Fig. 5.4-6). 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. 5.4a). The same result was obtained with the UV riboprobe derived from rainbow trout (rtUV, Fig. 5.4c). In
contrast, both the coho-derived blue opsin riboprobe (coBL, Fig. 5.4b) and that obtained from rainbow trout (rtBL, Fig. 5.4d) labeled all single cones, including a minority of corner cones. In peripheral regions, where UV opsin expression has been reported in adult salmon (Chapter 4), both UV opsin riboprobes (coUV, Fig. 5.4e, and rtUV, Fig. 5.4f) labeled 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. 5.5a,c) and radial (Fig. 5.5e,g) sections. In contrast, both blue opsin riboprobes labeled all single cones, whether centre or corner cones, as observed in tangential (Fig. 5.5b,d) and radial (Fig. 5.5f,h) sections. In these experiments (Fig. 5.4, 5.5), labeling by rtBL was generally more pronounced than that by coBL, likely because treatment with proteinase K in the in-situ protocol was longer for rtBL (10 min, as per Allison et al., 2003) than for coBL (5 min, as per Chapter 2). As well, the difference in length between riboprobes may have contributed to these results.

Further experiments that varied time of proteinase K treatment in the in-situ hybridization protocol showed that the same labeling pattern was obtained with analogous riboprobes. Serial sections of ventral retina in adult rainbow trout showed that coUV did not label any cone regardless if proteinase K incubation time was 5 min (Fig. 5.6a,d) or 13 min (Fig. 5.6b,e), and this was the same result as obtained with rtUV (Fig. 5.6c,f). All single cones labeled, however, with the blue riboprobe (e.g. rtBL, Fig. 5.6g,h). In general, longer proteinase K treatment enhanced both specific and background labeling without altering the pattern of cones labeled.
The darker appearance of some non-labeled 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-labeled double and single cones exhibited some darkness but at different "levels" of the (oblique) cross-section (e.g. Fig. 5.6a,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 labeling based on any of the following label attributes: colour, texture (appearance) and positioning.

5.3.3 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. 5.7). All single cones in the dorsal retina labeled exclusively with the UV riboprobe (rtUV, Fig. 5.7a,c) while no cone labeled with the blue riboprobe (rtBL, Fig. 5.7b,d). In the lower half of the ventral retina, UV riboprobe labeling was not as intense as in the rest of the retina (Fig. 5.7e,g) and several cells labeled faintly with the blue riboprobe (rtBL, Fig. 5.7f,h). Further analyses of the peripheral ventral retina showed that UV opsin mRNA expression appeared first (i.e. labeling was close to the undifferentiated growth zone, Fig. 5.8a) and was followed in time by blue opsin expression (labeling further away from the undifferentiated growth zone, Fig. 5.8b). Cone densities were highest in the ventral retina (Fig. 5.9a). These results are consistent with published reports on various salmonid species using coho-derived riboprobes (Chapters 2-4).

The retina of larger alevins (also termed parr, e.g. Veldhoen et al., 2006; Allison et al., 2006) showed a pattern of stratified labeling from ventral to dorsal retina (Fig. 5.9b, 5.10). Double labeling experiments with the rainbow trout riboprobes demonstrated
coexpression of UV and blue mRNA in single cones undergoing the transformation from UV to blue spectral phenotype (see also Chapter 2). 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. 5.10a,b). Co-expressing cones were most common in the centro-dorsal retina (Fig. 5.10a,b) at the advancing front of the single cone transformation (Fig. 5.9b). 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. 5.10c). Single cones expressing blue opsin mRNA (Fig. 5.10d) or in the process of switching opsins (Fig. 5.10h) were present in the nasal and temporal periphery, though most cones in these peripheral areas labeled solely with the UV riboprobe. Cones in the mid (Fig. 5.10e,f) and distal (Fig. 5.10g) dorsal retina labeled exclusively with the UV riboprobe. These distinct labeling patterns revealed a progression in UV opsin down-regulation from ventral to dorsal retina (Fig. 5.9b).

In the smolt, corner cones continued to be present in a large area of dorsotemporal retina (Fig. 5.9c). None of the cones labeled with the UV opsin riboprobe (rtUV, Fig. 5.11a) and all labeled with the blue opsin riboprobe (rtBL, Fig. 5.11b). Sections from the mid-dorsal (Fig. 5.11c,d) and temporal (Fig. 5.11e) retina showed that the single cones labeled 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. 5.12b). Double cones were green/red pairs that alternated their labeling pattern around the square mosaic unit (Fig. 5.12a). This double cone labeling pattern was the same in the adult retina (Fig. 5.12c,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. 5.9d). None of these single cones labeled with the UV opsin riboprobe (rtUV, Fig. 5.13a) but all labeled with the blue opsin riboprobe (rtBL, Fig. 5.13b). This labeling pattern was consistent throughout the entire (non-peripheral) retina including areas without corner cones (Fig. 5.13c-f). As in the smolt, cone densities were highest in the ventro-temporal and dorso-nasal regions of the retina (Fig. 5.9c,d).

5.3.4 Photoreceptor visual pigments

Our visual pigment absorbance measurements from individual photoreceptors (Fig. 5.14) corroborated the in-situ hybridization results. All the single cones measured (n=56) in the young alevin (Fig. 5.14a) had a UV visual pigment with average maximum absorbance ($\lambda_{\text{max}} \pm \text{SD} = 373 \pm 6$ nm), and double cones (n=25) with $\lambda_{\text{max}} \pm \text{SD} = 494 \pm 7$ 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 average $\lambda_{\text{max}} \pm \text{SD} = 437 \pm 5$ nm (Fig. 5.14a), and double cones (n=35) with average $\lambda_{\text{max}} \pm \text{SD} = 523 \pm 6$ nm (green member) and 578 ± 10 nm (red member) (Fig. 5.14b).

Compared to 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 both stages and had average $\lambda_{\text{max}} \pm \text{SD} = 510 \pm 5$ nm (Fig. 5.14b). These averages indicate retinas based on a mixture of vitamin A$_1$ and A$_2$ (Hárosi, 1994).
5.4 Discussion

5.4.1 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 (Chapter 3) 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 (Chapters 2-4).

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 first blue opsin mRNA expression in the ventro-temporal retina of salmonid embryos and subsequent progression toward the ventral and, later, toward the dorsal retina (Chapter 3). 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 which, together with the presence of TRβ2 receptor, induces a predominant expression of green opsin (MWS) in dorsal cones (Ng et al., 2001; Roberts et al., 2006; Applebury et al., 2007). The resulting ventrodorsal gradient of decreasing SWS1 opsin expression in the mouse is somewhat opposite to that in the juvenile rainbow trout, and is likely mediated by similar diffusible factors.
5.4.2 Chromatic organization of single cones in the rainbow trout retina

In accordance with previous findings from other salmonid species (Novales Flamarique, 2005; Chapters 2-3), 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. 5.10). 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, 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 these cones 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.

5.4.3 Consistency of riboprobe labeling reconciles literature findings

The labeling results obtained with our coho-derived riboprobes and those obtained from rainbow trout (similar probes to those used by 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, 2006) that 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, 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 analyzed retinas that comprised a variety of mosaic configurations (Fig. 5.9, 5.10). Yet, in their work, these authors present tangential micrographs from the ventral retina exclusively (Fig. 2B,C in 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., 2003) and the corner cones express UV opsin (in the centro- to mid-ventral retina; Fig. 2C of Allison et al., 2003). Curiously, the authors do not state where their sections originate but, instead, proclaim the labeling pattern as representative of the entire retina. In a later manuscript (Allison et al., 2006), a micrograph (Fig. 3B) is presented from the dorsal retina of a parr fish in which single cones are labeled with a UV opsin antibody. Careful analysis of this figure (see Fig. 5.15) shows both centre and corner cones labeled, 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, 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. Hárosi, 1994), but a change in opsin type or mosaic structure resulting from such variations in rearing environment has never been documented (see Novales Flamarique, 2005). Second, we have examined the retina 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, 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
comments by 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) have 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 TRβ2) 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 rearrangement 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. (2006) may have labeled with BrdU other photoreceptor nuclei 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 labeling 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, 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 opsins. Given the actions of steroid ligands in the retinas of other vertebrates (Prabhudesai et al., 2005; Roberts et al., 2005, 2006; Srinivas et al., 2006; Mader and Cameron, 2006; Applebury et al., 2007), we suspect that thyroid hormone will modulate UV and blue opsins expression (an indication of which was given by Veldhoen et al., 2006) but will not induce single cone apoptosis (as concluded by 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 opsins (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.
5.5 Figures and Figure Legends

Figure 5.1: Sequence alignment of *O. mykiss* UV opsin mRNA with published salmonid UV riboprobes

The sequence alignment (MultAlin, Corpet, 1988) shows the positions of our rtUV and coUV riboprobes with respect to *O. mykiss* UV opsin mRNA (GenBank accession #AF425074) and that of the UV riboprobe used by Allison et al. (2003).
Figure 5.2: Sequence alignment of *O. mykiss* blue opsin mRNA with published salmonid blue riboprobes

The sequence alignment (MultAlin, Corpet, 1988) shows the positions of our rtBL and coBL riboprobes with respect to *O. mykiss* blue opsin mRNA (GenBank accession #AF425075) and that of the blue riboprobe used by Allison et al. (2003).
Figure 5.3: 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, (c,d) square mosaic with and without corner cones from the centro-ventral and centro-nasal parts of the retina, respectively. An asterix (*) indicates a corner cone, a white arrow 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) = 25 μm holds for all panels.
Figure 5.4: Labeling with coho- and rainbow trout-derived riboprobes

Micrographs of sections from the post-juvenile 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, a, and rtUV, c) do not label any cones while the blue riboprobes (coUV, b, and coBL, d) label all single cones, regardless of position in the square mosaic. (e,f) Serial sections from the dorso-nasal periphery show multiple cells labeled by the UV riboprobes (coUV, e, and rtUV, f). Black arrow heads point to unlabelled single cones, black arrows point to single cones labeled with the blue riboprobes, and white arrows point to single cones labeled with the UV riboprobes; rpe, retinal pigment epithelium. The riboprobe used per section is specified at the bottom left of each panel. Other symbols and nomenclature as in Fig 5.3. Magnification bar (in a) = 25 μm holds for all panels.
Figure 5.5: Micrographs of sections from the adult retina following \textit{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, a, and rtUV, c) do not label any cones while the blue riboprobes (coUV, b, and coBL, 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 labeling by the UV riboprobes (coUV, e, and rtUV, g), and labeling of all single cones by the blue riboprobes (coBL, f, and rtBL, h). Note that, in the light-adapted retina, the double cone ellipsoids (see white arrow heads 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 5.4. Magnification bar (in a) = 25 \textmu m holds for all panels.
Figure 5.6: Variable proteinase K incubation times result in similar labeling

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 labeled by the blue riboprobe (rtBL) after 10 min of proteinase K treatment. The riboprobes used and the time of proteinase K exposure (in parenthesis) are shown at the bottom left of each panel. Note that proteinase K exposure times used by 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 5.4. Magnification bar (in a) = 25 μm holds for all panels.
Figure 5.7: 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 labeled by the UV riboprobe (a,c) and none label with the blue riboprobe (b,d). (e-h) Serial sections from the lower ventral retina show that most single cones are labeled by the UV riboprobe (e,g) but some also label with the blue riboprobe (f,h). Abbreviations: d, double cone; other symbols and nomenclature as in Fig 5.4. Magnification bar (in a) = 25 µm holds for all panels.
Figure 5.8: Progression of retinal development

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. Labeling by the UV riboprobe (rtUV, a) appears closer to the ugz than labeling by the blue riboprobe (rtBL, b). Symbols and nomenclature as in Fig 5.7. Magnification bar (at the bottom right of the figure) = 25 μm.
Figure 5.9: Retinal maps of cone distributions in the retina of rainbow trout from the alewijn to the adult (n=10 per map).
Figure 5.10: 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 5.7. Magnification bar (in a) = 25 μm holds for all panels.
(a,b) Serial radial sections from the distal nasal retina show that no cone labels with the UV riboprobe (a) and all single cones label with the blue riboprobe (b). (c-e) Tangential (c) and radial (d) sections from the mid-dorsal retina show that all single cones label exclusively with the blue riboprobe, as do single cones from the temporal retina (e). The sections shown in (c-e) were double labeled with the UV and blue riboprobes, but only the blue riboprobe showed labeling. Symbols and nomenclature as in Fig 5.7. Magnification bar (in a) = 25 μm holds for all panels.
Figure 5.12: Micrographs of sections from post-juvenile and adult retinas following double label in situ hybridization

(a) Tangential section from the centro-temporal retina of the smolt showing that each member of a double cone expresses an 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 post-juvenile, 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 labeling pattern of double cones as in the post-juvenile retina. Symbols and nomenclature as in Fig 5.7. Magnification bar (in a) = 25 µm holds for all panels.
Figure 5.13: 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 labeled by the UV riboprobe (a) but all single cones label with the blue riboprobe (b). (c-f) Tangential sections, double labeled with the UV and blue riboprobes, show exclusive labeling 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 5.7. Magnification bar (in a) = 25 μm holds for all panels.
Figure 5.14: 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 post-juvenile retina contain a visual pigment maximally sensitive to blue light (B). (b) Green (G) and red (R) visual pigments present in the double cones of the post-juvenile (one pigment per member), and rhodopsin pigment (r) contained in the rods.
Figure 5.15: Both center and corner cones in Allison et al. (2006) are labeled with the UV opsin antibody

These three panels (a-c) are enlarged sections of Fig. 3B (on the left) in Allison et al. (2006) showing both centre (black arrow) and corner (white arrow) cones labeled with the UV opsin antibody (green fluorescence). The double cone partitions (white arrowheads) are deduced from the shapes of the double cone ellipsoids (in red).
CHAPTER 6
THYROID HORMONE MEDIATES THE UV TO BLUE CONE TRANSFORMATION IN SALMONID FISHES

6.1 Introduction

Thyroid hormone has been implicated in the regulation of opsin expression in various organisms (Mader and Cameron, 2006; Roberts et al., 2006; Veldhoen et al., 2006). In the mouse, the ontogeny of opsin expression is such that the blue (SWS1) opsin is expressed prenatally while the green (MWS) opsin is expressed at around post-natal day 10 as blue opsin expression begins to subside. Recent studies using mice lacking a photoreceptor-specific thyroid hormone receptor, TRβ2, or mice with TRβ2 that is ligand binding defective showed that the change in blue and green opsin expression is mediated by thyroid hormone and TRβ2 (Ng et al., 2001; Applebury et al., 2007). Consistent with these observations, injection of T3 into wild type mice or TRβ2-deficient mice showed that T3 decreases blue opsin and increases green opsin expression, and that its effect is mediated by TRβ2 (Roberts et al., 2005). In salmon, thyroid hormone treatment also induced downregulation in UV opsin mRNA and upregulation in blue opsin mRNA as assessed by quantitative RT-PCR (Veldhoen et al., 2006). However, the effects of thyroid hormone on the chromatic organization of photoreceptors and on cone differentiation in salmonid fishes have not been explored.

In salmonid fishes, UV opsin is expressed first during embryonic development and blue opsin is expressed near the time of full yolk sac absorption (Chapters 2 and 3).
As in the mouse, the increase in blue opsin coincides with a decrease in UV opsin. This change in UV and blue opsin expression begins at the embryonic stage following a steady increase in thyroid hormone from hatching to the time of full yolk sac absorption (Sullivan et al., 1987). Such correlation parallels the reduction of blue opsin and concomitant increase of green opsin expression in the mouse retina following intraocular injection of thyroid hormone for several days (Roberts et al., 2005). To reveal whether thyroid hormone could induce the UV to blue opsin switch in the salmonid retina, we treated alevin fish (i.e. fish that have not undergone the UV to blue opsin switch) with this compound and analyzed opsin expression by in situ hybridization. Furthermore, to assess whether the thyroid hormone-mediated opsin switch took place at the level of transcription, the effects of thyroid hormone on UV and blue opsin promoter activity were examined in cell transfection assays.

6.2 Materials and Methods

6.2.1 Animals

Wild stock coho salmon (Oncorhynchus kisutch) and rainbow trout (O. mykiss) were obtained from the Capilano River and the Fraser Valley Trout hatcheries (BC, Canada). The weights and lengths of the fish used are presented in Table 6.1. These fish had just absorbed their yolk sac at the beginning of treatments and the majority of single cones overwhelmingly expressed UV opsin (see Results, Chapters 2 and 3). Fish were held in 10 L containers at a temperature of 6 °C under a 12 h dark: 12 h light regime. All holding and experimental procedures were approved by the Animal Care Committee of Simon Fraser University, which follows the guidelines set by the Canadian Council for Animal Care.
6.2.2 Thyroid hormone treatment

L-thyroxine (T₄, Sigma) dissolved in 0.1 M NaOH was added to the water to a final concentration of 300 µg/L. Treatment was performed daily for two weeks, and half of the water in each tank was changed every day. Control fish were treated identically with the exception that only the vehicle (0.1 M NaOH) was added to the water. Control and thyroid hormone treated fish were sampled at the end of two weeks following the start of treatments. Previous experiments have indicated that treatment of rainbow trout with thyroid hormone for two weeks is sufficient to induce changes in cone opsin expression (Veldhoen et al., 2006).

To test whether exposure to thyroid hormone was sufficient to sustain changes in opsin expression, we stopped treatment for six weeks after first exposure and compared the retinas of these fishes with those sampled after the two weeks of thyroid hormone treatment.

We also examined the effects of thyroid hormone on cell cultures transfected with UV or blue opsin promoter-reporter constructs. Thyroid hormone (T₃) was applied at concentrations of 10, 25, 50, 75, and 100 nM. If thyroid hormone mediated the UV to blue opsin switch in the single cones of salmonid fish, then we expected a downregulation of UV opsin and upregulation of blue opsin in both in vivo and cell culture experiments; such dynamics would mimic those taking place during natural development (Chapters 2 and 3). Thyroid hormone (T₃) was used for the cell culture study because the cells may have lacked the enzyme (deiodinase type II) needed to convert L-thyroxine (T₄) to the biologically active T₃.
6.2.3 Tissue preparation for histology

The procedure for retinal tissue preparation was the same as detailed in Chapter 2. The retinas were cut into four quarters corresponding to ventral, nasal, dorsal, and temporal areas.

6.2.4 Preparation of opsin riboprobes and in situ hybridization

Species-specific riboprobes for the detection of UV and blue opsin expression, and the in situ procedure used were the same as detailed in Chapter 5.

6.2.5 TUNEL

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, ApopTag Peroxidase In Situ Apoptosis Detection, Chemicon International) was performed on cryosections to assess the effect of thyroid hormone on programmed cell death in single cone photoreceptors. Cryosections were incubated with 10 mM citrate buffer for 5 min, followed by 3% hydrogen peroxide for 5 min, and with terminal deoxynucleotidyl transferase reaction buffer for 60 min. TUNEL labeling was detected using a DIG antibody conjugated to peroxidase and visualized using diaminobenzidine. Salmon embryos, which have high levels of apoptotic cells, were used as a positive control for the TUNEL procedure.

6.2.6 Cell culture

CHSE (Chinook salmon embryonic cells, generously provided by Dr. Robert Devlin, West Vancouver Laboratories) were maintained in L15 medium (Invitrogen) with 10% (v/v) fetal bovine serum (FBS), penicillin G (100 units/ml), and streptomycin (100 μg/ml), at 20 °C. For thyroid hormone treatment experiments, CHSE cells were cultured
in the presence of serum and 10, 25, 50, 75, and 100 nM of T₃ (Sigma). For control experiments, an equivalent amount of vehicle (0.1 M NaOH) was added to the cells. Further details on the treatments are provided below.

6.2.7 Plasmid constructs for transfection

A gene fragment of 500 base pairs (bp) from the 5’ flanking region of the UV opsin and blue opsin genes, starting from the translation start site and proceeding upstream, were isolated from an Atlantic salmon BAC genomic DNA library (kindly provided by Dr. William Davidson, Simon Fraser University). The probes used to screen the DNA library had the same sequence as that used for in situ hybridization (Chapter 2). The positive clones were isolated, cultured and sequenced. The identity of the sequences was confirmed by comparing to the NCBI database (BLAST, Altschul et al., 1997). It has been shown that the first 500 bp upstream sequence of either the UV or blue opsins is sufficient to drive proper spatial expression of a reporter protein in zebrafish (Takechi et al., 2003), human (Glushakova et al., 2006), and mouse (Akimoto et al., 2004). The 500 bp fragment was cloned into a pCRII-TOPO vector (Invitrogen), and the identities of the cloned inserts were confirmed by sequencing (UBC Sequencing laboratory). The fragment was then subcloned into a firefly luciferase reporter vector (pGL3-basic; Promega) in the sense orientation.

6.2.8 Transient transfection and luciferase assay

To examine whether thyroid hormone regulation of UV and blue opsin expression occurred at the transcriptional level, CHSE cells were transfected with UV/blue opsin promoter-luciferase constructs and treated with thyroid hormone. Transient transfection
of CHSE cells was performed using Lipofectamine 2000 reagent (Invitrogen). Each transfection contained 0.2 μg of promoter-firefly luciferase construct, 0.02 μg of pRL-TK (Promega), and 0.5 μl of the transfection reagent. The pRL-TK vector contains the Renilla luciferase reporter gene under the control of thymidine kinase basic promoter and serves as an internal control for transfection efficiency. After incubation for six hours, thyroid hormone (T3) or vehicle (0.1 M NaOH) was added to each well. Transfected cells were cultured for 48 hours before harvesting for the luciferase reporter assay. Both firefly and Renilla luciferase activities were assayed with 20 μl of crude cell lysate using the Dual-Luciferase Reporter Assay (Promega) and a TD-20/20 luminometer. Each transfection was done in triplicates and repeated three times.

6.3 Results

6.3.1 Effects of thyroid hormone on UV and blue opsin expression

The alevin coho salmon had a square mosaic in which the vast majority of single cones (both center and corner cones) labeled exclusively with the UV opsin riboprobe (Fig. 6.1a, Fig. 6.2). A few center cones in the ventro-nasal periphery labeled with the blue opsin riboprobe. A one-way ANOVA performed on the four retinal quadrants revealed that the number of blue opsin-expressing cones in the alevins treated with thyroid hormone for two weeks was statistically different from that in controls (ventral: F=45.690, p=0.001; temporal: F=95.664, p<0.0001; dorsal: F=43.419, p=0.001; nasal: F=119.989, p>0.0001) (Fig. 6.2b). Thyroid hormone-treated alevins had both center cones and corner cones expressing blue opsin, and these were distributed throughout most of the retina (Fig. 6.1d).
A one-way ANOVA revealed that the number of cones labeled with the UV opsin riboprobe in thyroid hormone-treated alevins was significantly less than the corresponding controls (Fig. 6.1a,b, 6.2a), except in the ventral region (ventral: F=3.399, p=0.125; temporal: F=26.047, p=0.004; dorsal: F=177.064, p<0.0001; nasal: F=14.347, p=0.013). The remaining UV riboprobe-labeled cones in thyroid hormone-treated fish retinas were corner cones, though some center cones labeled with the UV riboprobe could be found in the central retina. Thyroid hormone thus increased the numbers of blue opsin-expressing cones and decreased the numbers of UV opsin-expressing cones.

The number of cones labeled by the UV and blue opsin riboprobes in the thyroid hormone-treated retina exceeded the total number of single cones found in the control retina, suggesting that a population of single cones in the treated retina coexpressed UV and blue opsins. The average number of single and double cones measured histologically was the same between control and thyroid hormone-treated alevins (Table 6.2, Fig. 6.3). Moreover, the number of cones labeled with the blue riboprobe in the thyroid hormone-treated retina was greater than the difference in UV riboprobe-labeled cones in the control and the thyroid hormone-treated retinas. This implies an upregulation of blue opsin in some of the residual UV cones. Double in situ labeling with the UV and blue riboprobes confirmed the presence of dual-expressing cones in the thyroid hormone-treated retina (Fig. 6.4) providing direct evidence that thyroid hormone induced a switch in opsin mRNA expression from UV to blue.

To assess whether some of the decrease in UV riboprobe labeling in the thyroid hormone-treated fish could be ascribed to cone apoptosis, we analyzed some of these retinas by histology and TUNEL immunocytochemistry. In all but the ventral retinal
quadrant of control and treated fish examined, the square mosaic was full with a double cone to single cone ratio ~1 (Table 6.2, Fig. 6.3). Correspondingly, we found no evidence of cone apoptosis by TUNEL labeling (Fig. 6.5). These results show that the decrease in UV opsin mRNA expression induced by thyroid hormone is due to a switch in opsins and not to a loss of corner cones.

6.3.2 Thyroid hormone effects are similar in coho and rainbow trout

The majority of single cones in the young rainbow trout alevin labeled with the UV riboprobe and none labeled with the blue riboprobe. A few blue center cones were found in the ventral retina of control fish (Fig. 6.6b). Thyroid hormone produced similar changes as those observed in the retina of coho salmon, namely the density of cones labeled with the blue riboprobe increased by a factor of 150 and that of UV riboprobe labeled cones decreased by a factor of 6. The blue riboprobe labeled both center and corner cones in the thyroid hormone-treated retina (Fig. 6.6d, h). The UV riboprobe in this retina also labeled both cone types but the majority were corner cones, except in the ventral retina where none of the single cones labeled with the UV riboprobe (Fig. 6.6f).

6.3.3 Thyroid hormone effects are stage-dependent

During natural retinal development of salmon, single cones undergo the UV to blue opsin switch at around the time of full yolk sac absorption. This leads to a smolt retina where all single cones express blue opsin and this persists into adulthood (Chapter 4). Thyroid hormone-treated alevins left untreated for six weeks had a vast majority of single cones expressing UV opsin mRNA (Fig. 6.7 b, f) and a small minority of cones expressing blue opsin mRNA dispersed throughout the ventral retina (Fig. 6.7d, h).
These results are similar to those of control alevin where the majority of single cones were labeled with the UV riboprobe (Fig. 6.7a, e); only a few center cones in the centro-temporal retina were labeled with the blue riboprobe (Fig. 6.7c, g). In addition, as in the control alevin, some corner cones had disappeared from the ventral retina of post-thyroid hormone treated fish (Table 6.3). Thyroid hormone treatment at the early alevin stage did not result in permanent UV opsin downregulation and blue opsin upregulation as occurs during natural development (Chapter 4).

6.3.4 Possible down-regulation of UV opsin promoter activity by thyroid hormone

The blue opsin promoter activity was not affected by T₃ (Fig. 6.8b), but that of the UV opsin promoter was reduced (Fig. 6.8b, e). The strongest suppression of T₃ was observed at 50 nM; the luciferase activity at 50 nM was significantly less than that of controls but it was not significantly less than that achieved with other treatments (10 and 25 nM). The suppression was less at 75 and 100 nM (Fig. 6.8c), suggesting that ligand saturation or competition for binding sites may occur at higher T₃ concentrations.

6.4 Discussion

6.4.1 Differentiation effects of thyroid hormone on the expression of UV and blue opsins

Treatment with thyroid hormone of salmon alevins led to a significant decrease in UV opsin mRNA expression and an increase in blue opsin mRNA expression. These changes manifested themselves in the numbers of single cones expressing either of these opsin mRNAs. In addition, double in situ labeling further revealed that these changes occurred through a switch in opsins within individual cones, and that thyroid hormone
induced a premature transformation of UV to blue cones in the retina of coho and rainbow trout.

The effect of thyroid hormone on UV opsin expression may have occurred at the transcriptional level through regulation of the UV opsin promoter. No effect was detected on the blue opsin promoter. The lack of thyroid hormone effects on the blue opsin promoter in the transfection study suggests that the actions of thyroid hormone on this promoter are indirect and may be mediated by proteins that are missing from the CHSE cells. The suppressive effect of thyroid hormone on the UV opsin promoter does not necessarily prove that the effect is direct, but that the required factors involved are present in the CHSE cells. The effect of thyroid hormone on the opsin promoters measured in the CHSE cells might not be an accurate reflection of the in vivo effect. The cells were extracted from chinook embryos, which would consist of pluripotent cells, and incubated at 20 °C. Opsin switching takes place in well-differentiated photoreceptors residing in the retina of fish maintained at cooler water temperatures (~10 °C).

Nonetheless, cell transfection study is a convenient method to examine promoter activity.

Analysis of the opsin promoter sequences using MatInspector (Genomatix software, version 7.4.4) revealed a putative thyroid hormone response element (TRE) located in the first 250 bp of UV opsin upstream sequence (Fig. 6.9) and none in the blue opsin (Fig. 6.10). Transfection studies with TRE-deleted UV opsin constructs will reveal whether this element has any functional significance. MatInspector analysis and chromatin immunoprecipitation assay on the rainbow trout UV opsin promoter illustrated the presence of NFκB and c-jun binding sites at around -600 bp (Dann et al., 2004).
These two proteins are often involved in cell apoptosis. A putative TRE was not found by that study possibly due to the different version of the MatInspector program used.

During salmon natural ontogeny, transformation of UV to blue cones commences in the ventro-temporal retina of the young alevin, and this phenomenon sweeps dorsally expanding to the rest of the retina (Chapters 2 and 3). This spatial progression of UV to blue cone transformation was not observed in the alevins treated with thyroid hormone for two weeks, and these fish had blue labeled cones throughout the retina. It is possible that the thyroid hormone-mediated upregulation of blue opsin starts immediately after initiation of treatment, resulting in near completion of the UV to blue cone transformation throughout the retina after two weeks of treatment. Another possibility is that the concentration of exogenous thyroid hormone might be too high, resulting in saturation of receptors and type III deiodinase, thereby disrupting a graded distribution throughout the retina. Type III deiodinase, a thyroid hormone degrading enzyme, is distributed in a dorso-ventral gradient in the retina of xenopus (Marsh-Armstrong et al., 1999) and it is responsible for establishing the functional gradient of thyroid hormone during metamorphosis of this animal. In the mouse, a gradient of thyroid hormone also exists at the time of green (M) opsin onset despite a uniform distribution of the thyroid hormone receptor (TRβ2) (Roberts et al., 2005). Thus the gradient of thyroid hormone is thought to be set up by type III deiodinase. Determining the levels of type III deiodinase in salmonid retina will be helpful in assessing its role in directing the spatial progression of UV to blue cone transformation.

Our results corroborate our earlier findings showing that UV and blue opsin expression is not linked to the position of the single cone in the retinal mosaic of
salmonid fishes (Chapters 2-5). The specificity of our probes has been established in previous chapters through bioinformatics analysis of probe sequences and a series of experiments that examined adjacent pieces of retina by in situ hybridization and microspectrophotometry analysis of visual pigment absorbance (Chapter 2). In contrast to earlier reports on rainbow trout that claimed that thyroid hormone induced a decrease in UV sensitivity through loss of corner cones (Browman and Hawryshyn, 1992, 1994), we demonstrate that thyroid hormone induces changes to opsin expression, not apoptosis of corner cones.

We did not observe cone apoptosis by TUNEL labeling. These observations are consistent with the works of Ng et al. (2001) and Cornish et al. (2004) who showed that changes in photoreceptor subtypes are not associated with cell death. The absence of corner cone loss in fish treated with thyroid hormone up to four weeks raises the hypothesis that cone transformation and corner cone loss are uncoupled processes (see Chapter 5) and that thyroid hormone does not induce apoptosis of corner cones as reported during natural development of salmon (Kunz et al., 1994).

6.4.2 Effects of thyroid hormone on center and corner cones

Thyroid hormone upregulated blue opsin and downregulated UV opsin preferentially in center cones. This may due to the presence of a center cone-specific factor that facilitates thyroid hormone signaling, such as RXRγ, which is a retinoic acid receptor required to suppress S opsin in mouse (Roberts et al., 2005). This receptor heterodimerizes with TRβ2 receptor to regulate the pattern of opsin expression cooperatively (Oro et al., 1992; Yao et al., 1992). The S (blue-sensitive) opsin of mammals belongs to the family of opsins that also encompasses the salmonid UV opsin
(in the SWS1 opsin gene family). It is possible that the thyroid hormone-mediated
downregulation of UV opsin in salmon is also mediated by RXRγ, and that this factor is
preferentially expressed in center cones.

RORβ is a retinoid-related orphan receptor that also controls opsin expression. In
contrast to RXRγ, monomers of RORβ in mouse activate S opsin promoter activity and
the effect is synergized by a photoreceptor-specific transcription factor, Crx (Srinivas et
al., 2006). It is possible that, in salmon retina, RORβ is expressed in both center and
corner cones initially to induce UV opsin expression but then becomes restricted to corner
cones as thyroid hormone levels increase. It is also possible that RORβ competes with
RXRγ such that expression of one receptor excludes the expression of the other. Such
mutual antagonistic interactions occur in Drosophila during photoreceptor specification
(Doroquez and Rebay, 2006). RORβ might play a dual role in UV corner cones,
maintaining UV opsin expression and cell survival; mice lacking RORs have been shown
to undergo accelerated programmed cell death (Jetten et al., 2001). Localization of RXRγ
and RORβ in salmonid retinas will be helpful in assessing the roles of these transcription
factors in opsin regulation and photoreceptor apoptosis in these fishes.

6.4.3 Opsin transcription is mediated by thyroid hormone

During the life of salmon, there are three time points where the amount of plasma
thyroid hormone is elevated: around the time of yolk sac absorption (Sullivan et al., 1987;
Greenblatt et al., 1989), at smoltification (a process of physiological changes that
prepares the fish to enter salt water; Hoar, 1988), and at sexual maturation (Ueda et al.,
1984). Thyroid hormone-regulated effects are mediated through thyroid hormone
receptors which bind to the promoter of targeted genes and affect transcriptional activity.
Since hormone receptors are often positively regulated by their ligands (Machuca et al., 1995), thyroid hormone receptors may be at their highest levels of expression at times when thyroid hormone is high in the blood plasma of salmonid fishes.

Alevins treated with thyroid hormone have a majority of single cones that express blue opsin. The alevin is a life stage when thyroid hormone receptors are present (Jones et al., 2002) and their levels may increase due to positive feedback by thyroid hormone (Machuca et al., 1995). This leads to a model for opsin regulation in the single cones of salmonid fishes (Fig. 6.11). High levels of thyroid hormone and its receptors lead to suppression of UV opsin expression but activation of blue opsin expression. The ability of liganded thyroid hormone receptor to exert opposing effects, activating blue opsin and inhibiting UV opsin transcription, may be attributed to its associated partner that influences the recruitment of co-activators or co-repressors. Consistent with this, recent studies in the mouse have shown that TRβ2 complexes with RXRγ to repress S opsin expression but that RXRγ is not needed for TRβ2-mediated upregulation of M opsin (Roberts et al., 2005).

During low levels of thyroid hormone, the unliganded receptor would not be expected to activate UV opsin expression, as seen in the pre-treated alevins that had an ubiquitous expression of UV opsin in all single cones. It has been shown that TRβ2 is not necessary for the expression of S opsin in the mouse retina (Applebury et al., 2007) and, in fact, TRβ2 is downregulated at the time of S opsin onset (Roberts et al., 2006). Furthermore, mice lacking TRβ2 had a retina with all cones expressing S opsin, showing that TRβ2 plays no role in S opsin upregulation. The unliganded receptor may not activate UV opsin, but it may suppress blue opsin as thyroid hormone receptors are
known to suppress targeted gene transcription in the absence of ligand (Harvey and Williams, 2002). Determining levels of thyroid hormone receptor, TRβ2, in salmonid retinas with respect to the onset of UV and blue opsins will clarify its role in opsin regulation.

Thyroid hormone receptors turn over rapidly (Dace et al., 2000), so these receptors and their ligand are unlikely to regulate opsin expression continuously. The stable long-term repression of UV opsin and activation of blue opsin as seen during natural ontogeny of salmon was not achieved in our study. Upon termination of thyroid hormone treatment, opsin expression in the retina returned to the control state implying that, although thyroid hormone was the factor that directed downregulation of UV opsin and upregulation of blue opsin, previous thyroid hormone exposure was not sufficient to sustain expression. There might be factors responsible for maintaining opsin expression that are not active at this life stage, and that may direct chromatin remodeling required to achieve a permanent state of opsin expression.

Permanent changes to gene expression are thought to be mediated by changes in histone and DNA methylation that lead to a condensed, closed chromatin state (Grunstein, 1990; Kornberg and Lorch, 1999). The window of opportunity for opsin regulation by thyroid hormone may therefore be restricted to a time prior to when permanent changes in gene expression occur. Consistent with a time-dependent role of thyroid hormone, this hormone is ineffective in changing opsin expression in mice once the repression of S opsin has been established (Roberts et al., 2006). Induction of either hyperthyroidism or hypothyroidism had no effects on opsin expression in adult mice.
(Applebury et al., 2007) or postmetamorphic winter flounder (Mader and Cameron, 2006) once photoreceptor cells had fully differentiated.
6.5 Tables

Table 6.1: Body weight and length of salmon alevins

For any given group, the treatment and control statistics are not significantly different (t-test with alpha = 0.05). n=10 for each group.

<table>
<thead>
<tr>
<th>Fish and Treatment</th>
<th>Weight (+/- SD) (g)</th>
<th>Length (+/- SD) (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coho TH</td>
<td>0.37 (0.088)</td>
<td>3.89 (0.24)</td>
</tr>
<tr>
<td>Coho control</td>
<td>0.36 (0.091)</td>
<td>4.01 (0.30)</td>
</tr>
<tr>
<td>Rainbow trout TH</td>
<td>0.11 (0.039)</td>
<td>2.73 (0.29)</td>
</tr>
<tr>
<td>Rainbow trout control</td>
<td>0.11 (0.016)</td>
<td>2.69 (0.31)</td>
</tr>
<tr>
<td>Coho post-treatment</td>
<td>1.13 (0.35)</td>
<td>4.95 (0.58)</td>
</tr>
<tr>
<td>Coho control</td>
<td>1.17 (0.23)</td>
<td>5.10 (0.47)</td>
</tr>
<tr>
<td>Rainbow trout post-treatment</td>
<td>1.04 (0.28)</td>
<td>4.70 (0.34)</td>
</tr>
<tr>
<td>Rainbow trout control</td>
<td>1.01 (0.24)</td>
<td>4.65 (0.27)</td>
</tr>
</tbody>
</table>

Table 6.2: Average ratio of double to single cones in TH-treated coho

Average ratio of double to single cones (d/s) for each retinal quadrant examined in control and thyroid hormone-treated alevin coho (see Fig. 6.3, n=3).

<table>
<thead>
<tr>
<th>Retinal Location</th>
<th>Control</th>
<th>Thyroid Hormone-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral</td>
<td>1.65 (0.48)</td>
<td>1.86 (0.19)</td>
</tr>
<tr>
<td>Temporal</td>
<td>1.03 (0.05)</td>
<td>0.96 (0.06)</td>
</tr>
<tr>
<td>Dorsal</td>
<td>0.97 (0.03)</td>
<td>0.99 (0.07)</td>
</tr>
<tr>
<td>Nasal</td>
<td>1.00 (0.01)</td>
<td>0.96 (0.14)</td>
</tr>
</tbody>
</table>

Table 6.3: Average ratio of double to single cones in post-treated coho

Average ratio of double to single cones (d/s) for each retinal quadrant examined in control and post-thyroid hormone treated alevin coho (see Fig. 6.7, n=3).

<table>
<thead>
<tr>
<th>Retinal Location</th>
<th>Control</th>
<th>Thyroid Hormone-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral</td>
<td>1.46 (0.45)</td>
<td>1.31 (0.32)</td>
</tr>
<tr>
<td>Temporal</td>
<td>1.25 (0.39)</td>
<td>1.16 (0.26)</td>
</tr>
<tr>
<td>Dorsal</td>
<td>0.96 (0.05)</td>
<td>0.97 (0.10)</td>
</tr>
<tr>
<td>Nasal</td>
<td>1.31 (0.41)</td>
<td>1.29 (0.41)</td>
</tr>
</tbody>
</table>
6.6 Figures and Figure Legends

Figure 6.1: Micrographs of sections of control and thyroid hormone-treated alevin coho retinas

(a,c) Tangential sections from the dorsal retina of control alevin show a square mosaic in which all corner cones (white arrows) and center cones (asterisk) label with the UV riboprobe (a) and none label with the blue riboprobe (c).

(b,d) Tangential sections from the dorsal retina of treated alevin show a square mosaic in which only corner cones label with the UV riboprobe (b) and the majority of single cones label with the blue riboprobe (d). White arrowheads indicate double cone partitions; white arrows show corner cones and asterisks show center cones. Scale bar (in a) = 15 \mu m holds for all.
Figure 6.2: Quantification of UV and blue opsin-labeled cones in control and thyroid hormone-treated alevin coho

(a) The number of single cones labeled with the UV opsin riboprobe in the thyroid hormone-treated fish (purple bars) is significantly less than in the control (blue bars), except in the ventral quadrant. (b) None of the single cones in the control retina were labeled with the blue opsin riboprobe, except in the nasal quadrant (blue bar). See Table 6.2 (n=3).
Figure 6.3: Micrographs of sections showing cone mosaic formations in the retina of control and thyroid hormone-treated alevin coho

(a, c, e, g) Tangential sections from ventral (a), temporal (c), dorsal (e), and nasal (g) retina of control alevin.  (b, d, f, h) Tangential sections from ventral (b), temporal (d), dorsal (f), and nasal (h) retina of thyroid hormone-treated alevin. All quadrants in both control and thyroid hormone-treated fish have a square mosaic complete with corner cones, except in the ventral (a, b) quadrant. White arrowheads indicate double cone partitions; white arrows show corner cones; black arrows show absence of corner cones and asterisks show center cones. Scale bar (in a) $\approx 15 \mu m$ holds for all.
Figure 6.4: Micrographs of retinal sections from thyroid hormone-treated alevin showing coexpression of UV and blue opsin induced by thyroid hormone

(a) Tangential section showing UV (white arrows) and blue opsin (black arrows) riboprobe labeling center and corner cones. Some single cones label with both riboprobes (blue arrows). (b) Radial section showing single cones labeled with the UV opsin riboprobe (white arrow), some labeled with the blue opsin riboprobe (black arrow), and some labeled with both riboprobes (blue arrow). d, double cone; RPE, retinal pigmented epithelium. Scale bar (in a) = 15 μm holds for all.
Figure 6.5: Micrographs of sections from thyroid hormone-treated alevins showing that thyroid hormone did not induce single cone apoptosis

(a) Tangential section shows a row mosaic in which most single cones are present but are not labeled by TUNEL (white arrows). (b) Radial section shows single cones are not labeled by TUNEL (white arrows). (c) Radial section of coho salmon embryo used as a positive control shows cells labeled by the TUNEL procedure (black arrow). White arrowheads indicate double cones partitions. Scale bar (in a) = 15 μm holds for all.
Figure 6.6: Micrographs of retinal sections from control and thyroid hormone-treated rainbow trout alevin

(a,c) The UV riboprobe labels only corner cones in the ventral retina of control alevin (white arrows) (a) and the blue riboprobe labels only center cones (white asterisk) (c). (e,g) All single cones in the dorsal retina are labeled with the UV riboprobe and none label with the blue riboprobe. (b,d) The UV riboprobe does not label any of the single cones in the ventral retina of treated alevin (b) while the blue riboprobe labels all of them (d). (f,h) A rare UV corner cone is found in the dorsal retina of treated alevin (f), where the majority of single cones label with the blue opsin riboprobe (h). Scale bar (in a) = 15 μm holds for all.
Figure 6.7: Micrographs of retinal sections from control and post-treated alevin coho showing that UV and blue opsin expression revert to the pre-treated state

(a,c) Radial sections from ventral retina of control alevin showing that all single cones label with the UV opsin riboprobe (a) and none with the blue riboprobe (c). (e,g) Labeling pattern is similar in the dorsal retina of the control alevin in that all single cones label with the UV (e) but not the blue riboprobe (g). (b,d) Tangential sections from the ventral retina of an alevin treated for two weeks with thyroid hormone and then left untreated for the following six weeks; the UV riboprobe labels all center cones and most corner cones have disappeared at this time (black arrows). A remaining corner cone is labeled with the UV riboprobe (b). The blue riboprobe does not label any single cones (d). (f,h) Tangential sections from the dorsal retina of post-treated alevin show that corner cones are still present and are labeled with the UV riboprobe, which also labels center cones (f). None of the single cones in the post-treated alevin are labeled with the blue riboprobe (h). Symbols and nomenclature as in Fig 6.3. Scale bar (in a) = 15 μm holds for all.
Figure 6.8: Effects of thyroid hormone on the UV and blue opsin promoters

(a) Diagram of the UV (left) and blue (right) opsin promoter luciferase reporter gene constructs (lines, upstream region of opsin; white boxes, +68 bp and +178 bp of the 5' untranslated region of the UV and blue opsin genes, respectively, positions are with respect to predicted transcription start site (+1); arrow, direction of transcription; blue boxes, luciferase reporter gene). (b) Luciferase gene activity of UV (U0.5) and blue (B0.5) opsin promoter construct in control or treated with 50 nM of TH. Bar graphs are mean ± standard error (n=5, transfection was done in triplicates and repeated 5 times). (c) CHSE cells were either treated with 0, 10, 25, 50, 75, or 100 nM of thyroid hormone. The luciferase activity of the control (0 nM) was set to 1.0. The highest suppression was achieved at 50 nM. Asterisks (*) indicate means that are statistically different from the control mean.
Figure 6.9: The nucleotide sequence of the Atlantic salmon UV opsin upstream region

The nucleotide sequence of the 5' flanking fragment of the UV opsin is shown with a potential thyroid hormone response element (TRE) identified by MatInspector. The TATA box is boxed, the predicted transcription start site is indicated as +1, and the translation start site is underlined. A few codons of the UV opsin gene are included and are fused in frame with the luciferase reporter to promote strong expression.
Figure 6.10: The nucleotide sequence of the Atlantic salmon blue opsin upstream region

Shown is the nucleotide sequence of the 5' flanking fragment of the blue opsin. The TATA box (boxed), the predicted transcription start site (+1), and the translation start site (underlined) are indicated. A few codons of the blue opsin gene are included and are fused in frame with the luciferase reporter to promote strong expression. The consensus sequence for the thyroid hormone response element is not identified in the blue opsin upstream region.
During the embryonic to alevin period when levels of thyroid hormone are low, expression of UV opsin may be activated by RORβ, as in the mouse; while expression of blue opsin may be inhibited by unliganded thyroid hormone receptors. As thyroid hormone levels rise near yolk sac absorption, thyroid hormone binds to its receptor which complexes with RXRγ to repress UV opsin expression. The liganded thyroid hormone receptor may homodimerize or complex with an unknown factor to promote blue opsin expression. Dotted line indicates the possibility of receptors affecting opsin expression indirectly.
CHAPTER 7
CONCLUSION

7.1 Overall Conclusion

Researchers have long used salmonid fishes as model organisms to investigate photoreceptor development. However, some of the fundamental details, such as the order of opsin expression and the distribution of opsins throughout development, have not been addressed. In my dissertation, I examined the spatial and temporal appearance of opsin expression and the organization of photoreceptors during the ontogeny of salmonid fishes, and I also investigated mechanisms underlying the regulation of opsin expression. I have established a comprehensive picture of how the chromatic organization of the cone mosaic is achieved in the retina of salmonid fishes.

7.1.1 Ontogeny of opsin expression in salmonid fishes

The order of opsin appearance in salmonid fishes is UV opsin, followed by rhodopsin, red opsin, green and blue opsin. The origin of opsin expression is in the centro-temporal retina. The single cones exhibit plasticity in opsin expression such that all single cones express a UV opsin at hatching but later transform into blue cones by opsin switch. This changeover of opsins begins in the centro-ventro-temporal retina and the cones undergoing this process coexpress both UV and blue opsins. The double cone (with a red and green member) and the rod do not exhibit opsin switches.

The UV to blue cone transformation event appears to be closely related to the loss of cone cells since both events overlap in retinal surface area and both progress from
ventral to dorsal retina. The loss of corner cones does not extend to the entire retina such
that these are retained in the dorsal region of the smolt retina. The remaining corner
cones and the majority of single cones in the smolt retina express blue opsin. In the adult,
UV cones are scarce and are found only in the dorsal peripheral retina. The
transformation event that starts in the young salmon alevin leads to an irreversible
downregulation of UV opsin and upregulation of blue opsin throughout the life of the
animal.

7.1.2 Regulation of opsin expression by thyroid hormone

Thyroid hormone signaling is known to affect photoreceptor development in the
vertebrate retina and the underlying molecular mechanisms are the subject of ongoing
study. Treatment of salmon alevins with thyroid hormone led to a significant decrease in
UV opsin mRNA expression and an increase in blue opsin mRNA expression. These
changes manifested themselves in the numbers of single cones expressing either of these
opsin mRNAs. These results provide direct evidence that: (1) these changes occurred
through a switch in opsin within individual cones, and (2) thyroid hormone induced a
premature switch from UV to blue opsin expression. The ability of thyroid hormone to
induce this premature switch implies that alevins already possess the intrinsic machinery
for responding to thyroid hormone signalling, but that the extrinsic signal (thyroid
hormone) is the factor that determines the onset of transformation.

Upon cessation of thyroid hormone treatment, expression of UV and blue opsin
returned to the control state suggesting that, although thyroid hormone was the factor that
directed downregulation of UV and upregulation of blue opsin, previous thyroid hormone
exposure was not sufficient to sustain expression. There might be factors responsible for
maintaining opsin expression that are not activated at this stage, and that might direct chromatin remodelling. Indeed, permanent changes to gene expression are believed to take place through changes in histone and DNA methylation that lead to a condensed, closed chromatin state (Grunstein, 1990; Kornberg and Lorch, 1999). The window of opportunity for thyroid hormone action may therefore be restricted by the timing of permanent changes to opsin gene expression. Analyzing opsin expression in older salmon such as parr and smolt treated with thyroid hormone may confirm the time frame of thyroid hormone action.

The effect of thyroid hormone on UV opsin expression appears to occur at the transcriptional level through regulation of the UV opsin promoter. Whether the effect is direct or indirect remains to be determined. In contrast to earlier reports on rainbow trout that invoked thyroid hormone inducing a loss of corner cones to explain decreased UV sensitivity (Browman and Hawryshyn, 1992, 1994), we demonstrated that thyroid hormone induces changes in opsin expression without concomitant apoptosis of corner cones.

7.2 UV Opsin Expressing Cones May Reflect the Ancestral Cone Type

The ancestral cone type likely expressed an SWS1 (UV sensitive) opsin (Yokoyama, 2000; Ebrey and Koutalos, 2001). In salmonid fishes, cichlids, mouse and humans, cones expressing SWS1 opsin with maximum sensitivity in the UV-violet region of the spectrum appear first (Chapters 2 and 3; Spady et al., 2006; Szél et al., 1994; Applebury et al., 2000; Cornish et al., 2004). Although direct proof for an opsin switch has only been obtained for salmonid fishes (this thesis), it is likely that a subset of the original SWS1 cones give rise to the other spectral cone phenotypes by opsin switch in
other species as well (Szél et al., 1994; Xiao and Hendrickson, 2000). Thus, salmonid fishes, a family of fishes with ancient origins (Groot and Margolis, 1991), likely develop UV opsins expressing cones first in accordance with a conserved mechanism of retinal development dating back to early chordates.

Other fishes (e.g. the zebrafish and flatfishes) develop middle and long wavelength opsins expressing cones first (Takechi and Kawamura, 2005; Evans et al., 1993; Forsell et al., 2001; Mader and Cameron, 2004). The reasons for this variation in first opsin expressed are unclear. Fishes that express longer wavelength opsins seem to have transparent eggs. These eggs are usually pelagic (as in the zebrafish) and they are primarily exposed to longer wavelength light during development because the egg shells preferentially absorb shorter wavelength light (Novales Flamarique, personal communication) and, for some species, because the eggs develop at depth where only longer wavelengths abound (e.g. the flatfishes, see Novales Flamarique, 2002). These rearing conditions contrast with those of salmon and mammals, which occur in the dark. It is possible that the change in first opsin expressed reflects the use of variable environmental light cues for retinal development in fishes/vertebrates with exposed embryos. In accordance with this hypothesis, light-dependent expression has been described for several genes involved in the phototransduction cascade including rhodopsin (Korenbrot and Fernald, 1989), arrestin (McGinnis et al., 1994) and pinopsin (Takanaka et al., 2002). As well, premature expression of green opsin can be induced in mouse pups exposed to light (McCaffery et al., 1996). It is to be noted here that the eyelid of the closed pup eye transmits longer wavelengths (McCaffery et al., 1996).
7.3 Developmental Role of UV Opsin

The timing of the transformation and loss of corner cones questions the importance of UV cones in the behaviour/ecology of salmonid fishes. Ultraviolet sensitivity mediated by UV cones is thought to improve contrast enhancement of zooplankton prey (Loew et al., 1993; Browman et al., 1994), and to aid in the detection of the polarization of light for prey contrast enhancement (Novales Flamarique and Browman, 2001) and for orientation (Hawryshyn et al., 1990). The observations from this thesis do not support these functions. For instance, the transformation occurs in the early alevin stage of salmon at a time when the fish are dependent on zooplankton for their sole food source. Furthermore, young salmon feed by attacking prey from below, thereby forming an image in the ventral retina. If UV cones were important for detecting prey, then one would expect the transformation and apoptosis events to proceed from the dorsal to the ventral retina, leaving UV cones in the ventral retina where the image is formed and where cone density (and hence visual acuity) is high.

The expression of UV opsin during early retinal development suggests a role in chromatic organization of the cone mosaic. Ultraviolet opsin is expressed first during photoreceptor development and it is downregulated sometime after the photoreceptor mosaic is established. Experiments that involve knocking down UV opsin should reveal any potential role of this opsin in determining opsin expression of neighbouring protocones. Studies in a metamorphic species, the winter flounder, have shown that cone differentiation and mosaic development are two independent processes in this species (Hoke et al., 2006).
7.4 Future Directions

7.4.1 Unravelling thyroid hormone signaling in directing opsin expression

Thyroid hormone-mediated regulation of opsin expression has been demonstrated for a few organisms including the mouse (Roberts et al., 2006), winter flounder (Mader and Cameron, 2006), and salmon (this thesis). Thus, the mechanism by which thyroid hormone controls opsin expression may be conserved in vertebrates.

To gain a better understanding of how thyroid hormone determines opsin expression in the fish retina, the levels of endogenous thyroid hormone, thyroid hormone receptors, and deiodinase enzyme activity should be examined throughout ontogeny. In the mouse, opsin changes are mediated by levels of TRβ2 receptor (Ng et al., 2001; Roberts et al., 2006; Applebury et al., 2007), while those occurring during metamorphosis of flounder are mediated by TRα (Mader and Cameron, 2006).

7.4.2 Fate of corner cones

The method by which corner cones disappear remains controversial as several studies could not detect apoptosis of photoreceptors after the yolk sac alevis stage (Julian et al., 1998; Candal et al., 2005). The disappearance of corner cones could possibly involve a transformation to rod photoreceptors. It has been shown that cones could be induced to develop rod-like characteristics and to partially suppress cone-specific gene expression under the influence of NRL, a basic motif leucine zipper transcription factor (Oh et al., 2007). The photoreceptors of a retinal degeneration mutant mouse that carries a deletion in the nuclear transcription factor, Nr2e3, exhibit morphology of a hybrid cell type that expresses both rod- and cone-specific genes (Corbo and Cepko, 2005). Thus, it
is possible that corner cones in the salmonid retina at the time of transformation could express NRL or NR2E3 lending some support for this hypothesis.

7.4.3 Concluding remarks

My work has contributed to the understanding of the dynamics in photoreceptor differentiation in salmonid fishes and the molecular mechanisms that underlie this process. I have established the UV to blue cone transformation as a general feature of retinal growth in Pacific salmonids and I have proven that this transformation event is regulated by thyroid hormone. My work has established the foundations upon which further investigations into the molecular mechanisms underlying regulation of opsin expression in salmonid fishes can be undertaken.
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